Regulation of the Fanconi Anemia Pathway by a CUE Ubiquitin-Binding Domain in the FANCD2 Protein

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Short title: Characterization of a FANCD2 CUE domain

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The Fanconi anemia (FA)-BRCA pathway is critical for the repair of DNA interstrand crosslinks (ICLs) and the maintenance of chromosome stability. A key step in FA-BRCA pathway activation is the covalent attachment of monoubiquitin to FANCD2 and FANCI. Monoubiquitinated FANCD2 and FANCI localize in chromatin-associated nuclear foci where they interact with several well-characterized DNA repair proteins. Importantly, very little is known about the structure, function, and regulation of FANCD2. Herein, we describe the identification and characterization of a CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation) ubiquitin-binding domain (UBD) in FANCD2, and demonstrate that the CUE domain mediates noncovalent binding to ubiquitin in vitro. We show that while mutation of the CUE domain destabilizes FANCD2, the protein remains competent for DNA damage-inducible monoubiquitination and phosphorylation. Importantly, we demonstrate that the CUE domain is required for interaction with FANCI, retention of monoubiquitinated FANCD2 and FANCI in chromatin, and for efficient ICL repair. Our results suggest a model by which heterodimerization of monoubiquitinated FANCD2 and FANCI in chromatin is mediated in part through a noncovalent interaction between the FANCD2 CUE domain and monoubiquitin covalently attached to FANCI, and that this interaction shields monoubiquitinated FANCD2 from polyubiquitination and proteasomal degradation.
Introduction

Fanconi anemia (FA) is a rare, recessive disease characterized by congenital abnormalities, bone marrow failure, hematological malignancies, and elevated cancer risk. FA is caused by biallelic mutation in any one of the following fifteen genes: FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C and FANCP/SLX4. The FA proteins together with BRCA1 function cooperatively in the FA-BRCA pathway to repair damaged DNA and prevent cellular transformation. Disruption of the FA-BRCA pathway leads to cellular hypersensitivity to the cytotoxic and clastogenic effects of DNA interstrand crosslinking agents.

The FA-BRCA pathway is activated following exposure to DNA damaging agents and during S-phase of the cell cycle. Activation of the pathway occurs when the core FA complex comprised of FANCA, -B, -C, -E, -F, -G, -L, and -M, and other proteins, assembles in the nucleus and monoubiquitinates the paralogous proteins FANCD2 and FANCI. FANCL, a RING domain-containing protein is the catalytic E3 ubiquitin ligase subunit of the FA core complex, while UBE2T is the E2 conjugating enzyme. Monoubiquitination of FANCD2 and FANCI targets these proteins to discrete chromatin-associated nuclear foci, where they interact with several key DNA repair proteins, including BRCA1, FANCD1/BRCA2, and RAD51. Following DNA repair, FANCD2 and FANCI are deubiquitinated by the USP1/UAF1 complex facilitating the release of these proteins from chromatin. Recent studies indicate that FANCD2 monoubiquitination is necessary for the recruitment of the FAN1 and SLX4/FANCP endonucleases to sites of DNA damage. Despite the critical functions of FANCD2 and FANCI in ICL repair, very little is known about their structure, function, and regulation.
Ubiquitin signaling is essential for a vast array of cellular processes including protein trafficking, histone modification, transcriptional regulation, as well as DNA repair. One way in which the ubiquitin signal is recognized and interpreted is through noncovalent interaction with proteins harboring ubiquitin-binding domains (UBDs). Several types of UBDs exist including the ubiquitin-associated motif (UBA), ubiquitin-interacting motif (UIM), ubiquitin-binding zinc finger domain (UBZ), and the CUE domain (for coupling of ubiquitin conjugation to endoplasmic reticulum degradation). The CUE domain is a 42-43 amino acid sequence identified by its similarity to a region of the yeast Cue1 protein. Importantly, several proteins harboring UBDs are themselves monoubiquitinated. The structural arrangement of a UBD and a monoubiquitinated residue residing on the same protein has the potential to promote noncovalent intra- or intermolecular interactions. Indeed, coupled monoubiquitination has recently been demonstrated to play a key role in the regulation of the translesion DNA synthesis (TLS) polymerase pol η.

As FANCD2 is known to be monoubiquitinated in response to DNA damage, we have long suspected that FANCD2 harbors a UBD. In this study we describe the identification of a CUE UBD in the amino-terminus of FANCD2 and demonstrate that this domain mediates noncovalent interaction between FANCD2 and ubiquitin. We demonstrate that mutation of the CUE domain leads to a decrease in protein stability that can be rescued by inhibition of the proteasome. Multiple distinct FANCD2 CUE mutants retain the ability to undergo DNA damage-inducible monoubiquitination and phosphorylation indicating that reduced protein stability is not a general consequence of protein misfolding. Importantly, we establish that the CUE domain is required for efficient interaction between FANCD2 and FANCI and is essential for the localization of monoubiquitinated FANCD2 and FANCI in chromatin. Consequently, FANCD2 CUE mutants
fail to correct the MMC hypersensitivity of FA-D2 (FANCD2<sup>−/−</sup>) patient cells. Taken together, our results suggest that the heterodimerization of monoubiquitinated FANCD2 and FANCI in chromatin is mediated in part through a noncovalent interaction between the FANCD2 CUE domain and monoubiquitin covalently linked to K523 of FANCI, and that this interaction shields monoubiquitinated FANCD2 from polyubiquitination and proteasomal degradation.

