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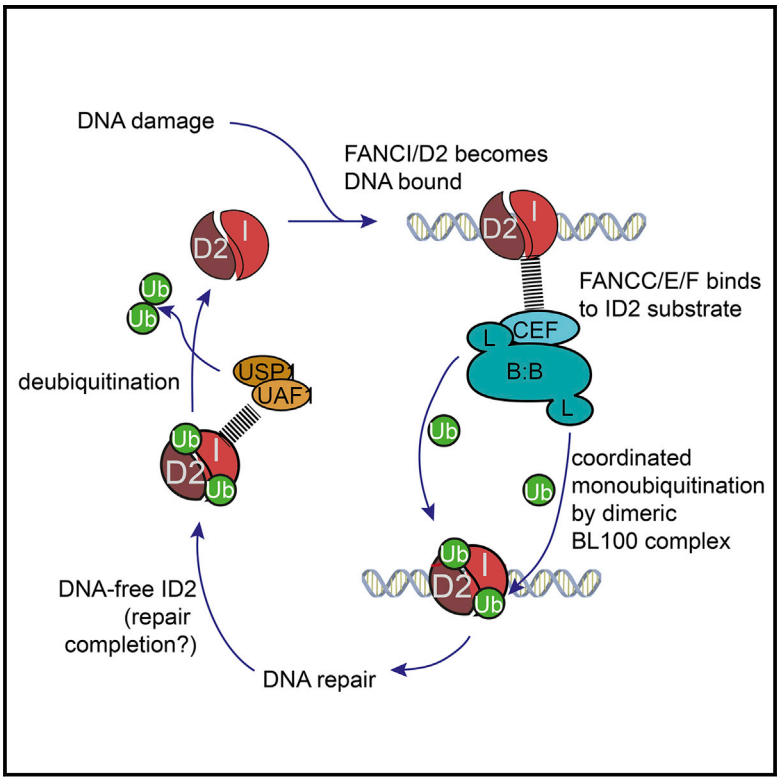
Molecular Cell

Mechanism of Ubiquitination and Deubiquitination in the Fanconi Anemia Pathway

Graphical Abstract



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In Brief

van Twest et al. report the biochemical reconstitution of FANCI:FANCD2 monoubiquitination by the Fanconi anemia core complex using only recombinant proteins. The authors uncover the mechanistic basis for temporal and spatial control of FANCD2:FANCI monoubiquitination that is critical for chemotherapy responses and prevention of Fanconi anemia.

Highlights

- Reconstitution of the Fanconi anemia (FA) pathway using recombinant proteins
- FANCB dimer coordinates FANCD2:FANCI monoubiquitination by two FANCL RING-ligases
- FANCC and FANCE provide FANCL specificity toward DNA-bound FANCD2:FANCI dimers
- Deubiquitination of FANCD2:FANCI by USP1:UAF1 occurs only when DNA is removed

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Mechanism of Ubiquitination and Deubiquitination in the Fanconi Anemia Pathway

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SUMMARY

Monoubiquitination and deubiquitination of FANCD2:FANCI heterodimer is central to DNA repair in a pathway that is defective in the cancer predisposition syndrome Fanconi anemia (FA). The "FA core complex" contains the RING-E3 ligase FANCL and seven other essential proteins that are mutated in various FA subtypes. Here, we purified recombinant FA core complex to reveal the function of these other proteins. The complex contains two spatially separate FANCL molecules that are dimerized by FANCB and FAAP100. FANCC and FANCE act as substrate receptors and restrict monoubiquitination to the FANCD2:FANCI heterodimer in only a DNA-bound form. FANCA and FANCG are dispensable for maximal in vitro ubiquitination. Finally, we show that the reversal of this reaction by the USP1:UAF1 deubiquitinase only occurs when DNA is disengaged. Our work reveals the mechanistic basis for temporal and spatial control of FANCD2:FANCI monoubiquitination that is critical for chemotherapy responses and prevention of Fanconi anemia.

INTRODUCTION

Fanconi anemia (FA) is a complex congenital disorder characterized by increased genome instability, early onset hematological presentations (including aplastic anemia, myelodysplastic syndrome, and acute myeloid leukemia), as well as embryonic developmental defects, and increased prevalence of some solid tumors (Walden and Deans, 2014). In particular, FA patient cells are extremely sensitive to chemotherapy or environmentally induced DNA interstrand crosslinks (ICLs) (Deans and West, 2011).

Central to the ICL sensitivity of FA is a defect in the monoubiquitination of FANCD2 protein that is required to recruit DNA repair proteins to the sites of stalled DNA replication (Ceccaldi et al., 2016). Most of the 19 different genes (*FANCA-FANCT*) that can be causative in FA are directly involved in the monoubiquitination reaction and the others participate up or downstream in the "FA pathway" (Wang and Smogorzewska, 2015). In particular, *FANCT* and *FANCL* gene products are the E2 and E3-RING ligase responsible for FANCD2 monoubiquitination (Meetei et al., 2003) and these proteins, together with an E1 enzyme and ubiquitin, are sufficient for low levels of in vitro FANCD2 monoubiquitination (Alpi et al., 2008). The structure of the FANCL:FANCT complex reveals details of the mechanism of E2 selectivity in this interaction (Hodson et al., 2014).

FANCL is a member of an "FA core complex" of proteins together with many other *FANC* gene products (Ceccaldi et al., 2016). The integrity of the FA core complex is critical for in vivo monoubiquitination of both FANCD2 and its heterodimeric partner FANCI (Smogorzewska et al., 2007). Deubiquitination of these proteins by the ubiquitin-specific protease USP1:UAF1 is also critical for ICL repair, and USP1 or UAF1 knockout causes an FA-like phenotype in mice (Kim et al., 2009; Nijman et al., 2005).

Importantly, FANCI and FANCD2 were confirmed as the only substrates of FA core complex in a mass spectrometry based screen (Renaudin et al., 2014), suggesting that this large protein assembly has only one essential function in cells. However, the absence of evolutionarily conserved domain motifs in core complex proteins other than FANCL has hampered our understanding of the biochemical role of these proteins within the complex. Various cellular experiments using overexpression systems, yeast-2-hybrid, and co-purification techniques have yielded some information about the critical interactions required for the integrity of the FA core complex (see reviews by Hodson and Walden, 2012; Walden and Deans, 2014). The described interactions are often contradictory, most probably because in patient-derived cell lines, the loss of FA core complex integrity leads to reduced expression of more than the mutated gene product (Meetei et al., 2004; Taniguchi and D'Andrea, 2002). There is, however, important genetic evidence for sub-complexes within the FA core complex (Huang et al., 2014). In particular, FANCB and FAAP100 subunits form a stable sub-complex with FANCL, that stimulates in vitro ubiquitination activity by ~5-fold over FANCL alone against FANCD2, but not FANCI (Rajendra et al., 2014).

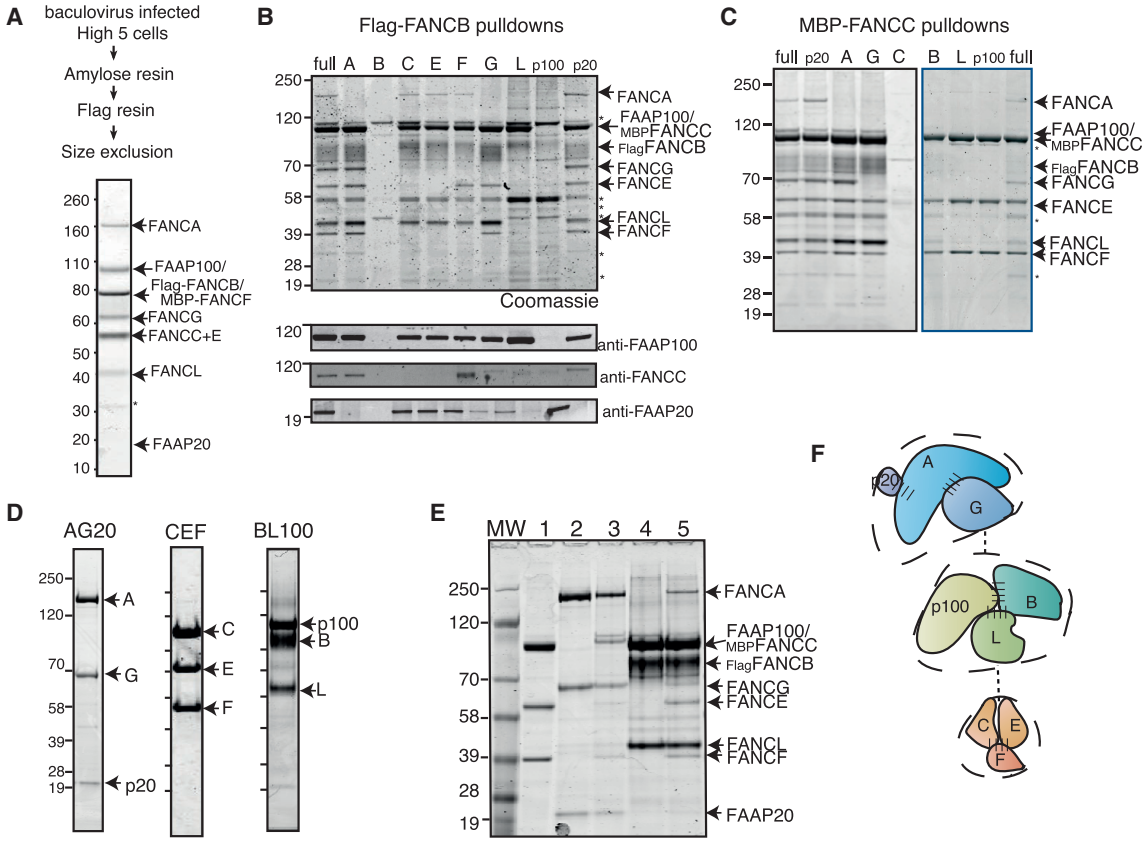

Figure 1. Subunit Composition and Protein:Protein Interactions within Recombinant FA Core Complex

(A) Schematic and Coomassie blue stained SDS-PAGE of purified recombinant FA core complex.

