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Fanconi anemia proteins FANCD2 and FANCI exhibit different DNA damage responses during S-phase

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ABSTRACT

Fanconi anemia (FA) pathway members, FANCD2 and FANCI, contribute to the repair of replication-stalling DNA lesions. FA pathway activation relies on phosphorylation of FANCI by the ataxia telangiectasia and Rad3-related (ATR) kinase, followed by monoubiquitination of FANCD2 and FANCI by the FA core complex. FANCD2 and FANCI are thought to form a functional heterodimer during DNA repair, but it is unclear how dimer formation is regulated or what the functions of the FANCD2–FANCI complex versus the monomeric proteins are. We show that the FANCD2–FANCI complex forms independently of ATR and FA core complex, and represents the inactive form of both proteins. DNA damage-induced FA pathway activation triggers dissociation of FANCD2 from FANCI. Dissociation coincides with FANCD2 monoubiquitination, which significantly precedes monoubiquitination of FANCI; moreover, monoubiquitination responses of FANCD2 and FANCI exhibit distinct DNA substrate specificities. A phosphodead FANCI mutant fails to dissociate from FANCD2, whereas phosphomimetic FANCI cannot interact with FANCD2, indicating that FANCI phosphorylation is the molecular trigger for FANCD2–FANCI dissociation. Following dissociation, FANCD2 binds replicating chromatin prior to—and independently of—FANCI. Moreover, the concentration of chromatin-bound FANCD2 exceeds that of FANCI throughout replication. Our results suggest that FANCD2 and FANCI function separately at consecutive steps during DNA repair in S-phase.

INTRODUCTION

Fanconi anemia (FA) is a recessively inherited multigene disease characterized by bone marrow failure and increased cancer susceptibility. Cells from FA patients exhibit spontaneous chromosomal instability and hypersensitivity to DNA interstrand crosslinks (DNA ICLs) (1–3). Fifteen FA genes have been identified and their protein products are thought to share a common pathway (3–5). Following various types of DNA damage and during every S-phase of the cell cycle, a nuclear 'core complex' containing eight FA proteins (FANCA, –B, –C, –E, –F, –G, –L and –M) mediates monoubiquitination of two downstream targets, FANCD2 and FANCI, followed by their recruitment to chromatin (6–10). Chromatin-bound FANCD2 and FANCI colocalize into nuclear foci that represent centers of ongoing DNA repair (9,11). DNA damage-induced chromatin binding of FANCD2 and FANCI is strictly replication dependent, suggesting that both proteins are recruited to replication forks stalled at sites of DNA damage (12,13). FANCD2 is required for the repair of DNA ICLs encountered by replication forks (13) and the subsequent fork restart (14). Recent studies attribute a specific role to the monoubiquitinated FANCD2 isoform ($FANCD2^{Ub}$) in recruiting the DNA repair factors FAN1 (Fanconi-associated nuclease 1) (15–18) and SLX4 (identical to FANCP; a holliday junction resolvase in complex with SLX1) (19–22) to ICL-containing chromatin, indicating that chromatin-bound $FANCD2^{Ub}$ acts as a docking platform for certain DNA repair nucleases. In addition to their monoubiquitination, FANCD2 and FANCI are also phosphorylated in response to DNA damage by the cell cycle checkpoint kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) (23–27). ATM phosphorylates FANCD2 following treatment with ionizing radiation, whereas ATR

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phosphorylates FANCD2 in response to replication-stalling agents including DNA ICLs. DNA ICLs also trigger phosphorylation of FANCI by ATR at several serines within an oligopeptide stretch containing six highly conserved serine/glutamine sites (the '6SQ stretch') (27,28). Intriguingly, the known modifications of FANCD2 and FANCI appear to have different functions: monoubiquitination of FANCD2—but not FANCI—is important for FA pathway function (9,10,27), whereas phosphorylation of FANCI—but not FANCD2—is crucial for FA pathway activation (8,26,27), suggesting that FANCD2 and FANCI may be controlled differently by FA core complex and the ATR/ATM pathways, respectively.

A subset of FANCD2 and FANCI molecules have been reported to interact in unsynchronized human cells (9,29) and this interaction is conserved in chicken and frog (13,27). Recombinant FANCD2 and FANCI are also able to interact *in vitro* (11,30) and the recently solved crystal structure of a FANCD2–FANCI heterodimer showed that FANCD2 and FANCI interact along an approximately 560-residue region on each protein. The monoubiquitination sites of both proteins and parts of the FANCI 6SQ stretch are positioned at the interaction interface (30). Interestingly, DNA-damage treatment does not appear to increase the FANCD2–FANCI interaction in human cells (9), hinting that FA pathway activation and the associated FANCD2/FANCI modifications may not be required for their interaction. Current FA pathway models depict the FANCD2–FANCI dimer as being constitutive or even inducible upon FA pathway activation (7,9,11,31), however, it is not known how FANCD2–FANCI dimer formation is regulated, or what the roles of the dimer versus the monomeric FANCD2 and FANCI proteins are. Here, we utilized the naturally cell cycle synchronous *Xenopus* egg extract system to investigate the dynamics of the FANCD2–FANCI complex during replication and in response to DNA damage. We show that the FANCD2–FANCI complex forms throughout the cell cycle independently of upstream regulatory FA core complex and ATR proteins. Following FA pathway activation, FANCD2 and FANCI dissociate in a manner dependent on FANCI phosphorylation. Subsequently, FANCD2 and FANCI are recruited to replicating chromatin in a consecutive fashion, indicating that separated FANCD2 and FANCI represent the active isoforms of both proteins. Our results support a novel hypothesis that FANCD2 and FANCI do not form a functional entity but instead have separate roles during replication and in response to DNA damage in S-phase.

MATERIALS AND METHODS

Preparation of *Xenopus* egg extracts

M- and S-phase extracts were prepared from *Xenopus* eggs according to the method of Murray, as previously described (12,32). To block the replication of circular single-stranded DNA (ssDNA), 50 μg/μl aphidicolin was added to S-phase egg extracts prior to addition of DNA.

Antibodies

FANCD2: polyclonal rabbit antibodies were raised against the N- and C-termini of *Xenopus laevis* FANCD2 (MVAKRKLSRSDDREESFT and SDKEIEGGDEDN EDEDSD). FANCI: polyclonal rabbit antibodies were raised against the N- and C- termini of *X. laevis* FANCI (MDQKILSLAAEEQNDGLQSC and DNEQAVTEEES QEPKKKRRRK). FANCA: rabbit polyclonal antibodies against FANCA were generated against the C-terminal region of *X. laevis* FANCA (amino acids 1205–1383) as described (12). FANCG: rabbit polyclonal antibodies were raised against *Xenopus* peptides DSLIKELEESAE EMQSEAVV (C-terminal) and ENKRGEEAVEHYL DLLALL. ATR: polyclonal rabbit antibodies were produced against *Xenopus* peptides AIRKAEPSLKEQIL EHESLG and ASAGAEEYN TTVQKPRQILC. Commercial antibodies were used against Flag (Sigma, F3165), Myc (Sigma, C3956; Covance, 14865101) and FAN1 (Abnova, H00022909-B01P).

DNA replication assay

Chromatin replication assay: replication of sperm chromatin in S-phase extracts was monitored as described before (12). ssDNA plasmid replication assay: S-phase egg extracts were supplemented with 50 ng/μl M13 circular ssDNA. Reaction aliquots were pulse labeled with [α-^{32}P]dGTP at the indicated time windows at 23°C. Replication reactions were stopped with 1% SDS/40 mM EDTA (pH 7.8) and digested with proteinase K (1 mg/ml) at 37°C for 1 h. DNA was extracted with phenol–chloroform and electrophoresed on a 1% agarose gel.

Preparation of DNA substrates

Plasmid DNA*:* Circular plasmid DNA (pBSKS) was prepared from *Escherichia coli* cultures using a QiaFilter Plasmid Maxi kit (QIAGEN). Double-stranded fragmented DNA: Circular plasmid DNA (pBSKS) was fragmented (14 DNA fragments) by digestion with restriction enzyme HaeIII (Invitrogen). M13mp18 single-stranded plasmid DNA was obtained from Bayou Biolabs. DNA substrates were used at a concentration of 50 ng/μl in egg extracts unless indicated otherwise.

Immunodepletion

Immunodepletions were performed essentially as previously described (12) (also described in Figure 1C). In brief, 200 μl of Sepharose 4B beads (50% slurry) were rotated overnight at 4°C with 500 μl of phosphate-buffered saline and 100 μl of protein-specific antibody (FANCA, FANCG, FANCD2, FANCI or ATR) or the corresponding control immunoglobulin G (IgG) antibody. Beads were pelleted from solution and washed four times in XB-buffer. For depletion, 200 μl of extract were added to 100 μl of dry conjugated beads and incubated on ice for 60 min. Subsequently, the extract–bead mix was centrifuged at 1800*g* for 5 min, and the extract was separated from the bead pellet. For quantitative removal of proteins from extracts, 1–2 depletion rounds were performed. Control IgG antibody was used for

