RAD18-mediated ubiquitination of PCNA activates the Fanconi anemia DNA repair network

Liyi Geng[1], Catherine J. Huntoon[1], and Larry M. Karnitz[1,2,3]

[1]Division of Oncology Research, [2]Department of Molecular Pharmacology and Experimental Therapeutics, and [3]Department of Radiation Oncology, Mayo Clinic College of Medicine, Rochester, MN 55905

The Fanconi anemia (FA) network is important for the repair of interstrand DNA cross-links. A key event in FA pathway activation is the monoubiquitylation of the FA complementation group I (FANCI)–FANCD2 (ID) complex by FA complementation group L (FANCL), an E3 ubiquitin ligase. In this study, we show that RAD18, another DNA damage–activated E3 ubiquitin ligase, also participates in ID complex activation by ubiquitylating proliferating cell nuclear antigen (PCNA) on Lys164, an event required for the recruitment of FANCL to chromatin. We also found that monoubiquitylated PCNA stimulates FANCL-catalyzed FANCD2 and FANCI monoubiquitylation. Collectively, these experiments identify RAD18-mediated PCNA monoubiquitination as a central hub for the mobilization of the FA pathway by promoting FANCL-mediated FANCD2 monoubiquitylation.

Introduction

Fanconi anemia (FA) is a human genetic disorder characterized by congenital abnormalities, bone marrow failure, chromosomal instability, cancer susceptibility, and a profound sensitivity to agents that produce interstrand DNA cross-links (ICLs). To date, 13 FA genetic complementation groups have been identified (Moldovan and D'Andrea, 2009). Consistent with the sensitivity to ICLs, the proteins encoded by the *FANC* genes repair these lesions. Although the molecular and biochemical functions of the FA pathway remain poorly understood, the following model has emerged. In response to ICLs and replication stress, FANCM and FAAP24 are recruited to replication forks stalled either by ICLs or other forms of replication stress (Ciccia et al., 2007; Kim et al., 2008). The FANCM–FAAP24 complex promotes chromatin loading of the FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FAAP100), which contains FA complementation group L (FANCL) protein, an E3 ubiquitin ligase. FANCL and UBE2T, its partner E2 ubiquitin–conjugating enzyme, catalyze the monoubiquitylation of both FA complementation group I (FANCI) and FANCD2, which form the FANCI–FANCD2 (ID) complex. Ubiquitylation of the ID complex targets it to the stalled replication fork or site of damage, where it promotes processing and repair of the ICL lesion (Räschle et al., 2008; Knipscheer et al., 2009; Kratz et al., 2010; Smogorzewska et al., 2010).

Correspondence to Larry M. Karnitz: karnitz.larry@mayo.edu

Abbreviations used in this paper: DRWD, double RWD; ELF, E2-like fold; FA, Fanconi anemia; FANCI, FA complementation group I; FANCL, FA complementation group L; ICL, interstrand DNA cross-link; ID, FANCI–FANCD2; PCNA, proliferating cell nuclear antigen; RWD, RING finger/WD repeat domain; S-PCNA; S-tagged PCNA; TLS, translesion synthesis.

ICLs and other forms of replication stress also activate the translesion synthesis (TLS) pathway (Lee and Myung, 2008), which is initiated by the recruitment of the RAD18–RAD6 complex, an E3 ubiquitin ligase and an E2 ubiquitin–conjugating enzyme pair. The RAD18–RAD6 complex ubiquitylates proliferating cell nuclear antigen (PCNA), a homotrimeric sliding clamp complex, on Lys164. PCNA ubiquitylation has a known role in the recruitment and regulation of TLS polymerases, which, because of large active sites, have reduced fidelity but increased ability to synthesize over lesions that stall the high fidelity DNA polymerases (Guo et al., 2009). Genetic data demonstrate that RAD18 and PCNA ubiquitylation also play critical roles in cells challenged with agents that induce ICLs (Yamashita et al., 2002; Tateishi et al., 2003; Nojima et al., 2005; Arakawa et al., 2006; Shen et al., 2006; Simpson et al., 2006; Saberi et al., 2007). However, these roles remain poorly understood. One function for ubiquitylated PCNA in the repair of ICLs is to recruit or regulate the TLS DNA polymerases REV1 and polymerase ζ, which are required for ICL repair (Sarkar et al., 2006; Shen et al., 2006). Nevertheless, PCNA ubiquitylation may have a previously unknown function in the regulation of the FA pathway. Consistent with this later possibility, we report in this study that RAD18-mediated PCNA ubiquitylation plays a pivotal role in FA pathway activation by regulating FANCL-mediated ubiquitylation of the ID complex.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 191 No. 2 249–257
www.jcb.org/cgi/doi/10.1083/jcb.201005101

Results and discussion

RAD18 and FANCD2 may be in a common pathway

Previous studies demonstrated that both RAD18 and FANCD2 play important roles in eukaryotic cells exposed to cisplatin and other DNA cross-linking agents (Yamashita et al., 2002; Nojima et al., 2005; Ross et al., 2005; Lee and Myung, 2008; Wagner and Karnitz, 2009; Hicks et al., 2010). In our experiments to understand the DNA repair pathways that affect the sensitivity of human tumor cells to cisplatin, a chemotherapy agent that causes ICL formation (Wang and Lippard, 2005), we depleted HeLa cells of FANCD2, a key participant in the FA repair pathway, and of RAD18, a regulator of the TLS pathway (Fig. 1 A, inset). As expected, siRNA-mediated depletion of RAD18 or FANCD2 individually sensitized cells to this cross-linking agent (Fig. 1 A and Fig. S1 A), whereas only RAD18 depletion sensitized to UV light (Fig. S1 B), which creates bulky DNA lesions that are bypassed by TLS polymerases after RAD18-mediated PCNA ubiquitylation (Tateishi et al., 2000; Kannouche et al., 2004; Watanabe et al., 2004). Surprisingly, however, codepletion of both proteins did not cause a more profound sensitization to cisplatin. Given that the RAD18 and FANCD2 depletions were both highly effective, this result raised the possibility that these proteins might act in the same pathway in cells treated with agents that cause ICL formation and prompted us look further for potential interactions between these pathways.