(B and C) Affinity tag purification of FA core complex using FLAG-FANCB (B) or MBP-FANCC (C) as the bait protein. In each lane, one FA gene has been omitted from the expression vector (A = FANCA, B = FANCB, C = FANCC, E = FANCE, F = FANCF, G = FANCG, L = FANCL, p100 = FAAP100, and p20 = FAAP20). The asterisks represent contaminant proteins that bind to affinity resin used. Western blots for FANCC and FAAP100 are shown as the two proteins run at identical position on SDS-PAGE gel, and FAAP20 cannot be clearly seen on Coomassie gels so it is also shown by western blotting.

(D) Coomassie blue stained SDS-PAGE of purified recombinant sub-complexes of FA core complex: AG20 = FANCA, FANCG, and FAAP20; CEF = FANCC, FANCE, and FANCF; and BL100 = FANCB, FANCL, and FAAP100.

(E) Reconstitution of full FA core complex by adding purified CEF (lane 1) and purified AG20 (lane 2) to FLAG-bead immobilized BL100. The flowthrough from the reaction (lane 3) and elutions without (lane 4) or with (lane 5) CEF and AG20 show that the recombinant core complex can be reconstituted from purified sub-complexes.

(F) Summary of the inferred direct protein:protein interactions within the FA core complex, based upon the results shown in (B)–(E). The dashed lines indicate groupings of sub-complexes, while the triple lines indicate putative direct protein interactions.

See also Figure S1.

Here, we provide biochemical evidence for the existence of three discrete sub-complexes that can be assembled into an active 9-protein FA core complex in vitro. We show that the FANCB:FAAP100:FANCL complex forms a dimeric center within the FA core complex where FANCB provides a template for the spatial arrangement of two FANCL molecules. A second sub-complex containing FANCC, FANCE, and FANCF bridges an interaction between the FANCB:FAAP100:FANCL complex and both FANCI and FANCD2 to catalyze the coordinated ubiquitination of both proteins in the dimer. Surprisingly, a third sub-complex of FANCA, FANCG, and FAAP20 can also bind stoichiometrically to the FANCB:FAAP100:FANCL complex, but is not required for in vitro ubiquitination. Finally, we show that the substrate must be DNA-bound for ubiquitination to occur, and that, unexpectedly, DNA binding also protects the complex from USP1:UAF1 mediated deubiquitination.

RESULTS

Purification of Recombinant FA Core Complex

In order to understand the assembly of the FA core complex, we co-expressed the nine constituent proteins (FANC-A, FANC-B, FANC-C, FANC-E, FANC-F, FANC-G, FANC-L, and FAAP20 and FAAP100) using the Multibac baculovirus expression system. A purification scheme was used to yield highly pure FA core complex that is homogeneous in mass (Figure 1A). Western blotting and mass spectrometry (MS) analysis revealed the presence of all subunits in the final purified complex (Figure S1).

To biochemically define interactions within the FA core complex, we next modified our Multibac expression vector system by omitting individual proteins from the complex. The affinity tags on FANCB or FANCC were then used to interrogate the assembly of the remaining constituents (Figures 1B and 1C). Using this approach, it is possible to infer direct interactions by showing which proteins are absent from FANCB and/or FANCC pull-downs. First, it is apparent that the FANCB, FANCL, and FAAP100 proteins are critical central components of the FA core complex. Many interactions are lost or destabilized when either of these proteins is lacking. For example, complexes lacking FAAP100 retain interactions between FANCB and FANCL (Figure 1B, lane p100), and between FANCC, FANCE, and FANCF (Figure 1C, lane p100), but FANCA, FANCG, and FAAP20 are no longer associated with the remainder of the complex. Second, the data reveal that FANCC and FANCE only participate in core complex formation when the other is also present (Figures 1B and 1C, lanes C and E). Third, a surprising observation is that an absence of FANCA, the most commonly mutated FA gene product (approx. 64% cases; Yuan et al., 2012), does not affect any of the other interactions within the complex, except for the inclusion of FAAP20 (Figures 1C and 1D, lane A). Despite this, FAAP20 deficient complexes ("p20" in Figures 1C and 1D) retain all other subunits, indicating that FAAP20 is not critical for FA core complex assembly in vitro and the same is observed for FANCF.

Reconstitution of the FA Core Complex from Three Sub-complexes

The ratio of subunits within the complex supports recent genetic evidence for sub-complexes—or modularity—within the FA core complex (Hodson and Walden, 2012; Huang et al., 2014). We noticed throughout the purification process that certain subunits often co-eluted in fractions that did not contain all proteins of the FA core complex. The subunits within these sub-complexes supported the interaction experiments outlined in Figure 1 and corresponded to the three sub-complexes proposed using genetic experiments (Huang et al., 2014). These are sub-complex AG20, consisting of FANCA, FANCG, and FAAP20; sub-complex BL100, consisting of FANCB, FANCL, and FAAP100; and sub-complex CEF, consisting of FANCC, FANCE, and FANCF. Based on these considerations, we separately co-expressed only the subunits of each of these sub-complexes. Remarkably, each sub-complex was stable and amenable to biochemical investigation (Figure 1D). Furthermore, the full FA core complex could be reconstituted in vitro from the independently purified sub-complexes (Figure 1E). This observation allowed us to ask which of the sub-complexes is integral to the ubiquitination reaction that is catalyzed by the E3-RING ligase FANCL and the E2 enzyme FANCT/UBE2T. A model for the direct interactions supported by our co-elution studies is presented in Figure 1F.

Using recombinant DNA-bound *Xenopus laevis* FANCD2:FANCI heterodimer as a substrate, we observed specific monoubiquitination activity of the BL100 sub-complex toward K521 of FANCD2 and, to a much lesser extent, on K565 of FANCI (Figures 2A and S2A). Ubiquitination of FANCD2 was appreciably faster than for reactions utilizing only FANCL purified in isolation (Figure S2B). Both the rate of ubiquitination and the maximum ubiquitination level achieved were substantially enhanced by the addition of CEF to BL100 (Figure 2B). In contrast to the results seen with addition of CEF, AG20 did not seem to be required for the stimulation of the in vitro ubiquitination reaction (Figure 2C).

In total, the BL100 and CEF complexes together showed approximately 250×-increased activity in monoubiquitination reactions compared to FANCL alone (Figure 2D). The most striking enhancement of ubiquitination activity by CEF was seen for the FANCI component of the heterodimer, although the level of ubiquitination plateaued at 4 hr at about 25% for FANCI rather than at ~100% for FANCD2. The lower levels of FANCI ubiquitination than FANCD2 is consistent with previous in vivo observations (Smogorzewska et al., 2007) and may be linked to a requirement for phosphorylation on FANCI (Ishiai et al., 2008). We observed that both FANCD2 and FANCI had a mixed phosphorylation status upon purification (see MS data; Figure S3); but found that λ-phosphatase treatment of ID2 had only a marginal effect on its ability to be in vitro monoubiquitinated (Figures S3A and S3B). Further, artificial phosphorylation of conserved SQ/TQ sites using recombinant ATM/ATR-family kinase DNA-PKcs did not lead to increased levels of FANCI monoubiquitination (Figures S3C and S3D).

A previous study of the DNA binding preferences of recombinant mouse ID2 complex revealed higher affinity for branched DNA molecules (Joo et al., 2011). As several groups have shown DNA binding by ID2 is required for its monoubiquitination both in cells and in vitro, we tested a panel of highly purified DNA structures for their ability to stimulate the reconstituted monoubiquitination reaction. Single-stranded (ss)DNA molecules, for which ID2 has the lowest affinity (Joo et al., 2011) showed either no ability (in the case of oligo-dT) or a relatively weak 1.8-fold ability (60-mer, 58% GC content) to stimulate FANCD2 monoubiquitination compared to no DNA. In contrast, the presence of equimolar junction containing molecules such as splayed arms and 5′-flaps increased FANCD2 monoubiquitination by 6- to 7-fold (similar to plasmid DNA), while 3′-flap and replication fork mimics molecules increase the ubiquitination by up to 12-fold. Somewhat surprisingly, a double-stranded (ds)DNA oligo-based DNA structure with no known junctions could also enhance the reaction, suggesting that free dsDNA ends may be enough to stimulate loading of ID2 and its consequent monoubiquitination by the FA core complex (Figure 2E).