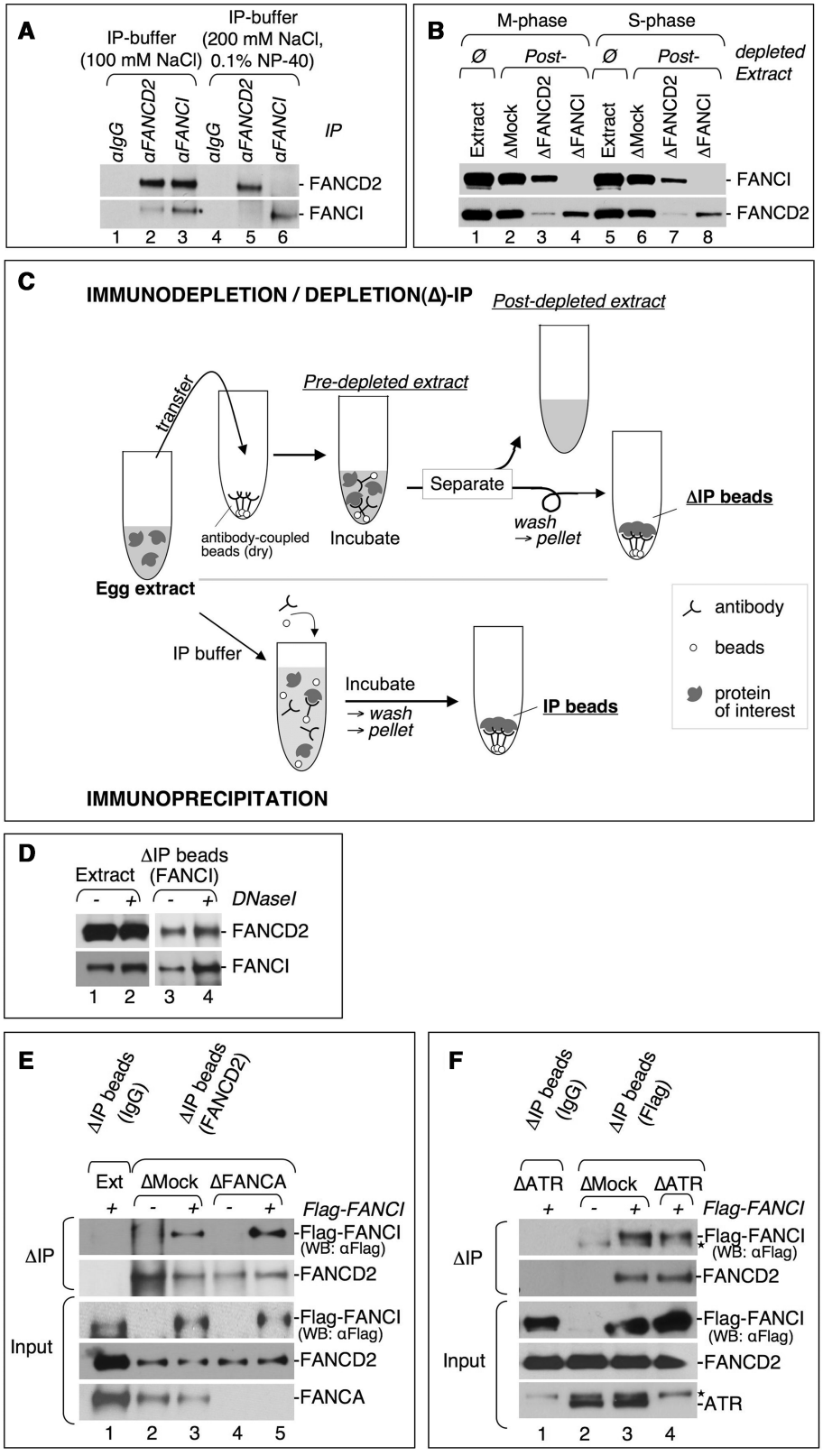

Figure 1. FANCD2 and FANCI form a weak complex throughout the cell cycle in a manner independently of the FA core complex or ATR. **(A)** FANCD2 and FANCI co-immunoprecipitate (co-IP) from S-phase extracts under low-stringency conditions. S-phase extracts were diluted in low-stringency buffer (lanes 1–3) or high-stringency buffer (lanes 4–6) (see also 'Materials and Methods' section), and incubated with rabbit IgG (lanes 1 and 4), FANCD2 antibody (lanes 2 and 5) or FANCI antibody (lanes 3 and 6), followed by incubation with sepharose beads. Beads were washed in the respective IP buffer and analyzed for bound FANCD2 and FANCI by SDS–PAGE and western blot. (**B**) FANCD2 and FANCI interact in M- and S-phase. *Xenopus* M-phase (lanes 1–4) or S-phase (lanes 5–8) egg extracts were mock- (lanes 2 and 6), FANCD2- (lanes 3 and 7), or FANCI-depleted (lanes 4 and 8). Depleted extracts were assayed for the presence of FANCD2 and FANCI by SDS–PAGE and western blot.

(continued)

mock-depletion. For 'depletion-immunoprecipitation' (depletion-IP or ΔIP) of FANCD2 or FANCI, only one depletion round was performed, followed by further processing of the reisolated bead pellet as described below.

Depletion-IP

Xenopus extracts were first subjected to one round of immunodepletion by incubating extracts with dry FANCI-, FANCD2-, FANCG-, Flag- or Myc-antibody coupled beads for 90 min on ice, followed by separation of beads and extract as described in the 'Immunodepletion' section (also described in Figure 1C). Subsequently, the reisolated beads containing antibody–protein complexes (named 'ΔIP beads') were washed four times in low-stringency IP buffer. Washed ΔIP beads were boiled in 1 × NuPAGE loading buffer (Invitrogen) and analyzed for bound proteins by SDS–PAGE and western blotting. To analyze the degree of FANCD2–FANCI co-depletion under different conditions, extract aliquots were taken from extract–bead mixes at the end of the immunodepletion incubation ('pre-depleted extract') and following separation of the ΔIP beads from the extracts ('post-depleted extract').

IP

IP from egg extracts (also described in Figure 1C): 50 µl of egg extracts were diluted in 700 µl of low-stringency IP buffer (100 mM NaCl, 20 mM HEPES pH 7.9, 1 mM DTT) or high-stringency IP buffer (200 mM NaCl, 20 mM HEPES pH 7.9, 0.1% NP40, 1 mM DTT). Affinity-purified FANCI antibody was added and the mix was rotated for 2 h at 4°C, followed by addition of 50 µl of 50% Sepharose A bead slurry and an additional rotation for 45 min at 4°C. IP beads were washed four times with high- or low-stringency IP buffer as indicated. Washed IP beads were boiled in 1 × NuPAGE loading buffer (Invitrogen) and analyzed for bound proteins by SDS–PAGE and western blotting.

IP of recombinant proteins *in vitro*: co-immunoprecipitation of $FANCD2_{WT}$ with $FANCI_{WT}$, $FANCI_{6S\rightarrow A}$ or $FANCI_{6S\rightarrow D}$ was essentially performed as described for the human protein homologs (11). Briefly, 6 pmol of purified Myc-tagged $FANCD2_{WT}$ was mixed with 6 pmol of purified Flag-tagged $FANCI_{WT}$, $FANCI_{6S\rightarrow A}$ or $FANCI_{6S\rightarrow D}$ in 100 µl of reaction buffer (150 mM KCl, 20 mM Tris–HCl, pH 7.6, 250 µg/ml BSA, 0.45% NP-40). After incubation on ice for 15 min, 10 µl aliquots were taken from each reaction (input). An anti-Myc antibody (Sigma) was added and the mixture was incubated on a rotator at 4°C overnight. Protein A Sepharose beads were added and incubated with the protein–antibody mix at 4°C for 1 h. Beads were washed twice with lysis buffer (50 mM Tris–HCl, pH8.0, 5 mM EDTA), twice with 300 mM NaCl and twice with 150 mM NaCl. Washed IP beads were boiled in 1 × NuPAGE loading buffer (Invitrogen) and analyzed for bound proteins by SDS–PAGE and immunoblotting.

Immunoblotting

Protein samples were separated on 3–8% gradient gels (Invitrogen) and transferred to Immobilon P membranes (Millipore). After being blocked in 5% milk for 1 h, membranes were incubated with the following primary antibodies: FANCD2 (1:2000), FANCI (1:1000), FANCA (1:1000), FANCG (1:2000), ATR (1:1000), Flag (1:3000) or Myc (1:1000). Horseradish peroxidase-conjugated rabbit secondary antibody (Jackson Labs) or mouse secondary antibody (Biorad) were used at dilutions of 1:10000 and 1:4000, respectively. Protein bands were visualized using an ECL Plus system (Amersham).

Preparation of chromatin fractions

At indicated time points, 50 µl of S-phase egg extracts containing 1000 sperm pronuclei/µl were diluted in chromatin isolation buffer (40 mM HEPES, 100 mM KCl, 20 mM $MgCl_2$, 0.2% Triton X-100) and purified by centrifugation through a 30% (w/v) sucrose cushion for 25 min at 6000*g* at 4°C. Chromatin pellets were analyzed by gel electrophoresis and immunoblotting.

Figure 1. Continued
One microliter of untreated M-phase extract (lane 1) or S-phase extract (lane 5) was used as a loading control. (**C**) Overview of Immunodepletion/Depletion-IP and Immunoprecipitation strategies. Immunodepletion/Depletion (Δ)-IP: Extracts were added to dry antibody-coupled beads and incubated on ice. Following incubation, beads were separated from the extract, washed in low stringency IP buffer and analyzed for bound protein (ΔIP beads). Aliquots were taken from the bead–extract mix just before separation of beads and extract (Pre-depleted extract) and from extracts after removal of beads (Post-depleted extract). Immunoprecipitation: Extracts were diluted in (low or high stringency) IP buffer first, followed by incubation with antibody and then beads (rotation at 4°C). Following incubation, beads were washed in the respective IP buffer and analyzed for bound protein (IP beads). (**D**) The FANCD2–FANCI complex persists in DNaseI-treated S-phase extracts. S-phase extracts were untreated (lane 1) or DNaseI-treated (lane 2), followed by ΔIP with FANCI antibody-beads. ΔIP beads were analyzed for the presence of FANCD2 and FANCI by western blot (lanes 3 and 4). (**E**) Recombinant Flag–FANCI and extract-endogenous FANCD2 interact in absence of FANCA. Input panels: S-phase extracts were undepleted (lane 1), mock- (lanes 2 and 3) or FANCA-depleted (lanes 4 and 5), and either untreated (lanes 2 and 4) or incubated with recombinant Flag–FANCI protein (lanes 1, 3 and 5) for 30 min. ΔIP panels: the different extracts were then subjected to ΔIP by incubation with beads coupled to rabbit IgG (lane 1) or FANCD2 antibody (lanes 2–5). ΔIP beads were assayed for bound FANCD2 and FANCI by SDS–PAGE and western blot. (**F**) Recombinant Flag–FANCI and extract-endogenous FANCD2 interact in absence of ATR. Input panels: S-phase extracts were mock- (lanes 2 and 3) or ATR depleted (lanes 1 and 4). Depleted extracts were untreated (lane 2) or incubated with recombinant Flag–FANCI (lanes 1, 3 and 4) for 30 min. ΔIP panels: the different extracts were then subjected to ΔIP by incubation with beads coupled to rabbit IgG (lane 1) or Flag antibody (lanes 2–4). ΔIP beads were assayed for bound FANCD2 and FANCI by SDS–PAGE and western blot [ATR depleted, Flag–FANCI-treated extracts instead of undepleted, Flag–FANCI-treated extracts were used for mock-depletion (lane 1) in Figure 1F]. The asterisk marks a non-specific band that is sometimes recognized by the Flag antibody (upper panel) and a non-specific band typically recognized by the ATR antibody (bottom panel).

