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Recruitment of DNA polymerase eta by FANCD2 in the early response to DNA damage

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How Fanconi anemia (FA) protein D2 (FANCD2) performs DNA damage repair remains largely elusive. We report here that translesion synthesis DNA polymerase (pol) eta is a novel mediator of FANCD2 function. We found that wild type (wt) FANCD2, not K561R (mt) FANCD2, can interact with pol eta. Upon DNA damage, the interaction of pol eta with FANCD2 occurs earlier than that with PCNA, which is in concert with our finding that FANCD2 monoubiquitination peaks at an earlier time point than that of PCNA monoubiquitination. FANCD2-null FA patient cells (PD20) carrying histone H2B-fused pol eta and wtFANCD2, respectively, show a similar tendency of low Mitomycin C (MMC) sensitivity, while cells transfected with empty vector control or pol eta alone demonstrate a similar high level of MMC sensitivity. It therefore appears that FANCD2 monoubiquitination plays a similar anchor role as histone to bind DNA in regulating pol eta. Collectively, our study indicates that, in the early phase of DNA damage response, FANCD2 plays crucial roles in recruiting pol eta to the sites of DNA damage for repair.

Introduction

In order to overcome or minimize the threat/damage to DNA posed by the constant bombardment of the human genome from both endogenous and exogenous sources,[1,2] DNA repair mechanisms, including the coupling of cell cycle arrest,[3,4] excision repair,[5,6] the FA pathway,[7-12] translesion synthesis[7,10,13-15] and others have to function at their optimal best. Deficiencies in any or several of the above DNA repair mechanisms may lead to genetic instability or carcinogenesis as a result of the alteration of genetic information by agents such as UV and those from endogenous and other exogenous sources.[1,4,16,17] Xeroderma pigmentosum (XP) is an autosomal recessive hereditary disorder with a cancer-prone phenotype that results from defective nucleotide excision repair (NER) or translesion synthesis (TLS) in the case of Xeroderma pigmentosum variant (XPV).[18-20] Based on cell perfusion experiments, XP has been classified into eight complementation groups (XP-A through XP-G, with the eighth group being XP-V). XP-A through XP-G are deficient in NER; however, XP-V is proficient in NER but deficient in translesion synthesis.[21-23] DNA polymerase eta is encoded by the XPV gene, which is capable of replicating DNA containing several UV photoproducts. As a consequence, a deficiency in this gene, as in the case of XPV, results in deficiencies in translesion synthesis, which ultimately predisposes affected individuals to cancer.[20,21]

Our current studies suggest that DNA polymerase eta and the FA pathway may be functioning in the same signaling pathway based on a similar phenotype displayed by both FA cells and pol eta mutated cells. FA is a rare genetic disease characterized by hypopigmentation, skeletal defects, progressive bone marrow failure and an early onset of cancer among others.[24-26] The FA pathway is involved in several biological processes including but not limited to DNA repair, protein ubiquitination and cancer progression.[27-30] The FA pathway comprises 15 or more proteins, including FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, O and P.[7,31] Defects in any of these FA proteins result in hypersensitivity to DNA damaging agents such as Mitomycin C (MMC), UV and diepoxybutane (DEB).[32-34] The removal of interstrand crosslinks (ICLs) by DNA repair pathways such as NER, homologous repair and translesion synthesis appears to be coordinated by the FA pathway.[7,30,35] As new strides are being made daily with regards to the involvement of the FA pathway in DNA damage repair, it might indeed be linked with a wider array of classic DNA repair mechanisms than is currently thought. There appears to be a crosstalk between the FA and translesion synthesis polymerase pathways, considering that FA cells and XPV cells show similar phenotypes, and also that FANCD2 knockdown affects pol eta

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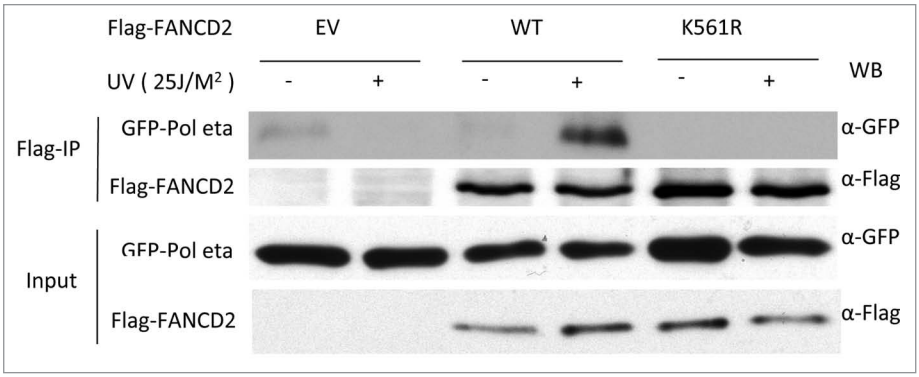