BL100 Complex Is a Dimer Required to Stimulate Ubiquitination of a Dimeric Substrate

Based on the central role for the FANCB:FAAP100:FANCL subunits for FANCD2 ubiquitination and for the integrity of the FA core complex, we analyzed this sub-complex in more detail. Using size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS), we found that the BL100 complex has a molecular mass of 465.5 kDa. This is equal to 197% of the sum of the predicted molecular mass of a 1:1:1 complex. Given the quantified stoichiometry of the subunits is equal based on PAGE, our data show that the BL100 sub-complex contains two copies each of FANCB, FANCL, and FAAP100 (Figure 3A; Table S1).

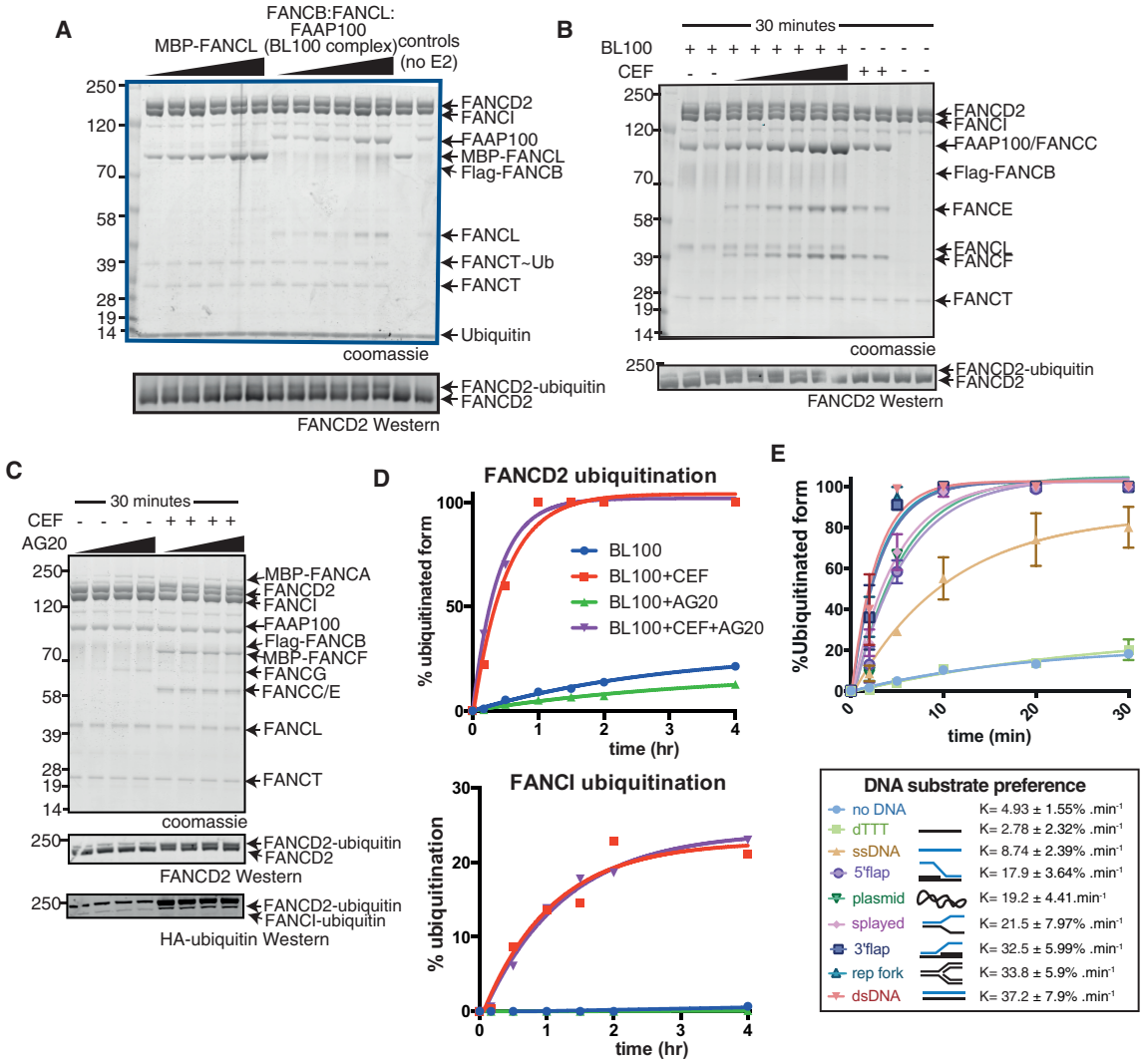

Figure 2. Sub-complexes within the FA Core Complex Stimulate Monoubiquitination of FANCD2 and FANCI

(A) Comparison of FANCD2 ubiquitination with increasing concentrations of MBP-FANCL or equimolar concentrations of BL100 in a 30 min reaction. The FANCD2 ubiquitination is observed on Coomassie blue stained gels or by anti-FANCD2 western blot. The controls including no FANCT for either reaction are also shown.

(B) Comparison of FANCD2 ubiquitination (as above) by BL100, with the addition of increasing concentrations of CEF complex. The controls including only CEF or no additional proteins are shown.

(C) Comparison of FANCD2 and FANCI ubiquitination (as above) by BL100 or BL100 and CEF, with the addition of increasing concentrations of AG20 complex.

(D) Summary of 4 hr time course experiments using combinations of BL100, CEF, and AG20 complexes. The percentage of ubiquitinated protein was calculated from anti-StrepII (FANCD2) or anti-FLAG (FANCI) western blots in a representative experiment.

(E) Summary of 30 min FANCD2 monoubiquitination time course experiments using ID2, BL100, CEF, and different purified DNA substrates (see Experimental Procedures). The points are shown as mean ± SE (n = 2) reaction constant for one-phase accumulation of monoubiquitinated FANCD2 = K (mean ± SE, with 10 degrees of freedom calculated from graphs shown).

See also Figures S2 and S3.

FANCB Is Critical for Dimer Formation in the FA Core Complex

Multimerization of RING-E3 ligases is common in ubiquitination regulation, and many dimerize through the RING domain directly. Although we could express and purify MBP-tagged FANCL, SEC of the protein resulted in void volume elution even in the presence of mild-solubilizing factors (Figure S4). This suggests that FANCL on its own is prone to formation of soluble aggregates.

In contrast, we could purify a stable FANCB and FAAP100 complex that lacked the FANCL component and did not aggregate. This B100 complex eluted only slightly later than the intact BL100 complex on a Superdex 200 column, corresponding to the predicted mass of a hetero-tetramer (Figure S5). As FANCB and FAAP100 were sufficient for the formation of a dimeric core, this argues against RING-domain mediated dimerization of the BL100 components, such as that observed for the RNF4

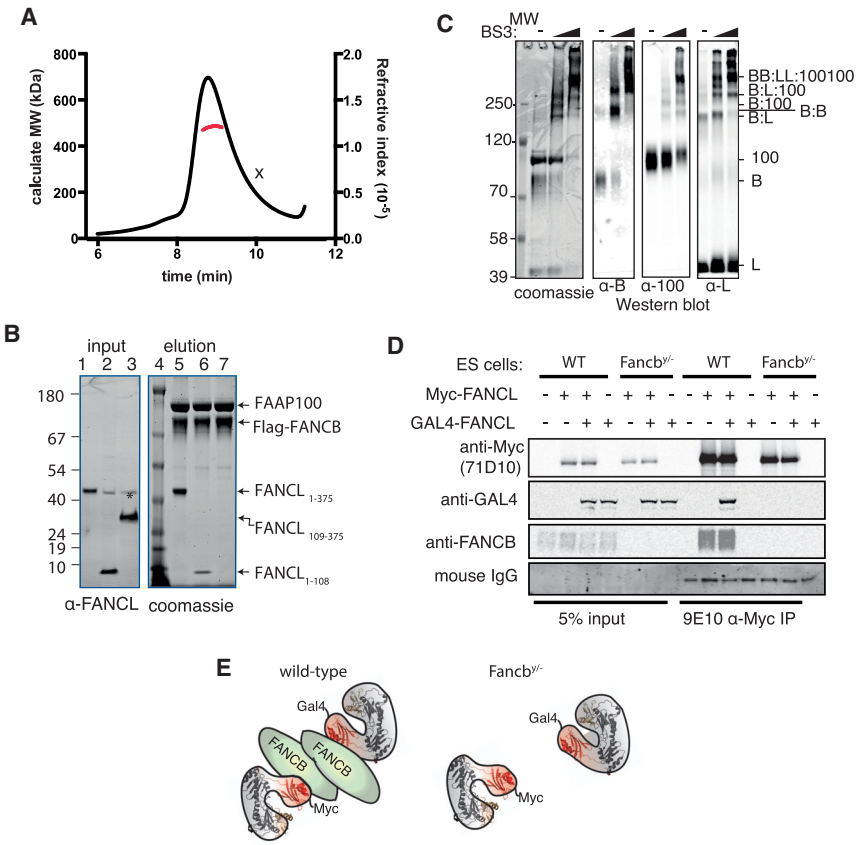

Figure 3. The FANCB: FANCL: FAAP100 Complex Is Dimeric, Mediated by Homodimerization of FANCB

(A) SEC-MALS was used to determine the mass of recombinant BL100 complex. The refractive index (black line, right axis) was used to measure the protein concentration, and the calculated molecular weight (red line, left axis) was determined by MALS (see Experimental Procedures). x marks the location of peak and mass if 1:1:1 complex was present.