**Methods**

**Cell culture and antibodies**

PD20 (FA-D2 (FANCD2<sup>−/−</sup>)), HeLa and COS-7 cells were grown in DMEM media supplemented with 12% v/v FBS, L-glutamine and penicillin/streptomycin. Stable FA-D2 cells were generated by infection with pMMP Moloney murine leukemia or pLenti6.2/V5-DEST (Invitrogen) virus harboring wild type or mutant FANCD2 cDNAs.<sup>4,25</sup> Stable cell lines were grown in DMEM media supplemented with either 1 μg/ml puromycin or 2 μg/ml blasticidin. The following antibodies were used: rabbit polyclonal antisera against FANCD2 (NB100-182; Novus Biologicals), FANCI (Dr. Patrick Sung, Yale University and A300-212A; Bethyl Laboratories), H2A (07-146; Millipore), and mouse monoclonal antisera against α-tubulin (MS-581-PO; Lab Vision) and V5 (R96025; Invitrogen).

**Immunofluorescence microscopy**

For immunofluorescence microscopy (IF) freely soluble cellular proteins were pre-extracted with 0.3% v/v Triton X-100 and cells fixed in 4% w/v paraformaldehyde and 2% w/v sucrose at 4°C followed by permeabilization in 0.3% v/v Triton X-100 in PBS. Fixed cells were blocked for 30 minutes in antibody dilution buffer (5% v/v goat serum, 0.1% v/v NP-40, in PBS) and incubated
with primary antibody for 1 h. Cells were washed three times in PBS and incubated for 30 minutes at room temperature with an Alexa fluor 488-conjugated secondary antibody. Nuclear foci were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software.

**Immunoprecipitation**

Cells were lysed in NETN100 (20 mM Tris-HCl pH 7.4, 0.1% v/v NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM Na$_3$O$_4$V, 1 mM NaF, supplemented with protease inhibitors), incubated on ice and sonicated briefly. 800 µg whole-cell lysates (WCL) were incubated with 3 µg of antibodies against FANCD2 (FI-17; Santa Cruz), V5 (R96025; Invitrogen) or mouse IgG (12-371B; Millipore).

**Plasmids and site-directed mutagenesis**

The *FANCD2-P204A*, -LP215AA, and -LL234AA cDNAs were generated by site-directed mutagenesis of the wild type *FANCD2* cDNA using the Quikchange Site-directed Mutagenesis Kit (Stratagene). The forward and reverse oligonucleotide sequences used are as follows: P204A FP, 5’-GATCATGCAGCTGATCAGTATTGCTGCAGAGAACCTGCAGCATGACATCAT-3’; P204A RP, 5’-ATGATGTCATGCTGCAGGTTCTCTGCAGCAATACTGATCAGCTGCATGACATCAT-3’; LP215AA FP, 5’-GAGAACCTGCAGCATGACATCATCACCAGCGCAGCTGAGATCCTAGGGGATTCCAGCAGTGAT-3’; LP215AA RP, 5’-ATCAGCGTGTGGGGAATCCTCCCCTAGGATATCAGCTGAGCTGAGCTGAGCTGAGCTGATGTCATGCTG

6
CAGGTTCTC-3’; LL234AA FP, 5’-
CACGCTGATGTGGGAAAGAAGTCAGTGACGCAGCGATAGAGAATCTTCACTCAC
TGTCCTCAATC-3’; LL234AA RP
GATTGGGCACAGTGAAGTTATTCCTATCGTCACTGAGGTTTCTTTTCCAC
ATCAGCGTG. The mammalian expression vector pDEST40 (Invitrogen) and the bacterial
expression vector pDEST49 (Invitrogen), allow for the expression of C-terminal H6/V5 fusion
proteins. pGEX-2TK (GE Healthcare) was used for the expression of N-terminal GST fusion
proteins.

Protein purification and ubiquitin-binding assays

6xHistidine (H6)/V5 fusion protein expression in E. coli BL21 (DE3) (Stratagene) was induced
with 0.2% L-arabinose for 10 h at room temperature. Cell pellets were collected, resuspended in
ice-cold lysis buffer (50 mM potassium phosphate pH 7.8, 400 mM NaCl, 100 mM KCl, 10%
v/v glycerol, 0.5% v/v Triton-X-100, 10 mM imidazole) with sonication. H6/V5 fusion proteins
were purified with Ni-NTA agarose (Invitrogen) according to the manufacturer’s instructions.
GST fusion protein expression was induced with 400 µM IPTG for 5 h at 30°C. Cell pellets were
collected, resuspended in ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA,
1mM EGTA, 10% v/v glycerol, 1% v/v Triton-X-100, 25 mM NaF, 10 µM ZnCl₂, pH 7.5)
containing protease inhibitors (Roche), sonicated and centrifuged for 15 min at 12,000 rpm.
Purified proteins were resolved on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and protein purity
assessed via Coomassie staining. For ubiquitin-binding assays between 4 and 40 ng of purified
protein, or 5 mg of cleared bacterial whole-cell lysates, was incubated with ubiquitin sepharose
(Boston Biochem) in lysis buffer for at least 1 h at 4°C. Beads were pelleted via brief
centrifugation and washed three times in lysis buffer. After the final wash, beads were heated in 2x NuPAGE LDS sample buffer (Invitrogen) and proteins resolved on NuPAGE 4-12% Bis-Tris gels.

