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ORIGINAL ARTICLE

A novel role for non-ubiquitinated FANCD2 in response to hydroxyurea-induced DNA damage

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Fanconi anemia (FA) is a genetic disease of bone marrow failure, cancer susceptibility, and sensitivity to DNA crosslinking agents. FANCD2, the central protein of the FA pathway, is monoubiquitinated upon DNA damage, such as crosslinkers and replication blockers such as hydroxyurea (HU). Even though FA cells demonstrate unequivocal sensitivity to crosslinkers, such as mitomycin C (MMC), we find that they are largely resistant to HU, except for cells absent for expression of FANCD2. FANCD2, RAD51 and RAD18 form a complex, which is enhanced upon HU exposure. Surprisingly, although FANCD2 is required for this enhanced interaction, its monoubiquitination is not. Similarly, non-ubiquitinated FANCD2 can still support proliferation cell nuclear antigen (PCNA) monoubiquitination. RAD51, but not BRCA2, is also required for PCNA monoubiquitination in response to HU, suggesting that this function is independent of homologous recombination (HR). We further show that translesion (TLS) polymerase PolH chromatin localization is decreased in FANCD2 deficient cells, FANCD2 siRNA knockdown cells and RAD51 siRNA knockdown cells, and PolH knockdown results in HU sensitivity only. Our data suggest that FANCD2 and RAD51 have an important role in PCNA monoubiquitination and TLS in a FANCD2 monoubiquitination and HR-independent manner in response to HU. This effect is not observed with MMC treatment, suggesting a non-canonical function for the FA pathway in response to different types of DNA damage.

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INTRODUCTION

The Fanconi anemia (FA) pathway is a DNA-repair pathway for DNA interstrand crosslinks (ICL). Patients with FA exhibit birth defects, bone marrow failure and a lifelong predisposition to cancer, such as acute myeloid leukemia and head and neck cancer. Cellular hypersensitivity to DNA cross-link agents, such as mitomycin C (MMC), is the hallmark of FA.[1–4] At least 16 genes, which when mutated, have been found to lead to FA.[5,6] The FA proteins FANCA, B, C, E, F, G, L and M form an upstream core complex with putative E3-ligase activity, whose target is the FANCD2-FANCI complex.[5,7] It is suggested that the FANCD1, J, N, O, P, Q proteins function downstream of activation of the FANCD2-FANCI complex. Monoubiquitination of FANCD2 by the core complex is essential for its chromatin localization, DNA-repair focus formation and function in repairing ICL. These events are implicated in recruitment of downstream proteins important for homologous recombination, several proteins which are part of the FA pathway. RAD51 is a key factor in homologous recombination (HR) by virtue of its activity of binding to single strand DNA and forming a nucleoprotein filament in cooperation with BRCA2,[8] which is itself the *bona fide* FA gene FANCD1.[9]

Translesion (TLS) is a mechanism for DNA damage tolerance or post-replication repair involving switching of the replicative DNA polymerase for specialized translesion polymerases, such as PolH, PolK and Rev1. By recruiting error-prone polymerases TLS allows replication machinery to bypass the replication-blocking lesions.[10,11] The recruitment of TLS polymerases is regulated by a post-translational modification of proliferation cell nuclear antigen (PCNA) *via* monoubiquitination. RAD18 is the E3-ligase responsible for PCNA monoubiquitination in TLS.[12] Upon monoubiquitination PCNA recruits TLS polymerases to chromatin. Our group and others have recently demonstrated RAD18 binding to FANCD2 in a manner critical for normal FA function,[13] whereas others have described the physical interaction of FANCD2-PCNA.[14] This study seeks to understand the relationship between these pathways.

The observation that FANCD2 colocalizes with RAD51 after exposure to DNA damage induced by MMC and HU indicates that FANCD2 and RAD51 may function together in repairing DNA damage.[15] The interplay between the FA pathway and the TLS pathway has also been demonstrated. The deubiqutinating enzyme USP1 is responsible for both FANCD2 and PCNA deubiquitination.[16,17] Also, PCNA and RAD18 can interact with FANCD2 and regulate FANCD2 monoubiquitination.[13,14,18] On the other hand, the FA core complex is required for efficient point mutagenesis and REV1 foci formation in response to UV irradiation, indicating that FA proteins may participate in regulating TLS in a way that is independent of FANCD2 monoubiquitination.[19] Interestingly, FANCD2 depletion inhibits TLS in Xenopus egg extracts.[20] The observation that FANCD2 interacts with PolH upon DNA damage induced by UV further demonstrates the interplay between FA pathway and TLS pathway.[21] These multiple levels of interaction suggest cooperativity amongst repair pathways that has the potential to fine tune response to DNA damage.

The colocalization of FANCD2 with RAD51 and PCNA after HU treatment indicates cooperativity.[15] To further understand the function and regulation of DNA-repair proteins we set out to study this relationship in response to distinct types of DNA damaging

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agents that stimulate the monoubiquitination response of FANCD2. DNA crosslinkers such as MMC, stimulate FANCD2 monoubiquitination, as well as confer hypersensitivity upon FA mutant cells. HU depletes the deoxyribonucleotide pool, resulting in replication fork collapse. HU also stimulates FANCD2 monoubiquitination, but the cytotoxicity effect on FA mutant cells has not been carefully explored. We find that almost all FA deficient cells are as resistant to HU as wild-type cells, whereas hypersensitive to MMC, in a manner that is dependent on FANCD2, regardless of its ability to be monoubiquitinated. We find that FANCD2 forms a complex with RAD51 and RAD18, which increases upon DNA damage induced by HU. We then show that HU can induce PCNA monoubiqutination in a FANCD2 and RAD51-dependent fashion but monoubiquitinated FANCD2-independent fashion. The impaired PolH chromatin localization in FANCD2 and RAD51 deficient cells further demonstrates that FANCD2 and RAD51 are important in regulating TLS in response to DNA damage induced by HU. Knockdown of polH results in HU sensitivity only. These data reveal a novel role of FANCD2 in the DNA damage response that does not depend on its monoubiquitination.

RESULTS

FANCD2 deficient cells are hypersensitive to HU

The FA pathway is a DNA-repair pathway specialized for repairing DNA ICLs. FA deficient cells are hypersensitive to crosslinking agents, such as MMC. MMC can induce FANCD2 monoubiquitination, a key step in FA pathway. However, FANCD2 monoubiquitination can also be induced by other type of DNA damaging agents, such as HU, UV and ionizing radiation.[22–24] HU is a replication inhibitor and can induce DNA double-strand breaks by causing replication fork arrest upon nucleotide pool depletion.[25,26] It has been shown that FANCD2 has an important role in protecting replication forks and in replication fork recovery.[27–29] In order to further understand FANCD2 function in response to different DNA damage agents we compared the MMC and HU hypersensitivity of FANCD2 deficient cells, FANCA deficient cells and FANCJ deficient cells. As expected, FANCD2 deficient cells, FANCA deficient cells and FANCJ deficient cells were all hypersensitive to MMC (Figure 1). However, only FA-D2 mutant cells expressing no FANCD2 were hypersensitive to HU, whereas FA-D2 cells containing FANCD2 monoubiquitination mutant K561R, FANCA deficient cells, which lack monoubiquitinated FANCD2, and FANCJ deficient cells were not (Figure 1a). These results suggest that non-ubiquitinated FANCD2 has an important but separate role in repair and response to DNA damage induced by HU. These data also clearly demonstrate that FA-J mutant cells are HU resistant, in contrast to earlier reports, but are consistent with much older data concerning cell cycle and HU in FA-A mutant cells.[30,31]

In order to confirm our results, we examined another FA phenotype: cell cycle arrest. Upon MMC exposure, FA-D2 mutant cells and FA-D2 cells expressing FANCD2 monoubiquitination mutant K561R protein displayed increased G2/M accumulation relative to corrected cells, as expected (Figure 1b). We also analyzed the cell cycle effect of HU on these same cells. Instead of G2/M phase HU arrests cells in S phase.[32] When cells were treated with HU, FA-D2 mutant cells displayed the highest amount of S phase arrest, whereas significantly less was observed in cells either expressing wild-type FANCD2 or monoubiquitination mutant FANCD2 (K561R) protein (Figure 1b). We confirmed this observation by measuring S phase cells only with BrdU labeled cells (Supplementary Figure A). We performed cell counts in parallel, demonstrating that growth kinetics of each cell line is not markedly different to explain the cell cycle differences (Supplementary Figure A). In FA-J cells we also observed increased G2/M accumulation relative to corrected cells when exposed to MMC (Figure 1c). However, FA-J mutant cells did not display higher S phase accumulation than corrected cells after HU exposure. These data confirm the observation that mutant cells with absent FANCD2 expression are more sensitive to HU than other mutant FA cells, as long as FANCD2 is present, regardless of its monoubiquitination status (Figure 1a). These data were further confirmed by using two siRNA separately transfected knockdown oligos directed against FANCI, the binding partner of FANCD2. Immunoblot demonstrated effective knockdown, and the resulting depleted cells were MMC sensitive but HU resistant, consistent with the idea that FANCD2 has a separable function in mediating resistance to HU (Figure 1d).