(B) FLAG-FANCB pull-downs when co-expressed with FAAP100 and full-length FANCL (1–375, lane 5), FANCL-ELF domain (1–108, lane 6), or FANCL DRWD-RING domain (109–375, lane 7). The lanes 1–3 show western blots of input extracts with polyclonal anti-FANCL antibody, while lanes 5–7 show elutions from FLAG-FANCB complex purifications. MW markers are shown in lane 4.

(C) Protein interactions within the BL100 complex were determined by mild crosslinking with increasing concentrations of BS3. Coomassie blue stained gels and overlayed western blots with specific antibodies reveal crosslinked interactions between the indicated components.

(D) Anti-Myc (mouse 9E10) immunoprecipitates from Myc-FANCL and GAL4-FANCL transfected or control transfected wild-type or Fancb-deficient ESCs, followed by western blot with the indicated antibodies.

(E) Schematic delineates data shown in (A)–(D). The FANCB is shown in green, and the FANCL-ELF domain is highlighted by red tint.

See also Figures S4 and S5 and Table S1.

ubiquitin ligase (Plechanovová et al., 2011). Further support for this conclusion comes from the observation that the N-terminal 108 residues of FANCL, containing its ELF (E2-like fold) domain, are sufficient for binding to the B100 complex and that a C-terminal RING-domain containing fragment is unable to participate in complex formation (Figure 3B). Together, these results support a conclusion that the dimeric FANCB: FAAP100 subunits form a scaffold to simultaneously orient in space two FANCL RING E3 ligases.

To identify which components of the BL100 sub-complex are directly involved in its dimerization, we used the homobifunctional NHS crosslinker BS3 for mild-crosslinking of the BL100 complex. Discrete bands running slower than the individual subunits represent crosslinked products (Figure 3C). By western blotting with specific antibodies, these bands correspond to FANCB:FANCL, FANCB:FAAP100, and FANCB:FANCB crosslinked products. It should also be noted that we observed no FANCL:FANCL crosslinked products, consistent with the conclusion that BL100 dimerization does not involve the FANCL RING domain.

Finally, to confirm that FANCL is part of a higher order complex organized by FANCB within cells, we co-expressed Myc- and GAL4-tagged FANCL in mouse embryonic stem cells (ESCs) and performed co-immunoprecipitation assays. An anti-Myc antibody could immunoprecipitate Myc-FANCL and GAL4-FANCL from wild-type extracts, confirming that higher order FANCL complex formation is not an artifact of recombinant protein production (Figure 3D). However, performing the same co-immunoprecipitation in Fancb-deficient ESCs lead to loss of the interaction between differently tagged FANCL molecules, and only Myc-FANCL was found in immunoprecipitates (Figures 3D and 3E).

From these biochemistry and cell-based findings, we conclude that FANCB is the protein within the FA core complex that drives dimerization, and that each FANCB monomer interacts independently with one molecule each of FANCL and FAAP100.

CEF Coordinates ID2 Ubiquitination by the BL100 Dimer

Given that both the BL100 complex and its key substrate ID2 form dimers, we tested whether the dimeric BL100 complex preferred dimeric ID2 as a substrate over monomeric FANCI or FANCD2 purified in isolation. In contrast to assays with dimeric ID2 substrate (Figure 2D), neither monomeric FANCI nor FANCD2 were appreciably ubiquitinated by BL100. In addition, the stimulatory effect of CEF on BL100 activity (Figure 2D) was also completely absent when using monomeric substrates (Figure 4A). This supports the conclusion that monoubiquitination of ID2 occurs predominantly in the context of the heterodimer (Longerich et al., 2014), and that the dimeric nature of BL100 enables the coordinated monoubiquitination of both FANCI and FANCD2.

As the CEF complex appears to stimulate the catalytic activity of BL100 complex only when the substrate is a heterodimer,

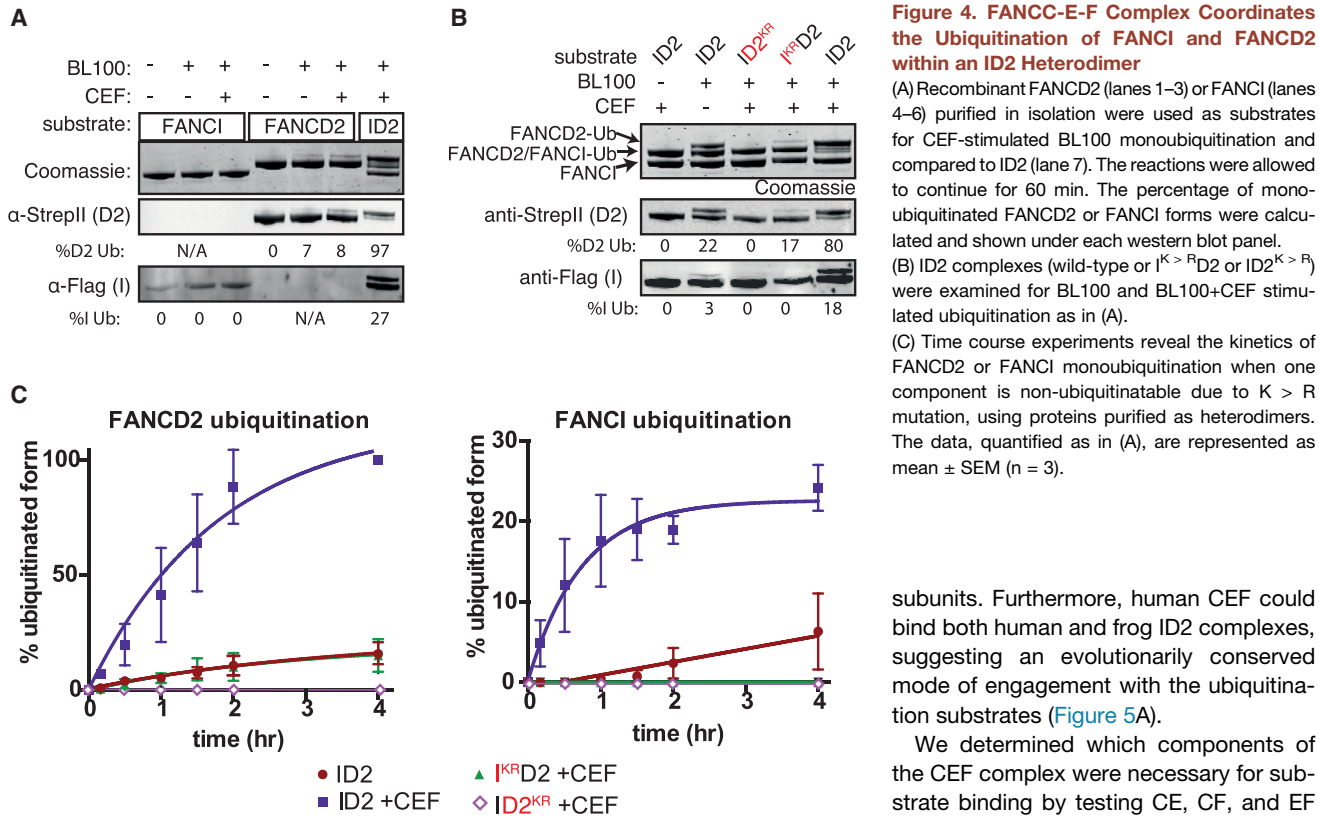

Figure 4. FANCC-E-F Complex Coordinates the Ubiquitination of FANCI and FANCD2 within an ID2 Heterodimer

(A) Recombinant FANCD2 (lanes 1–3) or FANCI (lanes 4–6) purified in isolation were used as substrates for CEF-stimulated BL100 monoubiquitination and compared to ID2 (lane 7). The reactions were allowed to continue for 60 min. The percentage of monoubiquitinated FANCD2 or FANCI forms were calculated and shown under each western blot panel.

(B) ID2 complexes (wild-type or $I^{K>R}D2$ or $ID2^{K>R}$) were examined for BL100 and BL100+CEF stimulated ubiquitination as in (A).