Figure 1. Monoubiquitination of FANCD2 is required for its interaction with Polymerase eta in response to UV irradiation. GFP-tagged pol eta was co-transfected with either wild type or ubiquitination site mutant (K561R) Flag-tagged FANCD2 plus empty vector (EV) into HeLa cells. Eighteen hours post-transfection, cells were split and followed by 25 J/M² UV treatment (+). The unirradiated samples were used as control (–). Cells were harvested 4 h after UV treatment by using 1% formaldehyde for crosslinking. FANCD2 protein complexes were immunoprecipitated by anti-Flag antibodies, and associated poly eta proteins were analyzed by western blotting using anti-GFP antibodies. Immunoprecipitated FANCD2 proteins were also analyzed by western blotting using anti-flag antibodies as control. 5% of whole cell extracts for each sample were used as input controls. Clearly, Flag-wtFANCD2, but not Flag-mtFANCD2, and GFP-pol eta can be detected in each other's IP eluent over a 2 min exposure for western [after overnight exposure, Flag mtFNCD2 and GFP-pol-eta can be seen in very faint bands in each other's IP-WB, indicating a very weak interaction between pol eta and K561RFANCD2 (not shown)].

focus formation and co-localization.[10] However, the exact nature of the relationship between these two pathways in DNA damage repair has barely been studied. Here we report how pol eta can execute FANCD2 function, providing novel insights into DNA damage repair as well as the tumor suppressor activity of the FA pathway.

Results

FANCD2 interacts with translesion DNA polymerase eta (pol eta) upon DNA damage. FANCD2 emerges to be an important tumor suppressor protein. However, it remains largely unclear how FANCD2 performs its tumor suppressor activity. We previously found that not only did the level of FANCD2 expression influence pol eta focus formation, but that their foci co-localized to each other. To address these new findings, which suggest that FANCD2 may play an important role in the regulation of pol eta function, we asked whether FANCD2 could interact with pol eta. We preformed reverse immunoprecipitation (IP) and western blotting using lysates prepared from HeLa cells transfected with Flag-wtFANCD2 or Flag-mtFANCD2 (K561R) in combination of GFP-fused pol eta, respectively. We found wtFANCD2, but not mtFANCD2, can interact with pol eta accompanied by background (data not shown). To clear the background, we used 1% fresh-made formaldehyde to crosslink cells before collecting cell lysate, as described by Kannouche,[36] noting the fact that interaction determined by a monoubiquitin motif is generally weak.[36] As shown in **Figure 1**, in Flag-IPed pellets prepared from UV-treated cells, we found GFP-pol eta co-IPed along with Flag-wtFANCD2. In striking contrast, we could not detect whether GFP-pol eta was pulled down along with Flag-mtFANCD2. Similarly, in GFP-pol-eta IP prepared from the same batch of cells, Flag-wtFANCD2, but not Flag-mtFANCD2, can co-IP along with GFP-pol eta. No bands of pol eta were detected in Flag-IP-western prepared from un-irradiated cells. Collectively, these results indicate that pol eta interacts preferentially with monoubiquitinated FANCD2 upon DNA damage, but not mtFANCD2 (K561R), which cannot be monoubiquitinated.

FANCD2 interacts with pol eta at ubiquitin binding domains or like domains known to be responsible for the interaction with PCNA. The mutations A, B, A + B, C, D and C + D at the ubiquitin binding domains of pol eta, can block its interaction with monoubiquitinated PCNA.[13,36,37] We thus wanted to know whether these ubiquitin-binding domains in pol eta are also required for interacting with monoubiquitinated FANCD2 in response to UV irradiation. To this end, we transfected HeLa cells with GFP-tagged wild type pol eta and various forms of mutants (A, B, A + B, C, D, C + D),[13,36,37] respectively with Flag-wtFANCD2, and performed reverse IP-westerns. GFP-wt-pol eta, but not any other forms of GFP-mt-pol eta, is detectable in the eluent of Flag-wtFANCD2-IP, while Flag-wtFANCD2 is only detectable in the eluent of GFP-IP prepared from cells transfected only with GFP-wt-pol eta (**Fig. 2**). These results suggest that monoubiquitnated FANCD2 interacts with pol eta through the same regions that are known to be responsible for interacting with PCNA.

