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A ubiquitin-binding protein FAAP20 links RNF8-mediated ubiquitination to the Fanconi anemia DNA Repair network

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SUMMARY

The Fanconi anemia (FA) protein network is necessary for repair of DNA interstrand crosslinks (ICLs), but its control mechanism remains unclear. Here we show that the network is regulated by a ubiquitin signaling cascade initiated by RNF8 and its partner, UBC13; and mediated by FAAP20, a component of the FA core complex. FAAP20 preferentially binds the ubiquitin product of RNF8-UBC13; and its recruitment to ICLs requires this ubiquitin-binding activity, RNF8 and UBC13. Both RNF8 and FAAP20 are required for recruitment of FA core complex and FANCD2 to ICLs, whereas RNF168 can modulate efficiency of the recruitment. RNF8 and FAAP20 are needed for efficient FANCD2 monoubiquitination, a key step of the FA network; RNF8 and FA core complex work in the same pathway to promote cellular resistance to ICLs. Thus, the RNF8-FAAP20 ubiquitin cascade is critical for recruiting FA core complex to ICLs and for normal function of the FA network.

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Detailed Experimental materials and methods can be found in Supplemental Information.

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Keywords

RNF8; FAAP20; RNF168; UBC13; Fanconi Anemia; ubiquitin

Introduction

DNA interstrand crosslinks (ICLs) are highly toxic lesions because they are absolute blocks to replication and transcription. In humans, the repair of ICLs requires a group of proteins in which mutations cause Fanconi anemia (FA), a genetic disorder characterized by developmental abnormalities, bone marrow failure, and cancer predisposition (Wang, 2007). FA patient cells display increased chromosome instability and cellular hypersensitivity in response to ICL-inducing agents. Fifteen genes defective in FA have been identified, and their products (FANC proteins) work with breast cancer susceptibility (BRCA) proteins to repair ICLs.

The FANC proteins can be classified into three groups. The first group includes the FA core complex, consisting of eight FANC proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) and four FA-associated proteins (FAAP100, FAAP24, MHF1 and MHF2) (Singh et al., 2010; Wang, 2007; Yan et al., 2010). In response to DNA damage or stalled replication, the core complex monoubiquitinates the second group of proteins, FANCD2 and FANCI, which form the “ID” complex and its monoubiquitination is necessary for repair of ICLs (Knipscheer et al., 2009). The monoubiquitinated ID complex also redistributes to DNA damage sites on chromatin, where it co-localizes with the third group of FA proteins FANCD1/BRCA2, FANCJ/BACH1/BRIP1, FANCN/PALB2. The monoubiquitinated ID complex may serve as a signal to recruit FANCP/SLX4 and nuclease FAN1 through interaction with their ubiquitin-binding zinc finger (UBZ) domains (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Yamamoto et al., 2011).

All three groups of FANC proteins are recruited to ICLs *in vivo* and *in vitro* (Ben-Yehoyada et al., 2009; Knipscheer et al., 2009; Shen et al., 2009; Yan et al., 2010), but the recruitment mechanism is poorly understood. Recruitment of the FA core complex has been reported to depend on ATR kinase, RPA (which binds the ssDNA and activates ATR), and nucleotide excision repair proteins XPA and XPC (Ben-Yehoyada et al., 2009; Shen et al., 2009). Three DNA binding components of the FA core complex (FANCM, MHF and FAAP24) have also been suggested to bind directly to forks stalled by ICLs and recruit the complex (Huang et al., 2010; Yan et al., 2010).

Here we show that FAAP20, a component of the FA core complex, preferentially binds lysine 63 (K63)- over lysine 48 (K48)-linked polyubiquitins *in vitro.* Both polyubiquitins have been observed in chromatin regions flanking DSBs and UV-induced DNA damage (Al-Hakim et al., 2010; Marteijn et al., 2009; Ulrich and Walden, 2010). RNF8 is the first E3 ubiquitin ligase that accumulates at damaged sites to build either K63- or K48-linked ubiquitin chains in damaged chromatin by working with different E2 ubiquitin conjugating enzymes. Specifically, it can cooperate with UBC13 to promote K63-linked ubiquitination of H2A-type histones in response to DSBs, UV and replication stress (Feng and Chen, 2012; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Sy et al., 2011; Wang and Elledge, 2007). The ubiquitinated H2A then recruits a second E3 ligase, RNF168, which works with UBC13 to further elongate and spread K63-linked polyubiquitin chains. This enables assembly of downstream repair proteins at damaged chromatin via ubiquitin-mediated protein-protein interactions. In this study, we describe a ubiquitin signaling cascade that is initiated by RNF8-UBC13 and mediated by FAAP20. We show that this

cascade is critical for recruitment of the FA core complex and FANCD2 to ICLs and also important for normal function of the FA network.

RESULTS

FAAP20 is a component of the FA core complex

We immunoprecipitated the FA core complex from HeLa nuclear extract with a FANCA antibody. Analyses of the immunoprecipitate by silver staining (Figure 1A) and mass spectrometry identified many known components of the FA core complex (FANCA, -B, -C, -E, -F, -G, -L, -M, FAAP100, MHF2), and the BLM complex (BLM, TOPIIIα, and RPA70). The results confirmed the association of the FA core complex and BLM complex in a super complex, BRAFT (Meetei et al., 2003). We also identified a 20 kDa polypeptide as LOC199990 (C1ORF86), an uncharacterized protein. We renamed it as FAAP20 (for Fanconi Anemia-Associated Protein 20 kDa).

Immunoblotting showed that a FAAP20 antibody recognized the corresponding polypeptide in the FA core complex immunoprecipitated with either anti-FANCA or FANCG antibodies (Figure 1B). In a reciprocal immunoprecipitation, FAAP20-associated polypeptides isolated by a Flag antibody from the extract of HeLa cells stably expressing Flag-tagged FAAP20 contained many FA core complex components (Figure 1C). Additionally, the profile of FAAP20 on gel-filtration chromatography overlapped with those of several FA core complex components (Figure S1A). Notably, the profile of FAAP20 was coincidental with those of FANCA and FANCG, arguing that these three proteins are likely present in a subcomplex.

The level of FAAP20 was significantly reduced in cells derived from a FANCA patient compared to that of cells from a healthy individual (Figure 1D, lanes 1 and 2); and this FAAP20 level was restored by re-induction of exogenous FANCA (Figure 1D, lanes 2 and 3). These data indicate that the stability of FAAP20 is dependent on FANCA, suggesting thatFAAP20 could interact with FANCA in the FA core complex.

FAAP20 is required for normal activation of the FA pathway and cellular resistance to ICLs

A key function of the FA core complex is to monoubiquitinate FANCD2 and FANCI in response to DNA damage. HeLa cells depleted of FAAP20 by two different siRNAs displayed reduced levels of monoubiquitinated FANCD2 and FANCI in response to mitomycin C (MMC) (Figures 1E and S1B), indicating that FAAP20 is a functional component of the core complex. The reduced ubiquitination was not due to inability of these cells to enter S-phase, because the S-phase population in cells depleted by siFAAP20-2 oligo was larger than that of cells treated with a control siRNA (Figure S1C). The FAAP20-depleted HeLa cells exhibited increased sensitivity to MMC (Figure 1F) as well as increased MMC-induced chromosomal breaks (Figure 1G). These are all characteristics of cells deficient in the FA core complex, suggesting that FAAP20 is important for normal function of the FA pathway and for cellular resistance to ICLs.

The FAAP20-depleted HeLa cells had a modestly reduced level of FANCA compared to cells treated with a control oligo (Figure 1E and S1B), again consistent with a need for FAAP20 for optimal stability of FANCA.

We further investigated the role of FAAP20 by inactivating this gene in chicken DT40 cells (Figure S1D, E and F). The levels of both monoubiquitinated FANCD2 and FANCI were reduced in three independent clones of *FAAP20*$^{-/-}$ DT40 cells when compared to wild type cells (Figures 1H, lanes 1–4; and S1G); and these were restored by re-introduction of human FAAP20 into these cells (Figure 1H, lanes 1, 2, 5 and 6). The results are consistent with the

data from HeLa cells that showed a requirement of FAAP20 for optimal monoubiquitination of FANCD2 and FANCI after DNA damage.

The *FAAP20*$^{-/-}$ DT40 cells did not exhibit significant sensitivity to cisplatin, which can induce ICLs (data not shown). This may not be a surprise because DT40 cells deficient in FANCA or FANCG (which may interact more directly with FAAP20 in a subcomplex; Figure S1A) are not very sensitive to crosslinking agents; unlike DT40 cells deficient in FANCL or FANCC, which are highly sensitive (Takata et al., 2009). It should be mentioned that cell survival is affected not only by the FA network, but also by other pathways; and measuring FANCD2 monoubiquitination and foci formation is a more direct readout for the FA network.

No FA patients have yet been found with mutations in FAAP20

We screened unclassified FA patients, which lacked a defect in any of the known FA genes, for FAAP20 mutations by both immunoblotting and exon-sequencing, but found no patient with a FAAP20 defect (data not shown).

FAAP20 contains a UBZ-type ubiquitin binding domain

FAAP20 orthologs are found in vertebrates, but not in invertebrates and yeast (Figure S2A). This feature of FAAP20 is shared by most FA core complex components, suggesting that the genes encoding them may have co-evolved.