**DNA damage assays**

Mitomycin C (MMC) survival assays and FACS analysis were carried out as previously described. For chromosome breakage assays, cells were treated with MMC for 24 h and harvested for chromosome preparations using standard conditions as described previously. Metaphase chromosomes were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software.

**Cellular fractionation and λ-phosphatase assay**

Soluble proteins were removed by extraction in cytoskeletal buffer (CSK) (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, and 0.5% v/v Triton-X-100) for 10 mins at 4°C. Pellets were washed once with CSK buffer, lysed in SDS sample buffer (2% w/v SDS, 50 mM Tris-HCl pH 7.4, 10 mM and EDTA), boiled for 15 minutes and sonicated. The λ-phosphatase assay was preformed as described previously.

**Results**

**FANCD2 contains a highly conserved putative CUE ubiquitin-binding domain**

To gain greater insight into the domain organization and structure of the FANCD2 protein, the 1451-amino acid sequence of FANCD2 was fragmented into ~150-amino acid segments and analyzed using Pfam and SMART databases. This approach yielded a low-homology hit between
an amino-terminus fragment of FANCD2 and the CUE domain of the *Saccharomyces cerevisiae* Vps9 protein (Figure 1A). A sequence alignment of this amino-terminus fragment of FANCD2 with several known CUE domains revealed high conservation of a proline residue and di-leucine motif, characteristic of CUE UBDs, as well as a number of other hydrophobic residues thought to be important for noncovalent interaction with ubiquitin (Figure 1B). A sequence alignment of this region of FANCD2 among vertebrates demonstrates strong evolutionary conservation (Figure 1C). Using PORTER secondary protein structure prediction software, the putative CUE domain was predicted to contain three consecutive alpha-helices, with the conserved proline and dileucine residues residing in helix one and three, respectively. Importantly, this arrangement is common among several CUE domains including that of the yeast Cue2 protein. Recently, the crystal structure of the murine Fancd2-Fanci (ID) complex was solved. In support of our secondary structure prediction, the crystallized ID structure demonstrates that the Fancd2 CUE domain does adopt a triple alpha helical arrangement (supplemental Figure 1A). Moreover, the CUE domain of Fancd2 is in close proximity to K522 of Fanci (equivalent residue to K523 of human FANCI) (supplemental Figure 1B), the residue that undergoes monoubiquitination, suggesting that the heterodimerization of FANCD2 and FANCI may be mediated by an interaction between the CUE domain of FANCD2 and ubiquitin covalently linked to FANCI K523.

**FANCD2 interacts with ubiquitin noncovalently**

To determine if FANCD2 does indeed interact noncovalently with ubiquitin, whole cell lysates (WCLs) from FA-D2 (FANCD2-/-) patient cells reconstituted with either wild type FANCD2 or FANCD2 K561R were incubated with ubiquitin- or protein G-conjugated sepharose. Both
nonubiquitinated and monoubiquitinated FANCD2 noncovalently bound to ubiquitin but not to protein G (Figure 2A, lower panel, lanes 1 and 4). In another approach, WCLs from HeLa cells transiently expressing V5-tagged wild type FANCD2 or FANCD2 K561R were incubated with ubiquitin-conjugated sepharose or SUMO-interacting motif (SIM)-conjugated sepharose, as a negative control. Once again FANCD2-V5 and FANCD2-K561R-V5 noncovalently bound to ubiquitin (Figure 2B, lanes 5 and 6). A weaker lower affinity interaction between FANCD2 and SIM was also detected (Figure 2B, lanes 8 and 9 and supplemental Figure 2A). Next we bacterially purified two FANCD2 fragments, FANCD2-1, encompassing amino acids 1-254 harboring the CUE domain, and FANCD2-3, encompassing amino acids 585-819, and incubated them with ubiquitin-sepharose. FANCD2-1 noncovalently bound to ubiquitin in a concentration-dependent manner, while FANCD2-3 did not (Figure 2C, lanes 3-6). Finally, we incubated bacterial lysates expressing GST-tagged FANCD2 solenoid 1 (S1) encompassing the CUE domain, as well as S1 harboring P204A and LL234AA missense mutations in the CUE domain (see below). Wild-type FANCD2 S1 bound efficiently to ubiquitin while ubiquitin binding was significantly compromised for both FANCD2 S1 CUE mutants, even taking into account the slightly reduced levels of expression of the CUE mutants (Figure 2D and supplemental Figure 2B). Taken together, these results demonstrate that FANCD2 can noncovalently interact with ubiquitin and that this interaction is mediated by the amino-terminus CUE domain. It is important to note that based on the absence of M and F residues preceding the conserved P, it is likely that the FANCD2 CUE domain represents a low affinity UBD similar to that of S. cerevisiae Cue1p, and unlike that of S. cerevisiae Vps9p (see Figure 1A).
Mutation of the CUE domain destabilizes FANCD2

