Monoubiquitination by the Fanconi Anemia core complex locks FANCI:FANCD2 on DNA in filamentous arrays

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Abstract

FANCI:FANCD2 monoubiquitination is a critical event for replication fork stabilization by the Fanconi anemia (FA) DNA repair pathway. It has been proposed that at stalled replication forks, monoubiquitinated-FANCD2 serves to recruit DNA repair proteins that contain ubiquitin-binding motifs. Here we have reconstituted the FA pathway in vitro to study functional consequences of FANCI:FANCD2 monoubiquitination. We report that monoubiquitination does not promote any specific exogenous protein:protein interactions, but instead stabilizes FANCI:FANCD2 heterodimers on dsDNA. This locking of FANCI:FANCD2 complex on DNA requires monoubiquitination of only the FANCD2 subunit. We further show that purified monoubiquitinated FANCI:FANCD2 forms filament-like arrays on long dsDNA using electron microscopy. Our results reveal how monoubiquitinated FANCI:FANCD2 is activated upon DNA binding and present new insights to potentially modulate monoubiquitinated FANCI:FANCD2/DNA filaments in FA cells.

Fanconi Anemia | ubiquitination | DNA repair | biochemistry

Introduction

Fanconi anemia (FA) is a devastating childhood syndrome that results in bone marrow failure, leukemia and head and neck cancers (1, 2). FA is caused by inheritance of one of 22 dysfunctional FA genes (FANCA-FANCW) (3). Absence of any one member of the pathway causes genome instability during DNA replication, which results in mutagenic (cancer-causing) DNA damage and hypersensitivity to chemotherapeutic (normal and cancer-killing) DNA damage (4). Central to the FA pathway is the conjugation of ubiquitin to FANCI:FANCD2 (ID2) complexes (5, 6). ID2 monoubiquitination is critical to prevention of bone marrow failure in FA, but it is currently unknown how ID2-ub differs in its function to ID2. Several proteins have been proposed to specifically bind FANCI\textsuperscript{Ub} or FANCD2\textsuperscript{Ub} but not the un-ubiquitinated proteins (7, 8). For example, FAN1 nuclease was proposed to interact with FANCD2\textsuperscript{Ub} via
its ubiquitin-binding domain (UBZ) (7), whereas recruitment of SLX4 endonuclease to the interstrand crosslink (ICL) site was shown to be dependent on FANCD2 ubiquitination (9). However, support for these interactions is limited to analysis of ubiquitination deficient (K>R) mutants, rather than evidence for direct ubiquitin-mediated protein interactions.

The retention of FANCD2 in chromatin foci is dependent on its monoubiquitination by a “core complex” of Fanconi anemia proteins (10). FANCI and the FA core complex are required to generate FANCD2-foci that mark the location of double strand breaks, stalled replication forks and R-loops (11-13) in the nucleus, and protect nascent DNA at these sites from degradation by cellular nucleases (14). The ubiquitinated form of FANCD2, and also its ubiquitinated partner protein FANCI, become resistant to detergent and high-salt extraction from these foci (15, 16), leading to speculation about the existence of a chromatin anchor or altered DNA binding specificity post-monoubiquitination (17).

A recent electron microscopy study revealed a DNA interacting domain that is required for FANCI:FANCD2 binding to DNA (18). The crystal structure of the non-ubiquitinated FANCI:FANCD2 shows that the monoubiquitination sites of FANCI:FANCD2 are buried and therefore inaccessible in the dimer interface of the complex (19), suggesting that DNA binding might be required to expose the ubiquitin binding sites. Based on biochemical analyses non-ubiquitinated FANCI and FANCD2 preferentially bind to branched DNA molecules which mimic DNA replication and repair intermediates (20-22), however how that activates monoubiquitination of FANCI:FANCD2 remains poorly understood. DNA is a cofactor for maximal ubiquitination (17, 23).

Here we have reconstituted the FA pathway using recombinant FA core complex and fluorescently labelled DNA oligomer substrates. We show that once monoubiquitinated, FANCI:FANCD2 forms a tight interaction with double-stranded containing DNA. We report the successful purification of monoubiquitinated FANCI:FANCD2 complex bound to DNA using an Avi-ubiquitin construct, and show that the monoubiquitination does not promote any new protein:protein interactions with other factors in vitro. Instead, we reveal a new role of monoubiquitinated FANCI:FANCD2 in forming higher order structures and demonstrate how monoubiquitinated FANCI:FANCD2 interacts with DNA to initiate DNA repair. Our work uncovers the molecular function of the pathogenetic defect in most cases of FA.