Alignment of FAAP20 sequences revealed a highly conserved zinc finger domain (Figure S2A) similar to the ubiquitin-binding zinc finger (UBZ) domain found in other DNA repair proteins, including FAN1 and FANCP/SLX4 (Figure 2A). Atomic absorption analyses of a recombinant GST-fusion protein containing the UBZ domain of FAAP20 confirmed the presence of a Zn atom in the wild type protein (GST-UBZ-WT), but not in a point mutant carrying a Cys to Ala substitution (GST-UBZ-C147A) (Figures 2A and S2B).

The UBZ domain of FAAP20 bound ubiquitin-conjugated agarose beads in a pull-down assay (Figure S2C). In contrast, neither GST protein alone nor two GST-fusion proteins carrying point mutations in the UBZ domain (C147A and D164A, respectively) bound to ubiquitin beads (Figure 2A and S2C). These data indicate that the UBZ domain of FAAP20 is indeed a ubiquitin binding domain.

Of the two UBZ mutations (Figure 2A), the C147A substitution inactivates Zn-binding (Figure S2B) and disrupts the tertiary structure of the domain. The D164A substitution mimics a similar mutation in the UBZ domains of Pol eta (D652) and Nemo (Q411), which disrupts the ubiquitin-binding surface without affecting tertiary structure (Figure 2A and B) (Bomar et al., 2007; Cordier et al., 2009). As expected, the D164A mutant retained the Zn atom in the structure (Figure S2B). Thus, our findings that the D164A mutant lost ubiquitin binding activity suggest that the ubiquitin-interface of the FAAP20 UBZ domain may be similar to that in other UBZ domains.

The UBZ domain of FAAP20 binds both K48- and K63-linked polyubiquitin chains

Proteins can be modified by either mono- or poly-ubiquitins linked through different lysine (K) residues, with different ubiquitin isoforms representing distinct signals for their host proteins. We found that a GST fusion protein containing the UBZ domain of FAAP20 bound both K48- and K63-linked polyubiquitin chains; but had no detectable binding for monoubiquitin (Figure 2C and D). As controls, GST-fusion proteins containing the UBZ domain with either C147A or D164A mutations had no binding activity for either form of

polyubiquitin (Figure 2E and F). The results suggest that FAAP20 can recognize both K48- and K63 -linked polyubiquitin, but not monoubiquitin.

We quantified the immunoblot images for each ubiquitin linkage and calculated the ratio between levels of the bound ubiquitin vs. those of the input (Figure 2D). The results showed that the ratio for each K63-linked chain is consistently higher than that for the respective K48-linked chain, indicating that the FAAP20-UBZ domain preferentially binds K63-linked chains.

The ubiquitin-binding activity is crucial for FAAP20 recruitment to ICLs

Two FA core complex components, FANCM and MHF1, are recruited to ICLs induced by laser-activated psoralen (Yan et al., 2010). Using the same method, we observed recruitment of GFP-tagged FAAP20 at ICLs within minutes upon laser activation (Figure 3A). Notably, no recruitment was detected for the GFP-FAAP20-D164A mutant that lacks ubiquitin-binding activity (Figure 3B and C).

We also performed eChIP, a chromatin-IP based assay that detects the recruitment of the FA core complex to an ICL on a non-replicating episomal plasmid (Shen et al., 2009). Flag-FAAP20 was recruited to the ICL, but the Flag-FAAP20-D164A mutant was not (Figures 3D and S3A). These data indicate that FAAP20 is recruited to ICLs through the ubiquitin-binding activity of its UBZ domain.

K63-linked polyubiquitin generated by RNF8-UBC13 signals FAAP20 recruitment

RNF8 is the first E3 ligase that accumulates at damaged DNA to build either K63- or K48-linked ubiquitin chains in the surrounding chromatin. Because both products of RNF8 can be bound by FAAP20, we investigated whether RNF8-dependent ubiquitination recruits FAAP20 to damaged sites. The accumulation of GFP-FAAP20 at ICLs was abolished in HeLa cells depleted of RNF8 by two different siRNAs (Figures 3E and F, and S3B). These data suggest that RNF8 and FAAP20 constitute a ubiquitin signaling cascade (RNF8-FAAP20 cascade) in which RNF8-mediated ubiquitination is a signal for FAAP20 recruitment.

K63-linked polyubiquitins often recruit other proteins through ubiquitin-mediated interactions, whereas K48-linked chains target substrates for proteolytic degradation. To examine whether K63-linked polyubiquitin is the signal for FAAP20 recruitment, we depleted UBC13, the ubiquitin conjugating enzyme specific for K63-linkage and a partner of RNF8, from HeLa cells by two different siRNAs. The recruitment of GFP-FAAP20 to ICLs was eliminated, as it was in RNF8-depleted cells (Figures 3E and F, and S3C), indicating that K63-linked polyubiquitin produced by RNF8-UBC13 is the primary signal for FAAP20 recruitment.

K63-linked ubiquitin chains formed at DSBs are stable and can last at least 4 hours, in contrast to K48-linked chains that are unstable and diminish within 1 hour (Feng and Chen, 2012). The GFP-FAAP20 remained at ICLs for at least 2.5 hours (Figure 3A), consistent with the notion that it recognizes the stable K63-linked chains.

RNF168 is largely dispensable for FAAP20 recruitment

RNF168 is the second E3 ubiquitin ligase that accumulates at damaged chromatin and amplifies K63-linked polyubiquitination initiated by RNF8-UBC13. Interestingly, the recruitment of GFP-FAAP20 to ICLs was observed in majority of cells depleted of RNF168 by two different siRNAs (83% and 78% for siRNF168-treated cells compared to 95% for control cells) (Figures 3E and F, and S3D). As a control, the recruitment of RNF168 itself to

ICLs was eliminated by the same siRNAs (see Figure 5D and E). Therefore, RNF168-mediated amplification of the K63 ubiquitin signal is largely dispensable for recruitment of FAAP20.

The ubiquitin-binding activity of FAAP20 is required for normal recruitment of the FA core complex and FANCD2 to ICLs

We studied whether the RNF8-FAAP20 cascade controls recruitment of the FA core complex and FANCD2 to ICLs. We observed recruitment of both FANCA and FANCD2 to ICLs (Figure 4A). The data are in agreement with earlier findings that FA proteins function at ICLs (Ben-Yehoyada et al., 2009; Knipscheer et al., 2009; Shen, 2009; Yan et al., 2010). Importantly, the recruitment of both FANCA and FANCD2 was strongly diminished in cells depleted of FAAP20 (Figure 4A and B), indicating that FAAP20 is required for normal recruitment of the FA core and ID complexes to ICLs. Notably, introduction of an siRNA-resistant version of FAAP20 into FAAP20 siRNA-treated cells partially rescued the recruitment of FA core complex and FANCD2, whereas the FAAP20-D164A mutant failed to rescue (Figures 4C, D and E; and S4A). In fact, even when cells were treated with control siRNA, those expressing FAAP20-D164A mutant had 90% reduction of FANCA and 40% reduction of FANCD2 recruitment compared those expressing wild type FAAP20, indicating that the UBZ mutant acts dominant-negatively to inhibit the recruitment process. The data suggest that the ubiquitin-binding activity of FAAP20 is needed for recruitment not only of itself (Figure 3B, C and D), but also of the FA core complex and FANCD2.

The ubiquitin-binding activity of FAAP20 is required for normal activation of the FA pathway

We investigated whether the ubiquitin-binding activity of FAAP20 is needed for FA pathway activation using *FAAP20*$^{-/-}$ DT40 cells. The *FAAP20*$^{-/-}$ cells had a lower level of monoubiquitinated FANCD2 (Figures 4F and S4B) as well as a reduced number of FANCD2 nuclear foci in response to MMC treatment (Figure 4G and H); introduction of wild type FAAP20 largely corrected both defects. In contrast, introduction of FAAP20 carrying either C147A or D164A substitutions failed to correct these defects (Figures 4F, G, H, and S4C, S4D). Because both mutants lack ubiquitin-binding activity, the data suggest that this activity of FAAP20 is required for optimal activation of the FA pathway.

A FAAP20 mutant that lacked the UBZ domain co-immunoprecipitated normally with FANCA (Figure S4E and F), suggesting that the failure of the UBZ domain mutants to restore the FA pathway is not due to their inability to assemble into the FA core complex. Another FAAP20 mutant lacking the N-terminal 65 residues failed to co-immunoprecipitate with FANCA (Figure S4E and F), indicating that this region is required for FAAP20 assembly into the core complex.

RNF8 and its ubiquitinated product accumulate at ICLs earlier than FA proteins

RNF8 accumulates rapidly at DSBs to promote K63-linked ubiquitination on H2A-type histones (Huen et al., 2007; Kolas et al., 2007; Marteijn et al., 2009; Wang and Elledge, 2007). We found that RNF8 and its product, ubiquitinated H2A, accumulated at laser-activated ICLs within minutes after photoactivation (Figure 5A and B). The appearance of RNF8 preceded that of ubiquitinated H2A (approximately 1 min vs. 3 min) (Figure 5A), consistent with a sequential process in which RNF8 is first recruited by an upstream signal and then ubiquitinates H2A at the site of damage.