To determine the function of the CUE domain we used a site-directed mutagenesis approach to generate mammalian expression constructs of the FANCD2 CUE missense mutants P204A and LL234AA (Figure 3A). Previous studies have demonstrated that mutation of the conserved proline and di-leucine residues impairs the ability of the CUE domain to noncovalently interact with ubiquitin. In addition, we generated a LP215AA CUE mutant as these residues also demonstrated a high degree of evolutionary conservation (see Figure 1C). PORTER secondary structure prediction analysis revealed that the triple helical supersecondary structure of the CUE domain should not be disrupted by these mutations. Equal quantities of wild type and CUE mutant FANCD2-V5 constructs were transfected into COS-7 cells and protein expression was assessed. Intriguingly, protein levels of all three FANCD2 CUE mutants were significantly reduced compared to that of wild type FANCD2 (Figure 3B and supplemental Figure 3). We also generated FA-D2 patient cell lines stably expressing wild type FANCD2 or the FANCD2 missense mutants K561R, P204A, LP215AA, and LL234AA. While both wild type FANCD2 and the K561R mutant displayed robust levels of expression, expression levels of all three CUE mutants were again reduced (Figure 3C). Reduced protein expression was consistently observed for multiple independently generated FA-D2 CUE mutant lines (results not shown). The FANCD2 CUE mutants remained competent for monoubiquitination however, indicating that protein folding and overall tertiary structure was not overtly affected. To determine if reduced protein stability was a consequence of increased protein turnover via proteasome-mediated degradation, COS-7 cells transiently expressing wild type FANCD2-V5 or the CUE mutants were treated with the proteasome inhibitor MG132 and protein levels measured. While MG132 treatment had little effect on wild type protein levels, a significant increase in FANCD2
LP215AA protein levels was observed (Figure 3D, lanes 11 and 12). Similar results were obtained for both FANCD2 P204A and LL234AA (Figure 3D, lanes 18 and 19 and results not shown). Furthermore, treatment with the protein translation inhibitor cycloheximide led to a rapid and pronounced reduction in FANCD2 LP214AA protein levels (Figure 3D, lane 14). Moreover, deletion of the entire CUE domain (amino acids 1-254) led to a marked destabilization of the protein (supplemental Figure 4). Collectively, these results suggest that the FANCD2 CUE domain is a major determinant of protein stability and may protect FANCD2 from rapid proteasome-mediated turnover.

**Disruption of the FANCD2 CUE domain does not impair DNA damage-inducible FANCD2 and FANCI post-translational modification**

A key step in the activation of the FA-BRCA pathway is the monoubiquitination of FANCD2 and FANCI. Therefore, we next examined the effect of mutation of the CUE domain on this important post-translational modification. For this experiment, and all subsequent experiments, to control for the effects of the large difference in wild type FANCD2 and CUE mutant protein expression levels, we included FA-D2 cells stably expressing wild type FANCD2-V5. Importantly, wild type FANCD2-V5 and the FANCD2 CUE mutants are expressed at similar levels in the FA-D2 cells (Figure 4A, compare lanes 1 and 2 to lanes 9-14). Moreover, wild type FANCD2-V5 undergoes DNA damage-inducible monoubiquitination and corrects the MMC-hypersensitivity of FA-D2 patient cells (see Figure 7). All FANCD2 CUE mutants remained competent for both spontaneous and MMC-inducible monoubiquitination (Figure 4A, lanes 9-14). Previous studies have shown that FANCD2 and FANCI monoubiquitination are interdependent. Consistent with these studies, mutation of FANCD2 K561 resulted in complete
abrogation of FANCI monoubiquitination (Figure 4A, lanes 5 and 6). In contrast, mutation of the CUE domain resulted in a modest reduction in MMC-inducible FANCI monoubiquitination compared with cells expressing wild type FANCD2 (Figure 4A, compare lanes 5 and 6 to lanes 9-14). However, similar levels of FANCI monoubiquitination were observed in FA-D2 cells expressing wild-type FANCD2-V5 (Figure 4A, compare lanes 1 and 2 to lanes 9-14), indicating that attenuated FANCI monoubiquitination was a consequence of reduced FANCD2 protein expression, and not mutation of the CUE domain per se. We also examined the ability of the FANCD2 CUE mutants to undergo DNA damage-inducible phosphorylation by comparing electrophoretic mobility in the absence and presence of λ-phosphatase. Mutation of the CUE domain had no discernible effect on DNA damage-inducible FANCD2 phosphorylation, as demonstrated by an increase in protein mobility following treatment of whole cell lysates with λ-phosphatase (Figure 4B, compare lanes 7 and 8). Taken together these findings demonstrate that while mutation of the FANCD2 CUE domain leads to a pronounced decrease in protein stability, tertiary protein structure is not overtly impacted by mutation of this domain.