(C) Time course experiments reveal the kinetics of FANCD2 or FANCI monoubiquitination when one component is non-ubiquitinatable due to K > R mutation, using proteins purified as heterodimers. The data, quantified as in (A), are represented as mean ± SEM (n = 3).

there is a possibility that it is involved in coordinating the monoubiquitination of both substrate proteins. To test this hypothesis, we used heterodimeric ID2 complex in which only one protein could act as a monoubiquitination substrate because the acceptor lysine of the respective other protein was mutated to arginine. In a reaction containing a non-ubiquitinatable $FANCI^{K>R}$ mutant, FANCD2 could be ubiquitinated by BL100, but the stimulation of the reaction by CEF complex was abolished (Figure 4B). The complete absence of FANCI monoubiquitination in the presence of non-ubiquitinatable $FANCD2^{K>R}$ suggests that FANCD2 monoubiquitination must precede FANCI monoubiquitination (Figures 4B and 4C). Together, these results suggest that the CEF complex can act as a sensor of FANCD2 monoubiquitination to stimulate the monoubiquitination of FANCI and drive further coordinated monoubiquitination of both members of the ID2 heterodimer.

FANCC and FANCE Are Substrate Adaptors for the Ubiquitination of ID2

One possible mechanism by which the CEF complex could stimulate BL100 activity would be by more efficiently recruiting ID2 substrate to the core complex. To investigate this hypothesis, we looked for direct protein:protein interactions between the CEF complex and ID2. Strikingly, we discovered that stable complexes of CEF and ID2 could be purified from baculovirus transduced cells expressing both complexes (Figure 5A). The CEF complex could bind FANCI and FANCD2 individually, suggesting the complex makes independent contact with both subunits. Furthermore, human CEF could bind both human and frog ID2 complexes, suggesting an evolutionarily conserved mode of engagement with the ubiquitination substrates (Figure 5A).

We determined which components of the CEF complex were necessary for substrate binding by testing CE, CF, and EF complexes for ID2 interaction. Pull-downs showed that only the CE complex could engage the ID2 heterodimer to the same extent as CEF. Therefore, FANCF is not critical for ID2 engagement (Figure 5B). We further found that FANCC:FANCE, but not FANCF are required for the stimulation of ubiquitination of ID2 by BL100 (Figure 5C). We did, however, observe consistently lower yields of recovered CE complex compared to CEF, suggesting that FANCF plays a role in stabilizing this protein complex.

As CEF binds both BL100 and ID2, our experiments indicate that the CEF complex is a molecular bridge between substrate and enzyme, required to greatly stimulate the ubiquitination reaction. As FANCC and FANCE mutations account for approximately 13% of FA patients, we explored several disease-associated variants (amino acid substitutions or small deletions) in these proteins for their effect on the ubiquitination reaction. Surprisingly, the FANCC and FANCE mutants that we tested could all form an intact FA core complex (Figure 5D). However, all failed to stimulate ID2 monoubiquitination beyond what was observed for BL100 only reactions (Figure 5E). From these experiments, we conclude that the CEF complex is a substrate adaptor for the FA core complex, and that FA disease pathology in patients with absent or abrogated FANCC and FANCE results from loss of substrate engagement (Figure 5F).

Core Complex Components Do Not Prevent Deubiquitination of ID2 by USP1:UAF1

Our biochemical investigation of the FA core complex suggests a rapid ID2 monoubiquitination reaction that proceeds in the absence of the replication machinery or specific DNA structures.

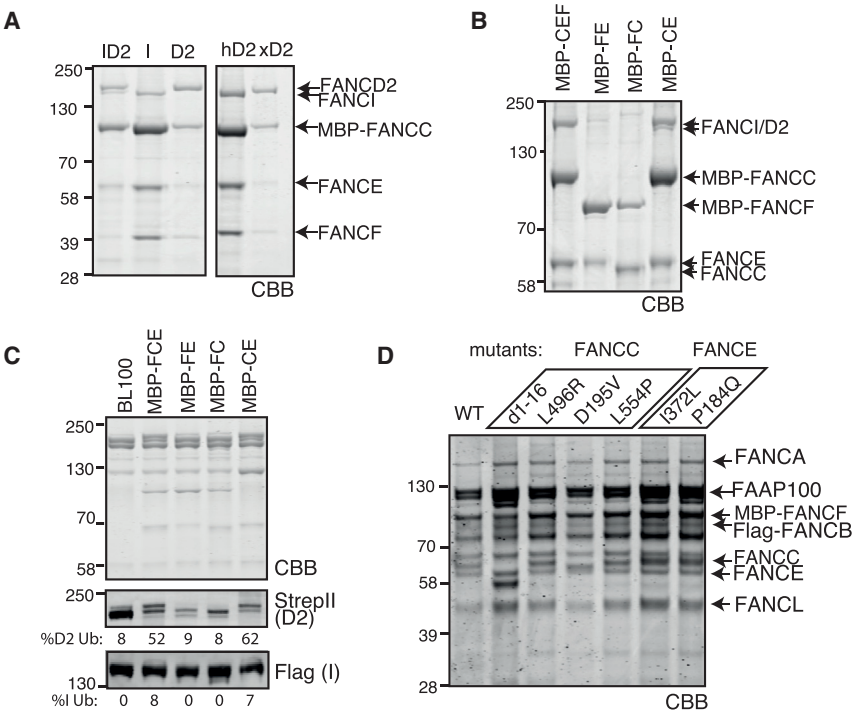

Figure 5. FANCC and FANCE Link the BL100 Complex to Substrate Binding

(A) MBP-CEF was co-expressed with FANCI, FANCD2, or ID2 complex and purified on amylose affinity resin (left). Also shown is the same experiment using human or *Xenopus* FANCD2 (right).

(B) MBP-FE, MBP-FC, or MBP-CF were used as capture complexes as per (A).

(C) ID2 complexes were examined for BL100 and BL100 + FE, FC, or CE stimulated monoubiquitination and compared to FCE. The percentage of monoubiquitinated FANCD2 or FANCI forms were calculated using western blots and ImageJ and shown under each western blot panel.

(D) FLAG-affinity purification via FLAG-FANCB of FA core complexes containing patient-associated FANCC or FANCE mutant proteins. All FA core complex components are present in all lanes.

(E) Mutant CEF complexes were examined for monoubiquitination of ID2 as per (C). The quantification of FANCD2 monoubiquitination is shown below each lane.

(F) Summary of data. CEF complex brings together the substrate ID2 and catalytic module BL100 to stimulate the reaction. The dotted lines indicate direct protein:protein interactions. The patient mutations in FANCC or E do not inhibit BL100 binding, but fail to act as substrate adaptors for ID2.

But in cells, ID2 deubiquitination is faster than the monoubiquitination reaction and is hypothesized to be involved at the conclusion of repair to switch off the FA pathway (Kim et al., 2009; Oestergaard et al., 2007; Yang et al., 2011). In cells, the ubiquitin-specific protease USP1 is targeted to ID2 by its heterodimeric partner UAF1, through an interaction with a SIM domain in the FANCI subunit (Yang et al., 2011). Our biochemical reconstitution of the monoubiquitination reaction allows us to answer a number of outstanding questions about its interaction with the deubiquitination reaction.

First, we showed that recombinant USP1:UAF1 can promote immediate deubiquitination activity against FANCD2 within the ID2 complex. USP1 alone was almost completely devoid of this activity, confirming previous cell-based experiments that indicated UAF1 targets ID2 to the ubiquitin protease (Figure 6A). Second, in order to understand how deubiquitination might be influenced by components of the FA core complex, we added USP1:UAF1 to our in vitro ubiquitination reactions. Efficient ubiquitination was prevented or reversed when USP1:UAF1 was added in the presence of BL100, BL100-CEF, or the full FA core complex (BL100-CEF-AG20) either during or after the reaction had reached maximum activity (Figure 6B). This result suggests that no component of the FA core complex can act as a direct antagonist of FANCD2 deubiquitination. However, USP1:UAF1 activity against ubiquitinated FANCI within the ID2 complex was considerably slower than for FANCD2 (Figure 6B). Furthermore, on closer analysis of extended time points, it was apparent that the ubiquitinated FANCD2 form never completely disappeared upon addition of USP1:UAF1 (Figure 6A). Time course experiments showed that, after the addition of USP1:UAF1, FANCD2 was rapidly deubiquitinated, but only until the ratio of ubiquitinated FANCD2: ubiquitinated FANCI approaches 1:1. Although FANCI also slowly started to become deubiquitinated at late time points, the ratio between ubiquitinated subunits continued to remain constant at 1:1 throughout the experiment (Figures 6C and S5). This indicates that the level of FANCI monoubiquitination inversely determines the level of FANCD2 deubiquitination by USP1:UAF1.