RAD18 is required for FANCD2 ubiquitination and chromatin binding

To test for a possible link between RAD18 and FANCD2, we asked whether RAD18 depletion affects FANCD2 monoubiquitylation, an essential step in the activation of the FA pathway that can be assessed by a decreased mobility of FANCD2 on SDS-PAGE. For these experiments, we used five independent RAD18 siRNAs: four of these siRNAs successfully depleted RAD18, and one caused only minimal depletion (Fig. 1 B). Strikingly, all four highly effective siRNAs reduced cisplatin-induced FANCD2 monoubiquitylation in K562 cells. In contrast, the ineffective siRNA did not disrupt FANCD2 monoubiquitylation. Similar results were seen in HCT-116 *RAD18*$^{-/-}$ cells (Fig. 1 C) and RAD18-depleted HeLa cells (Fig. S1 C). To verify that RAD18 depletion was indeed affecting FANCD2 monoubiquitylation, we showed that RAD18 depletion prevented the addition of transiently expressed HA-tagged ubiquitin to endogenous FANCD2 (Fig. 1 D). Furthermore, we found that RAD18 depletion also blocked the ubiquitylation of FANCI (Fig. S1 D) and reduced the cisplatin-induced binding of FANCD2 to chromatin, an event that depends on FANCD2 ubiquitylation (Wang et al., 2004), in K562 (Fig. 1 E) and HeLa cells (Fig. S1 E). Collectively, these results demonstrate that RAD18 plays a critical role in the ubiquitylation of ID complex members and recruitment of the complex to chromatin after DNA damage.

FANCL and RAD18 appear to function in a common pathway

We next explored the relative roles of RAD18 and FANCL in FANCD2 monoubiquitylation by depleting RAD18 only, FANCL only, or both E3 ligases and examining basal and cisplatin-induced FANCD2 monoubiquitylation. Basal and induced FANCD2 monoubiquitylation was dramatically and similarly disrupted in cells individually depleted of FANCL or RAD18 (Fig. 2 A). Notably, the codepletion of FANCL and RAD18 did not further reduce FANCD2 monoubiquitylation. Additionally, FANCL depletion did not diminish PCNA monoubiquitylation (Fig. S1 F), thus demonstrating that FANCL does not participate in this PCNA modification. Consistent with the effects of these depletions on FANCD2 monoubiquitylation, cells depleted of RAD18 alone, FANCL alone, or both had nearly identical sensitivities to cisplatin (Fig. 2 B and Fig. S2 A [Western blot]). Collectively, these results suggest that RAD18 and FANCL may function in a common pathway in human cells.

FANCD2 ubiquitination requires RAD18-mediated ubiquitination of PCNA on Lys164

The only known RAD18 substrate is PCNA, which is ubiquitylated by RAD18–RAD6 on Lys164. We therefore asked whether PCNA ubiquitination is required for cisplatin-induced FANCD2 monoubiquitylation by modifying a previously described approach (Niimi et al., 2008). Cells were transfected with an siRNA that depletes endogenous PCNA along with plasmids that express siRNA-resistant wild-type PCNA (PCNA^{WT}) or PCNA mutated at the ubiquitin acceptor site Lys164 (PCNA^{K164R}). The depletion of endogenous PCNA was highly effective (Fig. 2 C), and the transiently expressed S-tagged PCNA (S-PCNA) was expressed at levels comparable with endogenous protein. Importantly, both wild-type S-PCNA and S-PCNA^{K164R} had cycle profiles identical to cells cotransfected with empty vector and the luciferase control siRNA (Fig. S2 B), thus demonstrating that these PCNAs rescued replication. Analysis of these cells revealed that basal and cisplatin-induced FANCD2 monoubiquitylation was severely impaired in cells expressing PCNA^{K164R} compared with cells expressing wild-type PCNA or control cells transfected with empty vector and luciferase siRNA (Fig. 2 C). Moreover, the depletion of FANCD2 did not further sensitize cells expressing PCNA^{K164R} to cisplatin (Fig. S2 C). Collectively, these results show that RAD18 regulates FANCD2 ubiquitylation through the ubiquitylation of PCNA on Lys164.

The FANCL and FA core complex interacts with PCNA

One possible way for PCNA to participate in FANCD2 ubiquitination would be for PCNA to recruit FANCL to sites of DNA damage. Indeed, PCNA immunoprecipitates contained FANCL (Fig. 3 A) and the FA core complex members FANCA and FANCE (Fig. S2 D). We also found that FANCL coprecipitated with PCNA (Fig. 3 B) and that this interaction was not affected by DNaseI treatment (Fig. S2 E), demonstrating that it did not depend on DNA.

The FANCL double RING finger/WD repeat domain (RWD [DRWD]) domain binds PCNA

We next identified the regions of FANCL that are important for binding PCNA. Human FANCL was originally thought to have two domains: the N terminus (amino acids 1–306) was predicted to