The accumulation of RNF8 and ubiquitinated H2A at ICLs precedes that of FANCA and FANCD2 (1 and 3 min versus 5 and 10 min) (Figure 5A), suggesting that RNF8-initiated

ubiquitination signals (ubiquitinated H2A or other substrates) act earlier in a cascade to recruit FANC proteins to ICLs.

The recruitment of FANCA and FANCD2 to ICLs requires RNF8

The data above suggested a hierarchy in control of FA proteins: RNF8 is required for recruitment of FAAP20, which is in turn needed for recruitment of FA core complex and FANCD2. The data predict that RNF8 should also be required for recruitment of FA core complex and FANCD2. In agreement of this notion, depletion of RNF8 by two different siRNAs in HeLa cells not only disrupted induction of ubiquitinated H2A, but also substantially reduced the accumulation of FANCA and FANCD2 at ICLs (Figure 5B and C). These data suggest that RNF8 acts upstream of FA core complex and FANCD2 to promote their recruitment to ICLs.

RNF168 affects efficiency of the recruitment of FANC proteins to ICLs

Like RNF8, RNF168 also accumulated at ICLs, and its appearance was after RNF8 but before FANCA and FANCD2 (Figure 5A and D). However, depletion of RNF168 by two different siRNAs had only a modest effect on recruitment of FANCA and FANCD2: about 50% reduction for the former and 20% for the latter (Figure 5E). This is in contrast to the depletion of RNF8, which reduced recruitment of FANCA and FANCD2 by about 90% and 80%, respectively. These data are reminiscent of the findings that RNF168 depletion does not significantly affect recruitment of GFP-FAAP20 (Figure 3E and F). Thus, while RNF8 plays a critical role for recruitment of FANC proteins to ICLs, RNF168 only affects the efficiency of the recruitment.

RNF8 is dispensable for recruitment of FANCM to ICLs

We have previously shown that most FANCM and its DNA binding partner MHF (about 90%) do not associate with the FA core complex, but are present in the distinct FANCM-MHF complex that lacks FANCA and other FANC proteins. Moreover, the recruitment of FANCM-MHF to laser-activated psoralen ICLs only occurs in S-phase cells (Yan et al., 2010). This is in contrast to recruitment of RNF8, GFP-FAAP20, FANCA and FANCD2 that occurred in most (80% or more) of unsynchronized HeLa cells (Figures 3C and 5A; less than 20% of these cells are in S-phase (data not shown)). The data imply that recruitment of FANCM-MHF in the S-phase is different from the RNF8-dependent recruitment of FANCA and FANCD2, which can occur independently of cell cycle phase. In support of this notion, the recruitment of FANCM was unaffected in RNF8-depleted cells (Figure 5B and C), in contrast to recruitment of FANCA and FANCD2, which was disrupted. Together, the data suggest that RNF8-mediated ubiquitination is dispensable for FANCM recruitment during S-phase.

RNF8 promotes efficient activation of the FA network and works in the same pathway as the FA core complex in cellular resistance to ICLs

Our findings that RNF8 is required for recruitment of FANC proteins to ICLs prompted us to study if it is also needed for activation of the FA network. We found that HeLa cells depleted of RNF8 by two different siRNAs exhibited a reduced level of monoubiquitinated FANCD2 (Figure 6A), and a decreased number of FANCD2 nuclear foci in response to MMC (Figure 6B and C). The reduced FANCD2 monoubiquitination was not due to inability of the depleted cells to enter S-phase, because the percentage of S-phase cells in RNF8-depleted cells was comparable to that of control cells (Figure S5A). Moreover, RNF8-depleted cells displayed increased sensitivity to MMC (Figure S5B and C). These features resemble those of cells deficient in FA core complex, and suggest that RNF8 is required for normal function of the FA pathway.

Importantly, cells doubly-depleted of both RNF8 and FANCA exhibited MMC sensitivity similar to that of single gene depleted cells (Figure 6D and E), suggesting that RNF8 and FA core complex act in the same pathway to resist MMC-induced DNA damage.

The RNF8-FAAP20 cascade is required for recruitment of FA core complex and FANCD2 to DSBs

The FA network can be activated by not only ICLs but also other DNA damage, including DSBs (Garcia-Higuera et al., 2001). We asked if FANC proteins are recruited to DSBs, and if they do, whether their recruitment also depends on the RNF8-FAAP20 cascade. We found that both FANCA and FANCD2 are recruited to laser-activated DSBs (Figure S6A and B). Importantly, recruitment of both FANC proteins was strongly abrogated in cells depleted of either FAAP20 (Figure S6A and B) or RNF8 (Figure S6C and D). The data suggest that the RNF-FAAP20 cascade may be part of a general pathway that governs recruitment of FANC proteins to multiple forms of DNA damage.

Discussion

RNF8 and FAAP20 constitute a ubiquitin signaling cascade

Phosphorylation of several subunits of the FA core complex by ATR and its downstream kinase CHK1 has been shown to regulate monoubiquitination of FANCD2, a key function of the core complex (Collins et al., 2009; Wang et al., 2007) (Figure 7). Here we show that the core complex is also governed by a ubiquitin signaling cascade, which is initiated by RNF8 and its partner UBC13; and mediated by FAAP20, a component of the FA core complex. RNF8 and UBC13 act upstream of this cascade, because depletion of either protein abolished the recruitment of GFP-FAAP20 to ICLs. Conversely, FAAP20 functions downstream, as FAAP20 can bind K63-linked polyubiquitin, the product of RNF8-UBC13, *in vitro*; and the FAAP20 mutant defective in this ubiquitin-binding activity failed to accumulate at ICLs *in vivo*. Consistent with the proposed role of RNF8 in regulating FA network, our epistasis analyses showed that RNF8 and FANCA work in the same pathway to resist MMC-induced cell killing.

The RNF8-FAAP20 cascade is essential for recruitment of FA core complex to ICLs and for efficient activation of the FA pathway

Our time-course analyses revealed a sequential accumulation process of repair proteins at ICLs (Figure 5A). This sequential process suggests a model of how RNF8 regulates the recruitment of FANC proteins (Figure 7). In response to ICLs, RNF8 is recruited to the damage site where it works with UBC13 to catalyze K63-linked ubiquitination of histone H2A and possibly other substrates in chromatin flanking the lesions. The ubiquitinated H2A (and perhaps other ubiquitinated molecules) then interacts with the UBZ domain of FAAP20 to recruit the FA core complex to damaged chromatin. Finally, FANCD2 is recruited, possibly by interacting with the FA core complex already bound at the ICLs. This model is supported by the siRNA data showing that RNF8 depletion disrupted accumulation of all downstream proteins at ICLs, and that FAAP20 depletion reduced FANCA and FANCD2 recruitment. It is also supported by the evidence that the ubiquitin-binding activity of FAAP20 is needed for recruitment of FANCA and FANCD2.

The RNF8-FAAP20 cascade also plays a regulatory albeit non-essential role in modulating the efficiency of FANCD2 monoubiquitination, a key step of the FA network. This is evidenced by observations that HeLa cells depleted of either RNF8 or FAAP20, and DT40 cells inactivated of FAAP20, all displayed reduced levels of MMC-induced FANCD2 monoubiqutination and redistribution to nuclear foci.

FANCM is recruited to ICLs through a pathway independent of RNF8

Why is the RNF8-FAAP20 cascade essential for recruitment of FA core complex to psoralen-activated ICLs but non-essential for MMC-induced FANCD2 monoubiquition? One explanation is that there exists another pathway independent of the RNF8-FAAP20 cascade that can also promote FANCD2 monoubiquitination (Figure 7). It should be pointed out that ICLs generated by psoralen and MMC are structurally different so that they may be detected by distinct mechanisms, leading to different responses (Muniandy et al., 2010). The psoralen ICLs distort DNA duplex so that they can be rapidly detected by DNA damage sensors in any phase of the cell cycle to activate the RNF8-FAAP20 cascade. In contrast, MMC ICLs do not significantly distort DNA duplex, so they may remain largely undetected until S-phase when they block progression of replication forks. The blocked forks may be recognized by FANCM-MHF (and possibly FAAP24), which recruits the FA core complex through direct protein-protein interactions. In agreement of this notion, the recruitment of FANCM-MHF to ICLs of psoralen occurred only during S-phase (Yan et al., 2010), and was unaffected when RNF8 was depleted (Figure 5B and C). Thus, at least two pathways regulate the FA core complex recruitment: one is replication-independent, and the other one is replication-dependent. Only the first one depends on the RNF8-FAAP20 cascade, whereas the latter one depends on FANCM and its DNA-binding partners. Cells deficient in either pathway are partially defective in FANCD2 monoubiquitination. We predict that only when both pathways are disrupted, will FANCD2 monoubiquitination be eliminated.