FANCD2 monoubiquitination peaks earlier than PCNA monoubiquitination upon UV irradiation. The studies so far indicate that FANCD2 is partly capable of regulating DNA pol eta. Given the facts that both FANCD2 and PCNA can regulate pol eta focus formation, and the monoubiquitinated forms of both proteins preferentially interact with pol eta, we asked how these two regulators are coupled into cellular responses upon DNA damage. To demonstrate the relationship between monoubiquitinated FANCD2 and PCNA, we examined the time course of monoubiquitination of FANCD2 and PCNA in U2OS and 293T cells after UV irradiation. Our western blotting data (**Fig. 3**) revealed that the monoubiquitination of FANCD2 occurs as early as 30 min after UV treatment and reaches the peak at the 4 h point after UV treatment. In contrast, the monoubiquitination of PCNA cannot be detected until 8 h after UV irradiation. The monoubiquitination of these two proteins are hallmark of activation of the FA pathway and DNA damage repair. The early response to DNA damage by FANCD2 suggests that FANCD2 might initiate the polymerase switch and DNA damage repair before PCNA in translesion synthesis (TLS).

The interaction between monoubiquitinated FANCD2 and pol eta occurs earlier as compared with that between

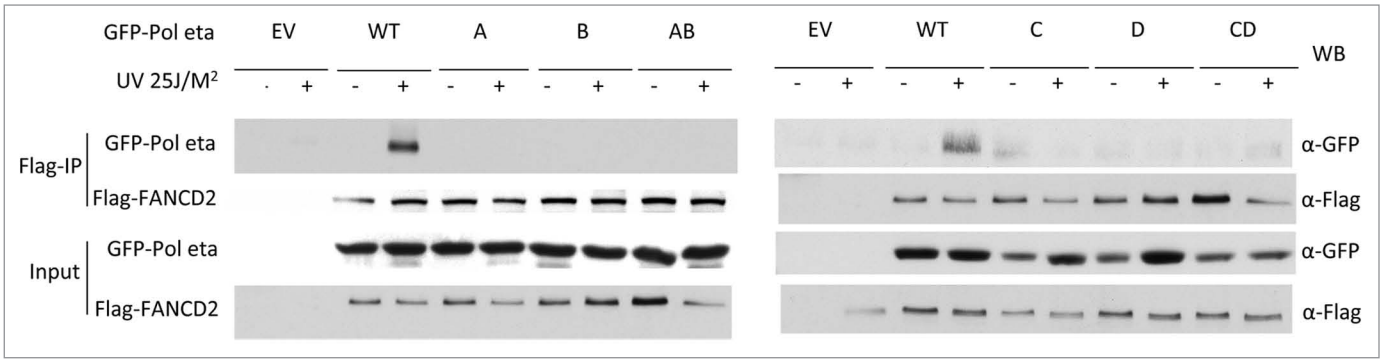

Figure 2. Mutations in ubiquitin binding domains of polymerase eta can block its interaction with FANCD2 in response to UV irradiation. Flag-tagged FANCD2 (wild type) was co-transfected with either wild type or mutant GFP-tagged polymerase eta in HeLa cells. Empty vectors (EV) of GFP tag and Flag tag constructs were used as controls. UV irradiation, protein complex crosslinking, co-immunoprecipitation with anti-Flag antibodies and western blotting are as described above. The level of GFP-tagged pol eta attached with FANCD2 complexes was detected by western blotting by using anti-GFP antibodies. 5% of whole-cell extracts for each sample were used as input controls.

monoubiquitinated PCNA and pol eta. To further differentiate the nature of the interactions of pol eta with monoubiquitinated FANCD2 and PCNA, we explored their interactions in a time course manner, given the earlier monoubiquitination of FANCD2 upon UV irradiation (**Fig. 4**) in comparison with PCNA. After treatment with 25 J/M² UV, 293T cells transfected with GFP-tagged pol eta were harvested at time 0, 3, 8 h and subsequently followed procedures to prepare lysate for IP-western. When we IPed pol eta from soluble cell extracts with anti-GFP antibodies, we found both FANCD2 and PCNA clearly co-IPed with pol eta using lysates prepared from cells 8 h post-UV irradiation. As a control, neither FANCD2 nor PCNA were found to co-IP with pol eta from untreated samples. Remarkably, FANCD2, but not PCNA, can be sufficiently co-IPed with pol eta using the lysate prepared from cells treated with UV light at the same dosage, collected 3 h after treatment. These data provide the first evidence that FANCD2 responds to DNA damage earlier than PCNA by its monoubiquitination and subsequent recruitment of pol eta for DNA damage repair.