The CUE domain is required for FANCD2 and FANCI nuclear foci formation and chromatin localization

Following exposure to DNA damaging agents, FANCD2 and FANCI assemble in discrete chromatin-associated nuclear foci to facilitate DNA repair. To determine if the FANCD2 CUE domain is important for FANCD2/I nuclear foci formation, FA-D2 cells stably expressing wild type or mutant FANCD2 were treated with MMC and FANCD2/I nuclear foci formation was assessed using IF. Both spontaneous and DNA damage-inducible FANCD2 nuclear foci formation were markedly impaired in cells expressing mutant FANCD2 (Figure 5A and
supplemental Figure 5A). For example, following MMC treatment ~66% percent of cells expressing wild type FANCD2 displayed nuclear foci compared with only ~10% of cells expressing FANCD2 P204A ($p < 0.001$). FANCI nuclear foci formation was also markedly impaired in FA-D2 cells reconstituted with the CUE mutants. For example, following exposure to MMC only approximately 10% of FA-D2 cells expressing FANCD2 P204A were positive for FANCI nuclear foci formation, in comparison with ~33% of cells expressing wild type FANCD2 ($p < 0.001$) (Figure 5A and supplemental Figure 5B). Importantly, reduced FANCD2 and FANCI nuclear foci formation was not solely a consequence of decreased levels of expression of the CUE mutants as foci formation was also significantly reduced compared with FA-D2 cells stably expressing similar levels of wild-type FANCD2-V5 (Figure 5A and supplemental Figures 5A and B). We next performed a chromatin fractionation experiment with FA-D2 cells stably expressing wild type FANCD2-V5 or FANCD2 P204A to corroborate our IF findings. Cells were incubated in the absence or presence of MMC for 12 hours, washed to remove the drug and allowed to recover in drug-free media. In agreement with our IF findings, despite similar total levels of FANCD2, we observed greatly reduced levels of monoubiquitinated FANCD2 and FANCI in chromatin fractions of FA-D2 cells expressing FANCD2 P204A, compared with cells expressing wild type FANCD2-V5 (Figure 5B, compare lanes 23 and 24 with lanes 11 and 12). Taken together, our results demonstrate that the FANCD2 CUE domain is required for the localization and/or stabilization of FANCD2 and FANCI in chromatin.

**The FANCD2 CUE domain is required for efficient interaction with FANCI**

Several reports have suggested that the stability of FANCD2 and FANCI are interdependent and that FANCD2/I monoubiquitination may act to stabilize the FANCI-FANCD2 (ID) complex.7,32
We hypothesized that mutation of the CUE domain might impair ID complex formation. To test this hypothesis, we examined the effect of mutation of the CUE domain on the ability of FANCD2 and FANCI to coimmunoprecipitate. FANCD2 immune complexes immunoprecipitated from FA-D2 cells reconstituted with wild type or mutant FANCD2 were examined for the presence of FANCI. Wild type FANCD2 immunoprecipitated the highest levels of both nonubiquitinated and monoubiquitinated FANCI (Figure 6A, IP: α-FANCI panel, lane 6). These results were not surprising given the robust level of FANCD2 expression in these cells (Figure 6A, Input: α-FANCD2 panel, lane 2). In order to gain a more meaningful understanding of the effect of CUE mutation on the FANCD2-FANCI interaction, we also included FA-D2 cells stably reconstituted with wild type FANCD2-V5, as wild type FANCD2-V5 and FANCD2-P204A are expressed at similar levels (Figure 6A, Input: α-FANCD2 panel, compare lanes 1 and 4) therefore, any difference in the ability of these proteins to immunoprecipitate FANCI is likely to be solely a consequence of CUE mutation and not an effect of protein expression. Importantly, wild type FANCD2-V5 and FANCD2-P204A immunoprecipitated similar levels of FANCD2 immune complexes (Figure 6A, IP:α-FANCD2 panel, compare lanes 5 and 8). However, in contrast, wild type FANCD2-V5 immunoprecipitated considerably higher levels of FANCI compared with FANCD2-P204A (Figure 6A, IP:α-FANCI panel, compare lanes 5 and 8). Interestingly, with the exception of the K561R mutant, monoubiquitinated FANCI was the predominant form immunoprecipitated, supporting previous studies suggesting that FANCD2/I monoubiquitination may stabilize ID complex formation.7,32 In an alternative approach, we transiently transfected COS-7 cells with wild type FANCD2-V5 or FANCD2-P204A-V5 and examined the association with endogenous FANCI. Despite lower expression levels of the FANCD2-P204A-V5 mutant compared with wild type FANCD2-V5, similar levels of FANCD2
immune complexes were precipitated (Figure 6B, α-V5 panel, compare lanes 5 and 6). However, similar to the results obtained above, considerably lower levels of FANCI coimmunoprecipitated with FANCD2-P204A-V5 compared with wild type FANCD2-V5 (Figure 6B, α-FANCI panel, compare lanes 5 and 6). Taken together, these results suggest that FANCI-FANCD2 binding may be facilitated, at least in part, by the CUE domain.