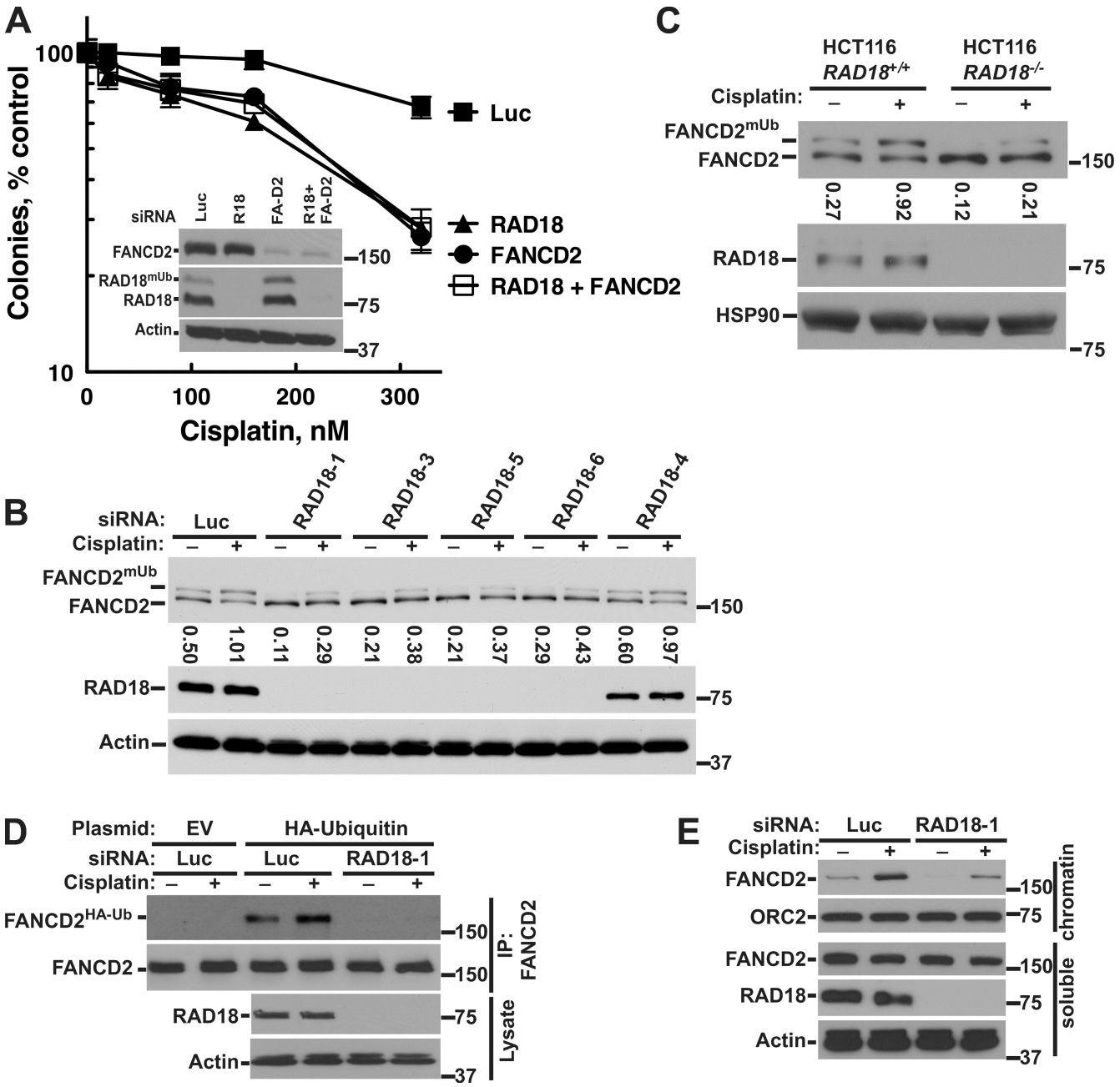

Figure 1. **RAD18 is required for cisplatin-induced FANCD2 monoubiquitination and recruitment to chromatin.** (A) HeLa cells were transfected with siRNAs that target Luciferase (Luc), RAD18-1 (R18), FANCD2 (FA-D2), or both. 2 d later, trypsinized cells were analyzed by immunoblotting (inset; note that monoubiquitylated FANCD2 is not resolved under these gel conditions) and replated, treated with cisplatin for 24 h, washed, and cultured for 8 d to allow colony formation. A representative experiment that has been repeated three times is shown ($n = 3 \pm$ SD; see Fig. S1 A for additional replicates). (B) K562 cells were transfected with siRNAs. 2 d later, the cells were treated with 30 µM cisplatin for 6 h (+), and lysates were immunoblotted. Monoubiquitylated FANCD2 (FANCD2^{mUb}) was detected by slower migration in SDS-PAGE. (C) *RAD18*$^{+/+}$ HCT-116 and *RAD18*$^{-/-}$ HCT-116 cells were treated with 30 µM cisplatin for 6 h and analyzed as in B. (D) K562 cells were cotransfected with siRNAs and plasmids that encode HA-tagged ubiquitin or empty vector (EV). 2 d after transfection, the cells were treated as in B. The lysate was immunoprecipitated (IP) with anti-FANCD2 antibody. The immunoprecipitates and starting lysates were sequentially immunoblotted to detect HA-ubiquitin covalently linked to FANCD2 (anti-HA mAb) and FANCD2. (E) K562 cells were transfected with siRNA, cultured, and treated with cisplatin as in B. Cells were separated into chromatin-bound and soluble fractions, which were analyzed by immunoblotting. Western blot markers are given in kilodaltons.

contain three WD40 domains, and the C terminus (amino acids 307–375) was predicted to contain a zinc-binding region that is essential for E3 ligase activity (Meetei et al., 2003). However, a very recent crystallographic structural analysis of *Drosophila melanogaster* FANCL has further refined our understanding of FANCL's structure (Cole et al., 2010). This study showed that the N terminus contains two separable domains, an E2-like fold (ELF) domain and a DRWD domain. To test which FANCL domain is important for PCNA binding, we examined binding to the entire N-terminal fragment (which contains the ELF and DRWD domains; amino acids 1–306), the individual ELF and DRWD domains, and the C-terminal RING domain. The N terminus (which contains the