In order to demonstrate that the FA-D2 cells containing the FANCD2(K561R) ubiquitination mutant have reduced DNA damage, we subjected the FA-D2 cell group to MMC and HU and analyzed the lysates for gamma-H2Ax signal, which is increased in proportion to increased DNA damage. As expected, FA-D2+FANCD2 cells expressed FANCD2 and responded to MMC or HU with FANCD2 monoubiquitination, whereas FA-D2+FANCD2 (K561R) cells expressed FANCD2 but were not monoubiquitinated in response to MMC or HU (Figure 1e, whole-cell lysate). In chromatin fractions the wild-type containing cells displayed increased monoubiquitinated FANCD2 with only modest increase in gamma-H2Ax signal (Figure 1e, lanes 11 and 12), and the FA-D2 mutant cells displayed marked increase in gamma-H2Ax signal and no increased K561R ubiquitination mutant protein on chromatin (lanes 17 and 18) in response to MMC or HU. Interestingly, the K561R ubiquitination mutant containing cells demonstrated increased signal only in response to MMC (lane 14 versus 15), suggesting that these cells are indeed proficient in the management of DNA damage induced by HU.

FANCD2-RAD51-RAD18 complex and RAD51-RAD18-PCNA complex interaction increase in response to HU

To further study the role of FANCD2 in HU-induced DNA damage we performed FANCD2 immunoprecipitation to test for binding partners with and without HU treatment. To rule out the possibility that DNA may have a role in co-precipitation we treated cell lysates with DNase I before performing FANCD2 immunoprecipitation. We found that RAD51 co-immunoprecipitated with FANCD2 and confirmed interaction of FANCD2 and RAD18, using Flag immunoprecipitation followed by FANCD2 immunoblotting (Figure 2a). Interestingly, FANCD2 monoubiquitination mutant also interacts with RAD51 and RAD18 (Figure 2a). We also observed that these interactions significantly increased with HU and MMC treatment (Figure 2a, lane 7 versus lanes 8 and 9, lane 16 versus lanes 17 and 18). RAD51 immunoprecipitation was performed, and RAD18 immunoblotting showed that RAD18 was co-immunoprecipitated by RAD51, with increased amount after HU treatment (Figure 2b), suggesting that FANCD2, RAD51 and RAD18 form a complex.

It has been shown that RAD18 was detected as non-monoubiquitinated and monoubiquitinated forms by Western blot in human cells.[33] Monoubiquitinated RAD18 was detected mainly in cytoplasm, whereas non-monoubiquitinated RAD18 was detected predominantly in the nuclei.[33] A recent paper shows that ubiquitination prevents RAD18 from localizing to sites of DNA damage, inducing PCNA monoubiquitination and suppressing mutagenesis.[34] Here we observed that FANCD2 and RAD51 predominantly interacts with non-monoubiquitinated RAD18 under our experimental conditions (Figures 2a and b), indicating that these interactions may have a role in PCNA monoubiquitination and DNA damage response. Interestingly, the interaction of RAD18 and RAD51 was only increased in the presence of HU (Figure 2b, lane 8) but not MMC (Figure 2b, lane 9), indicating that DNA-repair proteins differentially respond to different DNA damage agents.

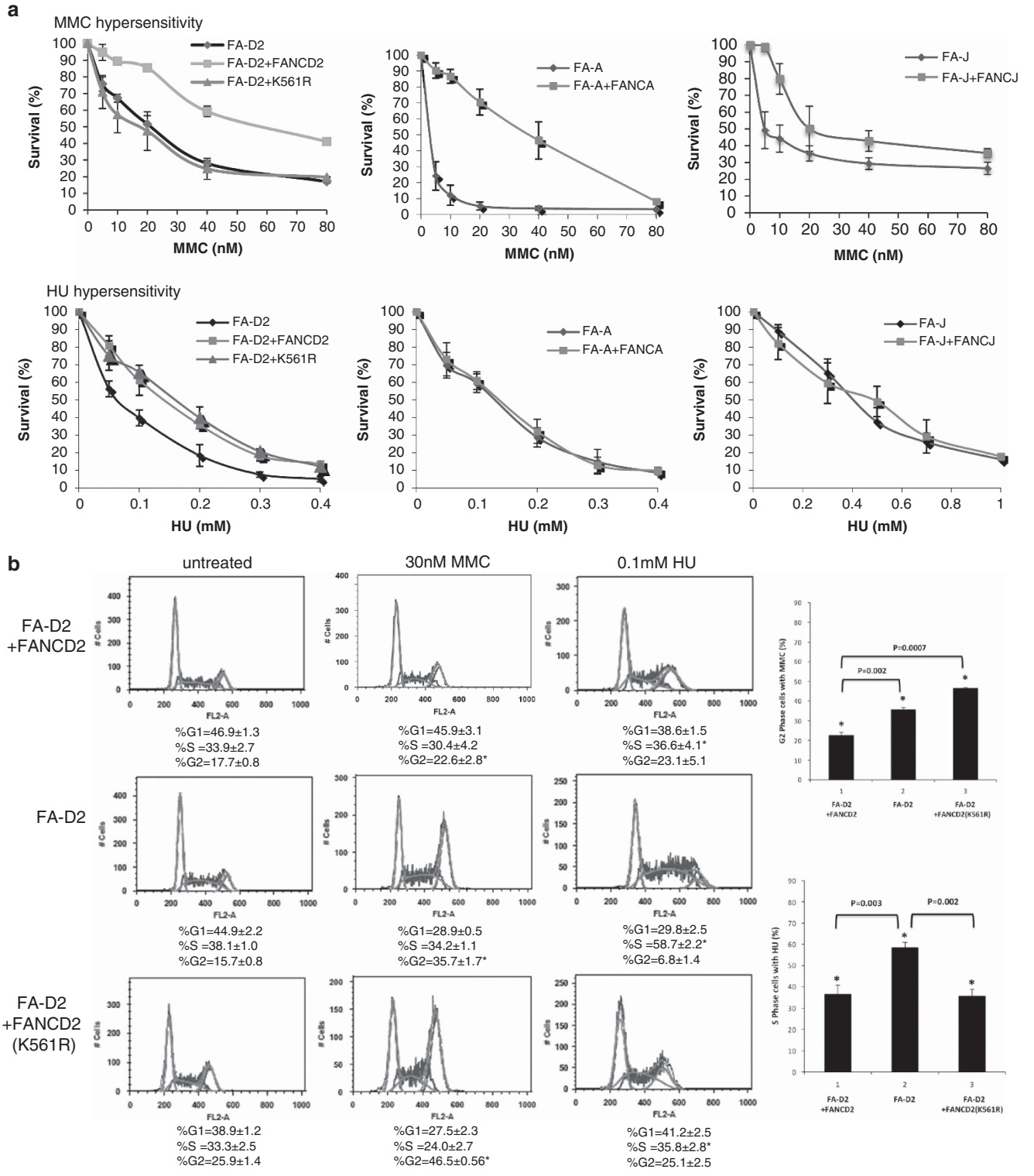

Figure 1. FANCD2 deficient cells are hypersensitive to HU. (**a**) FA-D2, FA-D2+FANCD2, FA-D2+FANCD2(K561R), FA-A, FA-A+FANCA, FA-J and FA-J+FANCJ cells were treated with MMC or HU with the concentrations as indicated in graph for 5 days. Cell survival was analyzed by crystal violet staining. (**b** and **c**) 100 000 of FA-D2, FA-D2+FANCD2, FA-D2+FANCD2(K561R), FA-J and FA-J+FANCJ cells from each treatment were collected after exposure to MMC or HU for 22 h. The cells were then processed, as in the Materials and methods section. Flow cytometry was performed to measure DNA content. (**d**) FANCI was knocked down in 293 T cells using two different siRNA sequences (Dharmacon, Lafayette, CO, USA), either individually or as a mixture of two sequences. The knockdown of FANCI was confirmed by immunoblot analysis. Ku86 was used as loading control. Then cells were subjected to survival assay for MMC or HU sensitivity as in **a**. (**e**) FA-D2, FA-D2+FANCD2, FA-D2 +FANCD2(K561R) cells were treated with 0.5 mM MMC or 0.5 mM HU. The chromatin fraction was subjected to immunoblot analysis for γH2AX level. Ku86 was used as loading control.