The Di-monoubiquitinated ID2 Complex Is Protected from Deubiquitination by USP1:UAF1

To test the hypothesis that USP1:UAF1 cannot act on ubiquitinated FANCI, we demonstrate that bead-immobilized catalytically inactive USP1(C90S):UAF1 can only bind the non-ubiquitinated form of FANCI (Figure 6D). Complexes where only FANCD2, and not FANCI, is ubiquitinated are bound by USP1:UAF1, as is the ID2 complex when neither protein is monoubiquitinated (Figure 6D). In the absence of any cellular factors, this finding suggests that a conformational change must occur in the ID2 complex when FANCI is monoubiquitinated. This shape change precludes the UAF1 binding site on FANCI, to prevent deubiquitination of either FANCI or FANCD2.

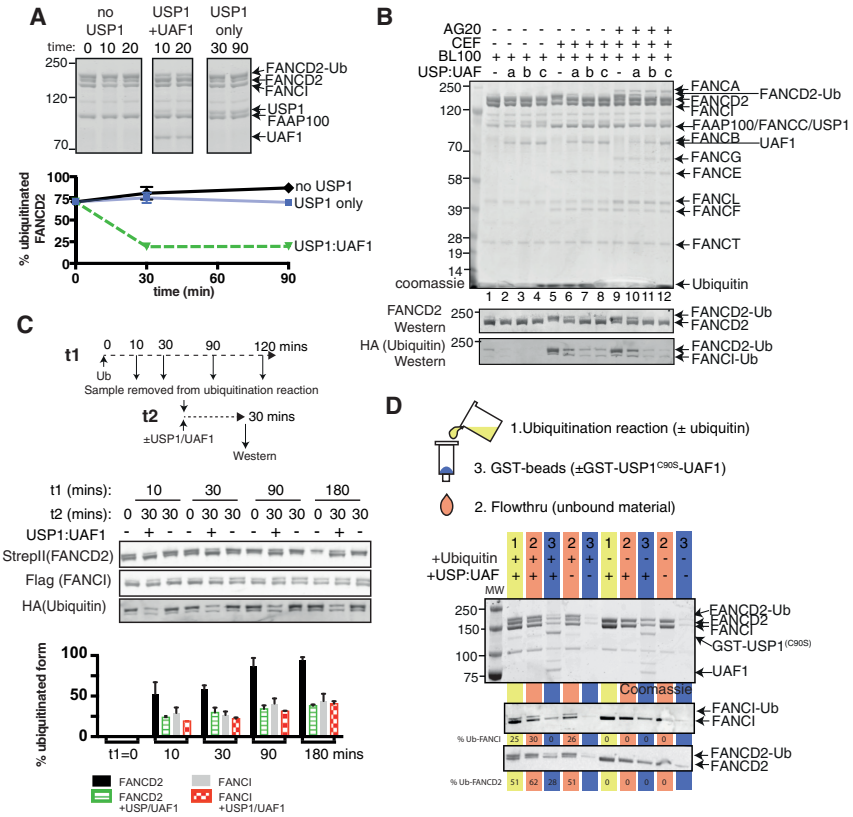

Figure 6. USP1:UAF1 Catalyzes Rapid Deubiquitination of FANCD2, but Not FANCI, within a DNA-Bound ID2 Heterodimer

(A) Completed monoubiquitination reactions (60 mins) were then incubated with USP1 or USP1:UAF1 for the indicated times and the percentage of monoubiquitinated FANCD2 calculated. (n = 3, mean ± SE).

(B) USP1:UAF was added at the beginning of the monoubiquitination reaction (lanes marked a), or at 30 min (lanes marked b), or 60 min (lanes marked c) of a 70 min ubiquitination reaction.

(C) Deubiquitination was measured in the absence of new additional ubiquitination, by desalting removal of ATP prior to addition of USP1:UAF1. The experimental detail is shown, and the percentage of monoubiquitinated FANCD2 and FANCI is shown as a graph to the right (n = 3, mean ± SE).

(D) FANCI:FANCD2 containing a mixture of monoubiquitinated, non-ubiquitinated, and di-ubiquitinated complexes was incubated with catalytically inactive USP1$^{(C90S\ G670A/G671A)}$:UAF1 immobilized on Glutathione beads. The percentage of monoubiquitinated and unubiquitinated FANCD2 and FANCI in input, flowthrough, and elutions was calculated.

See also Figures S6 and S7.

This co-regulation occurs because of FANCI:UAF1 interaction, because FANCD2-Ub alone cannot be bound and deubiquitinated by USP1:UAF1 if FANCI is absent from the reaction (Figure S7).

Removal of DNA Allows Deubiquitination of both FANCI and FANCD2

Our data that suggest the FA core and USP1:UAF complexes work together to achieve a simultaneous di-monoubiquitinated "on state" equilibrium (where both FANCI and FANCD2 are monoubiquitinated) or an "off-state" (where neither protein is monoubiquitinated). Given that DNA binding by ID2 is critical for monoubiquitination to occur (Longerich et al., 2014; Sato et al., 2012; Figure 2), we predicted that DNA might also regulate ID2 deubiquitination. To test this, the pan-nuclease Benzonase was added to the combined ubiquitination and/or deubiquitination reactions together with USP1:UAF1. In these assays, removal of DNA stimulated efficient deubiquitination of FANCI (Figure 7A). The deubiquitination activity of USP1:UAF1 in the absence of DNA also proceeded at a continuous rate toward zero for FANCD2 and at a slower rate against FANCI (Figure 7B). Furthermore, unlike in previous experiments with intact DNA, the di-monoubiquitinated ID2 complex became completely deubiquitinated when DNA was degraded, irrespective of the level of FANCI-Ub at the time of USP1:UAF1 addition (Figure 7B). For these experiments, the ubiquitination reaction was inhibited prior to USP1:UAF1 addition by depletion of ATP. Even after 90 min in the presence of the USP1:UAF1 protease, ID2 is stably maintained in a di-monoubiquitinated form, but it is then processed to non-ubiquitinated form in the time following DNA removal (Figure 7C). The kinetics of FANCI deubiquitination is considerably slower than for FANCD2, but the results demonstrate that the USP1:UAF1 protease can act on both members of di-monoubiquitinated-ID2, but only when it has disengaged from bound DNA.

DISCUSSION

In this study, we used biochemical reconstitution experiments to examine assembly and activity of the FA core complex and investigated the function of each subunit in the highly specific monoubiquitination of FANCI:FANCD2 substrates. As the cycle of FANCD2 monoubiquitination and deubiquitination is critical to the successful repair of DNA interstrand crosslinks, our findings have important mechanistic, biological, and clinical implications for our understanding of ICL repair and the genetic disorder Fanconi anemia.

First, we found that the FANCB and FAAP100 proteins sit at the center of the FA core complex, where they coordinate two FANCL E3-RING ligases. A parallel study (Swuec et al., 2017) reveals the architectural placement of these two FANCL molecules in the correct position for coordinated monoubiquitination of both members of the ID2 substrate by one BL100 assembly. Together, these findings provide a biochemical basis for previous findings in FANCI or FANCD2-patient cells, where monoubiquitination of the remaining intact member of this dimer was also defective (Smogorzewska et al., 2007). We also observed that FANCI or FANCD2 alone are extremely poor in vitro substrates

