Fanconi anemia (FA) is an autosomal recessive disorder characterized by aplastic anemia, cancer susceptibility, and cellular sensitivity to mitomycin C. Eight of the 11 cloned Fanconi anemia gene products (FANCA, -B, -C, -E, -F, -G, -L, and -M) form a multisubunit nuclear complex (FA core complex) required for monoubiquitination of a downstream FA protein, FANCD2. FANCL, which possesses three WD40 repeats and a plant homeodomain (PHD), is the putative E3 ubiquitin ligase subunit of the FA complex. Here, we demonstrate that the WD40 repeats of FANCL are required for interaction with other subunits of the FA complex. The PHD is dispensable for this interaction, although it is required for FANCD2 mono-ubiquitination. The PHD of FANCL also shares sequence similarity to the canonical RING finger of c-CBL, including a conserved tryptophan required for E2 binding by c-CBL. Mutation of this tryptophan in the FANCL PHD significantly impairs in vivo mono-ubiquitination of FANCD2 and in vitro auto-ubiquitination activity, and partially impairs restoration of mitomycin C resistance. We propose a model in which FANCL, via its WD40 region, binds the FA complex and, via its PHD, recruits an as-yet-unidentified E2 for mono-ubiquitination of FANCD2.

Fanconi anemia (FA) is an autosomal recessive disorder that leads to developmental abnormalities, progressive bone marrow failure, and tumorigenesis (1, 2). The most notable clinical features of FA are hematological, with high incidence of aplastic anaemia, myelodysplastic syndrome, and acute myeloid leukemia (2, 3). Developmental abnormalities are present in many other organ systems, including the skeletal, cardiac, renal, gastrointestinal, and reproductive systems (1, 2). Additionally, FA patients are predisposed to many types of cancer, including leukemias, and solid tumors of the head, neck, or gynecological systems (1).

Cells from FA patients are hypersensitive to DNA cross-linkers such as mitomycin C (MMC), diepoxybutane, or cisplatin (4). In comparison to wild-type cells, treatment of FA cells with genotoxins results in high levels of chromosome breakage, radial chromosomes (particular triradials), and other cytogenetic abnormalities that occur during metaphase (4). To date, 12 complementation groups of FA have been identified (5–14): (A, B, C, D1/BRCA2, D2, E, F, G, I, J, L, and M), and 11 FA genes have been cloned. Eight FA gene products (A, B, C, E, F, G, L, and M) form a core complex required for the mono-ubiquitination of FANCD2 (15).

Ubiquitination has many functional roles. While it was first described for its involvement in proteasome-mediated degradation, it has since been shown to regulate a diverse set of processes, including cellular trafficking and DNA repair (16). Ubiquitination is carried out by a cascade of three enzymes: the E1 activates a ubiquitin through ATP hydrolysis and then transfers the moietly, via a thioester linkage, to an E2 ubiquitin-conjugating (UBC) enzyme. The E3 ubiquitin ligase mediates transfer of the ubiquitin to the substrate and, thereby, confers specificity. Consistent with this role, there are several classes of E3s, each with a distinct mechanism of ubiquitin transfer.

HECT domain E3s bind ubiquitin via a thioester bond and then transfer it directly to the substrate (16). Alternatively, RING domain E3 ligases recruit both the E2 and the substrate, bringing them into close proximity and allowing direct transfer of the ubiquitin from the E2 to an isopeptide linkage with an internal lysine of the substrate. The E3/E2 interaction is mediated by the RING domain, while the E3/substrate interaction is mediated by a distinct substrate-recognition domain that varies with each ligase (17).

Several lines of in vivo evidence strongly suggest that the FA complex is the E3 ligase for FANCD2. Specifically, FANCD2 mono-ubiquitination is (a) lost in patient-derived cell lines lacking any single subunit of the complex (1); and (b) reduced in “wild-type” cell lines (HeLa, U2OS, 293T) following RNA interference knockdown of various members of the FA complex (11–13).