Results
Monoubiquitination does not promote association of FANCI:FANCD2 with a panel of proteins previously hypothesized to bind the ubiquitinated form
Mono-ubiquitinated FANCI:FANCD2 (henceforth IUbD2Ub) is the active form of the complex in repair of DNA damage. Many previous studies have speculated about the existence of DNA repair proteins that specifically associate with IUbD2Ub. A summary of these proteins is presented in Table 1. Using recombinant ID2 or IUbD2Ub prepared by a novel purification method (Figure 1a) (24) we sought to directly compare the binding of this panel of ID2-associated proteins. Each of the partner proteins was expressed using reticulocyte extracts (Figure 1b), and the majority bound to the ID2 complex as
predicted based on previously identified associations (Figure 1c). The strongest binding proteins in terms of fraction of protein recovered were SLX4, SMARCAD, FANCJ, PSMD4, SF3B1, TRIM25, MCM5 and BRE. Luciferase protein was used as a control 35S-labeled prey-protein, and this protein did not bind to ID2 (Figure 1c). Surprisingly, we discovered that none of the proteins showed any increased affinity for IUbD2Ub over ID2 (Figure 1c-d).

Table 1: List of proteins containing ubiquitin binding domain that are described or predicted to bind to ubiquitinated FANCD2.

| Protein | Function | Domain | Reference |
|---------|----------|--------|-----------|
| FAN1    | nuclease | UBZ4   | (25)      |
| SLX4    | nuclease | UBZ1   | (8)       |
| FAAP20  | FANCA partner | UBZ | (26) |
| RAP80   | BRCA1 partner | UIM  | (27)      |
| SMARCAD1| chromatin remodeler | CUE | (28)      |
| FANCJ   | helicase | -      | (29)      |
| PSMD4   | protease | UIM    | (30)      |
| SF3B1   | RNA binding protein | UBZ | (31)      |
| TRIM25  | E3 ligase | RING finger | (31) |
| MCM5    | CMG component | - | (31) |
| BRE     | BRCA1 partner | - | (32)      |
| BRCC    | BRCA1 partner | - | (32)      |
| SNM1A   | nuclease | UBZ    | (33)      |

Monoubiquitination locks FANCI:FANCD2 on DNA

An alternative explanation for the observed increased in association between ID2 and its associated proteins after DNA damage is that IUbD2Ub has an increased affinity for DNA, which brings the protein into closer proximity to these partners. The majority of ID2 associated proteins are chromatin localized. In order to explore the stability of IUbD2Ub on DNA, we performed in vitro monoubiquitination reactions in the presence of IR-dye700 labelled double-stranded DNA (dsDNA). As previously characterized (23), we observed DNA-dependent appearance of monoubiquitinated forms of FANCD2 and FANCI when using recombinant FA core complex components (Figure 2a-b). ID2 monoubiquitination readily lead to DNA mobility shifts using EMSA (electromobility shift assay) even at low concentrations, but this was not observed for the unmodified (apo)-ID2 complex in the absence of the enzymatically FA core complex, or when monoubiquination-defective K-to-R mutants of ID2 were used in the reaction (Figure 2c, lanes 2-4).
Previously, we and others showed that various different dsDNA-containing structures could robustly stimulate ID2 monoubiquitination (20, 34, 35), but that single-stranded DNA (ssDNA) does not. To determine if monoubiquitinated ID2 had increased affinity for other dsDNA containing structures, we repeated the monoubiquitination reactions in the presence of different IR-dye700 labelled DNA structures. Each of the dsDNA containing structures, led to increased ID2 monoubiquitination and increased retention of an EMSA shifted band (Figure 3). Conversely, ssDNA very weakly stimulated monoubiquitination but did not cause an EMSA shift.

Both FANCIub and FANCD2ub are associated with a “locked” protein:DNA complex

Previous studies reported that monoubiquitination of ID2 complex may lead to dissociation of the heterodimer to its individual subunits, as measured by loss of co-immunoprecipitation of FANCI with FANCD2 (35, 36). In contrast, we did not observe any Ub-mediated dissociation of ID2 in vitro. First, Western blotting of the EMSA gels confirmed that the gel shifted DNA band contains both FANCI and FANCD2 proteins (Figure 4a). Second, FANCIub still co-immunoprecipitated with FANCD2ub at the plateau of the in vitro ubiquitination reaction (Figure 4b).

To determine the contribution of each of FANCD2ub and FANCIub to the locking of ID2 complex to DNA, we used ubiquitination-deficient (KR) mutants in the ubiquitination reaction. FANCIKR:FANCD2WT or FANCIWT:FANCD2KR mutant results in decrease in EMSA shift, and FANCIKR:FANCD2KR did not bind to DNA (Figure 4c). However, this retention on DNA correlated with the extent of FANCD2 monoubiquitination retained by these mutant complexes. Western blotting the EMSA gels confirmed that both FANCD2 and FANCI are found in the EMSA shifted product, although in higher amounts when both proteins are capable of being monoubiquitinated (Figure 4d).