Plasmid construction and protein purification

Xenopus laevis FANCD2 and FANCI proteins were expressed using the Invitrogen Gateway system and SF9 insect cells essentially as previously described (33). For cloning and expression of Flag–FANCI and Myc–FANCD2, the pDEST10 vector (Invitrogen) was reconstructed to insert the Flag tag sequence 5′-GAC TAC AAA GAC GAT GAC GAC GAC AAG-3′ downstream of the pDEST10-encoded His tag sequence (His-Flag-pDEST10), or to insert the Myc tag sequence 5′-GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG ACA AGT-3′ downstream of the pDEST10-encoded His tag sequence (His–Myc-pDEST10). For expression of full-length *Xenopus* His–Myc-tagged FANCD2, the *FANCD2* coding region was subcloned from the pDONR201 vector into His–Myc-pDEST10. The wild-type *FANCD2*-pDONR201 construct was used as template to create the *FANCD2* monoubiquitination-dead mutant $FANCD2_{K562R}$ (corresponding to human $FANCD2_{K561R}$). For expression of full-length *Xenopus* His–Flag-tagged FANCI, the *FANCI* coding region was first cloned into pDONR201 [based on GenBank accession number GU144566 (13)], and subcloned into His–Flag-pDEST10. The wild-type *FANCI*-pDONR201 construct was used as template to create the *FANCI* phosphomimetic mutant, $FANCI_{6S\rightarrow D}$, and the FANCI phosphodead mutant, $FANCI_{6S\rightarrow A}$. Six codons encoding for serine (S) residues S557, S560, S556, S597, S618 and S630 on *Xenopus* FANCI (corresponding to serine residues S556, S559, S565, S596, S617 and S629 in human FANCI) were mutated to encode either for asparatic acid (D) residues ($FANCI_{6S\rightarrow D}$) or for alanine residues ($FANCI_{6S\rightarrow A}$) using a Stratagene site-directed mutagenesis kit. The *Xenopus* His-tagged proteins were expressed in SF9 cells using a Bac-to-Bac baculoviral expression system (Invitrogen). His-tagged proteins were purified using Ni-NTA beads under native conditions according to the Qiagen protocol.

RESULTS

Eighty percent of FANCD2 and FANCI form a complex throughout the cell cycle

To test if the FANCD2–FANCI interaction is cell cycle specific, we took advantage of the fact that naturally synchronous S- or M-phase extracts can be prepared from unfertilized *X. laevis* eggs (32,34). Moreover, since chromosomal DNA is removed during extract preparation, these extracts are initially DNA-free, allowing us to test whether interactions between FANCD2 and FANCI depend on the presence of DNA in the context of a fully functional DNA damage responsive network. IP of FANCD2 or FANCI under low-stringency buffer conditions (100 mM NaCl, no detergent) revealed that FANCD2 and FANCI interact in S-phase extracts (Figure 1A). However, the FANCD2–FANCI complex dissociated under standard IP conditions (200 mM NaCl, 0.1% NP40), indicating that the FANCD2–FANCI interaction is much weaker than interactions among FA core complex members that remain stably associated under the same IP conditions (35,36). To evaluate the percentage of complexed FANCD2–FANCI compared with monomeric FANCD2 and FANCI during the cell cycle, we performed quantitative immunodepletion of FANCD2 or FANCI from S-phase extracts (achieved by incubating bead-coupled FANCD2 or FANCI antibodies in egg extracts without further diluting the extracts). Quantitative removal of FANCD2 from S-phase extracts co-depleted ~80% of FANCI [also recently described by Knipscheer *et al.* (13)] and vice versa, quantitative depletion of FANCI co-depleted 80% of FANCD2 (Figure 1B). Similarly, 80% of FANCD2 and FANCI were co-depleted from M-phase extracts (Figure 1B), demonstrating that the majority of FANCD2 and FANCI molecules interact—although very weakly—throughout the cell cycle. Based on the observed intrinsic weakness of the FANCD2–FANCI complex, we used a modified IP protocol ('depletion-IP', see 'Experimental Procedures' and Figure 1C) for the remainder of this study to cause minimal interruption of the FANCD2–FANCI complex: instead of diluting extracts in IP buffer prior to the addition of antibody and beads, we performed a regular immunodepletion by incubating antibody-coupled beads in undiluted extracts first, followed by reisolation and subsequent washes of protein–antibody–bead complexes in low-stringency IP buffer. For simplicity, we will continue to refer to this method as 'immunoprecipitation' in the text; in the figures, depletion-IPs are marked as 'ΔIP' and re-isolated protein-antibody-beads are termed 'ΔIP beads'. To test if the FANCD2–FANCI interaction was truly DNA-independent and not due to residual contaminating chromosomal DNA in extracts, we co-immunoprecipitated FANCI with FANCD2 from untreated versus DNaseI-treated S-phase extracts and confirmed that FANCD2–FANCI complex stability was unaffected by the presence of DNaseI (Figure 1D). To test if FANCD2 and FANCI were able to interact *de novo* in our extract system, we incubated recombinant *Xenopus* Flag–FANCI or Myc–FANCD2 proteins in S-phase extracts, followed by IP with anti-Flag or anti-Myc antibodies, respectively. Recombinant Myc-FANCD2 interacted with endogenous FANCI and vice versa, recombinant Flag–FANCI interacted with its endogenous partner FANCD2 (Supplementary Figure S1A). Together, these results show that the majority of FANCD2 and FANCI molecules form a weak protein complex in a cell cycle- and DNA-independent manner.

The FANCD2–FANCI interaction does not depend on the FA core complex or the ATR kinase

The FA core complex and the DNA repair kinase ATR act upstream of FANCD2 and FANCI: the core complex mediates monoubiquitination of FANCD2 and FANCI during replication and following DNA damage (8–10,29), whereas ATR phosphorylates FANCD2 and FANCI in response to replication stress (24–26,28). Absence of ATR or FA core complex proteins from human cells or *Xenopus* extracts interrupts FA pathway activation (10,12,14,25,26,33). To determine if activation

of the FA pathway was required for *de novo* FANCD2–FANCI complex formation, we asked if recombinant Flag–FANCI was able to interact with endogenous FANCD2 in extracts that were depleted of FANCA (core complex member). FANCA-depleted extracts can be considered 'core complex-deficient' since FANCA depletion partly co-depletes other core complex members (36) and the core complex is non-functional in the absence of FANCA (37). Mock- and FANCA-depleted extracts equally supported *de novo* complex formation between recombinant Flag–FANCI and endogenous FANCD2 (Figure 1E and Supplementary Figure S1B), as well as between recombinant Myc–FANCD2 and endogenous FANCI (data not shown). To investigate if FANCD2–FANCI complex formation depended on ATR, we tested if recombinant Flag–FANCI was able to interact with endogenous FANCD2 in ATR- versus mock-depleted extracts. As shown in Figure 1F, *de novo* interaction between Flag–FANCI and FANCD2 was unaffected in absence of ATR. Thus, the interaction between FANCD2 and FANCI does not require a functional FA core complex and occurs independently of ATR.

FANCD2 and FANCI exhibit different monoubiquitination responses

Human and *Xenopus* FANCD2 and FANCI are monoubiquitinated (termed FANCD2^{Ub} and FANCI^{Ub}) in response to DNA damage during S-phase (9,10,12,13). We previously demonstrated that FANCD2^{Ub} formation can be triggered by adding small DNA damage structures to *Xenopus* S- or M-phase extracts (34). For example, FANCD2^{Ub} formation is strongly stimulated in the presence of circular double-stranded DNA (dsDNA) (mimicking naked dsDNA regions), dsDNA fragments [mimicking DNA double-stranded breaks (DNA DSBs)], or ssDNA (see also Figure 2A). In contrast, FANCI showed a more selective monoubiquitination response: FANCI^{Ub} formation was strongly induced by circular ssDNA, but only weakly induced by dsDNA fragments or circular dsDNA (Figure 2A). A time course study of FANCD2^{Ub} and FANCI^{Ub} formation in response to circular dsDNA and circular ssDNA revealed that onset and peak of FANCD2^{Ub} formation occurred significantly earlier than onset and peak of FANCI^{Ub} formation in response to either DNA substrate (Figure 2B and Supplementary Figure S2A; FANCD2^{Ub} peak: circular dsDNA: 20 min, circular ssDNA: 40 min; FANCI^{Ub} peak: circular dsDNA: very weakly at 180 min, circular ssDNA: 180 min). Similar results were obtained in the presence of fragmented dsDNA (data not shown) indicating that regardless of the nature of the pathway-activating DNA substrate, FANCD2 monoubiquitination occurs prior to FANCI monoubiquitination. It was previously shown that circular ssDNA—unlike circular dsDNA or dsDNA fragments—is an excellent template for DNA replication in *Xenopus* S-phase extracts: in a reaction mimicking lagging strand DNA synthesis, the ssDNA is primed at several sites followed by rapid elongation and synthesis of the complimentary DNA strand (38,39). To test if monoubiquitination of FANCD2 and FANCI was triggered by ssDNA *per se*, or instead correlated with replication of the circular ssDNA substrate, we analyzed circular ssDNA-induced FANCD2^{Ub} and FANCI^{Ub} formation in extracts that were untreated or treated with aphidicolin, an inhibitor of replicative DNA polymerases that completely blocked complementary strand synthesis (Figure 2C, inset). Monoubiquitination of FANCD2 and FANCI was completely abrogated in circular ssDNA-containing extracts treated with aphidicolin, indicating that in the presence of circular ssDNA, formation of FANCD2^{Ub} and FANCI^{Ub} is strictly tied to DNA synthesis, but occurs at different times during the replication process (Figure 2C and Supplementary Figure S2B). As expected, formation of FANCD2^{Ub} and FANCI^{Ub} in response to the essentially non-replicating DNA substrates (circular dsDNA and dsDNA fragments) was unaffected in the presence of aphidicolin (Supplementary Figure S2C), indicating that these two DNA substrates trigger monoubiquitination of FANCD2 and (weakly) of FANCI independently of ongoing DNA synthesis. Taken together, these results indicate that FANCD2 and FANCI have only partially overlapping DNA substrate specificities with regard to their monoubiquitination; moreover they show that FANCD2 monoubiquitination is temporally uncoupled from FANCI monoubiquitination in response to DNA damage as well as during normal DNA replication.