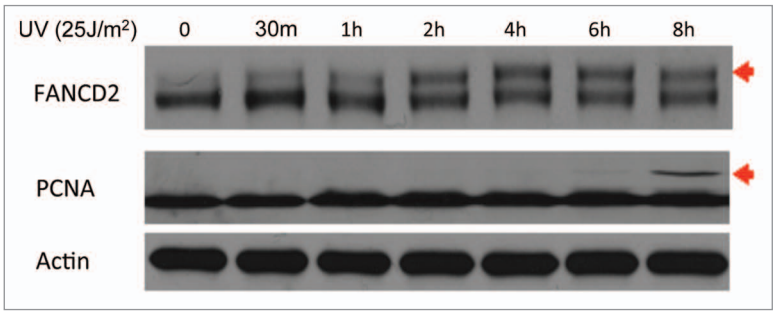

Figure 3. Monoubiquitination of FANCD2 peaks earlier than monoubiquitination of PCNA in response to UV irradiation. U2OS cells were UV irradiated at 25 J/M² at 30 min, 1 h, 2 h, 4 h, 6 h and 8 h time points (marked on the top of the image). Unirradiated samples (marked as 0) were used as a control. FANCD2 and PCNA were analyzed by western blotting using anti-FANCD2 and anti-PCNA antibodies. Bands of monoubiquitinated FANCD2 and monoubiquitinated PCNA were marked by a red arrow (←) on the right side of the image. Actin levels in each sample were shown as a loading control.

Protein complex profiles support the interaction of pol eta with FANCD2 occurring earlier than that with PCNA upon DNA damage. In response to DNA damage, FANCD2 and FANCI form a heterodimeric complex (ID complex) that relocates to DNA lesions, where it coordinates crosslink repair activities with downstream FA proteins (D1/J/N/O/P).[7] This FA complex acts as a landing pad for recruiting nucleases, helicases and polymerases to perform DNA repair processes.[7] However, understanding the molecular details of the FA complex with its coordinated nucleases and polymerase will provide invaluable insights into the DNA repair network. We, therefore, applied the U2OS cell nuclear extract (4 mg) directly to a sepharose 6B column and collected a series of fractions for western blotting analysis on distributions of FANCD2, pol eta and PCNA. The western results show that FANCD2 co-peaks with pol eta in samples prepared from cells collected at 3 h after UV treatment, whereas PCNA peaks in the fraction carrying protein complexes at a smaller size, away from the pol eta peak fraction. On the contrary, both PCNA and FANCD2 can be co-fractionated with pol eta when using lysates prepared from cells collected at the 8-h point after UV treatment (**Fig. 5**). Again, these data are consistent with the GFP IP results that indicate that pol eta can interact with both FANCD2 and PCNA in cells at the 8-h point after UV treatment, while pol eta only interacts with FANCD2 in cells at the 3-h point after UV treatment (**Fig. 4**).

H2B-pol eta, not pol eta alone, can offset PD 20 (FANCD2$^{-/-}$) cell sensitivity. A recent study showed that the monoubiquitination of FANCD2 is sufficient for its chromatin targeting and promotes DNA damage repair.[38] We propose that the interaction between FANCD2 and pol eta might recruit the pol eta complex to the targeted chromatin sites for repair, rendering cells lower drug sensitivity. We tested this hypothesis by expressing pol eta fused with a component of vertebrate chromatin, histone H2B. As seen in **Figure 6**, PD20 cells (FANCD2$^{-/-}$) transiently transfected with SF-H2B eta show a relatively low sub-G_1 percentage

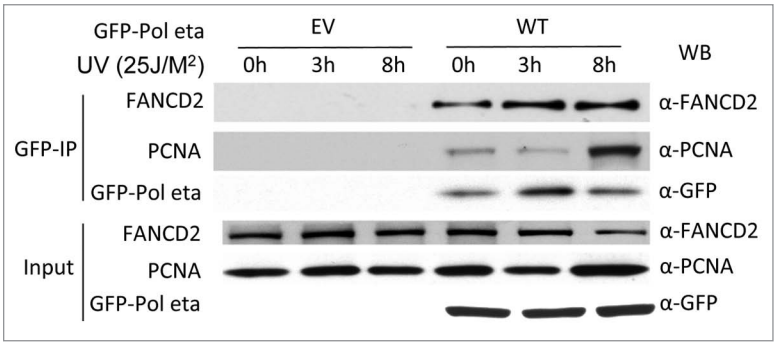

Figure 4. FANCD2 can interact with Polymerase eta earlier than PCNA in response to UV irradiation. GFP-tagged polymerase eta (pol eta) or empty vector (EV) was transfected into HeLa cells. Cell lysates were harvested at 3 h or 8 h after UV irradiation, and unirradiated samples were included as controls. Protein complex crosslinking, co-immunoprecipitation with anti-GFP antibodies and western blotting were as described above. The endogenous FNACD2 and PCNA proteins interacting with exogenous GFP-tagged pol eta were precipitated by anti-GFP antibodies and detected by western blotting using anti-FANCD2 and anti-PCNA antibodies. GFP antibodies can poll-down FANCD2, but not PCNA, from the lysates prepared from cells collected at the 3-h time point after UV treatment. The protein level of immunoprecipitated GFP-Pol eta was shown as controls. 5% of whole cell extracts for each sample were used as input controls.