**FANCD2 CUE mutants fail to rescue the MMC-hypersensitivity of FA-D2 patient cells**

FA patient-derived cells are characteristically hypersensitive to the cytotoxic and clastogenic affects of DNA interstrand crosslinking agents. Therefore, we next assessed the ability of the FANCD2 CUE mutants to rescue the MMC hypersensitivity of FA-D2 patient cells. To ensure that FANCD2 expression was not a confounding factor in our functional assays we again used FA-D2 cells stably expressing FANCD2-V5 as our wild type control. In a MMC cytotoxicity assay, while wild type FANCD2-V5 rescued the MMC hypersensitivity of FA-D2 cells, all three FA-D2 cell lines expressing the FANCD2 CUE mutants displayed intermediate MMC sensitivity (Figure 7A). For example, an approximate 2-fold ($p < 0.05$) reduction in % survival was observed for FA-D2 cells expressing FANCD2 P204A, compared to cells expressing wild type FANCD2, following exposure to 50 nM MMC (Figure 7A). Similarly, in a MMC clastogenicity assay, FA-D2 cells expressing the CUE mutants displayed intermediate sensitivity to the clastogenic effects of MMC (Figure 7B). For example, FA-D2 cells expressing FANCD2 LL234AA exhibited a ~2-fold ($p = 0.002$) increase in MMC-induced chromosome aberrations compared with FA-D2 cells expressing wild type FANCD2-V5. Furthermore, FACS analysis revealed an intermediate G2/M accumulation of FA-D2 cells expressing the FANCD2 CUE mutants, compared with FA-D2 cells expressing empty vector or FANCD2 K561R, following
exposure to MMC (supplemental Figure 6). Taken together, our results demonstrate that the FANCD2 CUE domain is necessary for efficient ICL repair.

Discussion

In this study we describe the identification and characterization of a CUE UBD in the amino-terminus of the FANCD2 protein. Despite the clear importance of FANCD2 in the repair of ICLs and the maintenance of chromosome stability, its structure, function, and regulation are quite poorly understood. Indeed, to date, only two functional motifs in FANCD2 have been identified and characterized, including a PCNA-interaction motif, or PIP box, and a carboxy-terminus EDGE motif (see Figure 1A).29,33 Thus, the CUE UBD represents the first bona fide domain to be identified in this important protein. Site-directed mutagenesis of residues predicted to play important roles in noncovalent ubiquitin binding revealed several observations about the importance of this domain. First, all missense CUE mutants examined, as well as a CUE deletion mutant, demonstrated reduced protein stability compared with wild type FANCD2 and the monoubiquitination defective K561R mutant. Reduced protein stability is unlikely to be an indirect consequence of protein misfolding as all the CUE mutants tested retained the ability to undergo DNA damage-inducible FANCD2 monoubiquitination and phosphorylation, suggesting that their overall structural conformation remained intact. Inhibition of the proteasome partially rescued the stability of the CUE mutants suggesting that an inability to noncovalently interact with ubiquitin may lead to ubiquitin-mediated proteolytic degradation of FANCD2. Second, mutation of the CUE domain reduced the ability of FANCD2 to interact with FANCI, suggesting that ID heterodimerization is facilitated at least in part by the CUE domain. Third, mutation of the CUE domain led to a dramatic reduction in the ability of both FANCD2 and FANCI to
assemble into discrete nuclear foci and to localize to chromatin, suggesting that ID complex formation is necessary for retention of both proteins in chromatin. Consequently, the CUE mutants failed to correct the MMC-hypersensitivity of FA-D2 patient cells underscoring the overall functional significance of this UBD. Underscoring the importance of this domain, several FA-D2 patient-derived mutations have been mapped to this region.34

The existence of a CUE UBD in FANCD2 gives rise to at least three plausible predictions regarding the behavior of FANCD2; 1) monoubiquitination of FANCD2 causes it to adopt a closed conformation whereby monoubiquitin covalently linked to K561 noncovalently interacts with the CUE domain on the same monomer, 2) monoubiquitination of FANCD2 leads to the formation of a FANCD2 homodimer facilitated through noncovalent interaction between the CUE domain and monoubiquitin covalently linked to K561 on an adjacent monomer, or 3) monoubiquitination of both FANCD2 and FANCI stimulates ID heterodimerization, facilitated in part by noncovalent interaction between the CUE domain and monoubiquitin covalently linked to FANCI K523 (see supplemental Figure 7). We favor the latter prediction for several reasons. For example, previous studies have demonstrated that monoubiquitinated FANCD2 and FANCI colocalize in discrete nuclear foci and physically interact.6,7 Here, we have demonstrated that mutation of the CUE domain reduces the ability of FANCD2 to interact with FANCI, most likely leading to destabilization of the ID complex. A partial disruption of ID complex formation may explain the intermediate ICL sensitivity phenotype observed for FA-D2 cells reconstituted with FANCD2 CUE missense mutants. In addition, as mentioned earlier, the crystal structure of the nonubiquitinated murine ID complex was recently solved.32 The dimensions of the tunnels and entrances of the ID structure are sufficiently large to accommodate ubiquitin and suggest that ubiquitin contacts both its conjugated protein and its heterodimerization partner. Importantly, the
authors note that the nonubiquitinated ID complex has a short half-life, and speculate that monoubiquitination may enhance ID complex stability.\textsuperscript{32} Our results are in agreement with this hypothesis and demonstrate that the FANCD2 CUE domain plays a significant role in this endeavor. Based on this model, one would speculate that FANCI also harbors a UBD and that an interaction with ubiquitin conjugated to FANCD2 K561 might also contribute to ID heterodimerization and stabilization. This could explain why mutation of the CUE domain only partially disrupts the interaction with FANCI; perhaps mutation of both UBDs is necessary to completely abolish ID complex formation. While this is an attractive hypothesis, to date, no UBDs have been uncovered in the FANCI protein.