The FANCD2–FANCI complex dissociates upon FA pathway activation

The current model of the FA pathway suggests that monoubiquitination of FANCD2—but not of FANCI—is crucial for FA pathway functions (8–10,26,27). To test if FANCD2^{Ub} formation affected the stability of the FANCD2—FANCI complex, we added circular dsDNA to S-phase extracts to specifically trigger formation of FANCD2^{Ub}. In response to the circular dsDNA substrate, FANCD2 monoubiquitination peaked at 1 h, with a complete return to the non-ubiquitinated FANCD2 isoform after 2 h (Figure 3A). To monitor behavior of the FANCD2–FANCI complex in dsDNA-containing extracts at 0 h (prior to FANCD2 monoubiquitination), at 1 h (peak of FANCD2 monoubiquitination) and at 2 h (after FANCD2 had returned to its non-ubiquitinated state), we immunodepleted FANCI from extracts at these three time points and assayed for co-depletion of FANCD2 (by analysis of FANCI-ΔIP beads). As shown in Figure 3A, the interaction between FANCD2 and FANCI was observed before and after induction of FANCD2^{Ub} at 0 h (lanes 1 and 4) and at 2 h (lanes 3 and 6), respectively, but was significantly reduced at 1 h when FANCD2 was mostly present in its monoubiquitinated state (lanes 2 and 5) (see also Supplementary Figure S3 for densitometry analysis of FANCD2 and FANCI protein bands shown in Figure 3A). Similarly, FANCD2^{Ub} did not co-deplete with FANCI in the presence of the DNA DSB-mimicking substrate (Figure 3B, compare lanes 2 and 5 with lanes 3 and 6). These results indicated that the interaction between FANCI and FANCD2 was interrupted while FANCD2

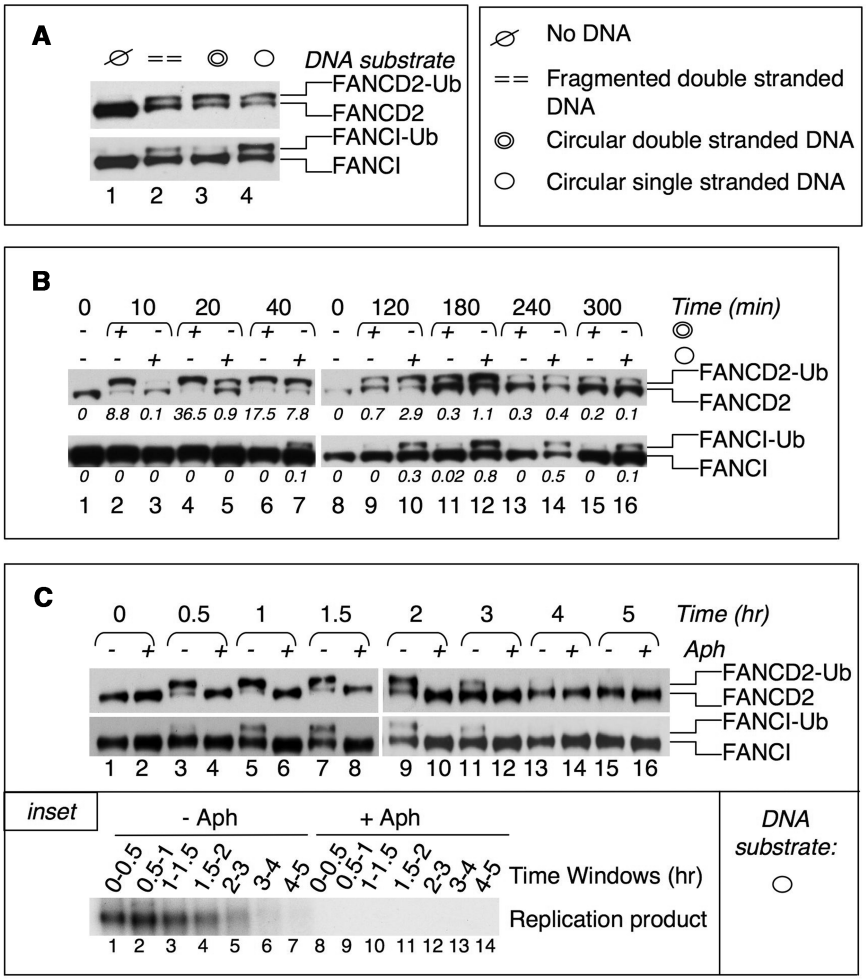

Figure 2. FANCD2 and FANCI exhibit different monoubiquitination responses. (**A**) FANCD2 and FANCI are not monoubiquitinated in response to the same DNA substrates. S-phase extracts were either untreated (lane 1) or treated with 50 ng/μl of fragmented dsDNA (lane 2), circular dsDNA (lane 3) or circular ssDNA (lane 4) for 1 h. Following incubation, 1 μl of extract was assayed for FANCD2 and FANCI by western blot. (**B**) Monoubiquitination responses of FANCD2 and FANCI follow a different time course. Extracts were either untreated (lanes 1 and 8) or treated with 50 ng/μl of circular dsDNA (lanes 2, 4, 6, 9, 11, 13 and 15) or circular ssDNA (lanes 3, 5, 7, 10, 12, 14 and 16) for the indicated time points. Following incubation, 1 μl of extract was assayed for FANCD2 and FANCI by western blot. The ratio of monoubiquitinated to non-ubiquitinated FANCD2 and FANCI is shown below each lane. A graphical presentation of $FANCD2^{Ub}$/FANCD2 and $FANCI^{Ub}$/FANCI ratios is provided in Supplementary Figure S2A. (**C**) Monoubiquitination of FANCD2 and FANCI is replication dependent in presence of circular ssDNA. S-phase extracts were supplemented with 50 ng/μl of circular ssDNA and either untreated (lanes 1, 3, 5, 7, 9, 11, 13 and 15), or treated with the replicative DNA polymerase inhibitor, aphidicolin (lanes 2, 4, 6, 8, 10, 12, 14 and 16) A graphical presentation of $FANCD2^{Ub}$/FANCD2 and $FANCI^{Ub}$/FANCI ratios is provided in Supplementary Figure S2B. Inset: the efficiency of replication inhibition in presence of aphidicolin was monitored in a parallel replication assay using aliquots of egg extracts that were supplemented with circular ssDNA and either untreated (lanes 1–7) or treated with aphidicolin (lanes 8–14).

was monoubiquitinated. To further investigate this, we performed several additional experiments. The depletion-IP results shown in Figure 3A and B were obtained using a mix of two FANCI antibodies directed against the N- and C-termini of FANCI. To test if one of these antibodies interrupted a newly formed interaction site between FANCI and $FANCD2^{Ub}$ (which may be caused by conformational changes of FANCD2 or FANCI upon FA pathway activation), we repeated the experiment shown in Figure 3A using separately purified N- or C-terminal FANCI antibodies to immunodeplete FANCI. If the N-terminal FANCI antibody was interrupting a putative FANCI–$FANCD2^{Ub}$ interaction, the C-terminal antibody should still co-deplete $FANCD2^{Ub}$ with FANCI and inversely, if the C-terminal antibody was interrupting FANCI–$FANCD2^{Ub}$, the N-terminal antibody should still co-deplete $FANCD2^{Ub}$ with FANCI. As shown in Figure 3C (iii) (analysis of FANCIΔIP beads), N- and C-terminal FANCI antibody selectively co-depleted FANCD2 but not $FANCD2^{Ub}$ from extracts stimulated with circular dsDNA. Concurrently, the ratio of $FANCD2^{Ub}$/FANCD2 in extracts depleted with either FANCI antibody ('post-depleted extracts') was increased compared with 'pre-depleted extracts' [Figure 3C (i) and (ii)], confirming that N- and C-terminal FANCI antibodies selectively