Mutant forms of ubiquitin can still lock ID2 onto DNA

We postulated that the altered affinity for DNA induced by monoubiquitination must result from either a conformational change in the ID2 heterodimer after monoubiquitination, or participation of the conjugated ubiquitin directly in protein:DNA or protein:protein binding. To help distinguish these possibilities we utilized mutants of ubiquitin that have previously been shown to mediate the known protein:ubiquitin or protein:DNA interactions in other ubiquitinated protein interactions (Figure 5a) (37). Each of these Ub mutants were conjugated to ID2 by the FA core complex with similar efficiency (Figure 5b) and their locking onto DNA was then measured. Mutations in surface patch 1 (F4A, D58A), surface patch 2 (I44A, V70A), a DNA binding residue (K11R) or a tail mutant (L73P) had no apparent effect on DNA locking (Figure 5c). This result suggests that no canonical surface or region of ubiquitin is critical for DNA locking of ID2, and instead ubiquitin conjugation to ID2 probably induces a conformational rearrangement of the heterodimer.

Purification of monoubiquitinated FANCI:FANCD2 complex bound to dsDNA reveals a filamentous architecture
In order to examine the architecture of purified recombinant IUbD2Ub complex in the presence of dsDNA plasmid, we utilized a recombinant Avi-tag ubiquitin construct containing a 3C protease site between the biotinylated Avi-tag and the N-terminus of ubiquitin (Figure 6a). This tagged ubiquitin is incorporated onto FANCI:FANCD2 by the FA core complex, allowing Avidin-Sepharose purification of monoubiquitinated ID2 that is then eluted by 3C protease cleavage. We recovered monoubiquitinated FANCI:FANCD2 complex only when FANCI is monoubiquitinated, suggesting that the N-terminus of D2-attached ubiquitin may be buried within the di-ubiquitinated complex, but the N-terminus of ubiquitin attached to FANCI is accessible for streptavidin binding (Figure 6b).

Using this purified protein, we compared FANCIub:FANCD2ub to unmodified FANCI:FANCD2 using electron microscopy (EM). Surprisingly, we observed that FANCIub:FANCD2ub forms filament-like oligomers when bound to dsDNA plasmid (Figure 6c). Such filaments were not observed in the unmodified FANCI:FANCD2 protein preparation in the absence of presence of plasmid DNA, nor in previous investigations of human or Xenopus FANCI:FANCD2 complexes studied by EM (Figure 6d-e and (38-40)).

When smaller DNA molecules were used as the substrate for ID2 binding, we either observed no filament-like structures (60bp, Figure 7a) or shorter filament-like structures (150bp, Figure 7b) compared to structures that were on average 7-8x longer than the characteristic double saxophone structure of ID2 heterodimer in the non-ubiquitinated state (Figure 7c).

The observation that filament length correlated with the size of DNA available for ID2 binding strongly suggested that the association between heterodimer subunits in the array was DNA-mediated. To test whether the array of IUbD2ub is also dependent upon binding to the same DNA molecule we examined the plasmid-stimulated ubiquitination reaction products after treatment with the non-specific endonuclease, Benzonase. It is apparent from EM images that addition of Benzonase breaks the long filaments formed by IUbD2ub complex into very short or heterodimer-sized units (Figure 7d). This finding is consistent with Benzonase cleaving exposed DNA between IUbD2ub units, leading to destabilization of the filamentous arrays. Together our results show that, in vitro, ubiquitination of ID2 leads to a ubiquitin- and DNA-stabilized filamentous structure.

**Single IUbD2ub heterodimers on short 60bp DNA have an altered architecture**

Due to variability in the length and shape of filament-like IUbD2ub structures on longer DNA molecules we have not been able to uncover the shape or subunit rearrangement of the individual units of the arrays. However, examination of IUbD2ub purified together with short 60bp DNA allowed us to collect sufficient images of individual particles for analysis. These particles were similar in size to non-ubiquitinated ID2, but it is clear from individual molecule and class average views that the IUbD2ub complex forms a distinct architecture from that of ID2 (Figure 8). In particular, the overall shape of individual particles and their class averages reveal a twisting that repositions the solenoid arms of one or both of the subunits bringing them into closer proximity. The conformational change induced appears
to reduce the size of ID2 in an X but not Y direction, similar to that predicted in a previously model prediction that placed DNA in a channel between FANCI and FANCD2 post DNA binding (17). These images support the view that monoubiquitination induces a conformational change in the ID2 complex that locks it upon DNA.