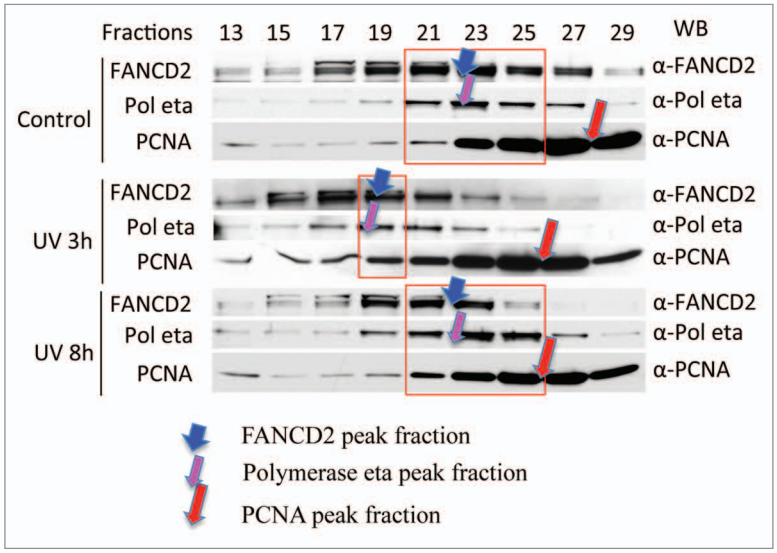

Figure 5. Pol eta is preferentially associated with FANCD2, but not with PCNA, in cells at the 3-h point after UV treatment. Nuclear extractions from U2OS cells were collected at the 3-h and 8-h points after UV irradiation. Samples from cells without UV irradiation were also used as controls. Four mg of NEs was used, respectively, for sepharose 6B chromatography. Western blotting analysis on the distribution of pol eta, FANCD2 and PCNA in these chromatographic fractions demonstrates co-fractionation of pol eta, mainly with FANCD2 at earlier time points (3 h), but unlikely with PCNA (marked by red lines), noting the peak distribution of PCNA (pointed by red arrow heads) far away from that of pol eta, whereas pol eta, FANCD and PCNA appear to work in concert in cells at 8 h time point after UV treatment. Fractions #9 and #31 correspond to known molecular weight markers Dextran Blue (2,000 KD) and thyroglobulin (700 KD), respectively.

upon MMC treatment compared with cells transiently transfected with SF-eta or empty vector, while wtFANCD2-reconstituted PD20 stable cells[8] displayed the lowest sub-G_1 percentage. As a control, untreated FANCD2-deficient cells transfected with either empty vector or wild type FANCD2 were found to have substantially low sub-G_1 percentages. Together, these results indicate that the H2B protein, to some extent, can functionally replace the monoubiquitinated FANCD2 protein. In other words, FANCD2, at least partly, plays a similar effect as H2B to tether pol eta at the sites of damaged DNA for repair, thus lowering the cell death percentage/sub-G_1 percentage.

Discussion

FANCD2 and pol eta foci have been shown to colocolize in response to DNA damage;[10] however, the nature of this co-localization has never been characterized. The current study aims at elucidating the nature and possible cooperative roles of pol eta and FANCD2 in DNA damage response. To this point, we performed reverse immunoprecipitation (IP) and western blot analysis using lysates prepared from HeLa cells transfected with Flag-wtFANCD2 or Flag-mtFANCD2 in combination with pol eta. Results show (**Fig. 1**) that only wtFANCD2 interacts with pol eta, supporting that the previously observed FANCD2 co-localization with pol eta[10] is a result of their interaction, and that FANCD2 may play a role in recruiting pol eta to DNA damage sites for translesion synthesis. Additionally, the observation that in UV-irradiated cells only wtFANCD2 but not the mtFANCD2 (K561R) interacts with pol eta suggests that pol eta appears to interact mainly with monoubiquitinated FANCD2 in response to DNA damage. Demonstrating this interaction for the first time is essential; however, it is equally imperative to determine which domain of pol eta that FANCD2 interacts with as this could have relevant therapeutic implications in DNA translesion synthesis and cancer therapy in general. The CUE domain or other ub-binding domains of pol eta are essential in its interaction with monoubiquitinated PCNA;[13,36,39] thus, these domains serve as possible candidate sites for interaction with FANCD2. Reverse IP-westerns of HeLa cells transfected with GFP-tagged wt pol eta or mt GFP-pol eta (without the ub-binding domains, respectively) combined with Flag-tagged FANCD2 revealed that only the GFP-wt eta, but not any mutant forms, was detectable in the eluent of Flag-wt FANCD2-IP (**Fig. 2**). These findings suggest that both PCNA and FANCD2 share the same interaction domains within pol eta.[37,40] Hence, these domains could be rational targets in therapeutic strategies by mitigating the repair ability of tumor cells to improve DNA damage drug sensitivity.