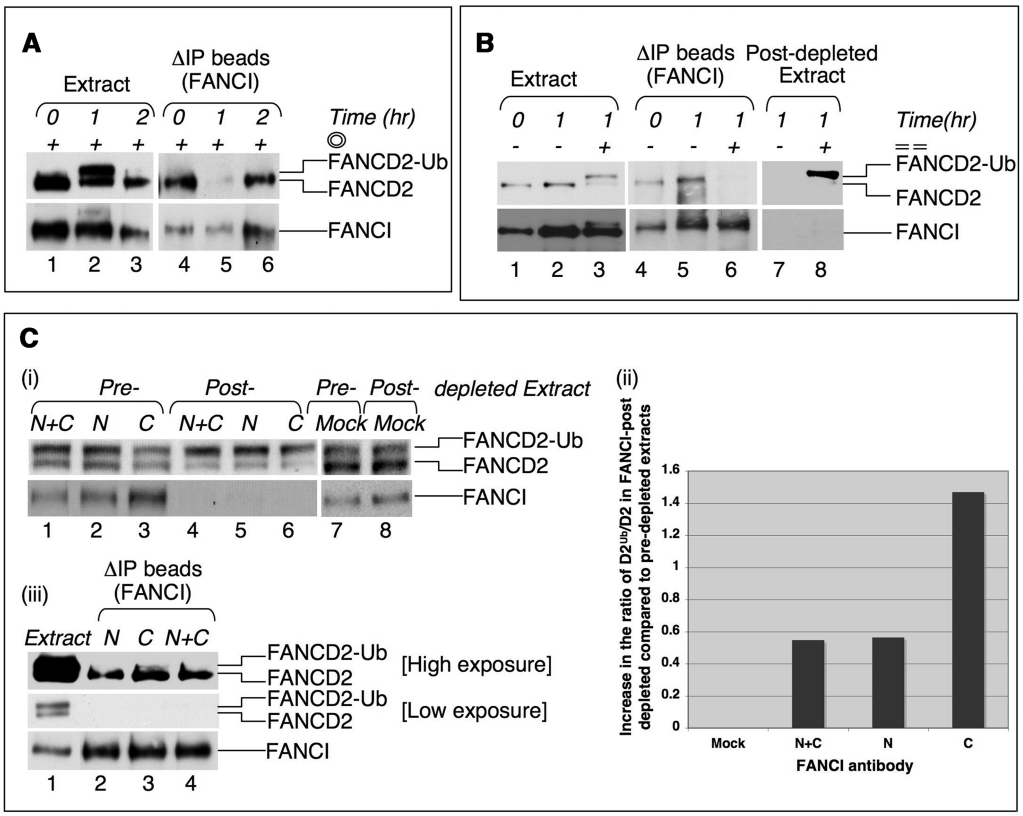

Figure 3. The FANCD2–FANCI complex dissociates in response to FA pathway activation. **(A)** DNA damage-induced dissociation and subsequent reassociation of FANCD2 and FANCI. S-phase extracts were incubated with 50 ng/μl of circular dsDNA for 0, 1 or 2 h (lanes 1–3, respectively). At the indicated time points, extracts were transferred to ice and subjected to ΔIP by incubation with beads coupled to FANCI antibody. ΔIP beads were assayed for bound FANCD2 and FANCI by SDS–PAGE and western blot (lanes 4–6). (**B**) the FANCI–FANCD2 complex dissociates in response to dsDNA fragments. S-phase extracts were either untreated, or treated with 50 ng/μl of fragmented dsDNA (lanes 1–3) for 1 h. At the indicated time points, extracts were transferred onto ice and subjected to ΔIP by incubation with beads coupled to FANCI antibody. ΔIP beads (lanes 4–6) and 'post-depleted' extracts (lanes 7 and 8) were assayed for the presence of FANCD2 and FANCI. (**C**) N- and C-terminal FANCI antibodies do not interrupt the FANCD2–FANCI complex. (i) S-phase egg extracts were incubated with 50 ng/μl of circular dsDNA for 60 min, transferred to ice, and subjected to ΔIP by incubation with beads coupled to antibodies against the FANCI N-terminus (N, lane 2), C-terminus (C, lane 3) or both (N+C, lane 1), or with beads coupled to rabbit IgG (lane 7). One microliter aliquots were taken at the end of the 90 min incubation time ('pre-depleted extract', lanes 1–3 and 7), followed by separation of extract ('post-depleted extract', lanes 4–6 and 8) and ΔIP beads [see (iii)]. Aliquots of pre- and post-depleted extracts were assayed for FANCD2 and FANCI by SDS–PAGE and immunoblot. The ratio of monoubiquitinated to non-ubiquitinated FANCD2 ($FANCD2^{Ub}$/FANCD2) was determined in pre- and post-depleted extracts and the ratio increase in post-depleted extracts compared with pre-depleted extracts was plotted and is shown in (ii). The ΔIP beads were assayed for the presence of FANCD2 and FANCI (iii). One microliter of undepleted, dsDNA-treated extract was used as a size control in (iii) (lane 1). (**D**) A *de novo* formed complex of Flag–FANCI and endogenous FANCD2 dissociates in response to fragmented dsDNA. Egg extracts were incubated with Flag–FANCI for 30 min, then untreated (lane 1) or treated with dsDNA fragments (lane 2) for an additional 30 min. DNA-free or DNA-containing extracts were subjected to ΔIP by incubation with Flag antibody-coupled beads. ΔIP beads were analyzed for bound FANCD2 and FANCI (lanes 4 and 5). ΔIP with Flag antibody-beads from untreated extracts (no Flag–FANCI, no DNA) was used as negative control (lane 3). (**E**) FANCI is not associated with the FAN1–$FANCD2^{Ub}$ complex. S-phase extracts were untreated (lane 1) or treated with 50 ng/μl of fragmented dsDNA (lane 2) for 1 h, and then subjected to ΔIP by incubation with FAN1 antibody-beads. ΔIP beads were assayed for bound FANCD2 and FANCI (lanes 4 and 5). Lane 3 shows a control ΔIP with mouse IgG. (**F**) A monoubiquitination-dead FANCD2 mutant dissociates from FANCI in response to dsDNA fragments. Egg extracts were incubated with Flag–$FANCI_{wt}$ alone (lane 1) or with Flag–$FANCI_{wt}$ and Myc–$FANCD2_{K562R}$ (lanes 2 and 3) for 30 min, then untreated (lane 2) or treated with dsDNA fragments (lanes 1 and 3) for an additional 30 min. DNA-free or DNA-containing extracts were subjected to ΔIP by incubation with Myc antibody-coupled beads. ΔIP with Myc antibody-beads from extracts lacking Myc–$FANCD2_{K562R}$ was used as negative control (lane 4). ΔIP beads were analyzed for bound FANCI and FANCD2 (lanes 4–6). (**G**) A phospho-dead FANCI mutant is unable to dissociate from FANCD2 in response to dsDNA fragments. (i) Egg extracts were incubated with Flag–$FANCI_{wt}$ (lanes 2 and 3) or Flag–$FANCI_{6S\rightarrow A}$ (lanes 4 and 5) for 30 min, then untreated (lanes 2 and 4) or treated with dsDNA fragments (lanes 1, 3 and 5) for an additional 60 min. DNA-free or DNA-containing extracts were subjected to ΔIP by incubation with Flag antibody-coupled beads. ΔIP beads were analyzed for bound FANCD2 and FANCI (lanes 6–10). ΔIP with Flag antibody-beads from DNA-treated extracts lacking recombinant FANCI was used as negative control (lane 6). (ii) The intensity of co-immunoprecipitated FANCD2 and FANCI protein bands shown in lanes 6–10 of Figure 3G (i) was determined by densitometry using Image J software. The relative intensity of each FANCD2 protein band compared with the corresponding FANCI protein band in the same lane was calculated. The relative amount of FANCD2 associated with Flag–$FANCI_{wt}$ or Flag–$FANCI_{S\text{-}A}$ in DNA-free extracts was set at a value of 100 and compared with the relative amount of FANCD2 associated with Flag–$FANCI_{wt}$ or with Flag–$FANCI_{S\text{-}A}$ in dsDNA fragment-treated extracts. [Densitometry of FANCD2 protein bands did include both FANCD2 isoforms (FANCD2 and $FANCD2^{Ub}$) where present (Figure 3G (i), lane 10)]. (**H**) A phosphomimetic FANCI mutant is unable to interact with FANCD2. DNA-free S-phase extracts were untreated (lane 4), or incubated for 30 min with Flag–$FANCI_{6S\text{-}D}$ (lane 5) or Flag–$FANCI_{wt}$ (lane 6). Extracts were subjected to ΔIP by incubation with Flag antibody-beads, and ΔIP beads were assayed for bound FANCD2 and FANCI (lanes 1–3). In all figures, the asterisk marks a non-specific band that is sometimes recognized by the Flag antibody.

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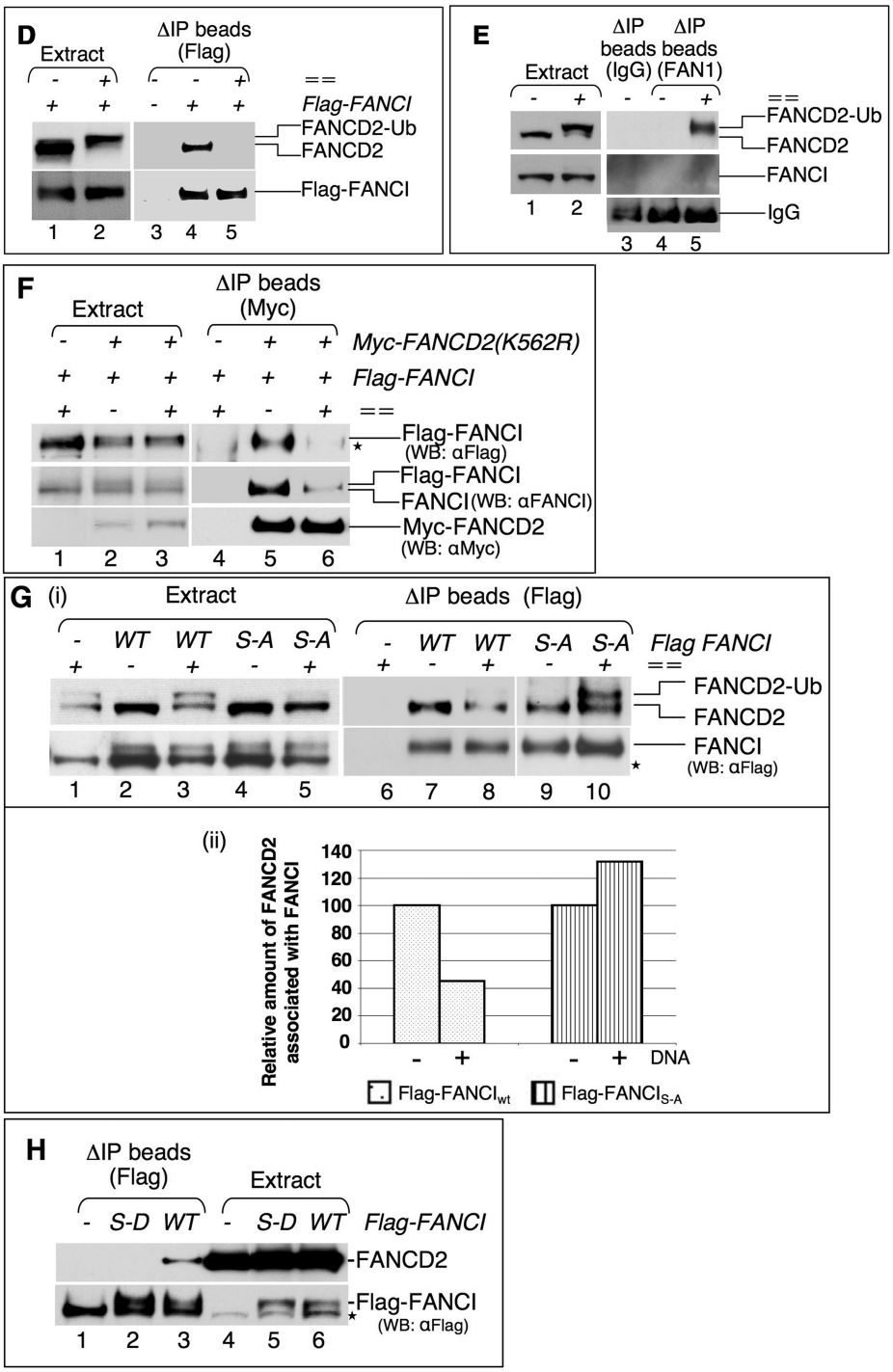