Discussion

The protection of stalled forks by DNA repair factors is essential for proper DNA replication and the maintenance of genome stability. The primary mechanism of replication fork stabilization at ICLs, and other blocking damage, utilizes the proteins of the FA-BRCA DNA repair pathway. Monoubiquitination of FANCI:FANCD2 by the FA core complex is the central event in this pathway. Here we showed that monoubiquitination directly locks the ID2 complex onto double-stranded DNA, and promotes a filament-like coating of long DNA molecules. This finding answers a long-standing question about the nature of the biochemical reaction that is absent in the majority of patients with FA (41, 42).

“Fork protection” involves (i) exclusion of cellular nucleases such as MRE11 and DNA2 from the stalled DNA replication fork, and (ii) specific recruitment of other factors that are able to restart DNA replication (14, 43, 44). The role of nuclease exclusion seems most important because inhibition of either of two nucleases, MRE11 or DNA2, can significantly alleviate the sensitivity of FA-BRCA mutant cells to replication stalling agents (14, 43). But in many studies, specific association of various repair factors with IUbD2Ub compared to ID2 have been described. These proteins mostly contain ubiquitin-binding domains, providing an impetus for the recruitment-based hypothesis (summary and references in table 1). However, because these previous studies focused on use of ubiquitination-deficient mutants, they could not address the underlying question of whether ubiquitin on either FANCI, FANCD2 or both proteins directly mediated the interaction. Now, we have shown that none of these proteins specifically bind recombinant purified IUbD2Ub compared to ID2. This finding was unexpected, and included proteins such as SLX4, MCM5, and FAN1, which have a demonstrated and essential role in the FA-pathway (10, 31, 45).

Instead, our data provide direct evidence that ID2 undergoes a conformational change after monoubiquitination, that leads to it becoming locked on double-stranded DNA. It is likely that in previous cell-based experiments specific interaction between SLX4 and FAN1 with FANCD2 but not FANCD2K561R was observed because FANCD2K561R does not become locked on DNA, even in the presence of active FA core complex. This finding is supported by the observation that FANCD2K561R also does not becomes chromatin localized at damage sites even after extensive DNA damage (ref). We propose that IUbD2Ub does not demonstrate restricted interaction with any specific protein partner. None-the-less, its retention in chromatin after ubiquitination would be much more likely to bring the complex into proximity of these other DNA repair factors, where it could still influence their activity.

Locking onto DNA occurs through a ubiquitin-mediated conformational change in the ID2 complex. A ubiquitin binding-domain (UBD) in FANCD2 has previously been shown necessary for the retention of
the protein in the chromatin faction, and for strong binding to FANCI (46). This UBD domain sits in the FANCD2 structure opposite to where ubiquitin is likely to reside after its conjugation on to FANCI by the FA core complex, and most likely mediates the locking function and conformational rearrangement. Relocation of a FANCD2 tower domain upon DNA binding most likely stimulates the conformational rearrangement that is then stapled in place by ubiquitin:UBD association. Other DNA binding proteins such as histone H2A show an increased association with DNA after monoubiquitination (47) and monoubiquitination also increases the DNA occupancy of transcription factors such as FOXO4 and CIITA (48). It’s possible that ubiquitin to UBD mediated locking is a general mechanism of protein:DNA target stabilization.

IUbD2Ub locked in nucleoprotein filaments

In addition to a conformational change in ID2 induced by monoubiquitination (that has also been concurrently discovered and reported by the Paveltich and Passmore labs (49, 50)) we found that monoubiquitinated IUbD2Ub formed large filament-like arrays when it was purified together with plasmid DNA, but not short 60bp DNA fragments. On average, the length of plasmid-associated structures is 7-8x that of that associated with 60bp DNA. Larger or longer arrays may potentially be obscured from view because the purification strategy makes elution exponentially more difficult with increasing numbers of conjugated ubiquitin-molecules. Steps to remove “aggregates” may have also inadvertently removed larger arrays. However, as the number of potential plasmid DNA binding sites for ID2 was in large excess the concentration of ID2 used to stimulate reaction, their appears to be some purpose to creation of these filamentous arrays.