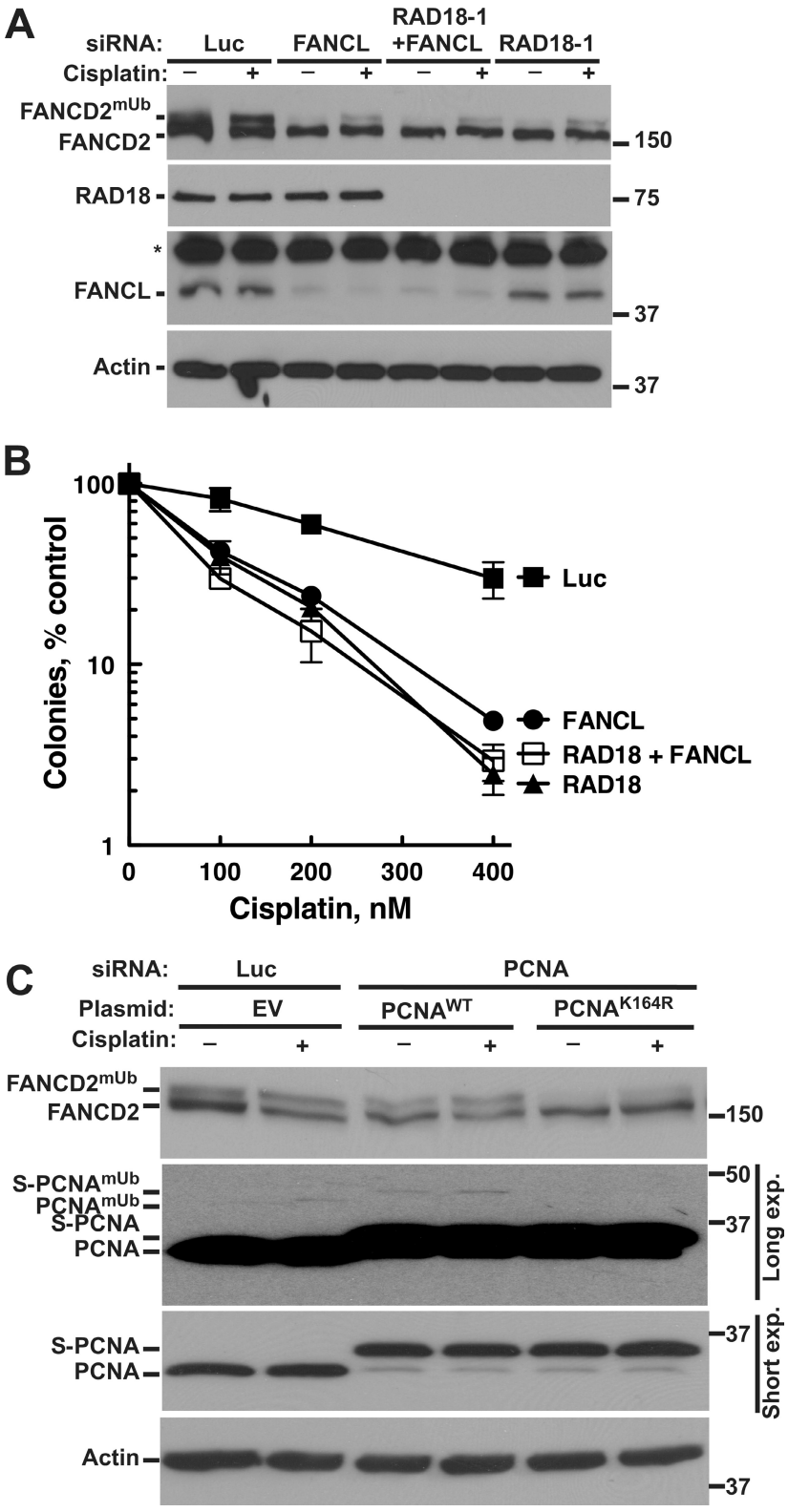

Figure 2. **RAD18 participates in cisplatin-induced FANCD2 monoubiquitylation through the ubiquitylation of PCNA on Lys164.** (A) K562 cells were transfected with siRNAs, cultured for 2 d, and treated with 30 µM cisplatin (6 h). Lysates were immunoblotted as indicated. *, nonspecific band. (B) HeLa cells were transfected with siRNAs. 2 d later, the trypsinized cells were analyzed by immunoblotting (Fig. S2 A), and the remaining cells were replated, treated with cisplatin for 24 h, washed, and cultured for 8 d to allow colony formation. A representative experiment of three independent experiments is shown ($n = 3 \pm$ SD). (C) K562 cells were cotransfected twice with siRNAs and plasmids that encode siRNA-resistant wild-type PCNA ($PCNA^{WT}$) or $PCNA^{K164R}$. 2 d after the second transfection, trypsinized cells were analyzed by immunoblotting and for cell cycle (Fig. S2 B). Short and long exposures of the PCNA immunoblot are shown to demonstrate PCNA loading and PCNA monoubiquitylation ($PCNA^{mUb}$), respectively. Western blot markers are given in kilodaltons.

ELF and DRWD domains) interacted with PCNA, whereas the C-terminal RING domain did not (Fig. 3 C). Further subdivision of the N terminus into the individual ELF and DRWD domains showed that the ELF domain did not bind PCNA, whereas the DRWD domain interacted with PCNA, thus demonstrating that the DRWD domain is important for the FANCL–PCNA interaction.

FANCL directly binds PCNA

To further probe the nature of the interaction between PCNA and FANCL, we immobilized GST or GST-PCNA on agarose beads and incubated the beads with lysates from cells expressing SFB-tagged FANCL. Consistent with the immunoprecipitation results, GST-PCNA specifically bound to FANCL (Fig. 3 D).

