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>1939S</sup>). FANCA<sup>1939S</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.

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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), including 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/FANC1 heterodimer (10). Monoubiquitinated FANCD2 is subsequently required for binding to SLX4/FANC1, 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, FANCA1939S, 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

FANCA1939S 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, 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 FANCA protein, a region implicated 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 FANCA1939S mutation blocks FAAP20 binding, we coexpressed Myc-tagged FANCA with Flag-tagged FAAP20 and tested for communoprecipitation. As predicted, unlike FANCAWT, the FANCA1939S protein failed to bind to FAAP20 (Figure 1E). Similarly, a GST-FANCA1939S 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 FANC D2 monoubiquitination and recruitment of the TLS for ICL repair (12). We next determined whether FANCA1939S could restore these functions in the FA pathway. Interestingly, FANCA1939S was able to complement FANC D2 monoubiquitination, albeit at a lower level than FANCAWT (Figure 2A). Consistent with the rescue of FANC D2 monoubiquitination, the FANCA-deficient GM6914 cells expressing FANCA1939S also exhibited DNA damage–inducible FANC D2 foci (Figure 2B and Supplemental Figure 2). This result confirms that the FANCA1939S mutant can support FA core complex assembly and FANC D2 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 FANCA1939S protein to correct the DNA cross-linker–inducible assembly of REV1 nuclear foci with that of the FANCAWT protein. Complemented FANCA-deficient GM6914 cells were exposed to psoralen plus UVA, a mechanism known to induce DNA ICLs. Interestingly, while FANCAWT supported the assembly of psoralen-UV–inducible REV1 foci, FANCA1939S 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 FANCA1939S protein, unlike FANCAWT, failed to rescue UVC-inducible point mutagenesis, as measured by the SupF assay (Figure 2D).

Taken together, these results indicate that FANCA1939S is a separation-of-function mutant. The FANCA1939S protein functions efficiently in the assembly of the FA core complex and the DNA damage–inducible monoubiquitination of FANC D2 protein, which is required for FANC D2 foci assembly and recruitment of the downstream endonuclease complex (11). However, the FANCA1939S 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...
lanes 5–8, and Supplemental Figure 3B). The MMC hypersensitivity of the DF2231 cells was functionally complemented with the cDNA-encoding FANCAWT 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 FANCAI939S 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-proteasome (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 FANCAI939S polypeptide (Figure 3B, lanes 5–8, and Supplemental Figure 3A) in DF2231 cells compared with the higher expression level of full-length FANCAWT 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 FANCAWT 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).

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contrast, the full-length FANCA WT 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 FANCAI939S 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 FANCAWT

![Diagram](https://via.placeholder.com/1526)

**Figure 2. The FANCAI939S 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 FANCAWT or FANCAI939S 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, ×60. Quantification of cells displaying more than 5 REV1 foci. Data shown are mean ± SEM from 3 independent experiments. *P < 0.05, compared with psoralen plus UVA–treated FANCAWT complemented GM6914 cells, 2-tailed t test (assuming unequal variance). (D) The mutation frequency in damaged (1,000 J/m² UVC) SupF plasmid recovered from GM6914 cells, complemented with vector, FANCAWT, or FANCAI939S. Data shown are mean ± SEM from 3 independent experiments. *P < 0.05, compared with UVC-treated FANCAWT complemented GM6914 cells, 2-tailed t test (assuming unequal variance).
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
specific for RNF4, RNF111, and/or FAAP20, and the stability of endogenous FANCA was measured (Figure 5A). Knockdown of FAAP20 resulted in a decreased cellular level of FANCA (Figure 5A, lane 2). Interestingly, concurrent knockdown of RNF4 and FAAP20, but not RNF111 and FAAP20, partially rescued the stability of FANCA, demonstrating that RNF4 promotes the degradation of FANCA protein when FAAP20 is unbound (Figure 5A, lanes 3–5). RNF4 is known to interact with SUMOylated MDC1 in response to DNA double-strand breaks (20). To test the interaction between RNF4 and FANCA, we performed coimmunoprecipitation of Myc-FANCA and Flag-RNF4. FANCA, similar to MDC1, physically associated with RNF4 under these conditions.