There is evidence that IUbD2Ub locked in nucleoprotein filaments exist in cells. Antibodies against FANCD2 have long been used as a marker of double strand breaks, stalled replication forks and R-loops because the protein forms large, intensely staining foci during S-phase that are increased after treatment with DNA damaging agents (11, 12, 51). We suspect that these intense foci are due to coating of DNA around damaged forks, potentially in filamentous arrays similar to those we observed by EM. Support for the large size and extent of DNA binding reflective of filamentous arrays also comes from chromatin immunoprecipitation and sequencing (ChIP-Seq) using anti-FANCD2 (13). FANCD2, and two other damage markers MRE11 and γH2AX, showed no specific localization in a bulk population of cells, but strongly localized adjacent to a Cas9-induced site-specific DNA break. Both γH2AX and FANCD2 produced a broad peak centred at the target site kilobases (kb) to megabases (mb) in length. In contrast, MRE11 is located within a very tight peak within ~100bp of the break. Chromatin within 1-2 kb of the DSB showed reduced occupancy by γH2AX, consistent with dechromatinization around break sites (52), but FANCD2 was present right up to the DSB. Accumulation of FANCD2 increases at the DSB early after cleavage, and accumulates more distant from the DSB progressively with time post-cleavage. This is suggestive of a polymerisation of the FANCD2 signal away from the break site, as hypothesised would occur for a protein that forms a growing filament at broken DNA (13). The conserved function of FANCD2 as a histone chaperone (53, 54) may even be directly linked to displacement of nucleosomes as filamentous arrays extend into break-adjacent chromatin.
In this study, we also observed direct association of two ID2 heterodimers by co-immunopurification only after the protein becomes monoubiquitinated. This approach, if performed in cells, could be used to further delineate the mechanism and cellular factors required for the extension of Iub:D2ub arrays during fork protection. Of particular interest will be determining the role of BRCA1 in locking and/or array extension. BRCA1:BARD1 was initially thought to be the E3 for FANCD2 monoubiquitination, because it co-immunoprecipitates FANCD2, and FANCD2 does not form nuclear foci after damage in BRCA1-deficient cells [31]. However, in various assays it was later shown that FANCD2 monoubiquitination does occur in BRCA1-deficient cells, but it is uncoupled from FANCD2 foci formation [32, 33].

How would a locked ID2 filament mediate fork protection?

Filamentous structures on DNA play a genome protective role in prokaryotes: eg DAN protein forms a rigid collaborative filament that reduces accessibility during anoxia (55), while the *Vibrio cholera* protein ParA2 forms protective filamentous structures on DNA during segregation (56). Structural characterization has demonstrated how these filaments function and, in the case of ParA2, can be targeted therapeutically (57). The coating of ssDNA by RPA in eukaryotes, also protect DNA from the activity of nucleases, and directs the specific activity of others (58-60). We propose that a FANCIub:D2ub filament may have a similar stabilising role on newly synthesised dsDNA at a stalled replication fork. This property would explain why stalled forks are prone to degradation in FA and BRCA patient cells (14, 43). In particular, we hypothesise that filamentous DNA-locked Iub:D2ub could prevent access to DNA by MRE11 and DNA2 nucleases and prevent aberrant ligation of broken DNA to other parts of the genome by non-homologous end-joining.

Second, the tight binding of FANCIub:D2ub to dsDNA, when localized to stalled replication forks, may also prevent the branch migration of replication forks and prevent their spontaneous or helicase-mediated reversal (61). Reversed forks are the substrate for degradation by DNA2 and WRN nuclease activities, providing a hypothetical link between the activities of FANCD2-monoubiquitination and the nuclease activity of DNA2 and WRN (62, 63).

Third, Iub:D2ub arrays may also locally suppress non-homologous end-joining (NHEJ) factors, and/or delineate the newly synthesized chromatin from unreplicated regions during the promotion of templated repair processes such as homologous recombination. FANCD2, FANCI, and components of the FA core complex were identified amongst relatively few other factors, in a genome-wide screen for genes that promote templated repair over NHEJ (64). Stabilization of RAD51 filaments, required for HR, is also an in vitro property of ID2 (65), suggesting Iub:D2ub filamentous arrays may exist adjacent to or coincident with RAD51 filaments in cells, in order to provide a polarity to the homologous recombination reaction without loss or gain of genomic sequences.

Role of FANCI-monoubiquitination

Fanci and Fancd2 have common and distinct functions in mouse models of Fanconi anemia (66), while the double knockout of FANCI and FANCD2 has an unexpectedly distinct phenotype compared to
single knockouts in human cells (67). But FANCI^{K523R} expressing cells are less sensitive to DNA damage than FANCI knockout in human cells (15), so what is the role of FANCI monoubiquitination? Previous studies demonstrated that FANCI monoubiquitination is always subsequent to FANCD2 monoubiquitination, both in cells (68) and in biochemical assays (23). FANCI also likely plays a role in recruiting the FA core complex to the substrate (69). In this study, we show that FANCI monoubiquitination is not necessary for locking of the ID2 complex onto DNA (Figure 4). However, in vivo it is likely that FANCI monoubiquitination plays a critical role in regulating deubiquitination of the ID2 complex. FANCI recruits the deubiquitinating enzyme USP1:UAF1 (70), which prevents trapping of monoubiquitinated FANCD2 at non-productive DNA damage sites, but only ID2^{Ub} but not I^{Ub}D2^{Ub} is a substrate (23). It is also clear from our EM investigations that FANCI must play an important role in the structural integrity of I^{Ub}D2^{Ub} filamentous arrays on DNA, possibly creating an asymmetry necessary for a specific polarity to array assembly.