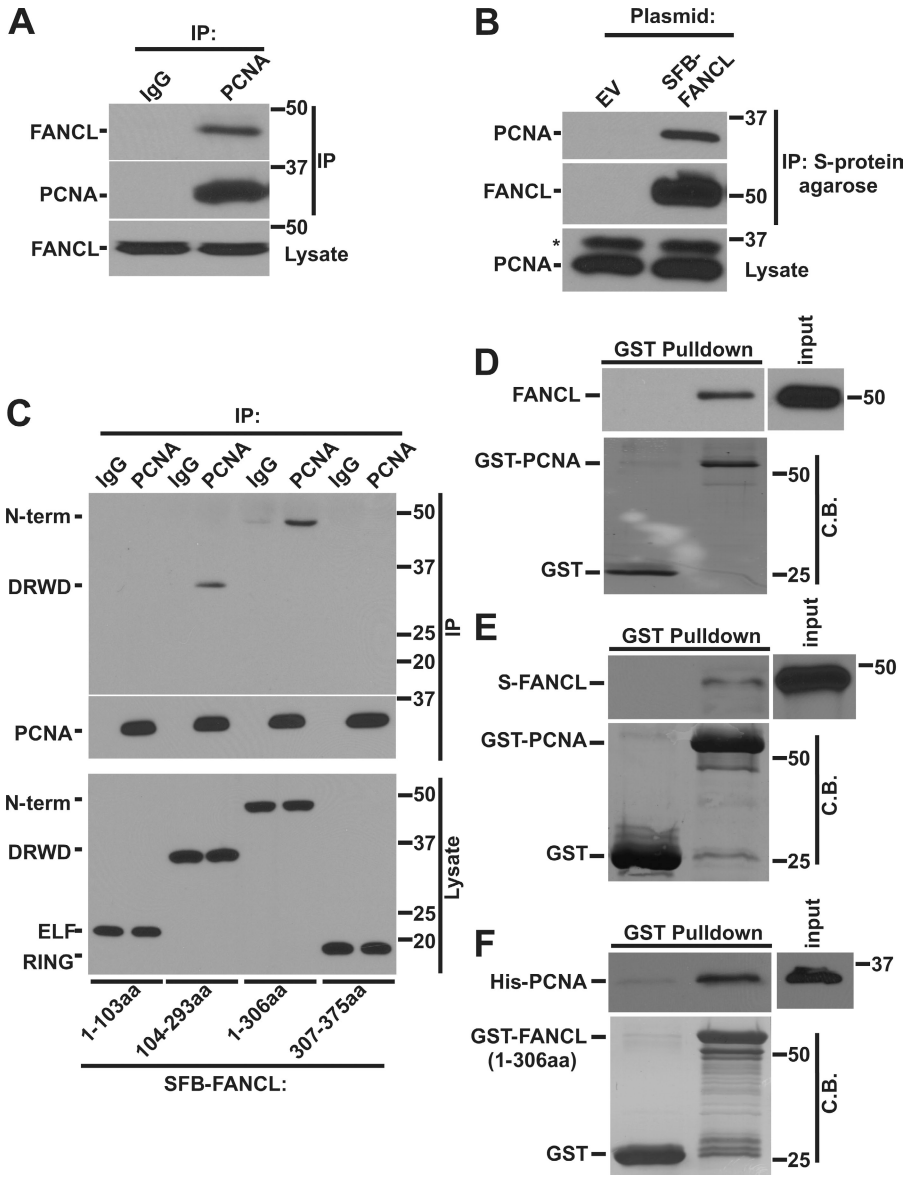

Figure 3. **FANCL binds PCNA via the FANCL DRWD domain.** (A) Cell lysates from K562 cells transiently expressing HA-tagged FANCL were immunoprecipitated with nonimmune (IgG) or anti-PCNA rabbit antisera. The precipitates were immunoblotted to detect FANCL (anti-HA) and PCNA. (B) Cell lysates from K562 cells transfected with empty vector (EV) or transiently expressing SFB-tagged FANCL were precipitated with S protein agarose. Precipitates were immunoblotted to detect PCNA and FANCL (anti–S peptide mAb, which recognizes the SFB tag). *, nonspecific band. (C) SFB-tagged versions of the following domains of FANCL were transiently expressed in K562 cells: ELF (amino acids 1–103), DRWD (amino acids 104–293), N terminus (amino acids 1–306), and RING (amino acids 307–375). Cell lysates were immunoprecipitated with nonimmune or rabbit anti-PCNA antiserum, and the precipitates were immunoblotted to detect the SFB tag (anti–S peptide mAb). (D and E) GST and GST-PCNA purified from *E. coli* were immobilized on GSH agarose, which was incubated with lysates from K562 cells transiently expressing SFB-FANCL (D) or S-tagged FANCL produced by in vitro translation (E). The washed beads were immunoblotted to detect FANCL (anti–S peptide mAb), and the membranes were stained with Coomassie blue (CB) to show GST and GST-PCNA loading. (F) GST and GST-FANCL (amino acids 1–306) purified from *E. coli* were immobilized on GSH agarose. Washed beads were incubated with *E. coli*–produced His_6-PCNA. The precipitates were immunoblotted to detect PCNA (anti-PCNA), and the membrane was stained with Coomassie blue to show GST and GST-FANCL (amino acids 1–306) loading. IP, immunoprecipitation. Western blot markers are given in kilodaltons.

To address whether FANCL directly interacted with PCNA, we first showed that in vitro–translated full-length FANCL bound specifically to PCNA purified from *Escherichia coli* (Fig. 3 E) and then showed that the N-terminal FANCL domain (amino acids 1–306), also produced in *E. coli*, bound PCNA (Fig. 3 F). Collectively, these results demonstrate that FANCL directly binds PCNA. Moreover, because the bacterially expressed proteins recapitulate the interactions, these results show that this interaction does not require the monoubiquitylation of PCNA by RAD18.

RAD18 and Lys164 PCNA ubiquitination are required for recruitment of FANCL to chromatin

The results presented thus far demonstrate two important points. First, RAD18-mediated PCNA monoubiquitylation is important for FANCD2 ubiquitylation. Second, PCNA binds FANCL, the E3 ubiquitin ligase for FANCD2. These observations, coupled with the fact that FANCL is recruited to chromatin after DNA damage (Matsushita et al., 2005; Alpi et al., 2008), prompted us to ask whether PCNA ubiquitylation affected the binding of FANCL to chromatin. As shown in Fig. 4 A, the depletion of RAD18 profoundly blocked the binding of FANCL to chromatin in response to cisplatin. Similarly, in cells expressing $PCNA^{K164R}$, cisplatin-induced binding of FANCL to chromatin was disrupted (Fig. 4 B), confirming that PCNA ubiquitylation on Lys164 plays a pivotal role in the recruitment of FANCL to chromatin.