Both PCNA and FANCD2 have been shown to regulate pol eta;[13,36,39] therefore, we investigated the interactive nature of both proteins. Co-IP studies of PCNA or FANCD2 with pol eta revealed that FANCD2 interacts with pol eta at an earlier phase in response to DNA damage (3 h after UV

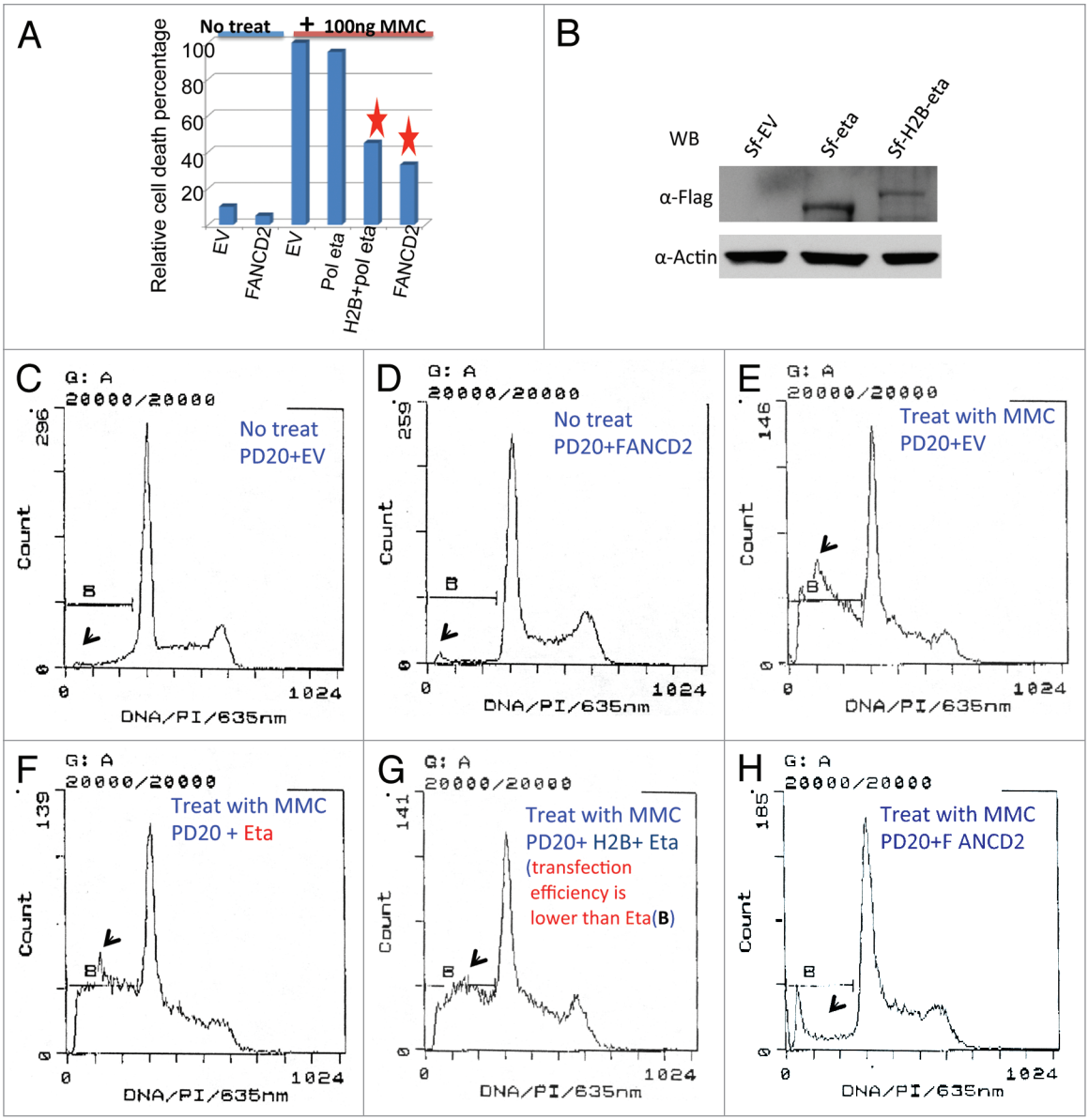

Figure 6. Ectopic expression of H2B-fused polymerase eta (H2B-Pol eta), but not pol eta alone, complements the MMC hypersensitivity of FANCD2-deficient cells (PD20). Cell death was analyzed in FANCD2-deficient cells (PD20) carrying with different plasmids. (**A**) Graph representation of the relative cell death rates of PD20 cells transfected with SF empty vector (EV), SF-FANCD2, SF-pol eta (about 30% transfection efficiency, % of green cells observed after transfection) and SF-H2B-Pol eta (about 15% transfection efficiency, % of green cells observed after transfection) with MMC (+) or without MMC (–) (the relative cell death rates for cells transfected with SF-eta and SF-H2B-eta were normalized with the transfection efficiency). A similar low rate of PD 20 cells carrying SF-H2B-eta as those with SF-wtFANCD2 indicates that the H2B-pol eta, but not pol eta alone, can partially rescue the MMC-induced cell death by showing a relative death rate down to 45% as compared with that of cells transfected with the SF empty vector (control, designated as 100%), noting that the relative cell death rate of PD20 cells transfected with SF-pol eta alone shows a similar level as