K63-linked polyubiquitin generated by RNF8-UBC13 signals FAAP20 recruitment

RNF8 can promote ubiquitination of both K63- and K48-linked chains at DSBs (Feng and Chen, 2012; Lok et al., 2011). We found that the UBZ domain of FAAP20 can bind both chains but has a preference for K63 linkage. Our findings that cells-depleted of UBC13, the E2 enzyme specific for K63-linked polyubiquitination, are completely deficient in recruiting GFP-FAAP20 to ICLs indicate that the K63 linkage is the primary signal. In support of this notion, inhibition of K63-linked polyubiquitination sensitizes cells to agents that induce ICLs (Chiu et al., 2006); and cells deficient in UBC13 are sensitive to ICL-inducing drugs (Zhao et al., 2007). The observation that K48 chains are bound by FAAP20 *in vitro*, but do not appear to be effective binding partners *in vivo* (because UBC13 depletion eliminated FAAP20 recruitment even though K48 chains would be present) suggests that recognition of K48 by FAAP20 may be suppressed *in vivo*, perhaps outcompeted by proteins with higher affinity for K48.

RNF168 affects efficiency of the recruitment of FA proteins to ICLs

RNF8 and RNF168 ubiquitin ligases work coordinately to catalyze K63-linked polyubiquitination at chromatin regions surrounding DSBs or UV-induced damage sites, and both are required for recruitment of several downstream repair molecules. Our data suggest that the RNF8-initiated K63-linked polyubiquitination signal is sufficient for recognition by the UBZ domain of FAAP20, leading to recruitment of FA core complex and FANCD2; further amplification of the signal by RNF168 is not necessary, but can increase the amount of the recruited FANC proteins. Notably, this selective dependence on RNF8 over RNF168 has been observed for accumulation of RAD51 recombinase at ssDNA lesions in response to replication stress induced by hydroxyurea (Sy et al. 2011). Perhaps, cells may have developed different ubiquitin-binding domains (UBDs) to distinguish different K63-linked polyubiquitin signals produced by RNF8 and RNF168. The FAAP20-UBZ domain may represent one type of UBDs that can bind shorter K63-linked chains generated by RNF8, whereas the UBDs in some other proteins may only recognize elongated K63 chains produced by RNF168.

The ubiqitin-binding activity of FAAP20 is essential for recruitment of FA core complex to ICLs and for optimal activation of the FA pathway

While our manuscript was under revision, three groups independently reported that FAAP20 is part of the FA core complex and required for optimal activation of the FA pathway (Ali et al., 2012; Kim et al., 2012; Leung et al., 2012), consistent with our findings. However, the conclusions regarding the ubiquitin binding activity of FAAP20 are controversial. First, Ali et al showed that FAAP20 does not bind monoubiquitin, in agreement with our data; whereas Kim et al proposed that FAAP20 binds Rev1, and this binding is enhanced by monoubiquitination of Rev1. However, examination of the data from Kim et al suggests that FAAP20 appears to bind better to non-ubiquitinated than monoubiquitinated Rev1 (the ratio between non-ubiquitinated vs. monoubiquitinated form was increased in their FAAP20 immunoprecipitate compared to the Pre-IP extract; their Fig. 4C, lanes 7 vs. 3); and FAAP20 with mutated UBZ domain can still bind Rev1 (their Fig. 4C, lane 8). One interpretation, which fits data from all groups, is that FAAP20 may bind non-ubiquitinated Rev1 through an ubiquitin-independent mechanism; and this binding may be decreased by monoubiquitination. Second, Ali et al using FAAP20-depleted HeLa cells concluded that the ubiquitin binding activity of FAAP20 is necessary for normal activation of the FA pathway, whereas Leung et al using FAAP20-inactivated HCT116 cells concluded that it is dispensable. Our data from FAAP20-knockout DT40 cells and FAAP20-depleted HeLa cells suggest that this activity is not only important for normal activation of the FA pathway, but also critical for recruitment of the FA core complex to ICLs. The lack of importance for this activity in HCT116 cells might be because that this cell line carries mutations in MRE11 and MLH1, which are involved in activation of ATR (Nam and Cortez, 2011), resulting in aberrant response to replication stress (Wen et al., 2008). We found that recruitment of FA proteins to ICLs in HCT116 cells is about 50% lower compared to that in HeLa cells (data not shown), suggesting that the RNF8-FAAP20 cascade may be inefficiently utilized and thus less important in this cell line.

In summary, our data suggest that the FA core complex is governed not only by phosphorylation but also by the RNF8-FAAP20 ubiquitin cascade. Our data that the recruitment of the FA core complex and FANCD2 to DSBs also depends on RNF8 and FAAP20 suggest that this cascade can respond to many forms of DNA damage.

EXPERIMENTAL PROCEDURES

Cell lines

Human HeLa and HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS). Chicken DT40 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% chicken serum, 10mM HEPES and 1% penicillin–streptomycin mixture in a 5% CO_2 incubator at 39.5°C. HeLa cells stably expressing Flag-FAAP20 WT and D164A mutant used in eChIP assay were kindly provided by Dr. AR. Meetei (Ali et al., 2012).

Antibodies

An anti-human FAAP20 antibody was raised against a chimeric protein containing a region of human FAAP20 (67–180 aa) fused to maltose-binding protein (New England Biolabs). An anti-chicken FANCA antibody was raised against a chimeric protein containing a region of chicken FANCA (1698–1944 aa) fused to maltose-binding protein. Anti-chicken FANCD2 and FANCI antibodies were generated in rabbits by injection of recombinant whole chicken FANCD2 protein (Yamamoto et al., 2011) and partial chicken FANCI protein (amino acids 1–125), respectively. An anti-RNF168 antibody was kindly provided by Dr. D. Durocher. Anti-RNF8 and anti-UBC13 antibodies were purchased from Abcam.

An anti-ubiquitinated histone H2A antibody was purchased from Millipore. An anti-ubiquitin antibody was purchased from Cell Signaling. Other antibodies have been previously described (Yan et al., 2010).

Protein Recruitment to laser-induced localized ICLs

We followed a previous protocol to detect proteins recruited at laser-induced localized ICLs (Muniandy et al., 2009). Briefly, cells were seeded in a 35 mm glass bottom culture dish (MatTek™) and were incubated with 6 μM trioxalen at 37°C for 20 minutes prior to laser treatment. Localized irradiation was performed using the Nikon Eclipse TE2000 confocal microscope equipped with an SRS NL100 nitrogen laser-pumped dye laser (Photonics Instruments, St Charles, IL) that fires 5 ns pulses with a repetition rate of 10 Hz at 365 nm, with a power of 0.7 nW, measured at the back aperture of the 60X objective. The laser was directed to a specified rectangular region of interest (ROI) within the nucleus of a cell visualized with a Plan Fluor 60X/NA1.25 oil objective. The laser beam was oriented by galvanometer driven beam displacers and fired randomly throughout the ROI until the entire region was exposed. Throughout an experiment, cells were maintained at 37 °C, 5% $CO_2$and 80% humidity using an environmental chamber. Cells were fixed immediately in freshly prepared 4% formaldehyde in PBS for 10 minutes at room temperature, followed by immunostaining.

Detecting proteins recruited to a site-specific psoralen-ICL by eChIP

The eChIP was carried out as described (Shen et al., 2009). Percentages of relative enrichment of FAAP20 at the ICL site were arrived by normalizing comparative concentration (from real-time PCR) of each sample with that of its input.

siRNA experiments

HeLa cells were transfected with siRNA oligos using Lipofectamine RNAi MAX (Invitrogen) according to manufacturer's protocol. The siRNA oligos used are listed in Supplemental Information.

Expression and purification of GST-fusion proteins from *E.coli*

The expression plasmid for GST-FAAP20-UBZ domain (pGEX-FAAP20-UBZ) was constructed by cloning a region of human FAAP20 that includes the UBZ domain into the *BamHI* and *EcoRI* sites of pGEX-2TK. *E. coli* Rosetta (Novagen) cells carrying pGEX-UBZ-wild type construct or its mutant versions were grown at 30°C to OD_{600} of 0.4–0.5, and 200μM $ZnCl_2$ (final concentration) was added to culture. When OD_{600} reached 0.6–0.8, cells were induced with 0.2mM IPTG at 30°C for 3 hrs. Cell pellets were resuspended in lysis buffer (25mM Sodium Phosphate pH 8.0, 300mM NaCl, 10% glycerol) containing lysozyme, DNase I, Benzonase and complete EDTA-free protease inhibitor cocktail (Roche). The mixture was sonicated, and the lysed cells were cleared by centrifugation at 16K RPM for 35 min. The supernatant was then incubated for 1 h with glutathione sepharose beads (GE Healthcare). After washing with 10 column volumes (CV) of lysis buffer followed by 10 CV of wash buffer (25mM Sodium Phosphate pH 8.0, 300mM NaCl), the bound GST-fusion proteins were eluted in 10 CV of elution buffer (25mM Sodium Phosphate pH 8.0, 150mM NaCl, 30mM Glutathione). Peak fractions were pooled and dialyzed with dialysis buffer (25mM Sodium Phosphate pH 7.0, 150mM NaCl).

In vitro ubiquitin binding assay

Equal amounts (10 μg) of purified GST-UBZ fusion proteins or GST protein alone were incubated with 10 μl of ubiquitin agarose (Boston Biochem) in binding buffer (25mM HEPES, pH7.9, 150mM NaCl, 20μM ZnCl2, 0.1% Tween 20, 5mM β-mercaptoethanol) at

4°C for 1 hr. After washing with binding buffer for four times, beads were boiled in SDS gel loading buffer and the samples were analyzed by Coomassie-blue staining.