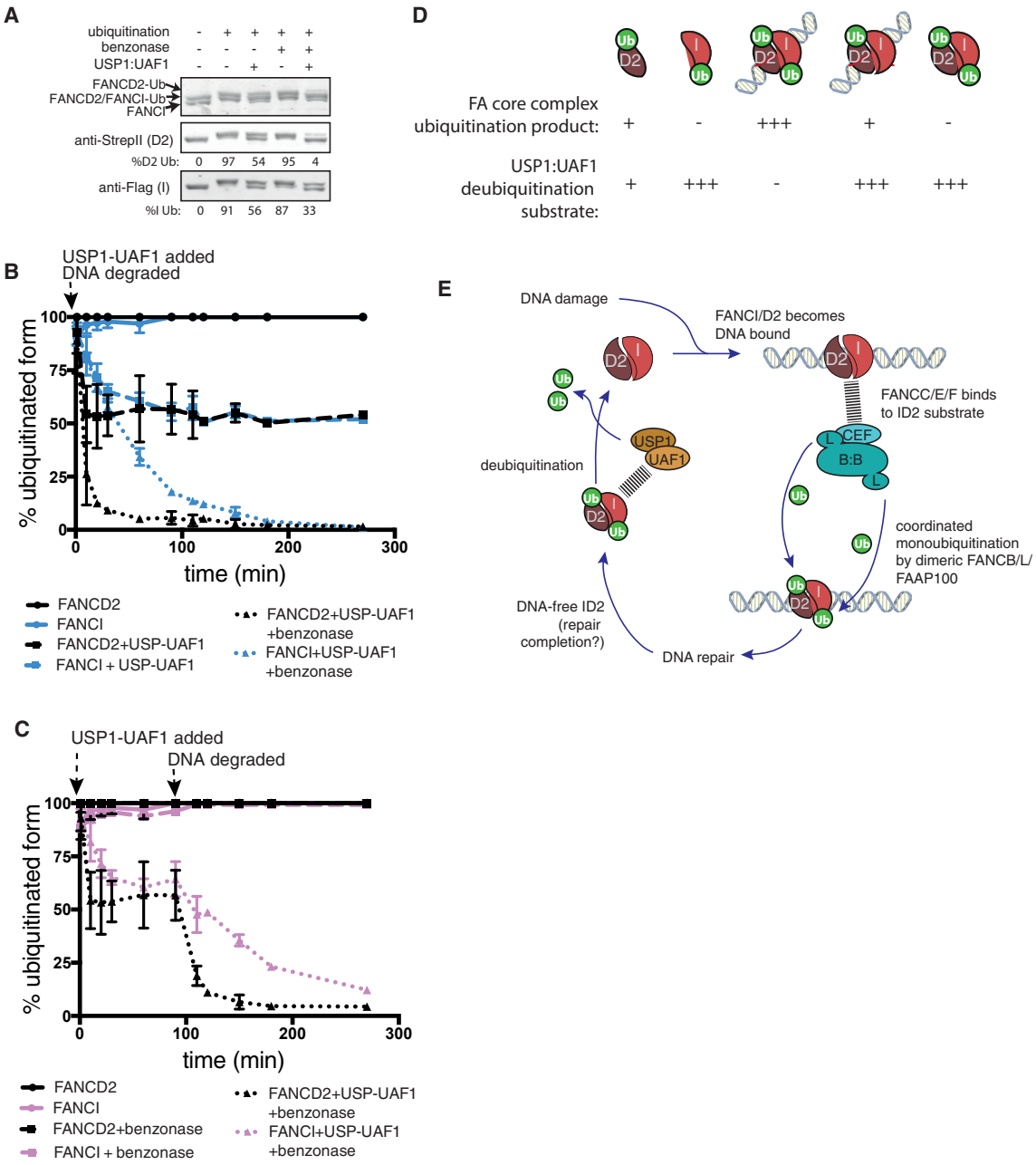

Figure 7. Removal of DNA Leads to Rapid Deubiquitination of FANCD2 and FANCI within the ID2 Heterodimer

(A) The pan-nuclease Benzonase was added to di-monoubiquitinated FANCI:FANCD2 complex ± USP1:UAF1.

(B and C) A time course following deubiquitination of FANCI and FANCD2 when Benzonase is added simultaneously with USP1:UAF1 (B) or after 90 min (C). The % ubiquitinated forms of FANCD2 and FANCI were quantified using western blots with anti-FLAG and anti-StrepII over a time course after reaction additions.

(D) Schematic of how the reciprocal substrate preference of the FA core complex and the USP1:UAF1 deubiquitinase enzyme control promotion of ubiquitination on DNA bound ID2 complex and deubiquitination on all other heterodimer or monomer forms.

(E) Overall model for how the FA core complex ubiquitin ligase and USP1:UAF1 ubiquitin hydrolase regulate the monoubiquitination of FANCI and FANCD2.

for the FA core complex, while the DNA-bound ID2 heterodimer can be ubiquitinated with 100% efficiency in a very short time frame. In addition, much of the FANCL-substrate promiscuity seen in previous studies that used FANCL purified in isolation is absent from the FA core complex reactions. In particular, other studies demonstrated high levels of FANCL autoubiquitination that is completely absent when using our intact FA core complex (Alpi et al., 2008; Sato et al., 2012; Figures 2, 3, 4, and 5). Likewise, we observed no in vitro activity of the intact core complex against another purported substrate FANCL substrate,

beta-catenin (Dao et al., 2012) (data not shown). We propose that the structural arrangement of the BL100 complex places the catalytic domains of the proteins in exactly the correct position for accessing a single lysine on each of FANCI and FANCD2 in the ID2 substrate. This arrangement precludes non-specific activity, but promotes coordinated ubiquitination activity necessary for activating the ICL repair pathway.

The FANCB subunit is one of the least studied subunits of the FA pathway, but we show that it forms the critical dimerization interface of the FA core complex. *FANCB* is X-linked and, like *FANCL*, its mutation is rare in FA, but associated with a more severe form that includes VACTERL-type developmental abnormalities (Holden et al., 2006; Vetro et al., 2015). The overlapping severe phenotypes (that are also seen in some *FANCD2* and *FANCI* patients) point to the most critical role of FANCB and FANCL at the center of the ubiquitination reaction. Our finding that FANCL binds FANCB through its ELF domain has important implications for understanding how the BL100 complex coordinates ubiquitination of both FANCI and FANCD2. In particular, in addition to binding FANCB, the ELF domain of FANCL also binds a second ubiquitin moiety (Miles et al., 2015). We speculate that the ELF of FANCL-molecule 2 binds ubiquitin conjugated to FANCD2 by FANCL-molecule 1, to stimulate its monoubiquitination of FANCI. This is because FANCD2 monoubiquitination precedes FANCI monoubiquitination in our experiments (Figure 2D). It is also is consistent with the slower kinetics of FANCI monoubiquitination in time course experiments, after DNA damage induction in cells (Sareen et al., 2012).

As the BL100 sub-complex sits at the center of the FA core complex, it is likely that other modular components, such as CEF or AG20, may have been independently added during evolution. The existence of such sub-complexes within the FA core complex has been hypothesized for some time (Garcia-Higuera et al., 2000; Hodson and Walden, 2012; Medhurst et al., 2006) and several phosphorylation events have been shown to be critical for establishment of the full FA core complex assembly in cells (Collins et al., 2009; Wang et al., 2007). While in a cellular context, these phosphorylation events may be critical for changing the localization of FANC proteins; our recombinant complex does not appear to require these phosphoresidues for normal assembly or activity. As such, ATR and Chk1 mediated phosphorylation of FANCA and FANCE, respectively, are more likely to be important in altering the physical location or stability of the sub-complexes, rather than directly mediate protein:protein interactions. However, future studies adding recombinant kinases or phosphomimic mutations may be useful in uncovering other mechanisms of biochemical regulation.

Notably, we have not observed any necessity for FANCA, FANCG, or FAAP20 (AG20 complex) in the stimulation of monoubiquitination reactions seen in our in vitro assays. This is surprising, as cells from FANCA−/− and FANCG−/− mice have no detectable FANCD2 monoubiquitination even after high-level induction of DNA damage (Cheng et al., 2000; Yang et al., 2001). This non-essential function of FANCA and FANCG result may explain the increased prevalence of FA patients with FANCA and FANCG mutations compared to other subtypes. It is possible that many causal FANCA or FANCG mutations have a less severe effect on ID2 monoubiquitination and therefore are not embryonic lethal like the mutations in other complementation groups. Instead, our data point to a more critical role for these proteins in the localization of the remaining catalytic ubiquitination machinery to damage sites (Huang et al., 2014). For example, it is known that FANCB and FANCL are mislocalized to the cytoplasm in FANCA null cells (Meetei et al., 2003, 2004), and DNA damage sensitivity associated with FANCA mutation is most severe in cells expressing mutant versions of FANCA that do not localize to the nucleus (Adachi et al., 2002). This has important ramifications for how we may think about treating FA through molecular means. A potential strategy may be to look for conditions, or small molecule activators, that promote nuclear accumulation of the BL100 complex in FANCA-defective cells. As FANCA- mutations account for over 60% of documented FA cases, such a strategy could activate the normal-functioning ubiquitination activity that is retained by a competent BL100+CEF complex in this main group of FA patients.

We have now unequivocally demonstrated a substrate receptor role for the CEF sub-complex. Other E3 ligase complexes also contain substrate receptors, including the F-box proteins in SCF complexes and Cdh1 in the anaphase-promoting complex (Kraft et al., 2005; Skowyra et al., 1997). Such adaptors provide greater specificity in controlling RING E3 ligases and can be shuffled to increase the diversity of substrates. Genetic and cell-based experiments suggest it is unlikely that the BL100 complex utilizes other substrate receptors (Huang et al., 2014) and each subunit within the CEF complex has a distinct role in substrate—ID2—engagement. Both FANCC and FANCE make contact with the ID2 heterodimer, and this explains why previous studies failed to see an effect of recombinant FANCE alone in stimulation of ubiquitination (Alpi et al., 2008). FANCF stabilizes FANCC and FANCE interaction, but is not critical for the assembly of the entire FA core complex. Instead, its function may be in regulating localized ubiquitination via interaction with the FANCM-anchor complex (Deans and West, 2009). In this manner, CEF could link ID2 engagement to locally elevated concentrations of FANCM on specific DNA structures associated with stalled replication forks (Coulthard et al., 2013). Our in vitro experiments suggest that some level of secondary structure or branching in the ID2 bound DNA is also beneficial, but not essential, to stimulate monoubiquitination. Although we still do not know the exact DNA structure(s) that activate the FA pathway in cells, the presence of several DNA binding activities in various FANC proteins may combine to concentrate ubiquitination and ID2 at a DNA damage lesion. This would create an exquisite control over the formation of concentrated ID2 foci,and limits the spurious activation of downstream pathways such as nuclease-mediated DNA cleavage.