those cells carrying SF-EV control. However, PD 20 cells carrying wtFANCD2 has a higher ability to rescue the cell death induced by MMC, in which the relative cell death rate is down to 33% as compared with that of SF-EV control (100%). (**B**) The ectopic expression of pol eta proteins in PD20 cells transiently transfected with SF empty vector, SF-pol eta and SF-H2B-Pol eta by western blotting using anti-flag antibodies. The actin level in each sample was used as loading control. SF-pol eta shows the transfection efficiency about 2-fold higher than that of SF-H2B-pol eta, which is consistent for the percentages of green cells observed (**A**). (**C–H**) panels show DNA histograms of PD20 cells carrying different plasmids as labeled in the figures, respectively, with or without treatment of MMC 100 ng/ml for 24 hr. The peak of cell death is marked by an arrow (<).

irradiation), while the PCNA interaction with pol eta was undetectable at the 3 h time point. This demonstrates for the first time that FANCD2 is essential in the early response to DNA damage repair and may be instrumental in the recruitment of PCNA during the late phase of DNA damage repair. As shown in **Figure** 7, we propose that the monoubiquitination of FANCD2 by the FA core complex in response to DNA damage functions at two distinct phases to promote the DNA repair process. In an early step, the localization and fixation of monoubiquitinated FANCD2 functions as a scaffold to recruit TLS polymerases such as pol eta