**Implications for understanding the deficiency of Fanconi anemia**

Onset of progressive bone marrow failure occurs at a median age of 7 in children with FA (71). Almost all of these patients lack FANCD2 and FANCI monoubiquitination, due to mutation in either FANCD2 or FANCI or one of the 9 other FANC proteins required for their monoubiquitination (10). The importance of the monoubiquitin signal is highlighted by the observation that up to 20% of patients acquire somatic reversion of the inherited mutation in a fraction of blood cells (72). These mutations restore monoubiquitination and prevent bone marrow failure. Our work suggests two potential strategies for treatment of FA: restoration of gene function, such as that which occurs in somatic revertants or, identification of novel mechanisms to stabilize an ID2:DNA-locked complex for fork protection by ubiquitin-mediated or innovative means. New small molecule activators or inhibitors of ID2:DNA locking could be therapeutics in FA or cancer-treatment. In vitro biochemistry has proven to be the most powerful tool in uncovering new functions of FANCD2-monoubiquination that had gone undiscovered for nearly 20 years. The approach is likely to be formidable in drugging the FA pathway in future studies.

**Materials and Methods**

**Protein purification**

Flag-FANCI and StrepII-FANCD2 were expressed using the pFastBac1 vector (Life Technologies). For FANCI:FANCD2 complex, Hi5 cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA, 10% glycerol and 1X mammalian protease inhibitor), and sonicated. Lysates were clarified by centrifugation at 20,000g and the supernatants were incubated with M2 anti-FLAG agarose resin for 2 h. The resin was washed 5x5 min incubation with wash buffer (20 mM Tris-HCl pH 8.0, 0.1 M NaCl, 10% glycerol), and the protein was eluted in the same buffer containing 100 μg/mL FLAG peptide. GST-UBE2T, Flag-BL100, MBP-CEF were purified as described (Van Twest, 2017). His-UBE1 was purchased from Boston Biochem.

**Biotinylated-Avi-ubiquitin purification**

His-Avi-ubiquitin was purified as described in (24).
In vitro ubiquitination assay

Standard ubiquitination reactions contained 10 μM recombinant human avidin-biotin-ubiquitin, 50 nM human recombinant UBE1, 100 nM UBE2T, 100 nM PUC19 plasmid, 2 mM ATP, 100 nM FANCI:FANCD2 complex wild type (WT) or ubiquitination-deficient (KR), in reaction buffer (50 mM Tris-HCl pH 7.4, 2.5 mM MgCl₂, 150 mM NaCl, 0.01% Triton X-100). 20 μL reactions were set up on ice and incubated at 25°C for 90 minutes. Reactions were stopped by adding 10 μL NuPage LDS sample buffer and heated at 80°C for 5 minutes. Reactions were loaded onto 4-12% SDS PAGE and run using NuPAGE ® MOPS buffer and assessed by western blot analysis using Flag (Jomar Life Research) or StrepII (Abcam) antibody.

In vitro Transcription/translation pull down of 35S-labeled proteins

Flag-tagged FANCI:FANCD2 and monoubiquitinated FANCI:FANCD2 was prepared by incubating purified FANCI:FANCD2 or monoubiquitinated FANCI:FANCD2 on Flag beads for 2 hr followed by extensive washes in buffer A (20 mM TEA pH 8.0, 150 mM NaCl, 10% glycerol). 35S-labeled proteins containing UBZ or other ubiquitin domains (Table 1) were generated using the TNT Quick Coupled T7 Transcription/Translation System (Promega) and 35S-labeled methionine (Perkin Elmer). 10 μL of TNT product was incubated for 4 hr at 4°C in buffer A with 100 ng Flag-tagged FANCI:FANCD2 or monoubiquitinated FANCI:FANCD2, 20 μL of Flag-beads (Sigma-Aldrich) in a 100 μL reaction. Beads were washed five times with buffer A and resuspended in LDS loading buffer. Proteins were separated by SDS-PAGE and visualized by autoradiography.