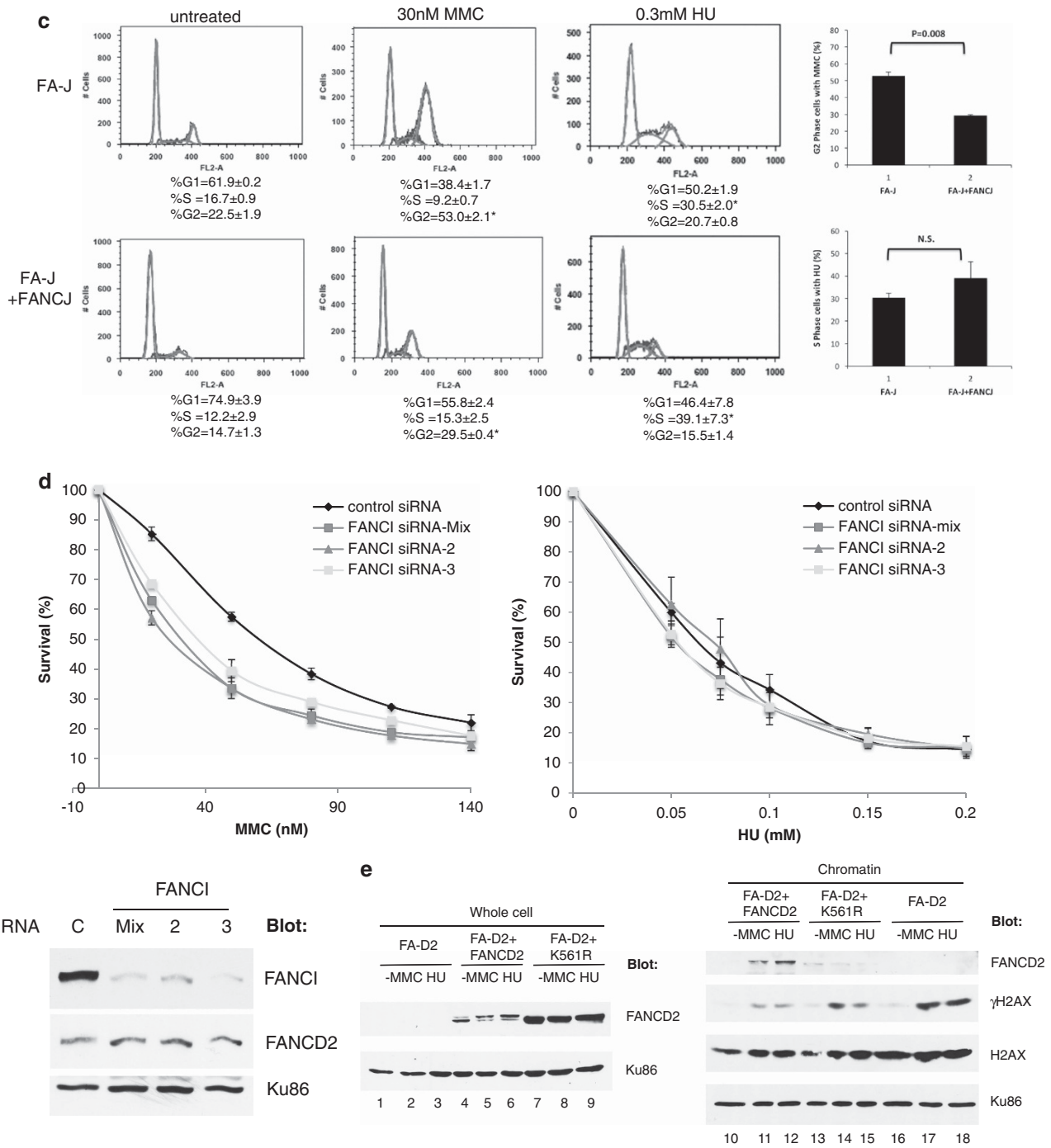

Figure 1. Continued

It has been previously shown that FANCD2 can interact with PCNA.[14] However, under our experimental conditions, interaction of FANCD2 and PCNA was not detected, suggesting lack of, transient, or weak interaction. We then tested if RAD51 could interact with PCNA. Indeed, in a PCNA immunoprecipitation assay we observed that RAD51 was co-immunoprecipitated with PCNA (Figure 2c). As in the FANCD2, RAD51 and RAD18 interactions, the interaction of RAD51 and PCNA was also increased in response to HU treatment (Figure 2c, lane 8). Immunoblotting of the fractions generated by gel-filtration chromatography of protein extracts from HU-treated cells demonstrated co-fractionation of FANCD2, RAD18, RAD51 and PCNA. All these four proteins were found in a 669 kDa protein complex in extracts from FA-D2+flag-FANCD2 cells (Figure 2d, fraction 21–27). We pooled these fractions and subjected the extracts to Flag immunoprecipitation. Subsequent immunoblotting demonstrated the co-precipitation of RAD18 and RAD51 (Supplementary Figure B). Again we observed that FANCD2 and RAD51 predominantly interacts with non-monoubiquitinated RAD18 (Supplementary Figure B) although both monoubiquitinated and non-monoubiquitinated RAD18 were fractionated (Figure 2d).

FANCD2 is required for increased interaction of RAD51 and RAD18

Given the increase in interaction of RAD51 and RAD18 in response to HU, we wanted to test the dependence of this

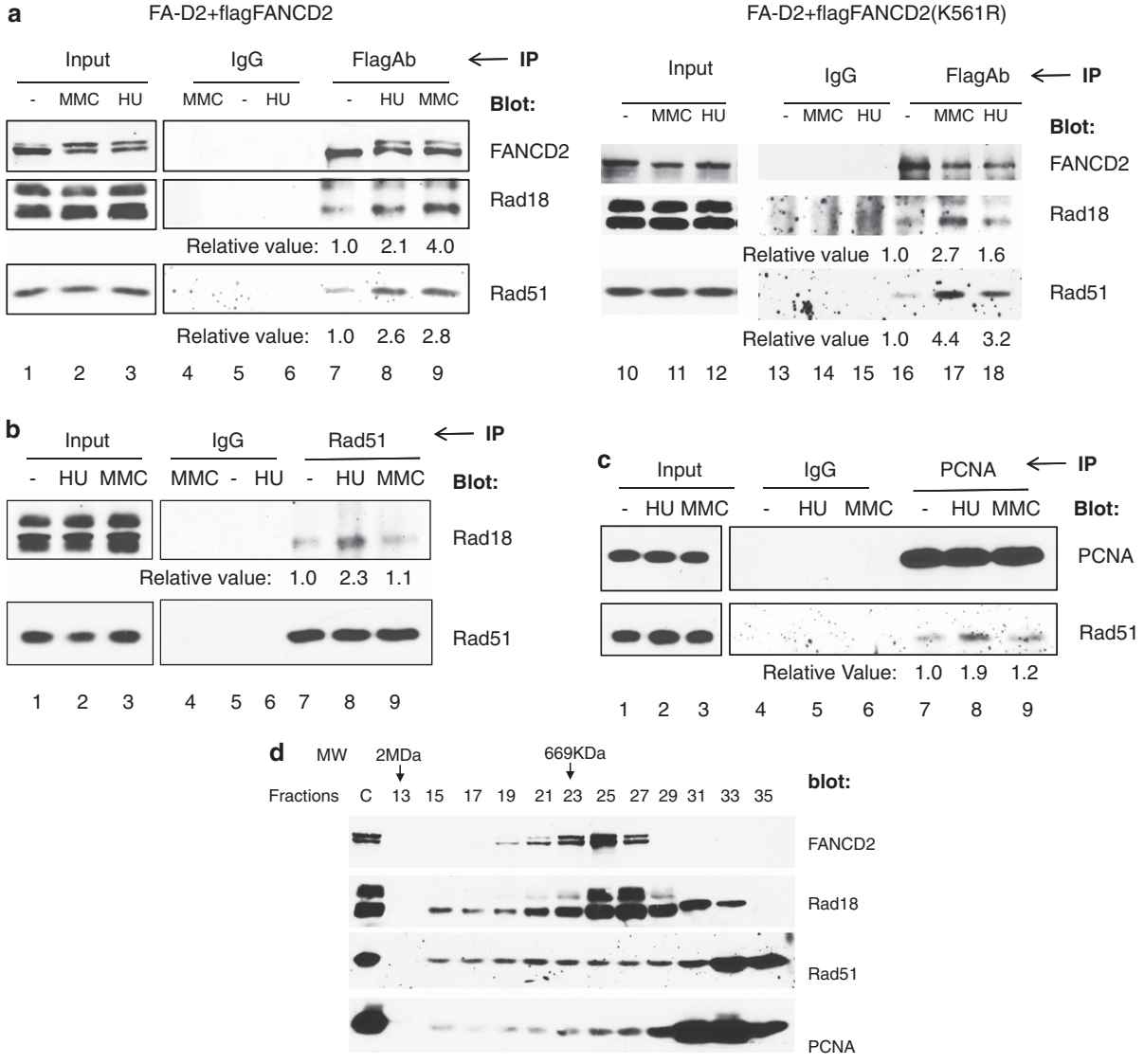

Figure 2. FANCD2-RAD51-RAD18 complex and RAD51-RAD18-PCNA complex interaction increase in response to HU. (**a**) FA-D2+Flag-FANCD2 cells were treated or not with 0.5 mM HU or 0.5 μM MMC overnight. Whole-cell extracts were immunoprecipitated with control IgG or anti-Flag antibody. The immunoprecipitation products were analyzed by immunoblot with FANCD2, RAD18 and RAD51 antibodies. (**b**) The 293 T cells were treated or not with 0.5 mM HU or 0.5 μM MMC overnight. Whole-cell extracts were prepared and immunoprecipitated with control IgG or RAD51 antibody. The immunoprecipitation products were analyzed by immunoblot with RAD18 and RAD51 antibodies. (**c**) The 293 T cells were treated or not with 0.5 mM HU or 0.5 μM MMC overnight. Whole-cell extracts were prepared and immunoprecipitated with control IgG or PCNA antibody. The immunoprecipitation products were analyzed by immunoblot with RAD51 and PCNA antibodies. (**d**) Gel filtration of FA-D2+flag-FANCD2 cell extracts (from cells treated with HU) was performed, and resulting fractions were immunoblotted for FANCD2, RAD18, RAD51 and PCNA. Lane C is loading control. All four proteins partially were co-fractionated.

increased interaction upon FANCD2. Therefore, we performed RAD51 immunoprecipitation in FA-D2 mutant cells lacking FANCD2 expression, FA-D2+ monoubiquitination deficient FANCD2 (K561R) cells, and FA-D2+wild-type FANCD2 cells. Our results showed that only in cells lacking FANCD2 expression was the interaction of RAD51 and RAD18 not enhanced by HU (Figure 3, lane 16), indicating that FANCD2 is a required component in increased RAD51 and RAD18 complex formation. Again, RAD51 predominantly interacts with non-monoubiquitinated RAD18. Interestingly, FANCD2 monoubiquitination deficient mutant also promoted the interaction of RAD51 and RAD18 in response to HU even though not as strongly as wild-type FANCD2 (Figure 3, lane 18), indicating that FANCD2 monoubiquitination is not critical for increased complex formation.