Figure 3. Continued.

co-depleted the non-ubiquitinated FANCD2 isoform. Occasionally, we observe that a small amount of FANCD2^{Ub} co-depletes with FANCI from dsDNA-stimulated extracts (Figure 3C (iii), lane 3), possibly representing a fraction of FANCD2 molecules that are not yet dissociated from FANCI. Next, we tested if *de novo* formed complexes composed of Flag–FANCI and extract-endogenous FANCD2 dissociated following addition of fragmented dsDNA to egg extracts. As shown in Figure 3D (Flag-ΔIP bead analysis), immunodepletion of Flag–FANCI with a Flag-specific antibody co-depleted FANCD2 from DNA-free extracts, but failed to co-deplete FANCD2^{Ub} from dsDNA-containing extracts, demonstrating that the newly formed Flag–FANCI–FANCD2 complex dissociated again upon FANCD2^{Ub} formation. Unfortunately, we were not able to test whether immunodepletion of FANCD2^{Ub} would also fail to co-deplete FANCI from extracts because none of

our *Xenopus*-specific FANCD2 antibodies are able to recognize the FANCD2^{Ub} isoform during immunodepletion. As an alternative approach, we tested if immunodepletion of a FANCD2^{Ub}-specific interactor, FAN1, was able to co-deplete FANCD2^{Ub}—but not FANCI—from dsDNA-stimulated egg extracts. Analysis of FAN1-ΔIP beads (Figure 3E) showed that FAN1 interacted selectively with FANCD2^{Ub} but not with the non-ubiquitinated FANCD2 or FANCI isoforms, demonstrating that the FAN1–FANCD2^{Ub} complex did not contain FANCI. Taken together, these results strongly indicate that activation of the FA pathway triggers dissociation of the FANCD2–FANCI complex in a step that coincides with FANCD2 monoubiquitination.

Lastly, we asked whether FANCG, a FA pathway member and interactor of FANCD2 in human cells (40), participated in the FANCD2–FANCI complex and its dissociation dynamics in *Xenopus* S-phase extracts. We found that a subset of *Xenopus* FANCG and FANCD2–FANCI molecules formed a complex in DNA-free extracts; moreover, we observed that FANCG retained its interaction with FANCD2 upon DNA substrate-triggered FANCD2–FANCI dissociation (Supplementary Figure S4), indicating that FANCG and FANCD2 cooperate during the DNA damage response but may act separately from FANCI.

Phosphorylation of FANCI is crucial for its dissociation from FANCD2

The fact that the monoubiquitinated isoform of FANCD2 was dissociated from FANCI hinted that FANCD2^{Ub} formation might contribute to the FANCD2–FANCI dissociation. We generated a *Xenopus* monoubiquitination-dead FANCD2 mutant (Myc–FANCD2$_{K562R}$) and tested its ability to dissociate from FANCI in response to dsDNA fragments in *Xenopus* extracts. As shown in Figure 3F, Myc–FANCD2$_{K562R}$ interacted with FANCI in DNA-free egg extracts, and dissociated from FANCI in response to dsDNA fragments, demonstrating that monoubiquitination of FANCD2 is dispensable for the FANCD2–FANCI dissociation. It was previously shown that monoubiquitination of human and chicken FANCD2 depends on prior phosphorylation of FANCI within a peptide stretch containing six SQ residues (6SQ-stretch) that are all conserved in *Xenopus* (13,27). Thus, we argued that phosphorylation of FANCI might be the actual molecular trigger for the dissociation of FANCD2 and FANCI while the immediately following FANCD2^{Ub} formation would just coincide with, but not be responsible for, dissociation of the FANCD2–FANCI complex. We generated two *Xenopus* FANCI mutant proteins: (i) a sextuple phosphorylation-dead FANCI mutant (Flag–FANCI$_{6S\rightarrow A}$) and (ii) a sextuple phosphorylation-mimetic FANCI mutant (Flag–FANCI$_{6S\rightarrow D}$). Both mutants are based on the sequence-homologous sextuple chicken FANCI mutants that caused suppression of FANCD2^{Ub} formation and constitutive FANCD2^{Ub} formation, respectively, in DT-40 cells (27). If phosphorylation of the 6SQ-stretch was crucial for FANCD2–FANCI dissociation, the Flag–FANCI$_{6S\rightarrow A}$ mutant should not be able to dissociate from endogenous FANCD2 in response to a DNA damage substrate. In contrast, the Flag–FANCI$_{6S\rightarrow D}$ mutant should not be able to interact with endogenous FANCD2 at all, even in absence of FANCD2^{Ub}-stimulating DNA substrates.

Addition of Flag–FANCI$_{6S\rightarrow A,}$ but not Flag–FANCI$_{wt}$ to *Xenopus* egg extracts suppressed dsDNA fragment-stimulated FANCD2^{Ub} formation [Figure 3G (i)], indicating that *Xenopus* Flag–FANCI$_{6S\rightarrow A}$ acts in a dominant negative manner to suppress FA pathway activation, similar to human FANCI$_{6S\rightarrow A}$ (27). Subsequent immunodepletion of Flag–FANCI$_{wt}$ or Flag–FANCI$_{6S\rightarrow A}$ from DNA-free or dsDNA-stimulated extracts revealed that the Flag–FANCI$_{6S\rightarrow A}$ mutant was unable to dissociate from endogenous FANCD2 in response to dsDNA fragments [Figure 3G (i) and (ii)]. In fact, Flag–FANCI$_{6S\rightarrow A}$ interacted even with monoubiquitinated FANCD2 [Figure 3G (i), lane 10] that still weakly formed in Flag–FANCI$_{6S\rightarrow A}$-containing extracts (likely mediated by the presence of endogenous wild-type FANCI) [Figure 3G (i), lane 5]. This result strongly suggests that phosphorylation of FANCI within the 6SQ stretch is in fact the molecular trigger for FANCD2–FANCI dissociation and required to maintain the physical separation of FANCI from FANCD2. In support of this finding, we found that the phosphomimetic Flag–FANCI$_{6S\rightarrow D}$ mutant was unable to interact with endogenous FANCD2 even in DNA-free extracts (Figure 3H). In further support, we observed that—unlike Flag–FANCI$_{wt}$ and Flag–FANCI$_{6S\rightarrow A}$—the Flag–FANCI$_{6S\rightarrow D}$ mutant was unable to interact with recombinant Myc–FANCD2 *in vitro* (Supplementary Figure S5). In summary, these results indicate that phosphorylation of FANCI at serine residues within the 6SQ-stretch is the molecular trigger of FANCD2–FANCI dissociation upon FA pathway activation.

FANCD2 binds chromatin prior to—and independently of—FANCI

FA core complex, FANCD2 and FANCI are recruited to chromatin in a replication-dependent manner (12–14). During replication, chromatin-bound FANCD2 exists exclusively in its monoubiquitinated state, while monoubiquitinated and non-ubiquitinated FANCI isoforms can bind chromatin (12,13). Interestingly, Wang *et al.* (14) demonstrated that the FA core complex binds replicating chromatin prior to FANCD2, suggesting a stepwise recruitment of FA core complex and FANCD2. Based on our findings that (i) FANCD2 was monoubiquitinated prior to FANCI (Figure 2B) and that (ii) FANCD2^{Ub} was dissociated from FANCI (Figure 3A–E), we asked if FANCD2^{Ub} is recruited to chromatin prior to FANCI. We added sperm chromatin to S-phase extracts and reisolated the chromosomal DNA at different time points during replication. FANCD2^{Ub} but not FANCI was detectable on chromatin during early replication stages, indicating that FANCD2^{Ub} is recruited to chromatin first, followed by FANCI (Figure 4A and B; Supplementary Figure S6A and B). We also noticed that relative to the respective protein concentration of

FANCD2 and FANCI in egg extracts, more available FANCD2 than FANCI molecules were recruited onto chromatin from early to late stages of replication (Figure 4A and B; Supplementary Figure S6A and B). Importantly, the ratio of chromatin-bound FANCI to total FANCI never reached the ratio of chromatin-bound FANCD2 to total FANCD2, even at 120 min when replication was completed (see Figure 4A, inset). This was a somewhat puzzling observation since our results above (Figure 1B) showed that the majority of FANCD2 and FANCI molecules are complexed prior to their activation. Thus, even considering that chromatin association of FANCI is delayed compared with FANCD2, our chromatin binding results indicate that following FANCD2–FANCI dissociation in the presence of chromatin, only a fraction of the freed FANCI molecules are actually recruited onto replicating and post-replication chromatin during S-phase.

Since FANCD2 bound chromatin prior to FANCI, we asked whether FANCD2 was able to bind chromatin in absence of FANCI. As shown in Figure 4C (lane 8), FANCI-depleted extracts (containing 15–20% residual FANCD2) were able to support chromatin recruitment of the residual FANCD2, although with a slower accumulation rate. In contrast, FANCD2-depleted extracts (containing 15–20% residual FANCI) did not support chromatin recruitment of residual FANCI (Figure 4C, lane 9). We tested if we could recapitulate this finding using recombinant wild-type FANCD2 and FANCI proteins and found that recombinant Myc–$FANCD2_{wt}$ was recruited to replicating chromatin in FANCI-depleted extracts (Figure 4D), whereas recombinant Flag–$FANCI_{wt}$ was unable to bind chromatin in FANCD2-depleted extracts (Figure 4E). Further analysis revealed that chromatin-bound FANCD2 in FANCI-depleted extracts is in its non-ubiquitinated state (Figure 4D, inset), indicating that monoubiquitination of FANCD2 is not required for its chromatin recruitment. Together, these results indicate that FANCD2 is able to bind chromatin independently of FANCI and FANCI-mediated monoubiquitination, whereas FANCI is completely dependent on FANCD2 for its chromatin recruitment.