Electrophoretic mobility shift assay

Oligonucleotides used to create fluorescently labeled DNA were IRDYE-700-labelled X0m1 (IDTDNA) and other oligos with the sequences shown in Supplementary Table 1. Assembly of the different DNA structures was performed exactly as previously described (Supplementary Table 2, (23)). 25 nM DNA substrates were incubated in 20 μL ubiquitination buffer containing 100 nM FANCI:FANCD2, 100 nM BL100, 100 nM CEF, 10 μM HA-ubiquitin (Boston Biochem), 50 nM UBE1 (Boston Biochem) and 100 nM UBE2T at room temperature for 90 min to initiate ubiquitination. The reaction was resolved by electrophoresis through a 6% non-denaturing polyacrylamide gel in TBE (100 mM Tris, 90 mM boric acid, 1 mM EDTA) buffer and visualized by Licor Odyssey system. Competitive EMSA were performed by adding cold dsDNA at concentrations 0, 0, 25, 2.5, 7.5, 25, 75, 250 and 1250 nM during or after the ubiquitination reaction.

Purification of monoubiquitinated FANCI:FANCD2 complex

Di-monoubiquitinated FANCI:FANCD2 complex was purified as described (24). DNA molecules of 60bp or 150bp (dsDNA from oligonucleotides) or 2.6kb (circular plasmid DNA) were used to stimulate the reaction for different experiments, as indicated.

Mass spectrometry analysis of monoubiquitinated FANCI:FANCD2 complex
Gels containing monoubiquitinated FANCI and FANCD2 bands were excised and in-gel digested with trypsin and subjected to LC/MS analysis on ESI-FTICR mass spectrometer at Bio21. The analysis program MASCOT was used to identify ubiquitination sites on FANCI and FANCD2.

**Negative stained electron microscopy**
Freshly purified monoubiquitinated or non-ubiquitinated FANCI:FANCD2 complex was applied to glow-discharged, carbon/formvar grids and allowed to adsorb for 60 s. Specimen was then stained with 2% uranyl formate for 60 s. Specimen were imaged at a magnification of 73,000 x with camera (corresponding to a pixel size of 1.9 A) in Tecnai 120 kV.

**Single-particle image processing**
Monoubiquitinated or non-ubiquitinated FANCI:FANCD2 particles were semi-automatically picked using XMIPP3 (73). The parameters of the contrast transfer function (CTF) for negative stained data was estimated on each micrograph using CTFFIND3 (74). Finally, reference free 2D alignment and averaging were executed using XMIPP3 or CisTEM (75).

**Acknowledgements**
We thank Alexandra Sobeck, Puck Knipscheer, Steve West, Johan de Winter, KJ Patel, Paul Hasty, Dario Alessi, Timothy Richmond, Beverlee Buzon, Andrew Blackford, Steve Jackson and Stephen Elledge for reagents. We thank Eric Hanssen from the Electron Microscopy facility, and Nick Shuai from the Mass Spectrometry facility, at Bio21 Institute, Melbourne. WT was supported by an Australian Government Research Training Scheme postgraduate scholarship. AJD is a Victorian Cancer Agency fellow. WMC is an NHMRC career development fellow and Maddie Riewoldt’s vision fellow (WC-MRV2016). MWP is an NHMRC Australia Senior Research Fellow. This work was funded by grants from the Fanconi Anemia Research Fund (to AJD and WC), Maddie Riewoldt’s Vision (to WC), the National Health and Medical Research Council (GNT1123100 and GNT1181110 to AJD and GNT1156343 to WMC), and the Victorian Government’s OIS Program.