FANCD2 and RAD51 are required for PCNA monoubiquitination in response to HU

Our immunoprecipitation data suggest that in response to HU, FANCD2-RAD51-RAD18 and RAD51-PCNA interactions are enhanced. Because RAD18 is responsible for PCNA monoubiquitination, one possible explanation is that FANCD2 and RAD51 participate in PCNA monoubiquitination, and in turn, TLS. Although PCNA ubiquitination can be triggered by HU it has not been shown that the ubiquitination is RAD18-dependent.[35] Because PCNA can be ubiquitinated by enzymes other than RAD18[36,37] we first tested whether HU-induced PCNA monoubiquitination depends on RAD18 under our experimental conditions using RAD18 − / − and wild-type cells. Immunoblotting of the chromatin fraction showed that HU treatment not only enhanced FANCD2 monoubiquitination but also enhanced PCNA

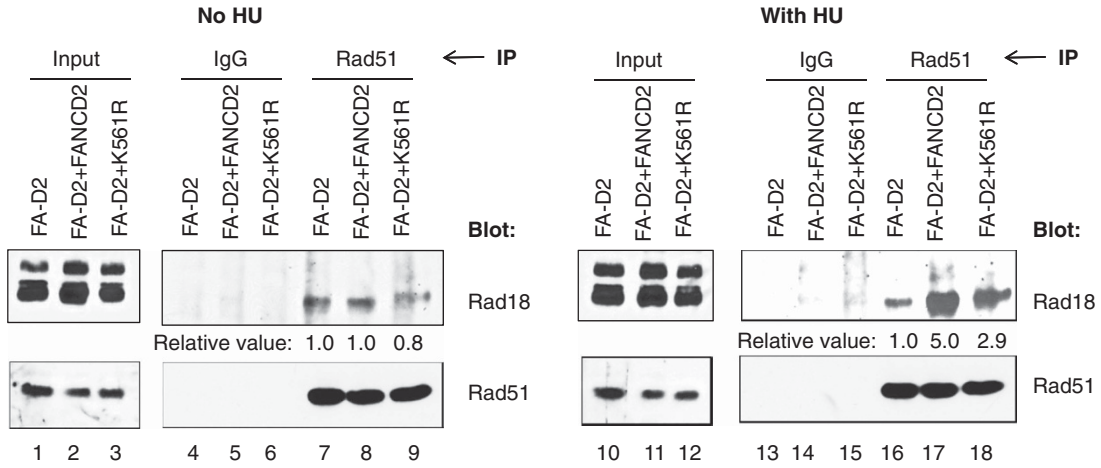

Figure 3. FANCD2 is required for increased interaction of RAD51 and RAD18 in response to HU. FA-D2, FA-D2+FANCD2 and FA-D2+FANCD2 (K561R) cells were treated or not with 0.5 mM HU overnight. Whole-cell extracts were prepared and immunoprecipitated with control IgG or RAD51 antibody. The immunoprecipitation products were analyzed by immunoblot with RAD18 and RAD51 antibodies.

monoubiquitination and that the enhanced PCNA monoubiquitination was dependent on RAD18 (Figure 4a). We then examined PCNA monoubiquitination in FANCD2 or RAD51 siRNA knockdown cells. The Dharmacon SmartPool siRNA (Dharmacon) was used to knockdown FANCD2 or Rad51 in 293 T cells. We found that in control knockdown 293 T cells PCNA monoubiquitination as measured by mean value of ub-PCNA was enhanced. However, the enhancement of monoubiquitinated PCNA was minimized in FANCD2 or RAD51 siRNA knockdown cells (Figure 4b, lane 12 and 18), suggesting that FANCD2 and RAD51 have important roles in PCNA monoubiquitination in response to HU. Interestingly, MMC treatment did not increase the level of monoubiquitinated PCNA. One of the possible explanations for these results might be that RAD18, RAD51 and FANCD2 complex formation occurs only in response to HU treatment (Figures 2a and 3). USP1 is the enzyme responsible for PCNA deubiquitination.[17] The USP1 level and chromatin localization were similar in control knockdown cells and RAD51 knockdown cells (Supplementary Figure C), suggesting that USP1 is not responsible for diminished PCNA monoubiquitination. Instead, FANCD2 and RAD51 may stimulate PCNA monoubiquitination through RAD18, as suggested by our immunoprecipitation data. The observation that FANCD2 monoubiquitination, which is also deubiquitinated by USP1,[16] was normal in RAD51 knockdown cells (Figure 4b, lane 5 and 6) further supports this idea. To confirm the specificity of knockdown results we repeated these experiments using two individually transfected siRNAs directed against FANCD2 and RAD51, respectively. Immunoblotting showed significant knockdown, as well as similar monoubiquitination response to HU (Supplementary Figure D). We also examined the PCNA in soluble fraction of cells. The level of PCNA was not changed in all cell lines and under different treatment conditions, and no ubiquitinated PCNA was observed (Supplementary Figure D).

These data also suggest that, given the differential effect of MMC and HU, the role of RAD51 in monoubiquitination of PCNA and thus HU resistance may not involve its canonical role in HR. To test this idea, we utilized the RAD51 inhibitor B02 and C34 cells, which contain the green fluorescent protein cassette and RFP cassette to measure HR and non-homologous end joining.[38] Exposure of C34 cells to B02 resulted in no change in monoubiquitinated PCNA response to HU. However, HR was impaired, suggesting a non-canonical role for RAD51 in the response to HU-induced DNA damage (Supplementary Figure E).

To confirm the result from the siRNA knockdown experiment and investigate the role of monoubiquitination of FANCD2 we tested PCNA monoubiquitination in FA-D2 mutant cells, FA-D2 cells expressing FANCD2(K561R), and FA-D2 cells expressing wild-type FANCD2. The results showed that FANCD2 deficient cells failed to enhance PCNA monoubiquitination in response to HU, whereas cells expressing wild-type FANCD2 could rescue PCNA monoubiquitination as measured by mean value of ub-PCNA (Figure 4c, lane 12). Interestingly, the cells expressing FANCD2 monoubiquitination deficient mutant could also rescue PCNA monoubiquitination, even though the level is not as high as wild-type FANCD2 (Figure 4c, lane 15). These data suggest that FANCD2 is required for enhancing PCNA monoubiquitination in response to HU, and FANCD2 monoubiquitination status is not critical for this activity.

The regulation of PCNA monoubiqutination by RAD51 is independent of BRCA2

RAD51 and its chromatin localization are very important for its function in HR. BRCA2 interacts with RAD51 and is responsible for loading RAD51 to chromatin at double-strand breaks for the performance of HR. To study the relationship between the role of RAD51 in regulating PCNA monoubiquitination and its relationship to HR, we tested for RAD51 chromatin localization and PCNA monoubiquitination in BRCA2 mutant cells EUFA423. EUFA423 cells are derived from an FA patient with complementation group D1 and have biallelic mutations (7691 insAT and 9900 insA) in *BRCA2* that result in two different truncated forms of BRCA2.[9] As expected RAD51 chromatin localization was impaired in BRCA2 deficient cells compared with wild-type cells, although total cellular RAD51 in BRCA2 deficient cells was unchanged. However, in response to HU monoubiquitinated PCNA was increased to a similar level in both BRCA2 deficient and wild-type cells, indicating that RAD51 chromatin localization is not required for regulating PCNA monoubiquitination, thus representing a function of RAD51 that is independent of HR (Figure 5a). We confirmed these data using an additional BRCA2 (−/−) cell line DLD-1 (Horizon Discovery; Supplementary Figure F). These data again show that BRCA2 is not required for hydroxyurea resistance. We tested for cell survival of BRCA2 (−/−)and BRCA2 wild-type cells to both MMC and HU. As expected, the BRCA2 mutant cells were hypersensitive only to MMC but not HU (Figure 5b).