DISCUSSION

Based on the findings that FANCD2 and FANCI can interact *in vivo* and form a heterodimer *in vitro* (9,11,13,27,29,30), current FA pathway models describe the FANCD2–FANCI interaction as constitutive and the FANCD2–FANCI complex as a monoubiquitination-activated entity. Our results indicate a different scenario where the FANCD2–FANCI complex undergoes dynamic changes in a replication- and DNA damage-dependent manner, enabling FANCD2 and FANCI to fulfill separate roles during DNA repair in S-phase (Figure 5).

Several of our findings demonstrate that the FANCD2–FANCI interaction takes place while the FA pathway is inactive. At least 80% of FANCD2 and FANCI are associated in *Xenopus* extracts that harbor a silent FA pathway in absence of DNA (34), and these silent extracts support *de novo* interactions between recombinant and endogenous FANCD2 and FANCI. Since FANCD2–FANCI complex formation is unaltered in FANCA- or ATR-depleted extracts, the FANCD2–FANCI interaction requires neither the physical presence of FA core complex or ATR, nor core complex- or ATR-mediated modifications of FANCD2 or FANCI. It seems therefore plausible that the FANCD2–FANCI complex is composed of unmodified FANCD2 and FANCI proteins that assemble independently of cellular factors that are important for FA pathway activation. In agreement with this model, FANCD2 and FANCI were previously shown to interact in a DNA damage- and FANCD2 monoubiquitination-independent manner in human cells (9); moreover, recombinant human or mouse FANCD2 and FANCI proteins (that are presumably not modified) interact directly *in vitro* (11,30).

Why did previous studies observe only a small population of FANCD2–FANCI complexes (or none at all) in undamaged human cells (9,29,41)? Firstly, these studies co-immunoprecipitated FANCD2 and FANCI under standard stringency conditions (9,29,41) that can interrupt weak protein complexes. Indeed, *Xenopus* FANCD2–FANCI dissociated under comparable conditions in egg extracts, demonstrating that their association *in vivo* is very weak. Secondly, since the FA pathway is activated during S-phase, and since FANCD2-FANCI dissociate upon pathway activation (Figure 3A–H), we would expect the percentage of FANCD2–FANCI complexes to be significantly lower in unsynchronized cell populations compared with FA pathway-inactive *Xenopus* extracts (12,34). In fact, we speculate that essentially all of FANCD2 and FANCI are complexed in egg extracts, and that immunodepletion *per se* interrupts some of these complexes, reducing co-depletion levels to only 80%.

Activation of the FA pathway revealed that $FANCD2^{Ub}$ formation is triggered by replicating and non-replicating DNA substrates, whereas significant $FANCI^{Ub}$ formation only occurred during replicative DNA synthesis, hinting that some DNA damage types require $FANCD2^{Ub}$ but not $FANCI^{Ub}$ for their successful repair. Notably, DNA damage- or replication-induced formation of $FANCI^{Ub}$ is generally weaker than formation of $FANCD2^{Ub}$, both in *Xenopus* extracts [Figure 2A–C; (13)] and in human cells (9,29); moreover a monoubiquitination-dead FANCI mutant partially complements DNA repair defects in FANCI-deficient human cells (9,27), supporting a model where $FANCI^{Ub}$ contributes only to the repair of a subset of DNA lesions.

Regardless of the activating DNA substrate, the responding onset and peak of $FANCD2^{Ub}$ always preceded that of $FANCI^{Ub}$, indicating that $FANCD2^{Ub}$ and $FANCI^{Ub}$ do not act simultaneously, but have subsequent roles during DNA repair. Monoubiquitination of both proteins in the presence of circular ssDNA was strictly replication dependent, not only confirming that ssDNA *per se* does not activate the FA pathway (34), but also emphasizing that this pathway functions to repair lesions other than DNA ICLs since—unless DNA

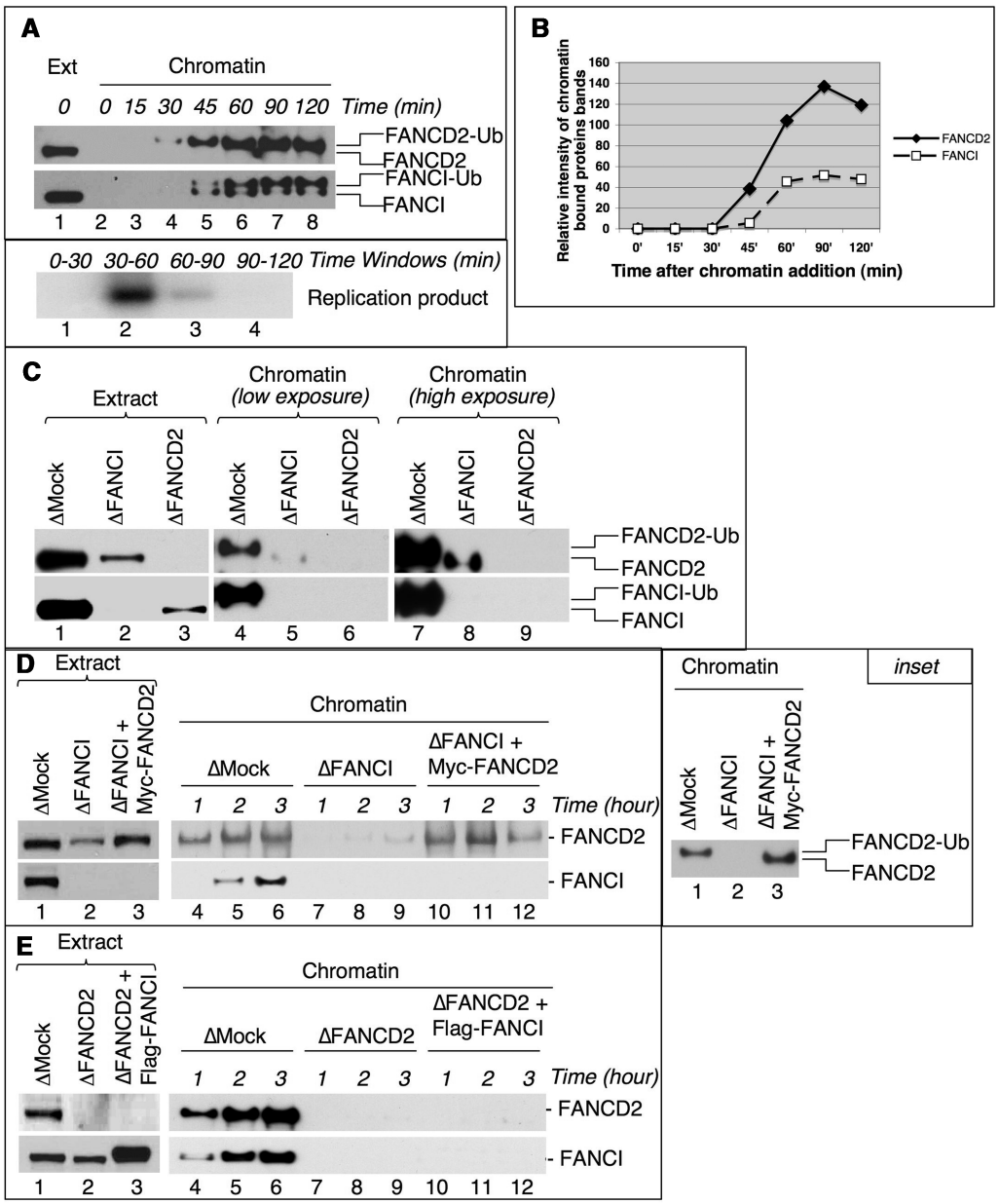

Figure 4. FANCD2 binds replicating chromatin prior to and independently of FANCI. **(A)** FANCD2 is recruited to replicating chromatin prior to FANCI. Sperm chromatin was added to S-phase extracts and reisolated at the indicated time points during replication. Chromatin fractions (lanes 2–8) were analyzed for bound FANCD2 and FANCI by SDS–PAGE and western blot. Lane 1: 1 µl extract (loading control). Inset: replication assay. Throughout the experimental procedure described in the legend to panel A, replication was monitored by pulsing replicating extract aliquots with [α-^{32}P]GTP at time windows as indicated. (**B**) The concentration of chromatin-bound FANCD2 is higher than that of FANCI. The intensity of chromatin-bound FANCD2 and FANCI protein bands shown in Figure 4A was determined by densitometry using Image J software. The relative intensity of each chromatin-bound protein band (lanes 2–8) compared with the protein band in the extract lane (lane 1) was plotted for each time point. (An independent repeat of this experiment is shown in Supplementary Figure S6A and B). (**C**) Residual FANCD2 binds chromatin in FANCI-depleted extracts. S-phase extracts were mock depleted (lane 1), FANCI depleted (lane 2) or FANCD2 depleted (lane 3). Sperm chromatin was added to the extracts and allowed to replicate. Chromatin was reisolated at 150 min (post-replication) and chromatin fractions were analyzed for bound FANCD2 and FANCI (lanes 4–6, short exposure; lanes 7–9, long exposure) by SDS–PAGE and western blotting. **(D)** Recombinant Myc–FANCD2$_{wt}$ binds replicating chromatin in FANCI-depleted extracts. S-phase extracts were mock depleted (lane 1), FANCI depleted (lane 2) or FANCI depleted (thus partially co-depleting FANCD2) and reconstituted with Myc–FANCD2$_{wt}$ (lane 3). Sperm chromatin was added to the extracts and allowed to replicate. Chromatin was reisolated at the indicated time points and chromatin fractions were analyzed for bound FANCD2 and FANCI (lanes 4–6: mock depleted; lanes 7–9: FANCI depleted; lanes 10–12: FANCI depleted and reconstituted with *Myc–FANCD2$_{wt}$*) by SDS–PAGE and western blotting. Inset: FANCI-depleted extracts (deficient in FANCD2^{Ub} formation) support chromatin recruitment of non-ubiquitinated Myc–FANCD2. The same chromatin fractions isolated from mock depleted (Figure 4D, lane 4), FANCI depleted (Figure 4D, lane 7) and FANCI depleted + Myc–FANCD2 (Figure 4D, lane 10) extracts were run on a lower percentage gel to allow separation of non-ubiquitinated and monoubiquitinated FANCD2 isoforms. (**E**) Recombinant Flag–FANCI$_{wt}$ is unable to bind replicating chromatin in FANCD2-depleted extracts. S-phase extracts were mock depleted (lane 1), FANCD2 depleted (lane 2) or FANCD2 depleted (thus partially co-depleting FANCI) and reconstituted with Flag–FANCI$_{wt}$ (lane 3). Sperm chromatin was added to the extracts and allowed to replicate. Chromatin was reisolated at the indicated time points and chromatin fractions were analyzed for bound FANCD2 and FANCI (lanes 4–6: mock depleted; lanes 7–9: FANCD2 depleted; lanes 10–12: FANCD2 depleted and reconstituted with Flag–FANCI$_{wt}$) by SDS–PAGE and western blotting.