Monoubiquitylated PCNA stimulates FANCL-catalyzed FANCD2 and FANCI ubiquitylation

The known function for PCNA Lys164 monoubiquitylation is to recruit and regulate TLS DNA polymerases in a process that requires the ubiquitin-binding domains in the TLS polymerases. We therefore initially looked for FANCL domains that could interact with ubiquitin; however, despite extended efforts, we were unable to find a region of FANCL that stably binds ubiquitin

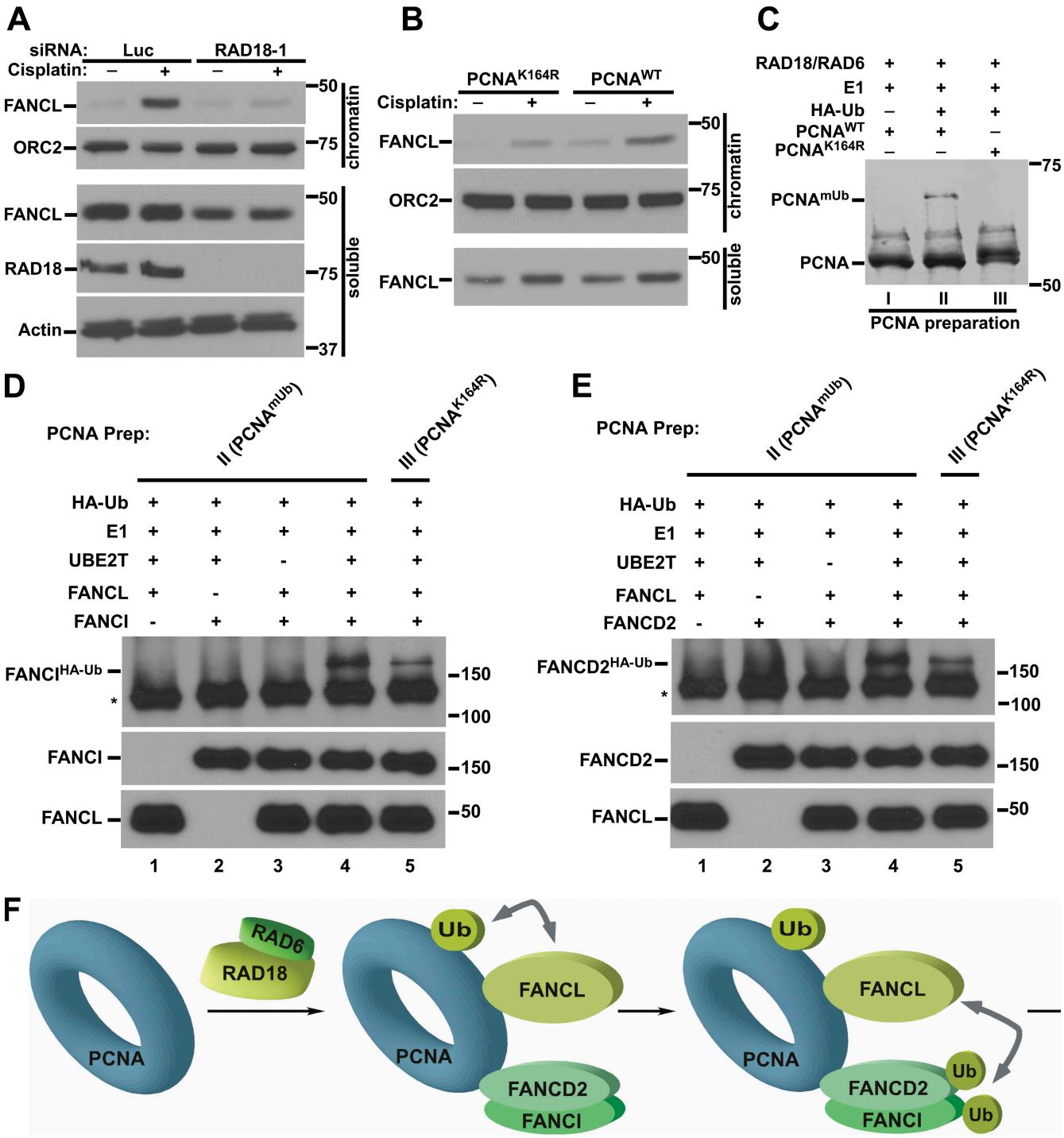

Figure 4. **Monoubiquitinated PCNA promotes FANCL chromatin binding and stimulates FANCL-mediated FANCD2 and FANCI monoubiquitylation.** (A and B) K562 cells were cotransfected with siRNAs and plasmids, cultured for 2 d, and treated with 30 µM cisplatin for 6 h. Chromatin-bound and soluble fractions were isolated and sequentially immunoblotted to detect FANCL (anti-HA mAb) or the loading controls ORC2 (chromatin-bound fraction) or actin (soluble fraction). (C) *E. coli*–produced GST-PCNA (wild type) and GST-PNCA^{K164R} were incubated with ubiquitin-activating enzyme E1, RAD18–RAD6 complex, and HA-ubiquitin (HA-Ub). PCNA preparations (I–III) were repurified by GSH agarose chromatography and analyzed by SDS-PAGE (Coomassie Blue stain). (D and E) The repurified PCNA preparations from C were analyzed for effects on FANCL activity. SFB-FANCL alone, SFB-FANCI alone, and the combination (D) or SFB-FANCL alone, SFB-FANCD2, and the combination (E) were copurified from K562 cells using S protein agarose. After extensive washing, the SFB-tagged proteins were eluted with S peptide and mixed with the indicated *E. coli*–produced proteins and PCNA preparations (II and III) from C under conditions that support in vitro ubiquitylation of FANCI and FANCD2. The reactions were analyzed by sequentially immunoblotting for ubiquitin (anti-HA mAb), FANCL, FANCD2, and FANCI (anti–S peptide mAb). *, nonspecific band. (F) Model of PCNA acting as a central hub in the activation of the FA pathway. Western blot markers are given in kilodaltons.

(unpublished data). Nonetheless, we reasoned that ubiquitylation of PCNA might affect the activity of FANCL. To test this hypothesis, we first set up in vitro PCNA ubiquitylation reactions (using the RAD18–RAD6 complex) and produced three PCNA preparations (Fig. 4 C): (I) wild-type PCNA incubated with all reaction components except ubiquitin, (II) wild-type PCNA incubated with all required components to promote PCNA ubiquitylation, and (III) $PCNA^{K164R}$, which cannot be ubiquitylated because it lacks the ubiquitin acceptor site. These PCNA preparations were tested for their ability to stimulate the FANCL-dependent ubiquitylation of FANCI and FANCD2 in in vitro reactions. In these reactions, FANCI and FANCD2 were modestly monoubiquitinated when $PCNA^{K164R}$ (preparation III; Fig. 4, D and E, lane 5; and Fig. S3, C and D [uncropped image]) or nonubiquitylated wild-type PCNA (preparation I; Fig. S3, A and B, lane 3) was added to reactions containing all other required components. In contrast, monoubiquitylated PCNA (preparation II) dramatically stimulated FANCI and FANCD2 monoubiquitylation (Fig. 4, D and E, lane 4), which was dependent on FANCL and UBE2T (Fig. 4, D and E, lanes 2 and 3). Therefore, these results demonstrate that PCNA ubiquitylated by RAD18–RAD6 can stimulate the FANCL-mediated monoubiquitylation of FANCD2 and FANCI.