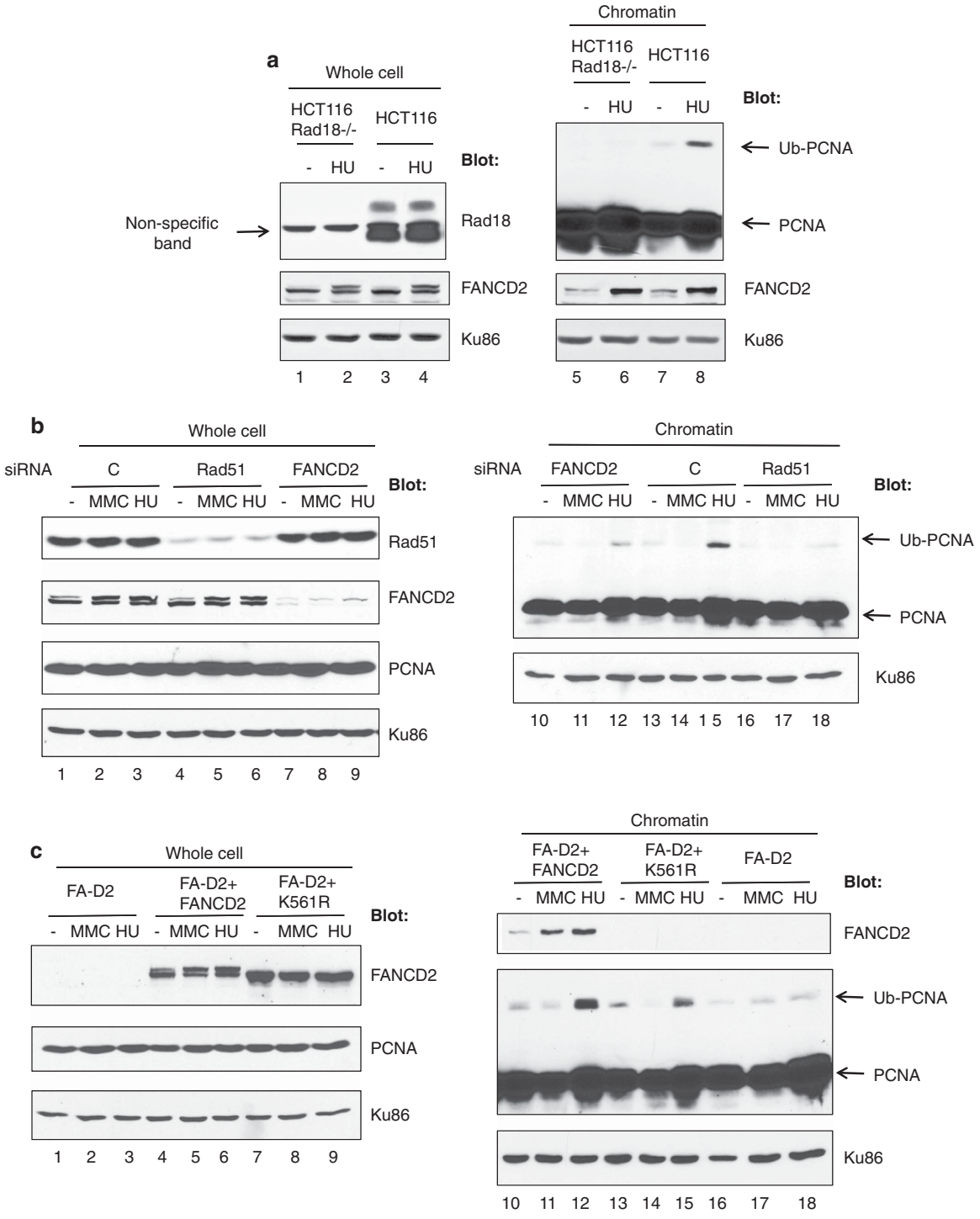

Figure 4. FANCD2 and RAD51 are required for PCNA monoubiquitination in response to HU. (**a**) RAD18 is responsible for PCNA monoubiquitination in response to HU. HCT116 and HCT116 (RAD18$^{-/-}$) cells were either treated or not with 0.5 mM HU overnight. The whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PCNA and FANCD2 antibodies. Ku86 was used as loading control. (**b**) PCNA monoubiquitination is decreased in FANCD2 or RAD51 siRNA knockdown 293 T cells. FANCD2 and RAD51 were knocked down by FANCD2 and RAD51 siRNA, respectively in 293 T cells. The knockdown cells were then treated or not with 0.5 μM MMC or 0.5 mM HU overnight. The whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with RAD51, PCNA and FANCD2 antibodies. Ku86 was used as loading control. Mean value of ub-PCNA is reported. (**c**) FANCD2, but not its monoubiquitination, is required for PCNA monoubiquitination. FA-D2, FA-D2+FANCD2 and FA-D2+FANCD2 (K561R) cells were treated or not with 0.5 μM MMC or 0.5 mM HU overnight. The whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PCNA and FANCD2 antibodies. Ku86 was used as loading control. Mean value of ub-PCNA is reported.

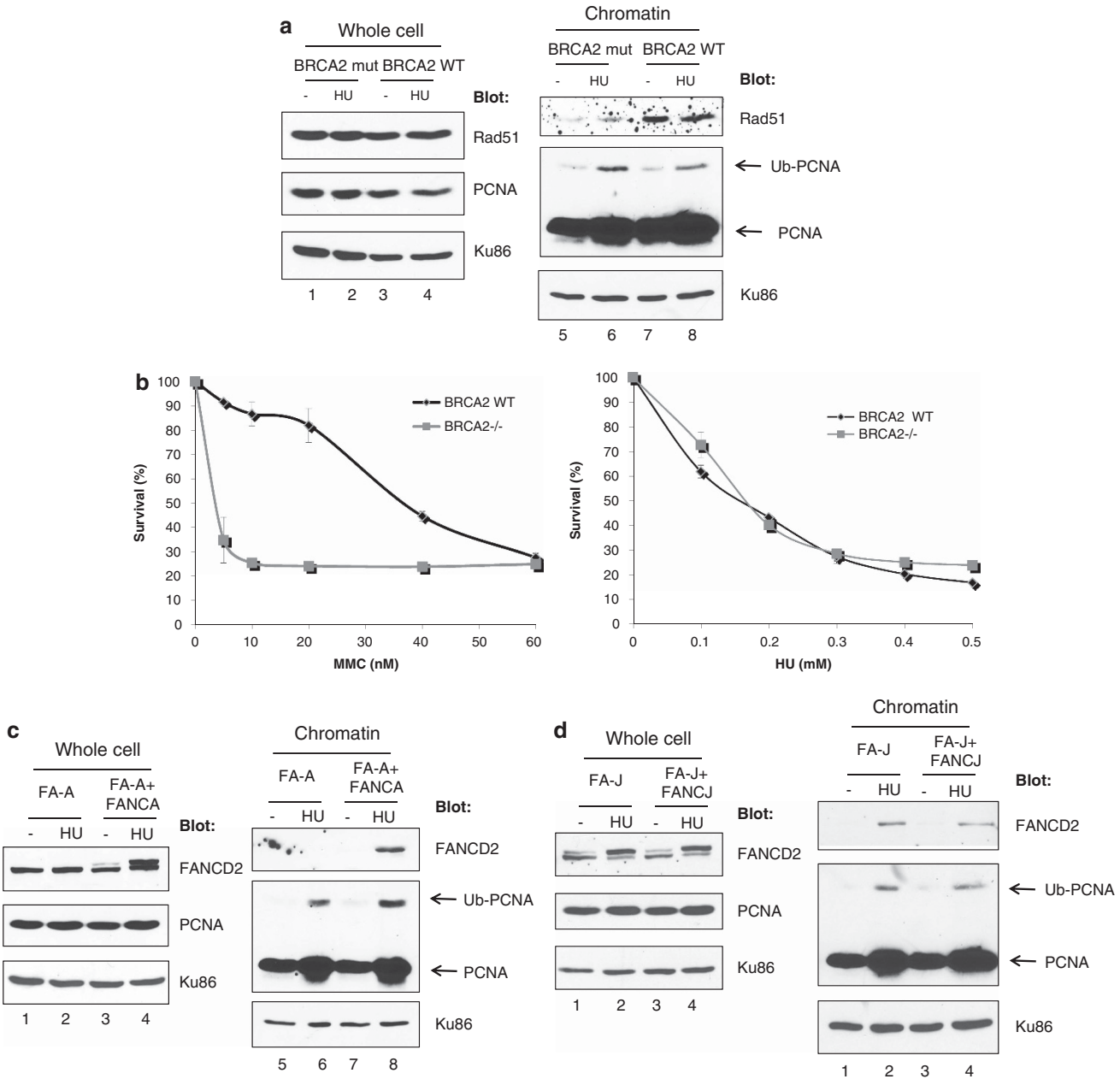

Figure 5. PCNA monoubiquitination is independent of BRCA2, FANCA and FANCJ in response to HU. (**a**) HU stimulates PCNA monoubiquitination in BRCA2-independent manner. BRCA2 deficient EUFA423 cells and EUFA423+BRCA2 cells were treated or not with 0.5 mM HU overnight. The whole-cell lysate and chromatin fractions were prepared and analyzed by immunoblot with PCNA and RAD51 antibodies. Ku86 was used as loading control. (**b**) BRCA2 wild-type DLD-1 and BRCA2 knock out DLD-1 cells were treated with MMC or HU with the concentrations as indicated in graph for 5 days. Cell survival was analyzed by crystal violet staining. (**c**) FANCA deficient cells exhibit normal PCNA monoubiquitination. FA-A and FA-A+FANCA cells were treated or not with 0.5 mM HU overnight. The whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PCNA and FANCD2 antibodies. Ku86 was used as loading control. (**d**) FANCJ deficient cells exhibit PCNA monoubiquitination. FA-J and FA-J+FANCJ cells were treated or not with 0.5 mM HU overnight. Whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PCNA and FANCD2 antibodies. Ku86 was used as loading control.