GST pull-down assay

Glutathione Sepharose beads (GE Health) with 10 μg of purified GST-UBZ fusion proteins or GST protein alone were incubated with different amounts of K63- or K48- linked polyubiquitin substrates (Boston Biochem) in binding buffer (described above) at 4°C overnight. Because the K63-linked polyubiquitin substrate contains a higher proportion of long chains than the K48-linked substrate, we used 10 μg of K48-linked polyubiquitin and 0.4 μg of K63-linked polyubiquitin in reactions of Figure 2C. This allows the level of K48-linked long chains to be comparable to that of the K63-linked long chains. In Figure 2E and F, 7 μg of K63- linked ubiquitin and 10 μg of K48-ubiquitin were used, respectively. After washing with the binding buffer for four times, beads were boiled in SDS gel loading buffer and the samples were analyzed by immunoblotting.

Gel filtration, Immunoprecipitation, and Protein identification

We followed the protocol as described (Yan et al., 2010). Briefly, we fractionated HeLa nuclear extract by Superose 6 gel filtration chromatography, pooled the peak fractions containing FANCA, and immunoprecipitated the core complex with a FANCA antibody. The eluted immunoprecipitates were subjected to silver-staining, mass spectrometry, and immunoblotting analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- FAAP20, a subunit of the FA core complex, binds the ubiquitin product of RNF8-UBC13.
- Recruitment of FAAP20 to ICLs depends on RNF8-UBC13 but not RNF168.
- RNF8 and FAAP20 form a ubiquitin cascade to recruit FA core complex to ICLs.
- RNF168 is not essential but can modulate the efficiency of the recruitment.

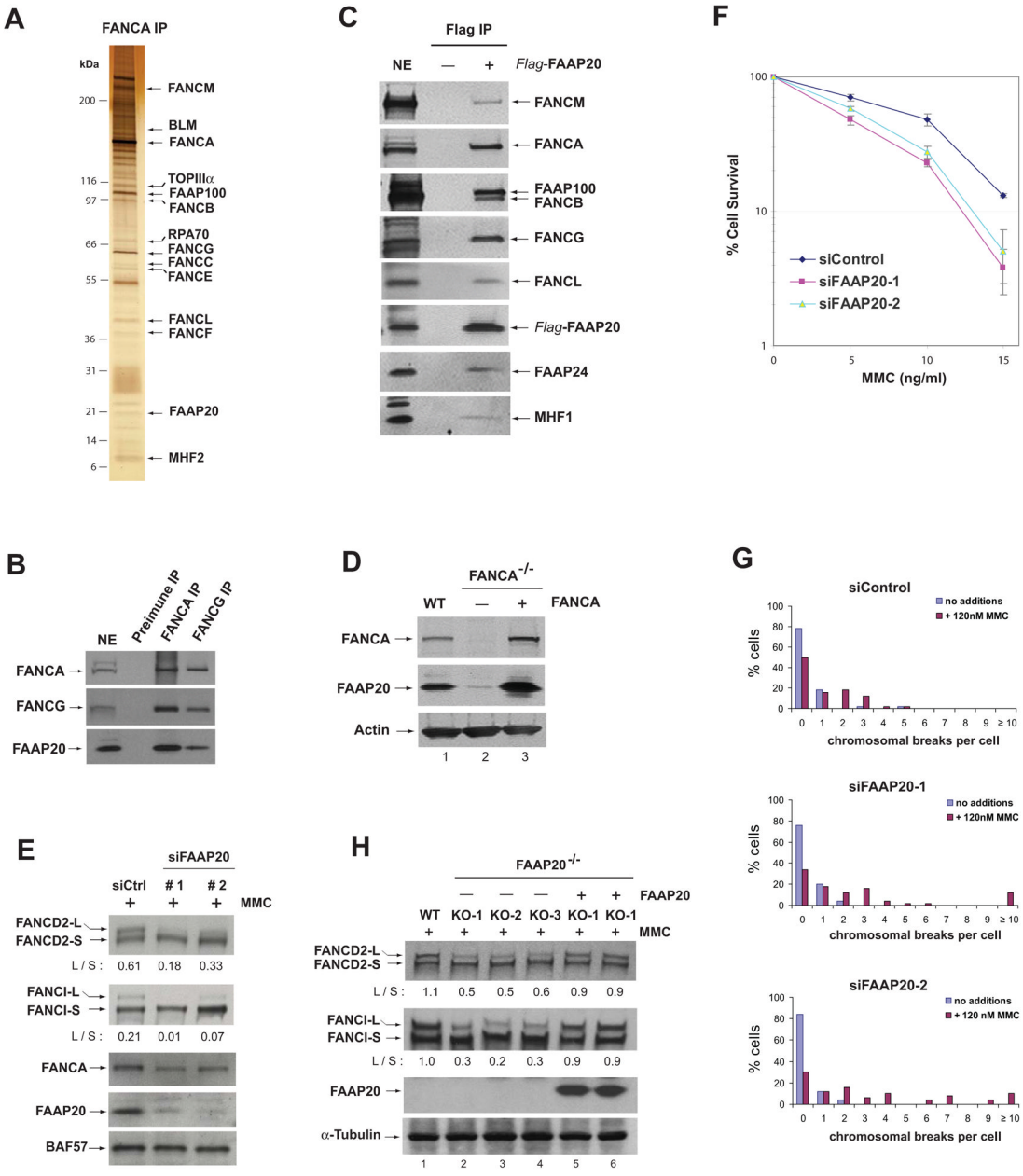

Figure 1. FAAP20 is required for normal activation of the FA pathway and cellular resistance to ICLs

(A) A silver-stained gel showing that the complex purified by a FANCA antibody from HeLa nuclear extract contained FAAP20 and other components of FA core and BLM complexes. IP indicates immunoprecipitation. **(B)** Immunoblotting shows that FAAP20 is present in the immunoprecipitates isolated from HeLa nuclear extract by FANCA or FANCG antibodies. Nuclear extract (NE) was used as a loading control. **(C)** Immunoblotting shows that FAAP20 co-immunoprecipitated with FANCA and other FA core complex components from HeLa cells stably expressing Flag-tagged FAAP20, but not from untransfected HeLa cells. A Flag antibody was used in IP. **(D)** Immunoblotting shows the level of FAAP20 in lysates of lymphoblastoid cells from a healthy individual (WT), a FANCA patient ($FANCA^{-/-}$), and the patient cell line complemented by expression of

exogenous FANCA. **(E)** Immunoblotting shows that HeLa cells depleted of FAAP20 by two different siRNAs have reduced levels of monoubiquitinated FANCD2 and FANCI in the presence of 60 ng/ml MMC for 20 hours. A non-targeting siRNA was used as a control (siCtrl). "L" (long) and "S" (short) represent monubiquitinated and non-ubiquitinated forms, respectively. The ratio between long and short forms (L/S) was obtained by using KODAK Molecular Imaging Software and shown below the blots. **(F)** Clonogenic survival assays of HeLa cells depleted of FAAP20 by two different siRNAs following the treatment with MMC. The mean surviving percentages with standard error of the mean (SEM) from three independent experiments are shown. **(G)** MMC-induced chromosomal aberrations in HeLa cells depleted of FAAP20 by two different siRNAs. Percentages of metaphases with and without aberrations after MMC treatment were compared between siControl and siFAAP20 cells using a two-sample Chi^2test. *P*-values for the differences between siFAAP20-1, siFAAP20-2 and siControl were 0.05 and 0.02, respectively.**(H)** Immunoblotting shows the levels of FANCD2 and FANCI in lysates from various DT40 cells (wild type [WT], *FAAP20*$^{-/-}$ cells of three independent clones [KO-1, KO-2, KO-3], and *FAAP20*$^{-/-}$ cells complemented with human FAAP20). Cells were treated with 500 ng/ml MMC for 6 hours (see also Figure S1).