The experiments presented in this study raise several discussion points. First, our findings differ from experiments in chicken DT40 cells, in which codisruption of *FANCC*, which encodes an FA core complex member, and *RAD18* caused greater sensitivity to cisplatin than did either individual gene disruption alone, suggesting that the FA and RAD18 pathways are not epistatic (Hirano et al., 2005). In contrast, our siRNA codepletion experiments suggest that the FA and RAD18 pathways may be epistatic in terms of cisplatin sensitivity (Fig. 1 A, Fig. 2 B, and Fig. S1 A). These disparate findings could reflect a difference between human and chicken cells in the regulation of the FA. However, it is worth noting that PCNA is still ubiquitylated in *RAD18*$^{-/-}$ cells and that cells expressing $PCNA^{K164R}$ are more sensitive to cisplatin than are *RAD18*$^{-/-}$ cells (Simpson et al., 2006), thus raising the possibility that the lack of epistasis could be explained by the residual PCNA ubiquitylation in *RAD18*$^{-/-}$ cells. Alternatively, this discrepancy may stem from our use of siRNAs, which, although very effective, do not ablate protein expression. Although we cannot definitively determine the epistatic relationship of the FA and RAD18 pathways in human cells in terms of cisplatin sensitivity, our experiments nonetheless clearly demonstrate in multiple human cell models that RAD18 and monoubiquitylated PCNA are required for FANCD2 ubiquitylation. Future studies that use genetically modified human cells will be required to determine whether the interactions between human and chicken FA pathways are significantly different.

Second, these findings suggest the following model (Fig. 4 F): upon replication fork stalling at cisplatin-induced DNA lesions, the RAD18–RAD6 complex ubiquitylates PCNA on Lys164. This posttranslationally modified PCNA stimulates activity of the PCNA-bound FANCL so that it promotes the monoubiquitylation of FANCD2. Although previous studies suggest that ubiquitylated PCNA plays an important role in regulating the TLS polymerases REV1 and REV3 (Sarkar et al., 2006; Shen et al., 2006), these results, taken in conjunction with the recent finding that FANCD2 has a PCNA interaction motif that is required for DNA damage–induced FANCD2 monoubiquitylation (Howlett et al., 2009), demonstrate that PCNA also serves as a central hub for the steps leading to the activation of the FA pathway in human cells.

Materials and methods

Cell culture, transfections, clonogenic assays, and cell cycle analyses

HeLa and K562 cells obtained from American Type Culture Collection were grown and transfected by electroporation as described previously (Arlander et al., 2003) and were used 48 or 72 h after transfection as indicated. Cell cycle analyses (Arlander et al., 2003) and clonogenic assays (Wagner and Karnitz, 2009) were performed as described previously. HCT-116 and HCT-116 *RAD18*$^{-/-}$ were obtained from T. Shiomi (National Institute of Radiological Sciences, Chiba, Japan) and grown as described previously (Shiomi et al., 2007).

Materials

Antibodies to the following antigens were used and obtained from the indicated sources: PCNA rabbit polyclonal antisera (provided by J. Chen, M.D. Anderson Cancer Center, Houston, TX), PCNA mAb (PC10 mAb; Santa Cruz Biotechnology, Inc.), RAD18 (Novus Biologicals), FANCD2 (GeneTex, Inc.), FANCA (Bethyl Laboratories, Inc.), FANCE (Bethyl Laboratories, Inc.), FANCL (provided by W. Wang, National Institutes of Health, Bethesda, MD), ORC2 (BD), β-actin (Sigma-Aldrich), HA tag (Covance), S tag (Hackbarth et al., 2004), and Flag tag (Flag M1 mAb; Sigma-Aldrich). Micrococcal nuclease was obtained from Worthington Biochemical. S protein agarose was obtained from EMD. Protein A–Sepharose was obtained from Sigma-Aldrich. Protein G–Sepharose was obtained from GE Healthcare.

The following duplex siRNAs (Thermo Fisher Scientific) were used: RAD18-1, 5′-GCUCUCUGAUCGUGAUUUA-3′; RAD18-3, 5′-GCAGUUUGCUUUAGAGUCA-3′; RAD18-4, 5′-AUAACCGCAUAUUAGAUGA-3′; RAD18-5, 5′-CCAAGAAACAAGCGUAAUA-3′; RAD18-6, 5′-GGAGCAGGUUAAUGGAUAA-3′; luciferase, 5′-CUUACGCUGAGUACUUCGA-3′ (Elbashir et al., 2001); FANCD2, 5′-GGUCAGAGCUGUAUUAUUC-3′ (Wagner and Karnitz, 2009); PCNA, 5′-GGAGAAAGUUUCAGACUAU-3′ (Merkerova et al., 2007); and FANCL, 5′-GACAAGAGCUGUAUGCACU-3′ (Meetei et al., 2003).