FANCA and FANCJ deficient cells exhibit PCNA monoubiquitination

FANCA is a component of the core complex of the FA pathway and is required for FANCD2 monoubiquitination. We expected that FANCA deficient cells would behave like FANCD2 monoubiquitination mutant cells with respect to PCNA monoubiquitination, as in Figure 4c. Indeed, in FANCA deficient cells FANCD2 was not monoubiquitinated, as expected, in response to HU. Consistent with data shown earlier using cells containing monoubiquitination deficient FANCD2, both FANCA deficient and proficient cells responded to HU by displaying PCNA monoubiquitination in response to HU, and FA-A cells, like monoubiquitination deficiet FANCD2 cells, showed slightly lower PCNA monoubiquitination than FANCA proficient cells (Figure 5c, lanes 6 and 8). Next, we analyzed another FA mutant cell line, FANCJ deficient cells, for PCNA monoubiquitination in response to HU. In FANCJ deficient cells PCNA monoubiquitination was similar to that in FANCJ proficient cells (Figure 5d), suggesting that FANCJ is not required for PCNA monoubiquitination. These results indicate that the effect of FANCD2 on PCNA monoubiquitination

is independent of both the upstream FA core complex and downstream FA effector proteins.

PolH chromatin localization is decreased in FANCD2 deficient cells and RAD51 siRNA knockdown cells

We have shown that FANCD2 and RAD51 are required for PCNA monoubiquitination in response to HU. PCNA monoubiquitination regulates the DNA polymerase switching in TLS.[39–41] To confirm HU-induced PCNA monoubiquitination has a role in TLS we tested for chromatin localization of TLS polymerases PolH and PolK in response to HU in FANCD2 or RAD51 pooled siRNA (Dharmacon) knockdown cells. In control knockdown cells PolH chromatin localization was increased in response to HU (Figure 6a, lane 15). However, in FANCD2 or RAD51 siRNA knockdown cells PolH chromatin localization failed to increase when treated with HU (Figure 6a, lanes 12 and 18). MMC treatment, in contrast, did not enhance the chromatin localization of PolH (Figure 6a, lane 14), consistent with the observation that PCNA monoubiquitination is enhanced by FANCD2 and RAD51 in response to HU but not MMC. These data confirm the roles of FANCD2 and RAD51 in regulating TLS through PCNA monoubiquitination in response to HU. Interestingly, PolK level on chromatin is unaffected in FANCD2 or RAD51 siRNA knockdown cells (Figure 6a). This may be because PolH has one ubiquitin binding domain and binds to monoubiquitinated PCNA, whereas PolK has two ubiquitin binding domains and binds to polyubiquitinated PCNA.[42,43] To confirm the specificity of FANCD2 and RAD51 knockdown we repeated knockdown experiment using two individually transfected siRNAs directed against FANCD2 and RAD51, respectively. Immunoblotting showed significant knockdown, as well as similar PolH chromatin localization in response to HU (Supplementary Figure G). The results from knockdown cells were confirmed in FANCD2 deficient cells (Figure 6b). In addition, the FANCD2 monoubiquitination mutant cells and FANCA deficient cells also promoted PolH chromatin localization in response to HU (Figures 6b and c), although not as strongly as wild type, in agreement with the PCNA monoubiquitination data. This again suggests that non-ubiquitinated FANCD2 can respond to HU and promote PCNA monoubiquitination and TLS. In order to demonstrate the specificity of polH in the context of HU, we performed siRNA knockdown using two different siRNAs directed against polH. Significant knockdown was achieved, as evidenced by immunoblotting (Figure 6d). Interestingly, PolH knockdown cells showed significant hypersensitivity to HU but not to MMC, consistent with the specific involvement of TLS-associated activities of PolH.

RAD51 deficient cells are hypersensitive to HU

We tested cellular sensitivity to MMC and HU in RAD51 knockdown cells. We demonstrated knockdown with pooled siRNA (Dharmicon) by immunoblot (Figure 6a). As expected, these cells showed hypersensitivity to HU and MMC (Figure 7). These results were confirmed by using two specific siRNA knockdown oligos, which we transfected separately into 293 T cells, confirming knockdown by immunoblotting. These resulting cells displayed the same sensitivity to both HU, as well as MMC (Supplementary Figure H). These data support the idea that RAD51 plays an important role in resolving the DNA damage induced by HU in a non-HR dependent fashion.

DISCUSSION

Although different DNA-repair pathways are responsible for different kinds of DNA damage, the components of one pathway may be important for repair of multiple types of DNA damage, especially for lesions as complex as ICL. Previously, we showed that mismatch repair proteins MSH2 and MLH1 could regulate FANCD2 monoubiquitination and ICL repair.[44] The E3-ligase RAD18 is important for PCNA monoubiquitination and TLS but also has an important role in FANCD2 monoubiquitination.[13,18] PCNA itself has been shown to regulate FANCD2 function.[14] In our work, we find that all FA mutant cells examined, except FA-D2-null mutant cells, which are largely devoid of FANCD2 protein, are nonetheless resistant to HU. These data are supported by the response to γH2Ax, which is increased in mutant FA cells in response to MMC but to HU only in FA-D2 deletion mutant cells. We then show that FANCD2 can interact with RAD18 and RAD51, even when non-monoubiquitinated, and RAD51 can also interact with RAD18 and PCNA (Figure 2), suggesting that FANCD2, RAD51 and RAD18 form a complex in a manner that regulates PCNA. These interactions are enhanced in response to HU (Figures 2b and 3). We further show that HU-induced PCNA monoubiquitination is RAD18-dependent (Figure 4a). This is distinctly different from FA-dependent TLS in MMC-induced repair, which is RAD18-independent.[45] More importantly, we find that FANCD2 and RAD51, but not BRCA2, are required for PCNA monoubiquitination and TLS in response to DNA damage caused by HU (Figures 4b and c). This represents a non-canonical role for RAD51, as the pharmacological inhibition of the HR function does not abolish PCNA monoubiquitination. The enhanced PCNA monoubiquitination and PolH chromatin localization are abrogated in either RAD51 knockdown cells or FANCD2 deficient cells (Figures 4b and c) supporting the idea that FANCD2, RAD51 and RAD18 form a complex to promote damage-specific TLS. It is likely this complex regulates PCNA monoubiquitination in response to HU. It has also been shown that FANCD2 and RAD51 have an important role in replication fork protection and that this FANCD2 mediated fork protection is epistatic with RAD51 function.[27] These data are also supportive of the idea that PCNA undergoes polymerase switching, as only PolH is induced by HU, but not MMC, and knockdown of PolH renders cells HU sensitive but not MMC sensitive. Taken together our data demonstrate that besides ICL repair, HR, and replication protection, FANCD2 and RAD51 together also have important and distinct roles in TLS in response to DNA damage caused by HU (Figure 8). Their function in PCNA monoubiquitination and TLS polymerase switching in response to HU may also have an important role in HU resistance. Our data that RAD51 knockdown cells are similarly hypersensitive to HU as FA-D2 cells support this idea, as do previous data that PCNA monoubiquitination mutant cells were hypersensitive to HU.[35]

Interestingly, the monoubiquitination of FANCD2 is dispensable for its role in response to HU, an agent primarily thought to lead to replication fork collapse. The function of non-monoubiquitinated FANCD2 may explain the fact that FA patients with absent FANCD2 protein expression have a more severe clinical phenotype than those with core complex protein mutations.[3] In an analogous fashion, FANCD2 deficient mice display higher magnitude of DNA damage than FANCG deficient mice.[46] We also did not observe the involvement of FANCD2 and RAD51 in PCNA monoubiquitination in the DNA damage response caused by MMC, and PCNA is only weakly monoubiquitinated in response to MMC, suggesting that the role of FANCD2 and RAD51 in PCNA monoubiquitination and TLS may be HU specific. Ho *et al.*[47] also showed that PCNA is only weakly monoubiquitinated in response to MMC. In addition, Howlett *et al.*[14] showed that FANCD2 is not required for PCNA monoubiqutination in response to UV damage. It has also been shown that in response to UV, FANCA and FANCG have important roles in regulating Rev1 foci formation in a PCNA monoubiquitination-independent manner.[19] These data indicate that different DNA damage may activate different DNA-repair pathways, even in the FA pathway, and conversely, different DNA-repair proteins cooperate to repair damage induced by different DNA damage agents.