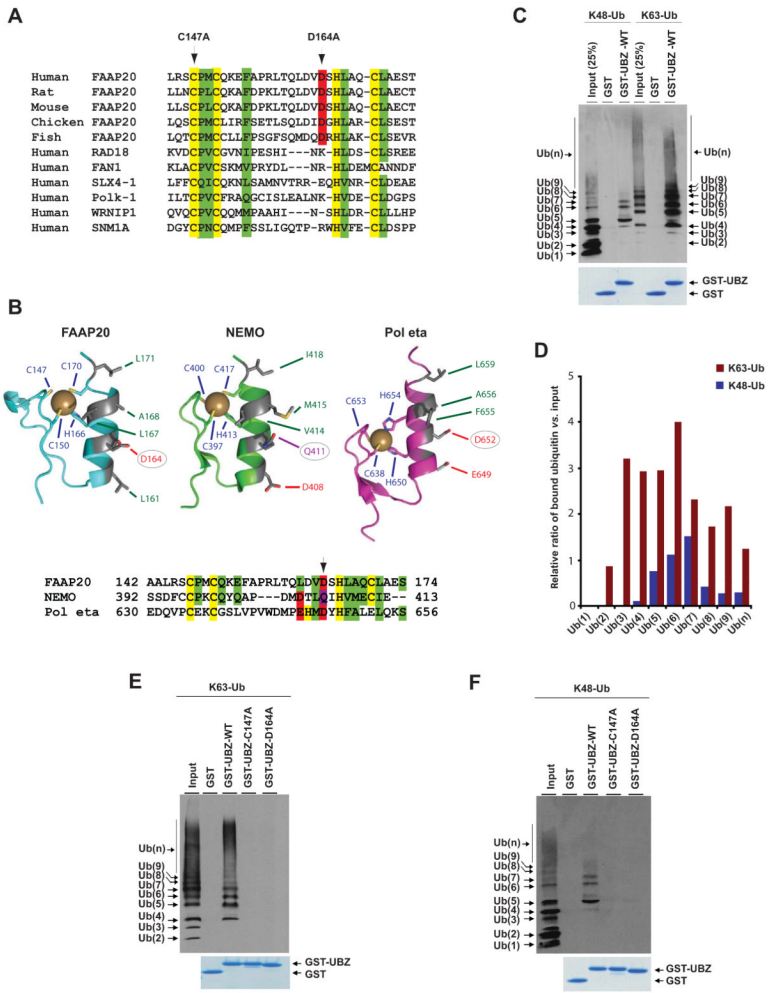

Figure 2. The UBZ domain of FAAP20 preferentially binds K63- over K48-linked polyubiquitin chains *in vitro*

(A) Sequence alignment of the UBZ domains of FAAP20 orthologs and several other proteins. Conserved residues of the zinc finger are marked yellow. A critical aspartate residue for ubiquitin binding is marked red. Other identical and similar residues are marked green. Two point mutants of human FAAP20 are indicated. **(B)** *Top panel:* A homology model to compare the UBZ domains of human FAAP20, NEMO, and Pol eta. Hydrophobic residues (green), acidic residues (red), polar uncharged (magenta), and the zinc coordinating residues (blue), are shown as sticks. *Bottom panel:* Sequence alignment of the UBZ domains. A critical residue at the ubiquitin-binding interface is indicated by an arrow. **(C)** GST pull-down coupled with immunoblotting shows the binding of both K48- and K63-linked polyubiquitin chains by a GST fusion protein containing the wild type (WT) UBZ domain of FAAP20 (GST-UBZ-WT), but not by GST protein alone. **(D)** A graph shows the ratio between levels of the bound ubiquitin *versus* those of input as reported in (C).The immunoblotting in (C) was quantified by KODAK Molecular Imaging Software. Notably, for each ubiquitin isoform, binding to K63 linked chains is consistently stronger than K48 chains. **(E and F)** GST pull-down coupled with immunoblotting shows the binding of K63- or K48-linked polyubiquitin by wild type (WT) UBZ domain, but not by GST-UBZ mutants (C147A and D164A, respectively) (see also Figure S2).

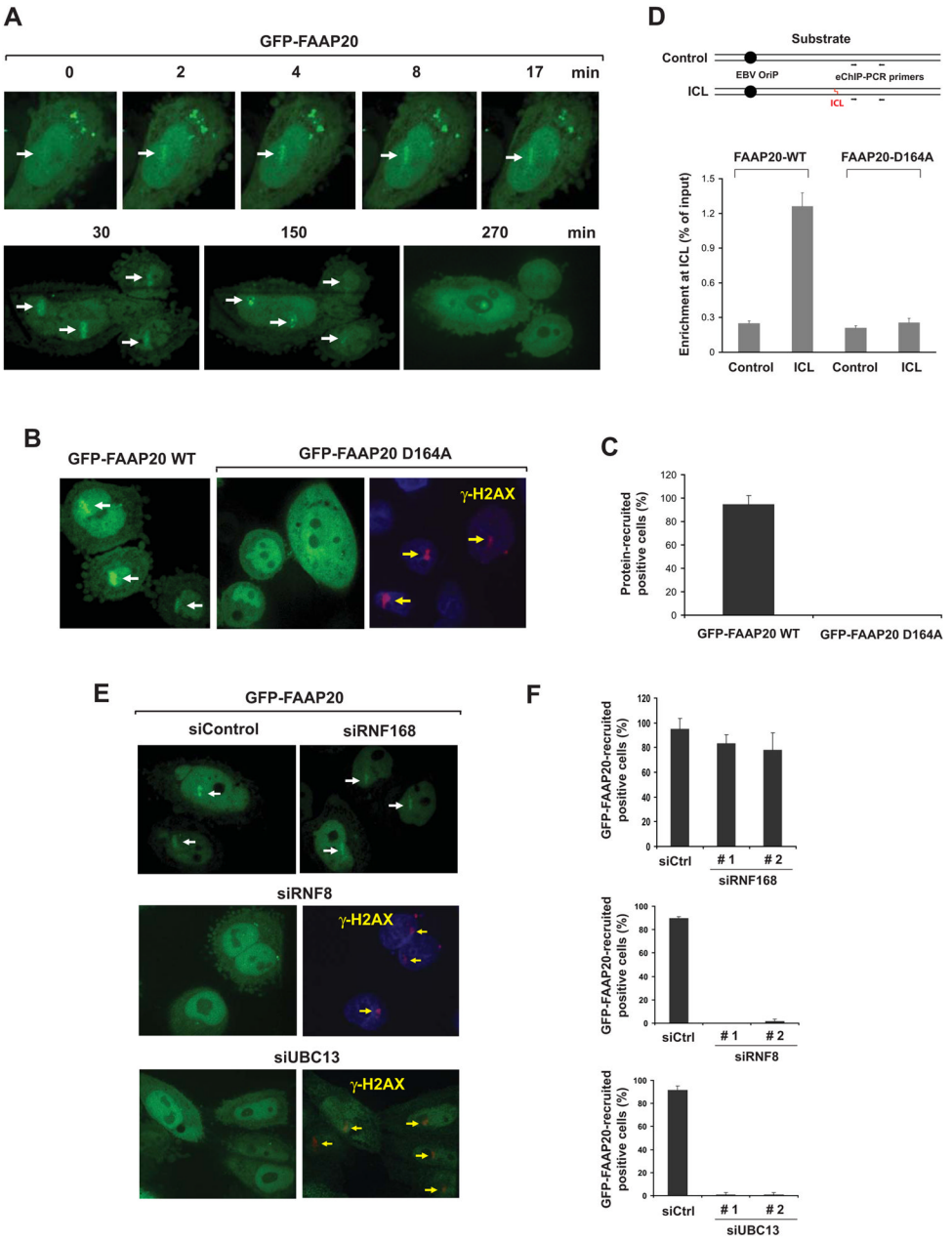

Figure 3. The recruitment of FAAP20 to ICLs requires its ubiquitin-binding activity, RNF8 and UBC13, but not RNF168

(A) Images showing that GFP-tagged FAAP20 protein in HeLa cells was recruited to psoralen-induced ICLs at various time points after laser activation. The arrows indicate positive recruitment signals. **(B and C)** Images (B) and a graph (C) show that GFP-FAAP20 wild type (WT) was recruited to ICL sites, but the GFP-FAAP20-D164A mutant was not. Immunostaining of γ-H2AX marks the areas targeted by the laser and also serves as a control to show a positive DNA damage response. Error bars in (C) are standard deviations. **(D)** *Top panel:* Schematic representation of the plasmid substrates used in the eChIP assay. The presence of psoralen-ICL is indicated. *Bottom panel:* A graph from eChIP shows that FAAP20 wild type (WT) protein was enriched about 4-fold at the ICL, whereas FAAP20-D164A mutant was not. Error bars represent standard deviations from three independent

experiments. **(E and F)** Images (E) and graphs (F) show the recruitment of GFP-FAAP20 to ICLs in HeLa cells transfected with two different siRNAs against RNF168, RNF8 or UBC13, respectively. Error bars in (F) are standard deviations (see also Figure S3).