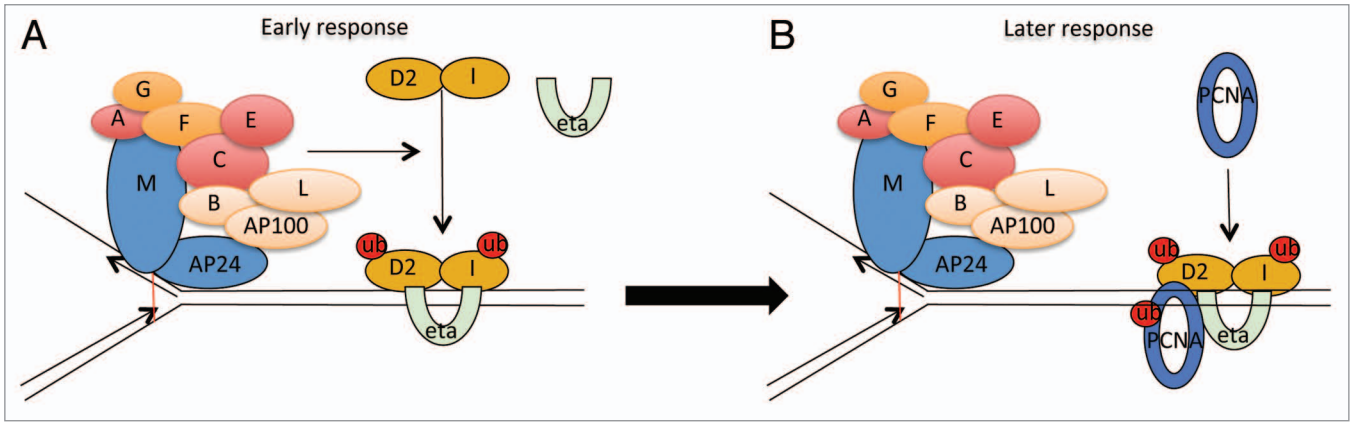

Figure 7. Ubiquitinated FANCD2 recruits Polymerase eta to target chromatin in response to DNA damage. (**A**) Our work indicates that peak/elevated-monoubiquitination of FANCD2, a hallmark of the activation FA pathway and promoted by the FA core complex, occurs early in response to UV irradiation. Monoubiquitinated FANCD2 can recruit the polymerase eta to DNA damage sites and enable TLS to take place. (**B**) Peak monoubiquitination of PCNA occurs later than the peak monoubiquitination of FANCD2 in response to DNA damage. The monoubiquitinated PCNA can work in concert with FANCD2 and pol eta at DNA damage sites on chromatin and promote the TLS processes.

and promotes DNA damage repair. In a later step, the monoubiquitinated FANCD2 cooperates with monoubiquitinated PCNA to facilitate the polymerase switching process, in which TLS polymerases are recruited to the DNA damage sites and exert a spatiotemporal regulatory role in the reaction of nucleotide insertion and extension beyond the lesion.[41,42] Together, this study advances our understanding of how cells can meticulously respond to genotoxic stressors. In addition, pol eta, mainly working in the TLS error-free pathway, unlike many other family members causing mutagenesis, plays an important role in maintaining genome stability, as demonstrated by XP-V.[18-20] Our finding of recruitment of pol eta by FANCD2 in the early response to DNA damage therefore provides a novel insight into how an impaired FA-BRCA tumor suppressor pathway contributes to the development of non-FA human cancer.[11]

Materials and Methods

Cell line and reagents. The PD20 cell line was a gift from the FA Research Foundation. Other cell lines used were all obtained from the American Type Culture Collection (ATCC). The pEGF-S-Flag-polymerase eta (called SF-eta) and SF-H2B-polymerase eta (H2B cDNA inserted into SF-eta) plasmids were generated sequentially through RT-PCR and inserting the coding region of pol eta or H2B cDNA in frame with cDNAs of fusion partners, respectively. GFP-pol eta (pcDNA3-GFP-fused pol eta), wild type and mutants, were provided by Dr I. Dikic (Institute of Biochemistry II) and Dr A.R. Lehmann (University of Sussex).

Transfection and protein complex crosslink. Plasmids were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) as previously described.[11] Cell lysates were collected 4 h after UV irradiation. Cells were rinsed twice in cold PBS followed with incubation for 5 min in buffer A. To crosslink the protein complex, cells were incubated on ice for 10 min in PBS with 1% formaldehyde. The reactions were stopped by a 5 min incubation with a 0.1 M glycine solution, and the cells were then rinsed twice with cold PBS, harvested and dissolved in IP lysis buffer [50 mM TRIS-HCl (pH 7.5), 200 mM NaCl, 5 mM EDTA. 0.2% Triton X-100 plus protease inhibitors]. DNase I treatment was performed in IP buffer with 10 units of RNase-free DNase I (Roche) on ice for 1 h.

Immunoprecipitation assay and western blotting. Dissolved proteins were incubated with monoclonal anti-Flag (Sigma) or anti-GFP antibodies (Santa-Cruz Biotechnology) overnight at 4°C, and followed by incubation with protein A-sepharose (Phamacia) for 2 h. The beads were washed with IP buffer and then boiled in SDS-lysis buffer for 10 min. The bound proteins were analyzed by western blotting using anti-GFP, anti-Flag, anti-FANCD2 (Novus Biologicals) or anti-PCNA (Santa-Cruze Biotechnology) antibodies.

Gel filtration. Gel filtration analysis was performed as described previously.[43] We isolated nuclear extract from U2OS cells with or without UV 25 J/M^2 irridiation. Hypotonic buffer with NP-40 was used to separate the cytoplasm and nuclei. The nuclear proteins were isolated by high salt extraction buffer [20 mM HEPES (pH 7.9), 420 mM Nacl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 m MEDTA, 0.5 mM dithiothreitol and protease inhibitors]. The nuclear extracts (4 mg) were directly applied to a sepharose 6B column (Sigma) equilibrated with column running buffer containing 20 mM HEPES (pH 7.9), 200 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol. We collected fractions of 1 ml and analyzed them (120 μl each) by SDS-PAGE [4–20% gradient Tris-glycine gel (Invitrogen)] and immunoblotting.

FACS analysis and other routine techniques were performed as described previously.[11]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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