Indeed, damage-specific regulation of TLS has been shown. Helicase-like transcription factor (HLTF) and SNF2 histone-linker

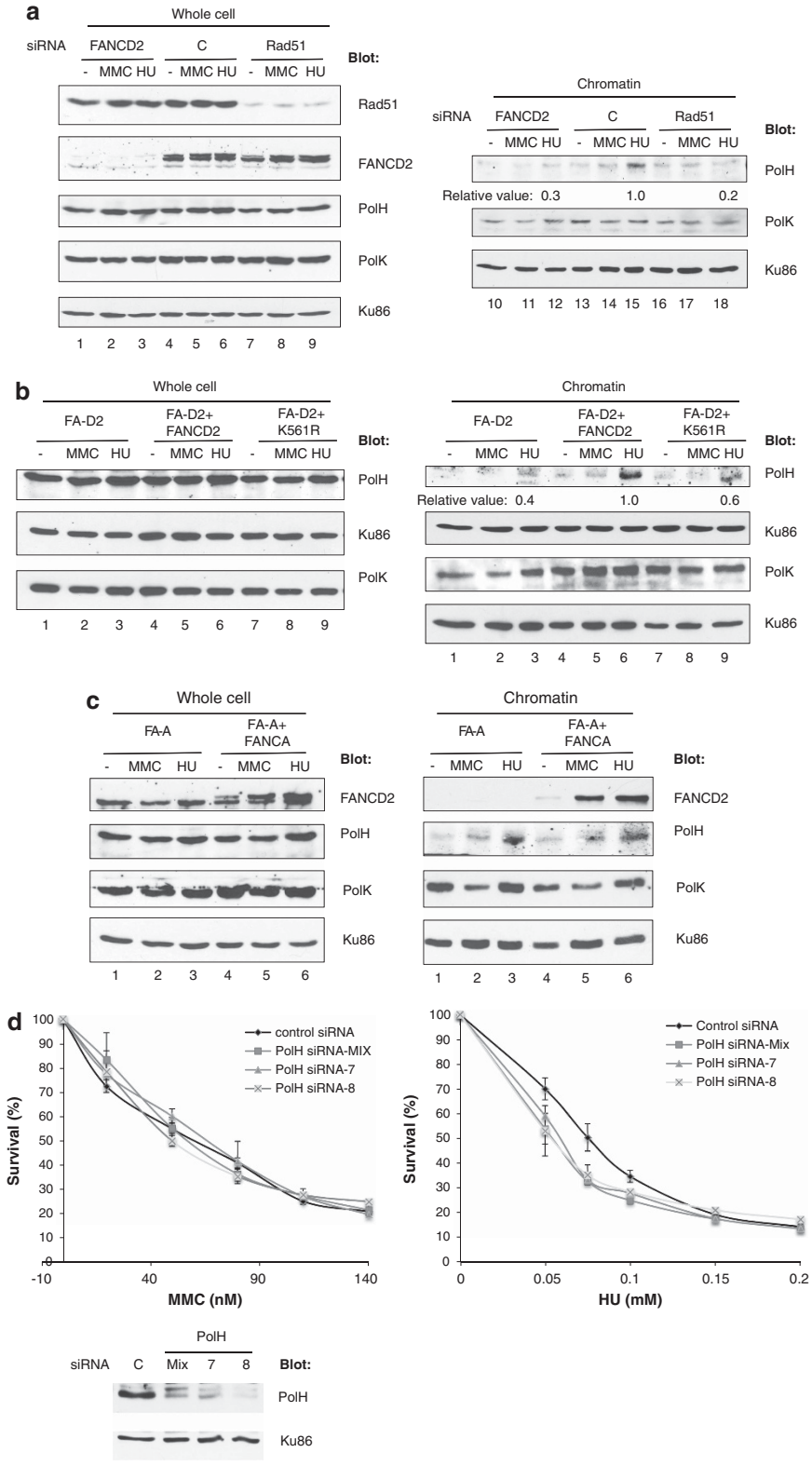

Figure 6. FANCD2 and RAD51 are required for PolH chromatin localization. (**a**) FANCD2 and RAD51 were knocked down by FANCD2 and RAD51 siRNA, respectively in 293 T cells. The knockdown cells were then treated or not with 0.5 μM MMC or 0.5 mM HU overnight. The whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PolH, RAD51, PolK and FANCD2 antibodies. Ku86 was used as loading control. (**b**) FA-D2, FA-D2+FANCD2 and FA-D2+FANCD2 (K561R) cells were treated or not with 0.5 μM MMC or 0.5 mM HU overnight. The whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PolH and PolK antibodies. Ku86 was used as loading control. (**c**) PolH chromatin localization in FA-A cells. FA-A and FA-A+FANCA cells were treated or not with 0.5 μM MMC or 0.5 mM HU overnight. Whole-cell lysate and chromatin fraction were prepared and analyzed by immunoblot with PolH, PolK and FANCD2 antibodies. Ku86 was used as loading control. (**d**) PolH was knocked down in 293 T cells using two different siRNA sequences (Dharmacon), either individually or as a mixture of two sequences. The knockdown of PolH was confirmed by immunoblot analysis. Ku86 was used as loading control. Then cells were subjected to survival assay for MMC or HU sensitivities (as in Figure 1a).

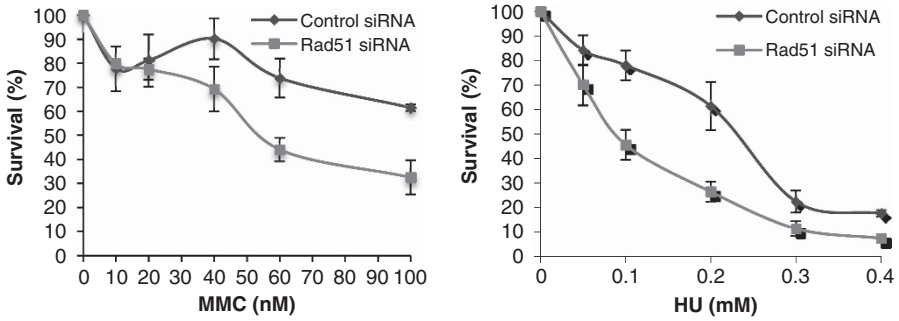

Figure 7. RAD51 deficient cells are hypersensitive to HU. RAD51 knockdown 293 T cells were treated with MMC or HU with the concentrations as indicated in graph for 5 days. Survival cells were analyzed by crystal violet staining (see Figure 4b for knockdown immunoblot).

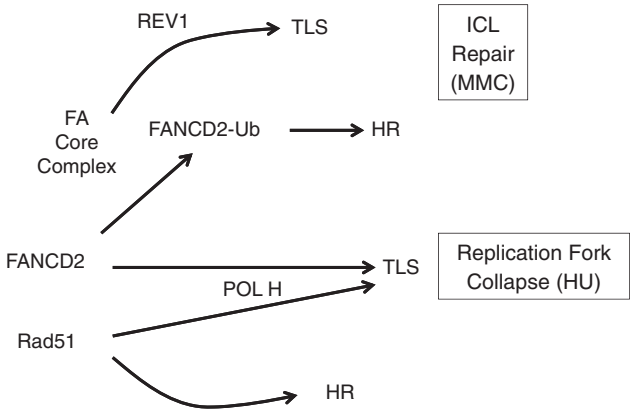

Figure 8. Multifunctionality of DNA-repair proteins. FA proteins are responsible for ICL repair. FA core complex is also important for Rev1 foci formation in response to UV in PCNA monoubiquitination-independent manner. FANCD2 also interacts with RAD51, RAD18 and PCNA and regulate PCNA monoubiqutination and PolH chromatin localization in response to HU. RAD51 is a key player for HR and TLS. It also interacts with FANCD2, RAD18 and PCNA and is required for PCNA monoubiquitination and PolH chromatin localization in response to HU.

PHD-finger RING-finger helicase (SHPRH) are Rad5 homologs involved in PCNA ubiquitination and TLS polymerase recruitment, contributing in different ways to specify DNA damage-induced mutagenesis. In response to the DNA damage caused by UV, HLTF enhances PCNA monoubiquitination and PolH recruitment and inhibits SHPRH function. However, in response to DNA damage caused by MMS, HLTF degradation is promoted, and the interaction of SHPRH with RAD18 and PolK is enhanced, leading to the recruitment of PolK.[43] Our data showed that PolH, but not PolK, chromatin localization is regulated by FANCD2 and RAD51 in response to HU. We speculate that HLTF may be used in FANCD2 and RAD51 regulated PCNA monoubiquitination and TLS.

A recent publication indicates that PolH can interact with monoubiquitinated FANCD2 also connects monoubiquitinated FANCD2 with PolH in UV damage,[21] supporting our observation that FANCD2 has an important role in recruiting PolH and TLS in response to HU. Although we show that FANCA and FANCD2 monoubiquitination are not required for this function in response to HU, the difference might be because of a differential response of cells to types of DNA damage. Interestingly, FANCA and FANCG, but not FANCD2, have been shown to have important roles in Rev1 foci formation in response to UV damage.[19] However, FANCG deficiency did not affect PCNA monoubiquitination or change PolH foci formation significantly in response to UV damage. We show that in response to HU damage FANCD2, but not FANCA, is important for PolH recruitment (Figure 6). PCNA monoubiquitination and TLS may be regulated differentially in response to different DNA damage.