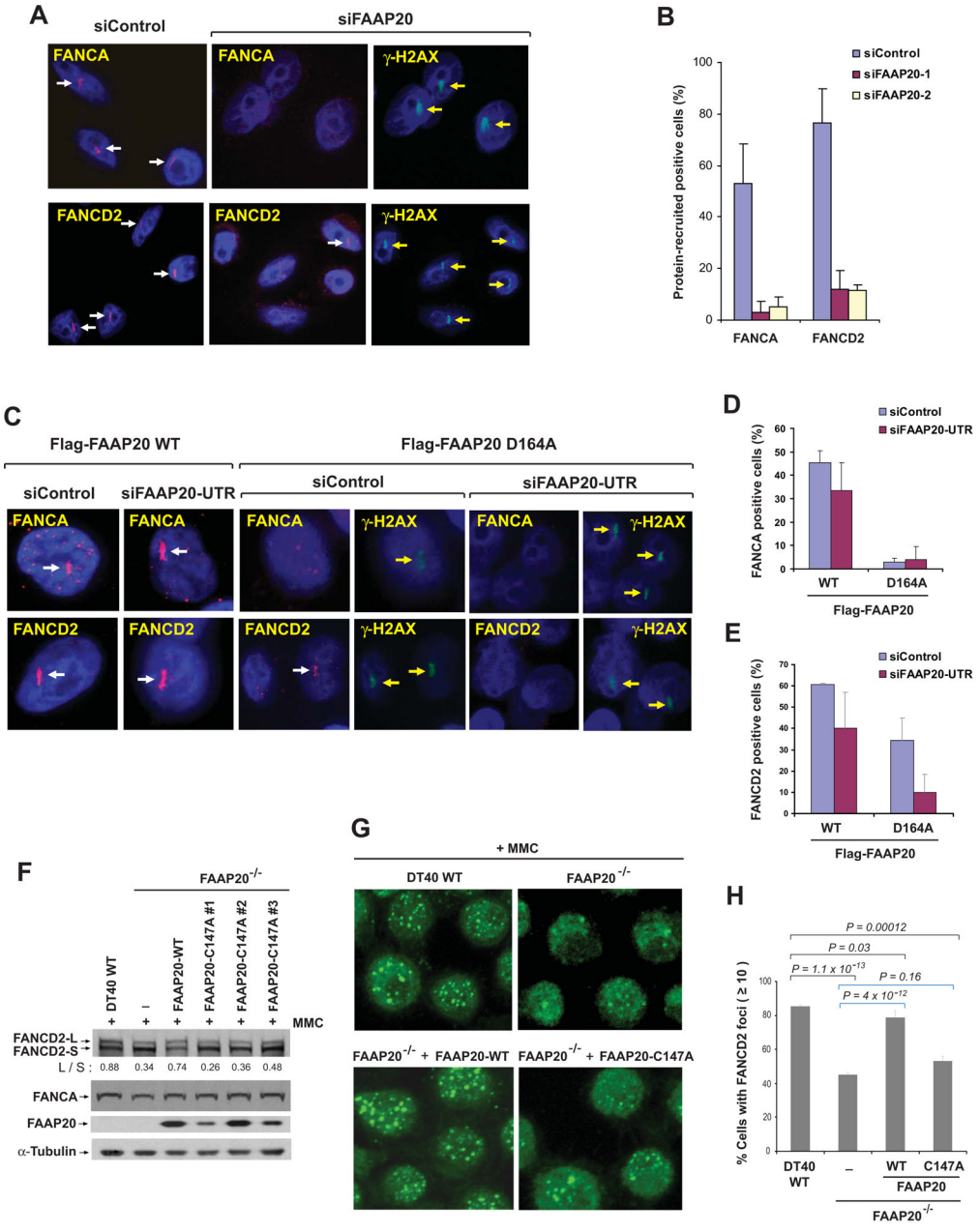

Figure 4. The ubiquitin-binding activity of FAAP20 is required for recruitment of FA core complex and FANCD2 and for normal activation of the FA pathway

(A and B) Images (A) and a graph (B) show that HeLa cells depleted of FAAP20 by two siRNAs are deficient in the recruitment of FANCA and FANCD2 to ICL sites. Immunostaining of γ-H2AX indicates the areas targeted by the laser and also serves as a control to show a positive DNA damage response. The positive recruitment signals are indicated by arrows. Error bars in (B) are standard deviations. **(C, D and E)** Images (C) and graphs (D and E) show the recruitment of FANCA and FANCD2 at ICLs in HeLa cells stably expressing an siRNA-resistant version of Flag-FAAP20 wild type (WT) or D164A mutant. These cells were treated with either a non-targeting siRNA (siControl), or a siRNA targeting the 3′-untranslated region of human FAAP20 (siFAAP20-UTR). Error bars in (D) and (E) are standard deviations. **(F)** Immunoblotting shows the levels of monoubiquitinated

(L) and non-ubiquitinated (S) FANCD2 in lysates from various DT40 cells (wild type [WT], *FAAP20*$^{-/-}$ cells and *FAAP20*$^{-/-}$ cells complemented with human FAAP20 wild type [WT] and C147A mutant version [three independent clones]). Cells were treated with 500 ng/ml MMC for 6 hours. **(G and H)** Immunostaining images (G) and a graph (H) show FANCD2 nuclear foci in various DT40 cells treated with MMC. The error bars in (H) are standard deviations. *P*-values between different cell lines are shown (see also Figure S4).

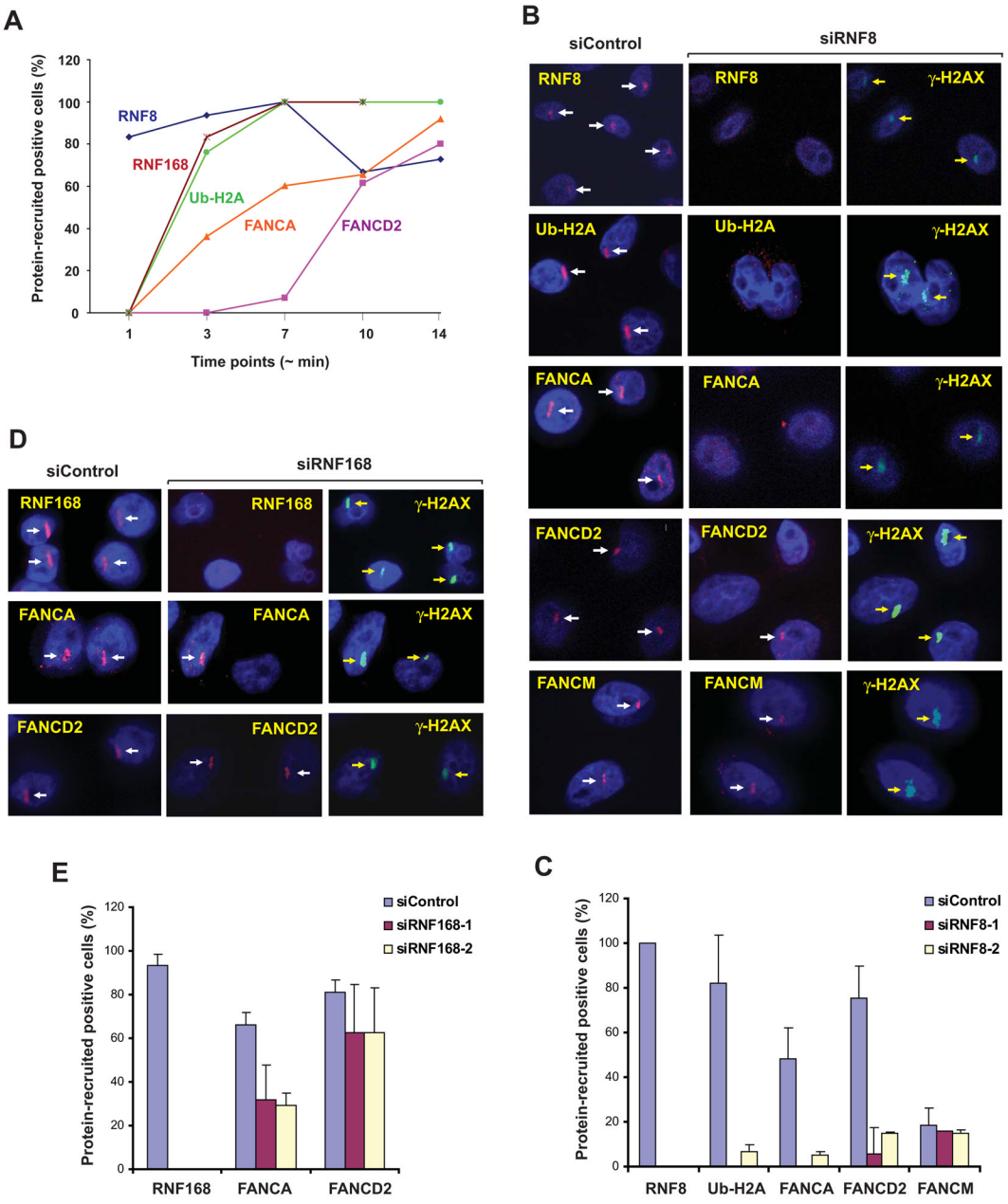

Figure 5. RNF8 is required for recruitment of FANCA and FANCD2 to ICLs, but not for FANCM recruitment

(A) A time-course study shows the sequential recruitment of RNF8, RNF168, Ub-H2A, FANCA and FANCD2 to psoralen-induced ICLs after laser activation. **(B and C)** Images (B) and a graph (C) show that HeLa cells depleted of RNF8 by two siRNAs are deficient in accumulation of ubiquitinated H2A (Ub-H2A), FANCA and FANCD2 at ICLs; but they are normal in accumulation of FANCM. Immunostaining of γ-H2AX marks the areas targeted by the laser and also serves as a control to show a positive DNA damage response. The recruitment signals are indicated by arrows. Error bars are standard deviations. **(D and E)** same as described in (B and C), except siRNAs targeting RNF168 are used.