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Figure 1. Mono-ubiquitination does not alter interaction of FANCI:FANCD2 with DNA repair proteins.
(a) 35S-labelled FAN1, SLX4, FAAP20, RAP80, SMARCAD, FANCJ, PSMD4, SF3B1, TRIM25, MCM5, BRE, BRCC, SNM1A or luciferase (control) inputs were expressed using reticulocyte.
(b) The inputs prepared from (c) were incubated with the indicated Flag-ID2 or (D) Flag-IubD2ub, followed by Flag pull-down and elution. The complexes were subjected to SDS-PAGE, and radiolabeled proteins were detected by autoradiography (representative experiment of n=2).
(d) Quantification showing percentage of ID2 or IubD2ub binding to inputs.
Figure 2. Monoubiquitination locks FANCI:FANCD2 on DNA.
(A) Schematic of the electrophoretic mobility shift assay (EMSA) using IRDye-700 labelled dsDNA.
(B) Coomassie stained SDS-PAGE gel showing monoubiquitination of FANCI:FANCD2 using recombinant FA core complex and IR-dye700 labelled dsDNA. 25, 50 and 100 nM of ID2 or l^mD2^m were incubated with 25 nM of the IR-dye700 dsDNA for 90 min. The percentage of FANCI or FANCD2 monoubiquitination were calculated and shown under SDS-PAGE gel.
(C) Monoubiquitination reactions from (B) were resolved on 6% native PAGE gel for EMSA analysis. The percentage of ID2 binding to DNA was calculated and shown under native PAGE gel.
Figure 3. Monoubiquitinated FANCI:FANCD2 binds to any type of dsDNA.
EMSA gels showing binding of monoubiquitinated or unmodified ID2 complex to different oligo-based DNA substrates. Above each panel, a schematic representing the tested DNA substrate is shown. 25, 50 and 100 nM of ID2 or ID2-ub were incubated with 25 nM of the indicated DNA substrate and the protein:DNA complexes were resolved on 6% PAGE gels (top). The percentage of DNA binding was calculated and shown under each EMSA gel. Coomassie stained SDS-PAGE gel (bottom) showing the ubiquitination reactions used in the EMSA. The percentage of FANCD2 ubiquitination was calculated and shown under each SDS-PAGE gel.
Figure 4. FANCD2 monoubiquitination is sufficient for FANCI:FANCD2 locking to DNA, but stimulated by FANCI monoubiquitination.

(a) Western blots of the EMSA gels containing 25, 50 and 100 nM of ID2\textsuperscript{+D2\textasciitilde ub}, ID2 or ID2\textsuperscript{-D2\textasciitilde ub} in the presence of 25 nM IRDye-700 labelled dsDNA (red). Left panels correspond to anti-FANCI antibody (green) and right panels correspond to anti-FANCD2 antibody (green).

(b) StreptII affinity purification of mono-ubiquitinated (+ATP) and non-ubiquitinated ID2 (-ATP).

(c) EMSA gels (top) and western blots (bottom) showing the monoubiquitination of 25, 50 and 100 nM ID2\textsuperscript{+D2\textasciitilde ub}, ID2 or ID2\textsuperscript{-D2\textasciitilde ub} in the presence of 25 nM IRDye-700 labelled dsDNA.

(d) Western blots of the EMSA gels from (C) showing FANCI (left, green) and FANCD2 (right, green) remained bound to IRDye-700 labelled DNA (red) after mono-ubiquitination.
**Figure 5.** Mutation in different ubiquitin patches do not affect ID2 mono-ubiquitination or DNA binding.

(a) Crystal structure of ubiquitin with ubiquitin mutant sites depicted (PDB: 1UBQ). Hydrophobic binding pockets are indicated in blue and pink.

(b) Western blots showing the time course ubiquitination assays of ID2 using wild-type ubiquitin, ubiquitin mutant F4A, V70A, I44A, D58A, K11R and L73P.

(c) EMSA gels showing 25, 50 and 100 nM monoubiquitinated ID2 binding to 25 nM IRDye-700 dsDNA using various ubiquitin mutants (top). Western blots of ID2 ubiquitination products were shown at the bottom and the percentage of FANCI and FANCD2 ubiquitination were shown at the bottom of each western blot panel.
Figure 6. Mono-ubiquitinated FANCl:FANCD2 complex assemble into filament-like arrays.
(a) Schematic of purification of monoubiquitinated FANCl:FANCD2 using Avi-ubiquitin.
(b) Coomassie stained SDS-PAGE gel (top) and western blots (bottom) showing the purification of monoubiquitinated FANCl:FANCD2 complex eluted using Prescision protease (lanes 1-7) compared to without Prescision protease (lanes 8-14).
(c-e) Representative negative-stained EM image of purified FANClub:FANCD2ub complex bound to 2.7 kb plasmid, unmodified FANCl:FANCD2 incubated with 2.7 kb plasmid and unmodified FANCl:FANCD2 complex. Pseudo-coloured regions are shown to highlight particular filament-like arrays in FANClub:FANCD2ub but not other samples.
Figure 7. Monoubiquitinated FANCI:FANCD2 forms filamentous oligomers along the length of dsDNA. (A-D) Representative EM image of monoubiquitinated FANCI:FANCD2 bound to (A) 60 bp dsDNA, (B) 150 bp dsDNA, (C) 2.7 kb dsDNA, and (D) 2.7 kb dsDNA and benzonase-treated.
**Figure 8. Electron micrographs of ID2 and I<sub>ub</sub>D<sub>2ub</sub>**

(A) Representative negative-stained electron micrograph and 2D class average of ID2.
(B) Representative negative-stain electron micrograph and 2D class average of I<sub>ub</sub>D<sub>2ub</sub>.