Plasmids

The FANCL expression plasmids were created by ligating PCR-amplified FANCL into vectors that append an N-terminal SFB tag (pSFB; Chini and Chen, 2006), an N-terminal S tag (pSPN; Hackbarth et al., 2004), or a C-terminal tandem HA tag (pcDNA3-HA2; Volkmer and Karnitz, 1999). The S-PCNA expression vector was constructed by ligating PCR-amplified PCNA into pSPC, which appends a C-terminal S tag (Hackbarth et al., 2004). S-PCNA K164R was derived from pSPC-PCNA by site-directed mutagenesis. PCNA expression plasmids resistant to the siRNA, four silent mutations (identified by underlines), were generated using the oligonucleotide 5′-CGCTAGTATTTGAAGCACCAAACCAGGAAAAGGTCTCCGACTATGAAATGAAGTTGATG-3′ and its reverse complement as primers for site-directed mutagenesis reactions (QuikChange; Agilent Technologies). The FANCI expression plasmids were constructed by ligating PCR-amplified FANCI into pSFB and pcDNA3-HA2 plasmids. The FANCD2 expression vector (pSFB-FANCD2) used in these experiments encodes amino acids 1–1,394 of FANCD2. The sequences of all final plasmids were verified to ensure the fidelity of all DNA manipulations. The plasmid pET25b-hRAD18-His_6hHR6B, which was used to express the RAD18–RAD6B complex in *E. coli*, has been described previously (Notenboom et al., 2007) and was provided by T. Sixma (Netherlands Cancer Institute, Amsterdam, Netherlands). The GST-PCNA and GST-FANCL (amino acids 1–306) *E. coli* expression plasmids were generated by ligating PCR-amplified PCNA and FANCL (amino acids 1–306) into pGEX-KG vector (American Type Culture Collection), respectively. The pET-24a(+)-His_6-PCNA plasmid was constructed by ligating a PCR-amplified fragment of PCNA into pET-24a(+) vector (EMD).

Cell lysis, cell fractionation, immunoprecipitations, and immunoblotting

For immunoblotting, cells were lysed in SDS-PAGE sample buffer or in 50 mM Hepes, pH 7.6, 1% Triton X-100, 10 mM NaF, 30 mM $Na_4P_2O_7$, 150 mM NaCl, 1 mM EDTA supplemented with 10 µg/ml leupeptin, 5 µg/ml pepstatin, 20 nM microcystin-LR, 5 µg/ml aprotinin, 1 mM Na_3VO_4, 10 mM 2-glycerophosphate, and 10 mM *N*-ethylmaleimide. The biochemical fractionation of

soluble and chromatin-bound proteins was performed as described previously (Geng et al., 2007). For immunoprecipitations, cells were lysed in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100 supplemented with 5 mM $CaCl_2$, 300 U/ml micrococcal nuclease, 10 mM *N*-ethylmaleimide, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 20 nM microcystin-LR, 5 µg/ml aprotinin, 1 mM Na_3VO_4, and 10 mM 2-glycerophosphate, incubated at 20°C for 20 min, and 3 mM EDTA was added. The lysates were centrifuged at 20,800 *g* at 4°C for 10 min, and the supernatants were immunoprecipitated. All immunoblots were imaged using ECL Western blotting substrate (Thermo Fisher Scientific) and exposure to x-ray film. Ratios of ubiquitylated FANCD2 to unmodified FANCD2 were determined by scanning and digitizing x-ray films using a Gel Doc XR system (Bio-Rad Laboratories) with Quantity One volume analysis software (Bio-Rad Laboratories).

GST pull-down assays

GST pull-down experiments were performed as described previously (Bienko et al., 2005) using proteins obtained as follows: GST, GST-PCNA, His_6-PCNA, and GST-FANCL (amino acids 1–306) were purified from *E. coli* using glutathione agarose (Thermo Fisher Scientific) or TALON Superflow metal affinity resin (Takara Bio Inc.) chromatography, S-tagged FANCL was produced with a coupled in vitro transcription/translation system (Promega), and SFB-FANCL was produced by transient expression in K562 cells.

In vitro ubiquitination assays

To prepare in vitro–ubiquitylated PCNA, 100 ng purified GST-PCNA or GST-$PCNA^{K164R}$ was incubated with 50 ng rabbit E1 ubiquitin–activating enzyme (EMD) and 350 ng hRAD18-RAD6 (prepared as described previously in Notenboom et al. [2007]) with or without 5 µg HA-tagged human ubiquitin (Boston Biochem) in ubiquitination reaction buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 2 µM $ZnCl_2$, 2 mM NaF, and 0.6 mM DTT) at 30°C for 1 h. The GST-tagged PCNA preparations were incubated with glutathione agarose at 4°C for 1 h and washed with 50 mM Tris, pH 7.4, 500 mM NaCl, and 1% NP-40 four times and once with ubiquitination reaction buffer.

For the in vitro FANCI and FANCD2 ubiquitylation assays, SFB-FANCL alone, SFB-FANCI alone, SFB-FANCD2 alone, or indicated combinations were transiently expressed in K562 cells. The cells were lysed in 2.0% NP-40, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 420 mM NaCl, and soluble proteins were purified using S protein agarose chromatography. Precipitates were washed four times in the lysis buffer, once with S peptide buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, and 150 mM NaCl), and eluted with 1 mg/ml S peptide in S peptide buffer. The purified SFB-tagged FANC proteins were mixed with or without 300 ng human recombinant UBE2T (Boston Biochem), 50 ng rabbit E1 ubiquitin–activating enzyme, 5 µg HA-tagged human ubiquitin (Boston Biochem), and the indicated in vitro–ubiquitinated PCNA preparations in ubiquitination reaction buffer, incubated at 30°C for 1 h, separated by SDS-PAGE, and immunoblotted with anti-HA antibody.

Online supplemental material

Fig. S1 shows that RAD18 is in the same pathway as FANCD2 and is required for ID ubiquitylation and chromatin binding. Fig. S2 shows that ubiquitylated PCNA and FANCD2 are in the same pathway and that FA pathway components interact with PCNA. Fig. S3 shows that ubiquitylated PCNA stimulates FANCL-mediated FANCD2 ubiquitylation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201005101/DC1.

We thank Dr. Yuichi Machida (Mayo Clinic, Rochester, MN) for providing FANCL cDNA, Dr. Toshiyasu Taniguchi (Fred Hutchinson Cancer Research Center, Seattle, WA) for providing FANCD2 cDNA, Dr. Titia Sixma for the RAD18–RAD6 *E. coli* expression vector, Dr. Weidong Wang for providing the anti-FANCL antiserum, Dr. Junjie Chen for the anti-PCNA rabbit antiserum, and members of the Karnitz Laboratory for helpful discussions.

This work was supported by the Ralph Wilson Medical Research Foundation and the Mayo Clinic.

Submitted: 19 May 2010
Accepted: 16 September 2010

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