We have also investigated the important contribution of deubiquitination to the regulation of ID2. In particular, we have discovered that the USP1:UAF1 complex has a reciprocal substrate preference to the FA core complex (Figure 7D). In particular, while UAF1 has previously been shown to bind to the SIM domain of FANCI (Yang et al., 2011), our results suggest that this interaction is obscured when FANCI is in the DNA bound monoubiquitinated form. Our data point to two roles for the USP1:UAF1 complex in the FA pathway: (1) fast removal of

ubiquitin from ubiquitinated-FANCD2 when FANCI has not been also ubiquitinated and (2) removal of ubiquitin from both FANCD2 and FANCI when DNA repair is completed and the ID2 complex is no longer DNA bound. These observations support the hypothesis that FANCD2 ubiquitination is dynamic within the ID2 complex and FANCI-ubiquitination commits to FA pathway activation. The severe FA-like phenotype of USP1−/− or UAF1+/− mouse cells (Kim et al., 2009; Park et al., 2013) suggest that the trapping of ID2 complexes in a ubiquitinated form, either during the dynamic exchange or at the conclusion of repair prevents new repair from being initiated. Our reactions also support a dominance of USP1:UAF1 deubiquitination reaction over the FA core complex monoubiquitination reaction (Figures 6 and 7). This points to either an additional factor or a particular DNA substrate that would continue to drive and/or protect ubiquitination activity in the presence of USP1:UAF1. In this way the dynamic regulation of ubiquitin addition and subtraction could be further limited to sites of stalled replication or DNA damage.

What happens to the ID2 complex (and indeed the disassembly of the FA core complex) post ubiquitination reaction is still somewhat of a "black box". The ubiquitination is essential for cutting one strand of DNA from either side of an ICL ("unhooking") by the XPF (FANCQ) nuclease (Klein Douwel et al., 2014). XPF is localized by SLX4 (FANCP) to cut the 5′-side and the 3′-side could be cut by FAN1 or SMN1A nucleases (MacKay et al., 2010; Wang et al., 2011). All three of these nucleases contain a ubiquitin binding domain, but it is not clear whether they engage ubiquitinated forms of FANCD2, FANCI, or another protein. The coordinated local action of all of these nucleases is also intricately linked to unloading of the replicative helicase (CMG) by BRCA1 (FANCS). Thus, an additional level of control exists post-ubiquitination to ensure that nuclease activity does not occur when a replication fork still has the opportunity to be recovered by non-cleavage means. Our in vitro system could be combined with in vitro replication tools to determine more details of these intricate controls.

In summary, our biochemical reconstitution of ubiquitination by the FA core complex has helped explain why such a complex machine is necessary for what appears, on face value, to be a relatively simple biochemical modification. We have uncovered a ubiquitination complex that is not dissimilar from other ubiquitination machines that contain core catalytic subunits, substrate receptors, and processivity factors, like the APC or SCF ubiquitin ligases (Figure 7E). As in these other multi-subunit ligases, the FA core complex has evolved to ubiquitinate a very specific substrate, with mechanisms of temporal and spatial control. Uniquely, however, the FA core complex has adapted these controls for coordinated monoubiquitination of two distinct proteins that must exist in a defined (DNA-bound and heterodimeric) conformation. Any change to this tight control results in severe sensitivity to ICLs, making our biochemical system an excellent tool to uncover small molecule sensitizers to ICL-based chemotherapy. Alternatively, we may also uncover ways that the highly specific reaction could be reactivated in the absence of one component and use this to address the bone marrow failure and cancer predisposition phenotypes of FA patients.

EXPERIMENTAL PROCEDURES

Cloning of Multibac Expression Vectors

Detailed descriptions of cloning using the Multibac system (Bieniossek et al., 2008) are provided in the Supplemental Experimental Procedures.

Antibodies

The following antibodies were used in this study: rabbit polyclonal antibodies against FANCA, FANCC, and FANCG (Fanconi Anemia Research Fund), FANCL (GTX100162, GeneTex), mouse Fancb (Aviva ARP60052) FANCE (Pace et al., 2002), GAL4-DBD (Santa Cruz), MBP-tag (New England Biolabs), StrepII tag (ab76949, Abcam), and FAAP20 and FAAP100 (Sigma); rabbit monoclonal antibodies against FANCB (D9W6S, Cell Signaling), FANCD2 (ab178705, Abcam), and Myc epitope (71D10, Cell Signaling); goat polyclonal against FANCF (EB10140, Everest Biotech); and mouse monoclonal antibodies against FLAG- (Sigma, M2) and HA- (12CA5) epitopes.

Protein Expression and Purification

FA Core Complex

FA core complex proteins, USP1:UAF1, or FANCD2:FANCI were produced using High Five cells infected with Multibac baculoviruses. UBE2T was produced in *E.coli*. Detailed descriptions of protein purifications are found in the Supplemental Experimental Procedures. Mass spectrometry was used to confirm the identity of the purified proteins.

In Vitro Ubiquitination and/or Deubiquitination Assays

Standard ubiquitination reactions contained 10 μM recombinant human HA-ubiquitin (Boston Biochem U-110-01M), 50 nM human recombinant Ube1 (CSIRO), 100 nM FANCT/UBE2T, 2 mM adenosine triphosphate, and in reaction buffer (50 mM Tris [pH 7.4], 2.5 mM MgCl2, 150 mM NaCl, 0.01% Tritonx100, and 1 mM DTT). Titration of FANCI:D2 (100 nM–1 μM) was done to establish optimal substrate concentration. Each reaction contained 165 nM FANCI:D2, 165 nM BL100, and 165 nM CEF, and 165 nM DNA. 30-μL reactions were set up on ice and incubated at 25°C. 165 nM USP1:UAF1 (Boston Biochem) was added at indicated intervals, in most cases after removal of ATP using a Zeba spin column (7,000 molecular mass cutoff) to simultaneously inhibit the monoubiquitination reaction. All reactions were stopped by adding 10 μL NuPage LDS sample buffer (Invitrogen) and heated at 80°C for 5 min. Samples were separated by SDS-PAGE using BOLT 4%–12% Bis-Tris or NuPAGE 3%–8% Tris-Acetate gels (Invitrogen).

Protein Analysis by SEC-MALS

Purified proteins and complexes were separated on an SRT-500 column (Sepax) in (20 mM Tris [pH 7.5]/50 mM KCl/100 mM NaCl/1 mM DTT at 0.35 mL/min) attached to a Shimadzu chromatography system. A DAWN HELIOS II Multi-Angle Light Scattering detector and an Optilab rEX Refractive Index detector recorded the light scattering and refractive index of the samples upon elution from the size exclusion column. The Wyatt software ASTRA was used to analyze the data collected. Standards used for size estimations are shown in Table S1.

BS3 Crosslinking

BL100 was crosslinked with bis(sulfosuccinimidyl) suberate (BS3, Pierce) at molar ratios of 1:0, 1:200, 1:600, and 1:1,800. Reactions were carried out in 20 mM Triethanolamine (pH 7.5), 150 mM NaCl, and 5% glycerol for 2 hr on ice then stopped by addition of Tris (pH 8.5) to 40 mM. Crosslinked complex was heated at 80°C for 5 min with NuPAGE LDS Sample Buffer (4X) (NOVEX Life Technologies) separated on 8% Tris-acetate gel (NOVEX Life Technologies). The crosslinked proteins were visualized with Coomassie brilliant blue R250 or dual FLAG/FAAP100 or FLAG/FANCL antibody staining of western blots using Li-Cor Odyssey far-red imaging.

Cell Based Immunoprecipitations

Fancb$^{y/-}$ or WT ESCs were a kind gift of Paul Hasty (University of Texas Health Science Center) and maintained as described (Kim et al., 2015). A total of 3 × 10^6 cells were transfected with 1 μg pDEST-Myc-FANCL and/or pDEST-GAL4-FANCL. At 48 hr later, cells were extracted in co-immunoprecipitation (coIP) buffer (20 mM Tris [pH 7.5], 150 mM KCl, 1 mM EDTA, and 1 mM AEBSF), briefly sonicated, and centrifuged at 20,000 × *g* for 1 hr at 4°C. Cleared lysates were incubated for 4 hr with 1 μg of 9E10 Myc-epitope antibody and immunoprecipitated with 25 μL of protein-G magnetic beads

(Pierce). Immunoprecipitates were washed and eluted with 1× LDS loading buffer and examined by western blotting.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.11.005.

AUTHOR CONTRIBUTIONS

Conceptualization, S.v.T., C.H., J.H., and A.J.D.; Methodology, S.v.T., V.J.M., C.H., J.J.O., J.H., and A.J.D.; Investigation, S.v.T., V.J.M., C.H., P.S., W.T., J.J.O., J.H., and A.J.D.; Writing - Original Draft, A.J.D.; Writing - Review & Editing, S.v.T., C.H., W.C., J.H., and A.J.D.; Funding Acquisition, W.C. and A.J.D.; Resources, S.v.T., V.J.M., P.S., and A.J.D.; and Supervision, W.C., C.H., J.H., and A.J.D.

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