The importance of interactions between ubiquitin and UBD containing proteins in the cellular DNA damage response has become increasingly evident. For example, the interaction between UBZ domains on Y-family polymerases and monoubiquitinated PCNA is essential for the polymerase switch that facilitates TLS.\textsuperscript{24,35} An intramolecular interaction between monoubiquitinated lysines in the carboxy-terminus of pol $\eta$ and a UBZ domain in its amino-terminus promotes a closed conformation that precludes its interaction with PCNA.\textsuperscript{24} Upon exposure to DNA damaging agents, pol $\eta$ is deubiquitinated leaving the UBZ free to interact with monoubiquitinated PCNA. Recent reports have also demonstrated a direct role for UBDs in the FA-BRCA pathway. The FAN1 and FANCP/SLX4 proteins harbor UBZ domains that are essential for cellular resistance to ICLs. Importantly, recruitment of these nucleases to sites of DNA damage relies on an interaction between their UBZ domains and monoubiquitin covalently linked to FANCD2.\textsuperscript{16-18,36} Furthermore, the newly identified FAAP20 protein has a UBZ4 domain that mediates binding to monoubiquitinated REV1 to promote TLS.\textsuperscript{37-39} Thus, FANCD2 represents the fourth FA pathway protein to harbor a functional UBD. While there are important
similarities between FANCD2 and pol η, there are also intriguing differences in the ubiquitin-dependent regulation of these proteins. For example, DNA damage stimulates FANCD2 monoubiquitination while it results in an inhibition of pol η monoubiquitination.\textsuperscript{4,24} Furthermore, while FANCD2 is monoubiquitinated on a single lysine residue K561, multiple lysine residues of the PCNA-interaction region of pol η can be monoubiquitinated.\textsuperscript{4,24} Indeed, Bienko \textit{et al} speculate that in a situation where monoubiquitination leads to an intramolecular association between a UBD and ubiquitin \textit{in cis}, monoubiquitination may not be restricted to a single lysine but rather may occur on any lysine residue in the region.\textsuperscript{24} Conversely, for homo- or heterodimerization processes facilitated by UBD-ubiquitin associations \textit{in trans}, as is the case for FANCD2 and FANCI, monoubiquitination of specific K residues may be critical. Consistent with this hypothesis, FANCI has been demonstrated to both stimulate FANCD2 monoubiquitination and to restrict FANCD2 monoubiquitination to K561 \textit{in vitro}.\textsuperscript{40}

Taken together our results establish a novel role for noncovalent ubiquitin signaling in the regulation of the FANCD2 protein, and add much needed insight into the structure and function of a poorly understood protein with important implications for bone marrow failure and cancer susceptibility.

\textbf{Acknowledgements}

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Authorship

Contribution: M.A.R. designed experiments, performed research, analyzed data, and wrote the manuscript. F.W.K, E.A.V., and M.M performed research and analyzed data. N.G.H. designed experiments, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


27. Taniguchi T, D'Andrea AD. The Fanconi anemia protein, FANCE, promotes the nuclear accumulation of FANCC. *Blood*. 2002;100(7):2457-2462.


Figure legends

**Figure 1. FANCD2 contains a putative CUE ubiquitin-binding domain.** (A) Schematic of the FANCD2 protein indicating the amino terminus CUE domain, the PCNA-interaction motif (PIP), and the carboxy terminus EDGE motif. (B) A ClustalW alignment of the amino terminus of FANCD2 with several known CUE domains. (C) A ClustalW alignment of the amino terminus of FANCD2 from several species demonstrates high conservation of this region. Asterisks indicate highly conserved amino acid residues known to be important for noncovalent interaction with ubiquitin.

**Figure 2. FANCD2 noncovalently interacts with ubiquitin.** (A) Whole-cell lysates from FA-D2 cells expressing wild type or mutant FANCD2 were incubated with ubiquitin- or protein G-conjugated sepharose and bound proteins resolved and immunoblotted with antibodies against FANCD2. *, Non-specific band. WCL, whole-cell lysate; Ub-seph., ubiquitin-conjugated sepharose; G-seph., protein G-conjugated sepharose. (B) Whole-cell lysates from HeLa cells expressing wild type or mutant FANCD2-V5 were incubated with ubiquitin- or SIM-conjugated sepharose and bound proteins resolved and immunoblotted with antibodies against V5. (C) Bacterially purified fragments of FANCD2 were incubated with ubiquitin-conjugated sepharose and bound proteins resolved and immunoblotted with antibodies against V5. (D) Bacterial whole-cell lysates expressing GST-Empty or GST-tagged wild type FANCD2 solenoid 1 (D2-S1 wt), FANCD2 S1 P204A (D2-S1 P-A), or FANCD2 S1 LL234AA (D2-S1 LL-AA) were incubated with ubiquitin-conjugated sepharose and bound proteins resolved and immunoblotted with antibodies against FANCD2.
**Figure 3. Mutation of the CUE domain impairs FANCD2 protein stability.** (A) Using site-directed mutagenesis, three CUE domain missense mutations were generated. (B) Whole-cell lysates from COS-7 cells expressing wild type or mutant FANCD2 were resolved and immunoblotted with antibodies against FANCD2, V5, and α-tubulin (TUB). (C) Whole-cell lysates from FA-D2 cells reconstituted with wild type or mutant FANCD2 were resolved and immunoblotted with antibodies against FANCD2 and tubulin. (D) COS-7 cells were transfected with wild type or mutant FANCD2. Forty-eight hours later cells were untreated or treated with 10 µM MG132 or 35 µM cyclohexamide (CHX). Pellets were collected at the indicated times and whole-cell lysates generated. Proteins were resolved and immunoblotted with antibodies against V5 and α-tubulin.