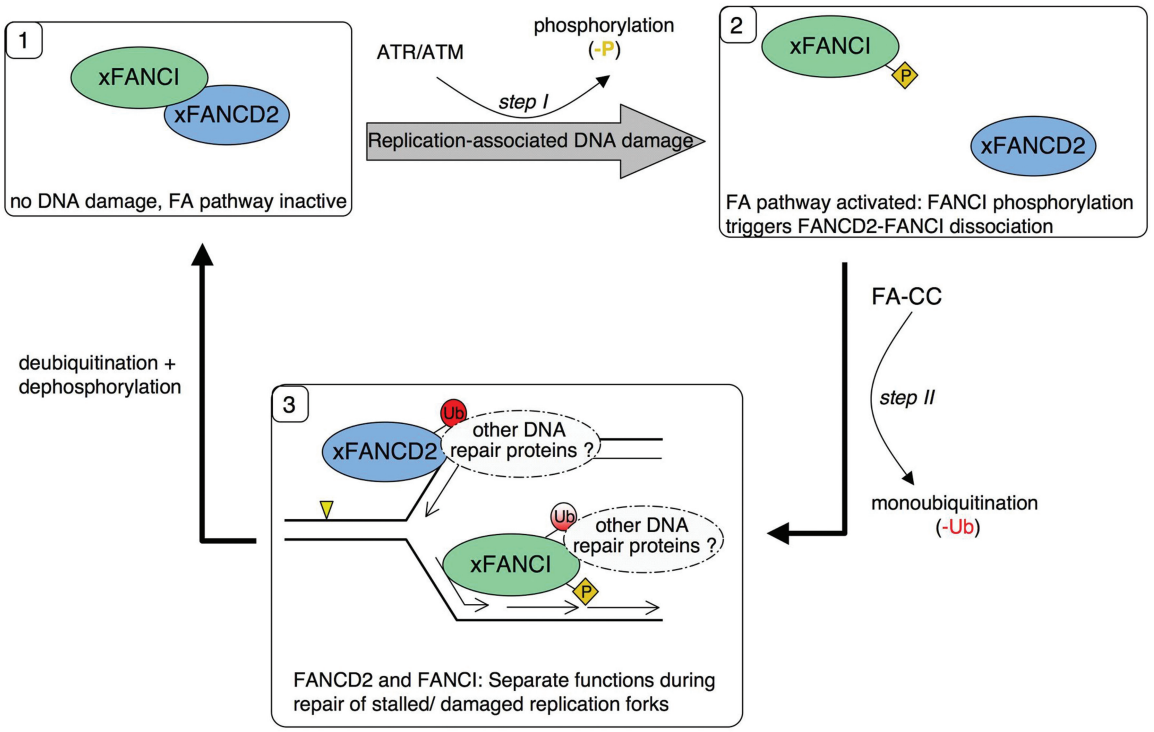

Figure 5. Dynamic FA pathway model. The FANCD2–FANCI dimer represents the inactive state of FANCD2 and FANCI (1). When DNA damage (yellow triangle) is encountered during DNA replication, the FA pathway is activated via ATR/ATM-mediated phosphorylation of FANCI. FANCI phosphorylation triggers dissociation of the FANCD2–FANCI dimer (2), immediately followed by monoubiquitination of FANCD2 by the FA core complex (FA-CC). Once dissociated, the monomeric FANCD2 and FANCI proteins are recruited separately to stalled replication forks where they have distinct functions during repair of stalled/collapsed replication forks. (3) One function of dissociated activated FANCD2 and FANCI may be to recruit different DNA repair protein complexes during their activated state. Once the DNA lesion is repaired, FANCD2 and FANCI modifications are removed and unmodified FANCD2 and FANCI return to their inactive, heterodimeric state (1).

interstrand crosslinking occurred within secondary hairpin structures—circular ssDNA should not contain ICLs. Contrasting our results, Sims *et al.* (29) showed synchronous FANCD2^{Ub} and FANCI^{Ub} induction after treatment of human cells with DNA damaging agents (e.g. MMC, UV light). Similarly, Knipscheer *et al.* (13) found synchronous FANCD2/FANCI monoubiquitination timing during DNA ICL repair in replicating nucleoplasmic *Xenopus* extracts. However, in both studies, cells or extracts were confronted with a variety of structurally heterogeneous DNA replication- and repair-associated intermediates. If some of these DNA intermediates stimulated FANCD2^{Ub} and FANCI^{Ub} responses differently, the overall ubiquitination responses of FANCD2 and FANCI would have appeared synchronous. In contrast, the DNA substrates used in our study—even the replicating ssDNA plasmid—represent a structurally more homogeneous pool of DNA molecules, which allowed us to detect the stepwise nature of the FANCD2 and FANCI monoubiquitination responses.

A surprising discovery was that FA pathway activation triggered dissociation of FANCD2 and FANCI. Our results indicate that a crucial modification known to occur early during FA pathway activation—the phosphorylation of FANCI within the 6SQ stretch—represents the molecular trigger for FANCD2—FANCI dissociation, immediately followed by monoubiquitination of FANCD2. This in turn suggests that the dissociated FANCI and FANCD2 isoforms represent the active state of both proteins, whereas the FANCD2–FANCI complex represents the inactive, 'resting' state. Intriguingly, a previous study showed results indicating a 'decreased' FANCD2–FANCI interaction after treatment of human cells with ionizing radiation [Smogorzewska *et al.* (9), Supplementary Figure S4C], suggesting that the DNA damage-induced FANCD2–FANCI dissociation step is conserved between human and frog. A subset of FANCD2–FANCI complexes contained the FANCD2-interactor FANCG that remained predominantly associated with the 'non-ubiquitinated' FANCD2 isoform upon FANCD2–FANCI dissociation (Supplementary Figure S4), hinting that the FANCG–FANCD2 complex functions separately from FANCD2^{Ub} or FANCI during DNA repair. This result supports human cell-based findings showing that non-ubiquitinated FANCD2 and FANCG participate in a functional protein complex during DNA repair (40), and hints at the existence of additional as yet unidentified members of the FANCD2–FANCI complex.

What consequences does the dissociation of FANCD2 from FANCI have? It allows the subsequently formed FANCD2^{Ub} to bind replicating chromatin earlier than either of the FANCI isoforms (non-ubiquitinated or ubiquitinated), indicating that FANCD2^{Ub} does act at

earlier DNA repair steps than FANCI. This in turn suggests that once separated from FANCI, FANCD2 can function independently of FANCI. In support of this idea, we found that FANCI-depleted extracts still support chromatin binding of endogenous residual FANCD2, as well as recombinant supplemented FANCD2, whereas the reverse is not the case. Our observation that endogenous or recombinant chromatin-bound FANCD2 is not monoubiquitinated in FANCI-depleted extracts adds to a growing body of evidence that both isoforms of FANCD2 (FANCD2 and $FANCD2^{Ub}$) can bind chromatin (9,16,41,42), and that monoubiquitination mediates other functions of FANCD2 such as recruitment of specific ubiquitin-binding nucleases (15–22,43). FANCI on the other hand may require the presence of already chromatin-bound FANCD2 or depend on a prior FANCD2-mediated DNA-processing step for its own chromatin recruitment. It should be mentioned that FANCI is able to bind naked DNA substrates in absence of FANCD2 *in vitro* (11); however, recruitment of FANCI to whole replicating chromatin may occur in a more regulated manner, requiring accessory factors that rely on the presence of chromatin-bound FANCD2.

Another unexpected discovery was the high concentration of chromatin-bound FANCD2 compared with FANCI throughout replication. Interestingly, Knipscheer *et al.* (13) estimated the total concentration of FANCD2 to be twice as high as that of FANCI in *Xenopus* nucleoplasmic extracts that are prepared from sperm nuclei previously replicated in S-phase extracts, supporting the idea that more FANCD2 than FANCI molecules are imported into S-phase nuclei and subsequently loaded onto replicating chromatin. Consequently, these data suggest that at least some DNA lesions require more FANCD2 than FANCI molecules (or no FANCI molecules at all) for their repair and support our hypothesis that chromatin-bound FANCD2 and FANCI do not act as a heterodimeric entity.

Nevertheless, the question remains whether $FANCD2^{Ub}$ and $FANCI^{Ub}$ can reunite to perform specific functions as a double-ubiquitinated heterodimer. Since our antibodies do not recognize native $FANCD2^{Ub}$ or $FANCI^{Ub}$ isoforms during immunodepletion—likely due to conformational changes of FANCD2 and FANCI upon monoubiquitination—we are currently unable to answer this question.

In summary, our results indicate a greater complexity of the FANCD2–FANCI relationship than previously appreciated. Instead of being constitutive as previously proposed, the FANCD2–FANCI complex dissociates in a dynamic DNA damage-sensitive manner, likely allowing these proteins to perform different functions at separate steps during repair of replication-associated DNA lesions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6 and Supplementary References [44–46].

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