Recruitment of RAD51 to chromatin by BRCA2 is required for its function in HR, which is important ultimately for ICL repair.[8] The colocalization of FANCD2 and RAD51 has been observed previously,[15] and it has been shown that FANCD2 deficient cells are defective in efficient HR repair.[48] However, here we show that RAD51 also has an important role in PCNA monoubiquitination and TLS in a HR-independent manner in response to HU. As RAD51 also has an important role in restarting HU-stalled replication forks without triggering HR,[49] the activities we observe in this investigation may represent an alternative regulatory pathway involving exclusively DNA replication. Alternatively, and perhaps more compellingly, this phenomenon may be a means of coordinating replication and repair, thus utilizing components in common to both pathways.

MATERIALS AND METHODS

Cell culture

The 293 T and C34 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Biowest, Miami, FL, USA) and pen/strep (Invitrogen, Grand Island, NY, USA). FA-D2 mutant cells PD20+pMMP vector, PD20+pMMP-Flag-FANCD2, PD20 plus FANCD2 pMMP-Flag-K561R mutant and FA-A mutant cells GM6914 and GM6914 plus pMMP-Flag-FANCA were cultured in DMEM containing 15% FBS and pen/strep. FA-J mutant cells and FA-J+FANCJ and BRCA2 mutant EUFA423 cells and EUFA423+BRCA2 cells cells were cultured in RPMI containing 20% FBS and pen/strep. BRCA2 knock out and wild-type colorectal adenocarcinoma DLD-1 cells were cultured in RPMI containing 10% FBS and pen/strep.

Immunoprecipitation

The 293 T cells and FANCD2 deficient and proficient cells were cultured on 15 cm^2 plates and treated with or without 0.5 μM MMC or 0.5 mM HU overnight prior to collecting. Following phosphate-buffered saline (PBS) washed, whole-cell lysate was prepared by adding 1 ml of the whole-cell lysis buffer (300 mM NaCl, 1.0% Triton X-100, 5 mM EDTA, 2 mM $NaVO_4$, 2 mM $Na_4O_7P_2$, 0.02% NaN_3, and 50 mM Tris, pH 7.4) with proteinase inhibitors. Following 10 s of sonication, extracts were cleared by centrifugation at 14 000 r.p.m. for 15 min at 4 °C. Supernatants were collected, and equal amount of extract (2 mg protein) were used for each immunoprecipitation. The lysates were treated with DNase I (20 u) for 15 min at room temperature and then 15 min at 37 °C. Then 2 μg antibody (FANCD2 (H-300), RAD51(C20), PCNA(PC10), or normal rabbit or mouse IgG, Santa Cruz, Santa Cruz, CA, USA) were added. After incubation overnight at 4 °C, protein G beads were added for another 4 h. Beads were washed four times, and the pellets were subjected to immunoblot analysis.

Immunoblot

Protein samples were suspended in SDS Loading Buffer (50 mM Tris-Cl pH6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 10% β-mercaptoethanol) and boiled for 5 min. Samples were run on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in TBS-T (TBS

+0.1% Tween-20) with 10% milk for 1.5 h and incubated in primary antibody (FANCD2 1:2000, RAD51 1:2000, PCNA 1:500, Ku86 1:5000, Santa Cruz, and RAD18 1:1000, Bethyl, Montgomery, TX, USA) for 1 h at room temperature in TBS-T+1% milk. After washing, membranes were incubated in secondary antibody (ECL anti-rabbit IgG or anti-mouse IgG-HRP, Amersham, Princeton, NY, USA) 1:5000 in TBS-T+1% milk for 1 h at room temperature. Blots were washed in TBS-T three times for 5 min each and developed by chemiluminescence (Supersignal West Pico Kit or Supersignal West Femto kit, Pierce, Rockford, IL, USA). The quantification of Western blot was carried out using ImageJ software (NIH, Bethesda, MD, USA). We measured mean values of each band with fixed area. The relative values of each band were shown. For ub-PCNA we measured mean values of each band, and the values were then corrected with mean values of PCNA. The final values were shown.

Chromatography

FA-D2+pMMP-Flag-FANCD2 cells were cultured on 15 cm^2 plates and treated with 0.5 μM HU overnight prior to collecting. Whole-cell lysate was prepared as in immunoprecipitation assay. Lysate was run through a P11 column, and the flow through was loaded on a Superose6 gel-filtration column (GE Healthcare, Piscataway, NJ, USA). Fractions were eluted in non-denaturing lysis buffer, collected and analyzed by SDS-polyacrylamide gel electrophoresis. Selected fractions were isolated and analyzed by immunoblot for FANCD2, RAD18, RAD51 and PCNA co-fractionation. Fraction sizes on column were standardized using molecular weight standards (GE Healthcare).

Cell fractionation

Procedures for permeabilization and subnuclear extraction are described previously.[50] In brief, after PBS wash cells on the 15 cm^2 plates were collected by scraping, and centrifuged for 5 min to obtain the pellet. The soluble fraction was extracted by suspending the cells in 1 ml of low-salt buffer (10 mM Hepes, pH 7.4, 10 mM KCl and 50 μg/ml digitonin) containing protease and phosphatase inhibitors (2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4) for 15 min at 4 °C. Permeabilized nuclei were recovered by centrifugation at 2000 r.p.m., 5 min at 4 °C. The supernatant was termed the soluble fraction. The nuclei were washed three times more in the same buffer. After wash, the nuclei were resuspended in 200 μl of low-salt buffer containing 40 units of DNase I (RNase free, Roche Diagnostics, Indianapolis, IN, USA) for 15 min at room temperature and then an additional 15 min at 37 °C, then pelleted again. Supernatant was set aside. The pellet was extracted once more with extraction buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 300 mM NaCl and 30 mM $Na_4P_2O_7$•$10H_2O$, 10 M NaF, and 1 mM EDTA) containing protease and phosphatase inhibitors for 15 min at 4 °C. Supernatant was collected at 14 000 r.p.m. in a microfuge for 10 min and pooled with the low-salt buffer+DNase I supernatant to form the chromatin fraction. Cellular fractions were analyzed by immunoblot.

siRNA knockdown

The 293 T cells were grown to 50% confluence in DMEM with 10% FBS. A total of 60 nM FANCD2 or RAD51 siRNA pool (siGENOME SMARTpool) and non-targeting control siRNA (Dharmacon) were transfected in 293 T cells using X-tremeGENE siRNA transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. For individual siRNA knockdown the sequences used are: FANCD2–18 (5′-GAACAAAGGAAGCCGGAAU-3′), FANCD2-2 (5′-GAUAAGUUGUCGUCUAUUA-3′), RAD51-7 (5′-CCAACGAUGUGAAGAAAUU-3′), RAD51-2 (5′-GAAGCUAUGUUCGCCAUUA-3′), PolH-7 (5′-GCACUUACAUUGAAGGGUU-3′), PolH-8 (5′-GCAAUUAGCCCAGGAACUA-3′), FANCI-2 (5′-GUAAGAGCCUGAACUAUAC-3′) FANCI-3 (5′-GAUGGGAGUUUGUGAGGUU-3′). The second transfection was performed next day after first transfection to ensure maximum knockdown. Whole-cell lysate and chromatin fractions were made from the cells 5 days after the first transfection and then analyzed by immunoblot.

Cell survival assay

Cells were grown in six-well plates with or without MMC or HU in the indicated concentrations for 5 days. After wash with PBS cells were fixed with 10% acetic acid and 10% methanol for 5 min then stained with 1% crystal violet for 3 min. After washing and allowing the plates to dry, the stain was dissolved with 0.1% SDS in methanol and read at a wavelength of 595 nm in a microplate reader.

Cell cycle analysis

Cells were collected and washed in PBS (Ca^{++}, Mg^{++}), then fixed with 70% ethanol overnight at 4 °C. After wash with PBS cells were subjected to RNase treatment (20 μg/ml) and propidium iodide staining (10 μg/ml) at 37 °C for 30 min. Cellular fluorescence was then quantified using a FACS Calibur flow cytometer. Histograms were analyzed with FlowJo software (Ashland, OR, USA). For S phase analysis Alexa Fluor 488 conjugated BrdU antibody was used (EMD Millpore). After overnight treatment of HU cells, they were incubated with 10 μM BrdU for 2 h. Collected cells were then washed with PBS and fixed with cold 70% ethanol overnight at 4 °C. After wash the cells were treated with 2 mM HCl at room temperature for 30 min. Cells were then washed twice with PBS and incubated with BrdU antibody (1:50) at room temperature for 20 min. Cells were washed once with PBS and then subjected to RNase treatment (20 μg/ml) and propidium iodide staining (10 μg/ml) at 37 °C for 30 min.

HR assay

C34 cells were grown in DMEM with 10% Tet free serum in six-well plate. Cells were pre-treated with 20 μM RAD51 inhibitor B02 (EMD Millipore, Billerica, MA, USA) for 2 h, then ligand Shield 1 (Clontech, Mountain View, CA, USA; 2 μl/ml) and triamcinolone acetonide (100 μM) were added to cells for overnight to induce double-strand break. Cells were washed and replaced with fresh media. Three days later the cells were collected and analyzed through fluorescence-activated cell sorting for green fluorescent protein (HR positive) and RFP (non-homologous end joining positive) cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)