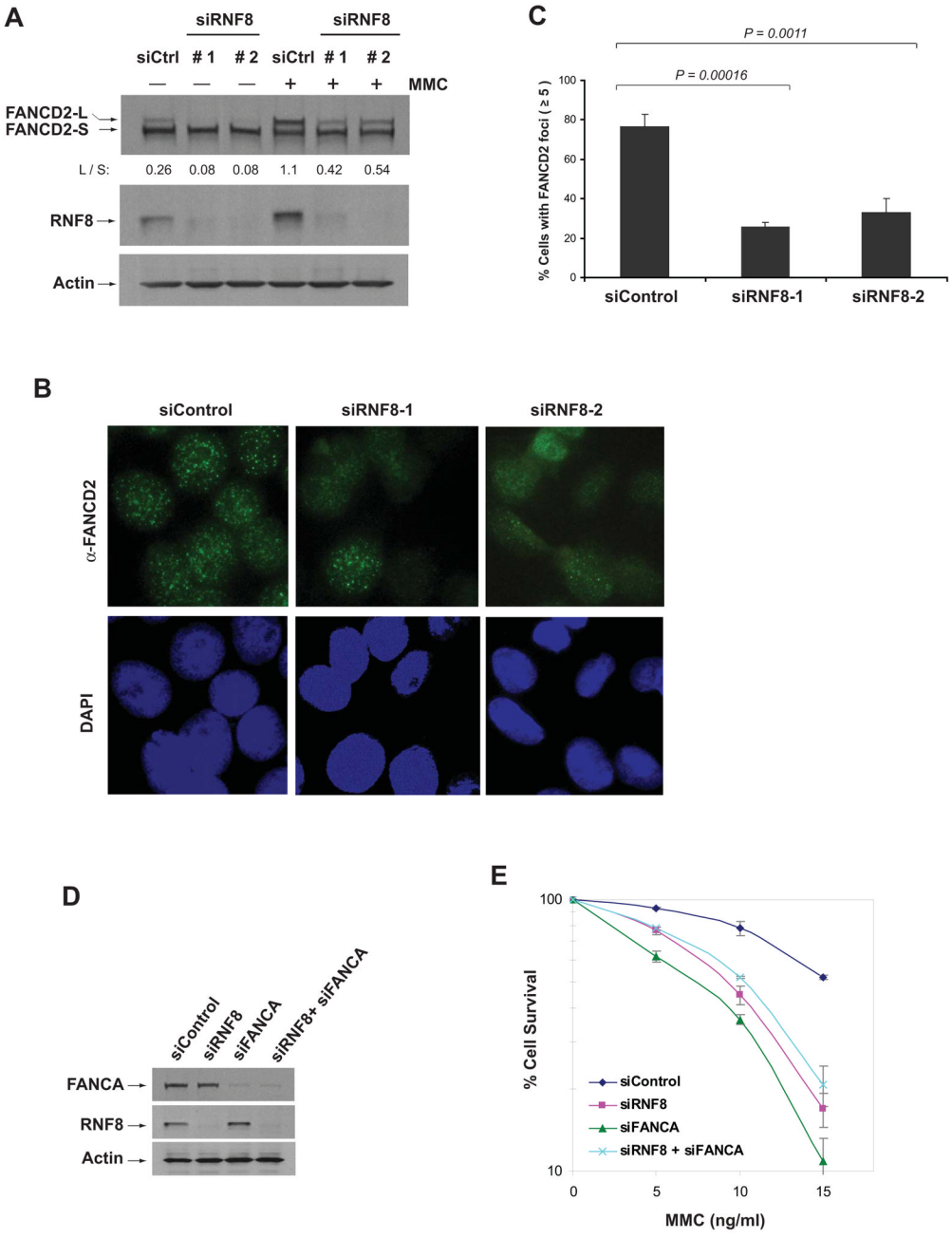

Figure 6. RNF8 is required for efficient FANCD2 monoubiquitination and foci formation, and works in the same pathway as the FA core complex in cellular resistance to ICLs

(A) Immunoblotting shows that HeLa cells depleted of RNF8 by two different siRNAs have a reduced level of monoubiquitinated FANCD2 in the absence or presence of MMC (60 ng/ml for 16 hours). **(B and C)** Immunostaining (B) and a graph (C) show that HeLa cells depleted of RNF8 by two siRNAs have a decreased number of FANCD2 nuclear foci in the presence of MMC. Error bars are standard deviations. *P*-values are shown in the top. **(D)** Immunoblotting shows the levels of FANCA and RNF8 in lysates from HeLa cells treated with various siRNAs as indicated. **(E)** Clonogenic survival assays of HeLa cells depleted of RNF8, FANCA or doubly-depleted of both by siRNAs following the treatment with MMC.

The mean surviving percentages with standard error of the mean (SEM) from three independent experiments are shown (see also Figure S5).

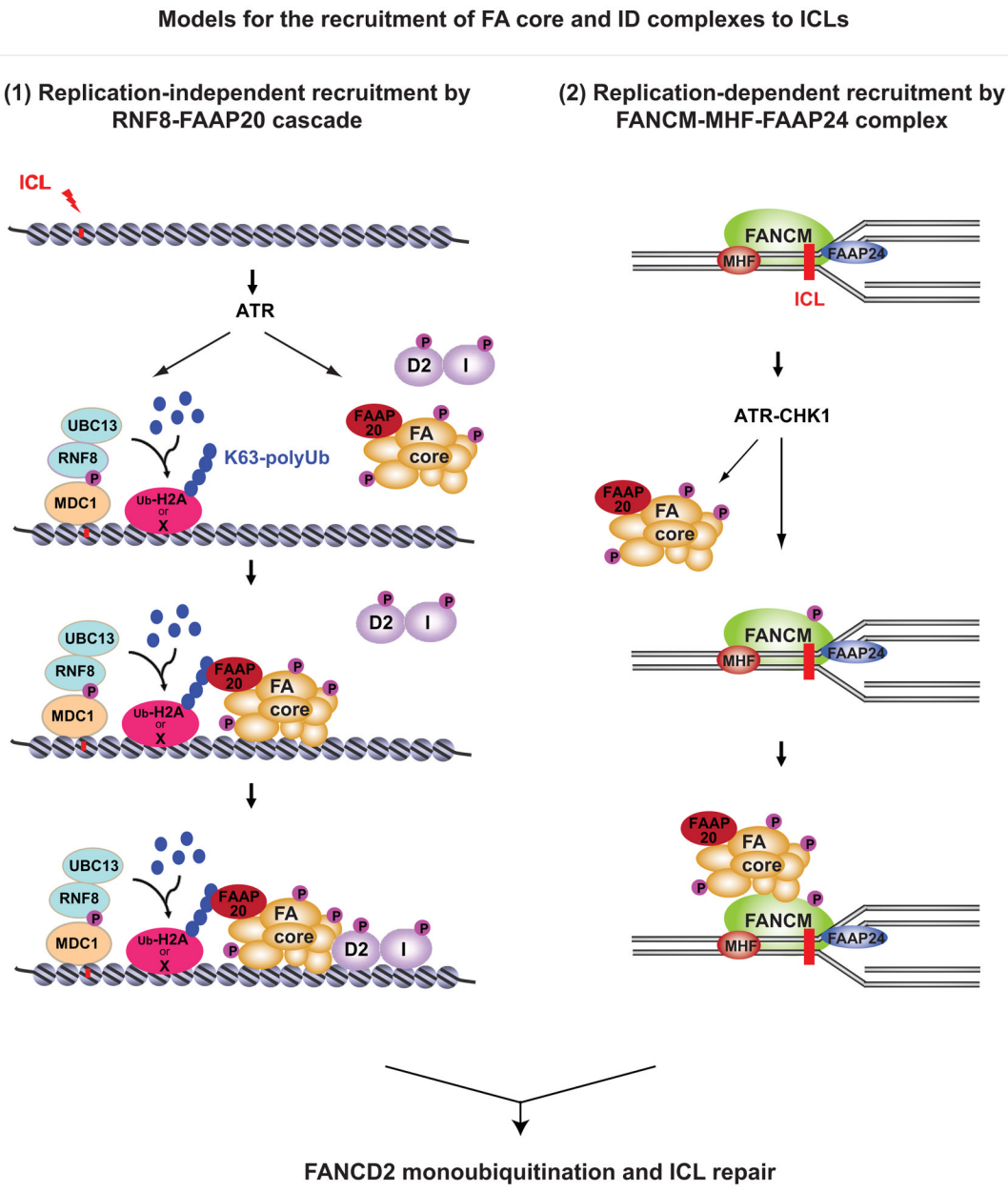

Figure 7. Models for the recruitment of FA core complex and FANCD2 to ICLs by two different pathways

The first pathway is replication-independent and controlled by the RNF8-FAAP20 cascade. In keeping with the literature on the activation of the DNA damage response by DSBs, ICLs may activate ATR, which can phosphorylate MDC1 and FANC proteins. RNF8 and UBC13 are recruited to initiate K63-linked polyubiquitination of histone H2A and other substrates (marked by "X") in surrounding chromatin, which are recognized by the UBZ domain of FAAP20 to trigger the recruitment of FA core complex. The FA ID complex is recruited possibly by interacting with the FA core complex. The second pathway is replication-dependent and governed by FANCM-MHF-FAAP24 complex. In S-phase, FANCM-MHF-FAAP24 complex recognizes ICL-stalled replication forks, activates ATR (Collis et al., 2008), and recruits the FA core complex by direct protein-protein interactions. Both

pathways lead to FANCD2 monoubiquitination, the key step of the FA network (see also Figure S6).