**Figure 4. Disruption of the FANCD2 CUE domain does not impair DNA damage-inducible FANCD2 and FANCI post-translational modification.** (A) FA-D2 cells were untreated or treated with 250 nM MMC. 12 h later pellets were collected and lysed and proteins resolved and immunoblotted with antibodies against FANCD2, FANCI, and tubulin. (B) FA-D2 cells were untreated or treated with 20 J/m² UV-C irradiation. Six-hours later pellets were collected, and whole-cell lysates (WCL) were generated. For each sample, an aliquot of the WCL was then treated with λ-phosphatase (λ-PP). Proteins were resolved and immunoblotted with antibodies against FANCD2, FANCI and α-tubulin.

**Figure 5. The CUE domain is required for FANCD2 and FANCI nuclear foci formation and chromatin localization.** (A) FA-D2 cells reconstituted with wild type or mutant FANCD2 were untreated or treated with 250 nM MMC for 12 h and then fixed and immunostained with
antibodies against FANCD2 and FANCI. Nuclear foci were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software. The percentage of nuclei with greater than 5 foci were scored and plotted in the indicated histograms. *** p < 0.001. (B) FA-D2 cells reconstituted with wild type or mutant FANCD2 were untreated or treated with 250 nM MMC for 12 h, released from treatment and pellets collected at the indicated time points. Pellets were divided and half was lysed in 2% SDS lysis buffer, sonicated and boiled (WCE). The remaining pellet was sequentially lysed in cytoskeletal buffer to extract soluble proteins (C) and then 2% SDS lysis buffer with sonication and boiling to extract proteins bound to the chromatin (N). Proteins were resolved and immunoblotted with antibodies against FANCD2, FANCI, α-tubulin, and H2A.

Figure 6. The FANCD2 CUE domain is required for efficient interaction with FANCI. (A) Whole-cell lysates from FA-D2 cells reconstituted with wild type or mutant FANCD2 were immunoprecipitated with antibodies against FANCD2 or mouse IgG. Bound proteins were resolved and immunoblotted with antibodies against FANCD2 and FANCI. (B) Whole-cell lysates from COS-7 cells transfected with wild type or mutant FANCD2-V5 were immunoprecipitated with antibodies against V5 or mouse IgG. Bound proteins were resolved and immunoblotted with antibodies against V5 and FANCI.

Figure 7. The FANCD2 CUE mutants fail to rescue the MMC-hypersensitivity of FA-D2 patient cells. (A) FA-D2 cells reconstituted with wild type or mutant FANCD2 were treated with the indicated concentrations of mitomycin C (MMC) for 7-10 days. Cells were fixed and stained with crystal violet and percent survival calculated and plotted. Each measurement was
performed in triplicate. The averages for three independent experiments were calculated and plotted. (B) FA-D2 cells reconstituted with wild type or mutant FANCD2 were incubated in the absence or presence of 8 or 16 nM MMC for 24 h and the numbers of chromosome aberrations including gaps and breaks, dicentrics, and complex chromosome aberrations, including radial formations, were scored. Metaphase spreads were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software. At least 80 metaphases were scored per treatment. Error bars represent the standard error of the means.
**Figure 1**

A. Diagram showing the CUE, PIP, and EDGE domains of FANCD2 (1451 aa).

B. Sequence alignment of CUE domains from various species:

- *C. elegans YR75*
- *F. rubripes QWNS29*
- *H. sapiens TOLLIP*
- *S. cerevisiae CUE1*
- *S. cerevisiae DON1*
- *C. pombe O14319*
- *S. cerevisiae VPS9*
- *L. friedlin O69961*
- *S. cerevisiae CUE2*
- *H. sapiens FANC D2*

C. Sequence alignment of FANCD2 from various species:

- *H. sapiens* aa 191-198
- *P. troglodytes* aa 153-160
- *C. familiaris* aa 191-198
- *M. musculus* aa 189-196
- *G. gallus* aa 80-87
- *X. laevis* aa 193-198
- *D. melanogaster* aa 190-198

**Legend:**
- **N**
- **C**
- **FANCD2 (1451 aa)**
- **CUE**
- **PIP**
- **EDGE**
Figure 2
**Figure 3**

A. FANCD2 CUE amino acids 201-238

```
SIAKENLQHDIIITSLEILGDSQHADVKELSDDLIEN
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C. FA-D2 α-D2

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D. COS-7

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- CHX
- MG132
- +
Figure 4
Figure 5
Figure 6
Figure 7

A

B

% Survival

Mitomycin C (nM)

0 40 80 120 160 200

Empty vector
D2-V5-wt
D2-P-A
D2-LL-AA
D2-LP-AA

0 nM MMC
8
16

Average chromosome aberrations per metaphase

FA-D2 +
Empty vector
D2-wt
D2-LP-AA
D2-LL-AA
D2-P-A

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Regulation of the Fanconi anemia pathway by a CUE ubiquitin-binding domain in the FANCD2 protein

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