Figure 4. The SUMO E3 ligase PIAS1 mediates FANCA SUMOylation at K921. (A) Alignment of FANCA from various species. The conserved K921 and I939 residues are indicated with asterisks. (B) 293T cells were transfected with HA-tagged SUMO or cotransfected with HA-tagged SUMO with Myc-tagged FANCAWT. FANCA SUMOylation was determined under denaturing conditions by anti-HA immunoprecipitation, followed by anti-Myc immunoblot. (C) 293T cells were transfected with Myc-tagged FANCAWT or FANCAK921R with HA-SUMO3 as indicated. FANCA SUMOylation was determined under denaturing conditions by anti-HA immunoprecipitation followed by anti-Myc immunoblot. (D) 293T cells were transfected with Myc-tagged FANCAWT or FANCAK921R. Cells were treated with 20 μM MG132 (lanes 2 and 3), and FANCA ubiquitination was examined under denaturing conditions by anti-myc immunoprecipitation, followed by anti-Myc immunoblot. (E) 293T cells were transfected with Myc-tagged FANCAWT alone or with HA-SUMO3 as indicated. Subsequently, cells were exposed to siRNAs against control, UBC9, PIAS1, or PIAS4. Protein expression was determined by immunoblot, and FANCA SUMOylation was examined under denaturing conditions by anti-HA immunoprecipitation, followed by anti-Myc immunoblot. (F) Immunoblot of FANCA derived from cell lysates of the indicated complemented GM6914 cells treated with cycloheximide (CHX) (20 μg/ml). (G) Complemented GM6914 cells were treated with increasing doses of MMC and plated for 4 days. Three independent experiments were performed.
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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 FANCAI939S in DF2231 cells (Figure 5B). Furthermore, RNF4 suppression increased the MMC sensitivity of GM6914 cells expressing FANCAWT but had little effect on the MMC sensitivity of GM6914 cells expressing the mutant FANCAI939S protein (Figure 5C). Interestingly, RNF4 suppression did not further increase the MMC sensitivity of GM6914 cells expressing FANCAK921R (Supplemental Figure 5D). Taken together, these results support the hypothesis that RNF4 functions as the STUbL for FANCA.

A normal function of RNF4 in the FA/BRCA pathway. We next hypothesized that RNF4 may promote the degradation of FANCAWT in normal (non-FA) human cells, albeit at a slower rate than the degradation of the mutant FANCAI939S protein in the cells from patients with FA. Indeed, RNF4-mediated degradation of FANCAWT 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 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 (FANCAI939S). The FANCAI939S 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–/–, RNF4–/–, or FANCC–/–/RNF4–/– 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–/–, RNF4–/–, or FANCC–/–/RNF4–/– DT40 cells exposed to 50 ng/ml MMC for 16 hours.

On the other hand, the FANCA I939S 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 I939S to bind to FAAP20 resulted in its enhanced degradation in the patient-derived cells. Interestingly, the instability of the FAANCA 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 FANC 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 FANCE 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 FANC 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 I939S, 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-
Ase–tagged (GST-tagged) FANCA variants were expressed in BL21 washed 6 times with 0.1% SDS in PBS before immunoblot analysis. Denatured proteins were incubated with anti-HA antibody–conjugated agarose beads (Sigma-Aldrich) or anti-FANCA antibody. Lysates were incubated with anti-HA antibody–diluted 10-fold in PBS containing 10 mM N-ethylmaleimide. Lysates were incubated with anti-HA antibody–conjugated agarose beads (Sigma-Aldrich), washed 6 times with 0.1% SDS in PBS before immunoblot analysis.

**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), β-actin (Cell Signaling), β-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′-CAGGGTATCGACAGTATCAA-3′; RN4-A, 5′-AGAATGACCTCCTCATGTT-3′; RN4-B, 5′-AACCGTGCTGGTAGACAGATA-3′; RN4-C, 5′-CCCTGTCTTCATGAAACGAAA-3′; FAAP20, 5′-CACGCTGAGCCGGACCTAT-3′; FANCA, 5′-CACGGTGGAGATATCAAAGAT-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); RNFI11, 5′-TAGAGATGACTCAAGGAGAA-3′; and FANCG, 5′-CCACGTTATCATCGACACTTA-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, SUMOylation assays were performed as previously described with DT40 cells were treated with 50 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/m²) 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 β-gal MBM7070 indicator strain plasmids via electroporation (GenePulsor X Cell, Bio-Rad) and plated onto LB plates containing 1 mM isopropyl-β-D-thiogalactoside, 100 μg/ml X-gal, and 100 μg/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 ± 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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