Additionally, FANCL was recently identified as the putative catalytic E3 ubiquitin ligase subunit of the FA core complex (11). It possesses three WD40 repeats and a plant homeodomain (PHD). In general, WD40 repeats mediate protein-protein interactions, while PHD fingers have been ascribed various functions, including phosphoinositide binding (18), chromatin association, and ubiquitin ligase activity (19). There is some debate, however, as to whether PHD E3s are, in fact, better categorized as RING finger variants (20). In vitro assays demonstrated auto-ubiquitination activity of FANCL in the presence of E1, E2, ATP, and ubiquitin, and this activity is lost upon mutation of a conserved zinc-coordinating cysteine that forms part of the PHD consensus sequence (11). This mutant also fails to restore FANCD2 mono-ubiquitination in vivo. It has also been suggested that the FANCL PHD is required for complex stability (11, 21). No study has shown in vitro ubiquitination of FANCD2 by FANCL.

The precise roles of each domain of FANCL are poorly understood. Therefore, this study carries out structure/function analysis of the domains of FANCL. Through mutagenesis, we show that the FA complex is bound and stabilized by the WD40 repeats of FANCL and that the PHD is dispensable for this interaction. We also demonstrate that a tryptophan conserved in PHD and RING-variant E3s is required for full activity of FANCL, both in vivo and in vitro. We propose a model in which FANCL, via its WD40 repeats, binds the FA complex and, via its...
PHD, recruits an as-yet-identified E2 for mono-ubiquitination of FANCD2.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions**—Epstein-Barr virus-transformed lymphoblasts were maintained in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum and grown in a humidified 5% CO₂-containing atmosphere at 37 °C. Wild-type (normal adult) lymphoblast lines (PD7 and GM02254) (22) and the FA lymphoblast line, EUFA868 (FA-L) (11), have been described previously.

**Plasmids**—The retroviral expression vector, pMMP-puro, was described previously (23). FANCL cDNA (11) (generously provided by J. de Winter and H. Joenje, Department of Clinical Genetics and Human Genetics, Free University Medical Center, Amsterdam, The Netherlands).
**FANCL WD40 Repeats Required for FA Core Complex Assembly**

### A

| Mutant | Linker Region | D2-Ub | Stabilization of FA Core Complex |
|--------|---------------|-------|----------------------------------|
| WT     | FLAG WD40-1, WD40-2, WD40-3, PHD | +     | +                                |
| ΔWD40-1| FLAG WD40-2, WD40-3, PHD | -     | -                                |
| ΔWD40-2| FLAG WD40-1, WD40-3, PHD | -     | -                                |
| ΔWD40-3| FLAG WD40-1, WD40-2, WD40-3, PHD | -     | -                                |
| 1-306  | FLAG WD40-1, WD40-2, WD40-3 | -     | +                                |
| 1-275  | FLAG WD40-1, WD40-2, WD40-3 | -     | -                                |

### B

**Vector**

1. FANCA
2. FLAG-FANCL

**ΔWD40-1**

1. FANCA
2. FLAG-FANCL

**ΔWD40-2**

1. FANCA
2. FLAG-FANCL

**ΔWD40-3**

1. FANCA
2. FLAG-FANCL

### C

**Input**

1. ΔWD40-1
2. ΔWD40-2
3. ΔWD40-3
4. 1-306

**IP**

1. ΔWD40-1
2. ΔWD40-2
3. ΔWD40-3
4. 1-306

### D

**MMC:**

1. Vector
2. ΔWD40-1
3. ΔWD40-2
4. ΔWD40-3
5. 1-306

**D2-L**

1. Vector
2. ΔWD40-1
3. ΔWD40-2
4. ΔWD40-3
5. 1-306

**D2-S**

1. Vector
2. ΔWD40-1
3. ΔWD40-2
4. ΔWD40-3
5. 1-306

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FANCL WD40 repeats of FANCL, but not the PHD, are required for interaction with the FA complex. A, schematic of FLAG-FANCL WD40 deletion and truncation mutants with a summary of FANCD2 mono-ubiquitination status, D2-Ub, and stabilization of the FA core complex. Each WD40 repeat, designated WD40-1, -2, and -3, was deleted individually. The region of residues from 275–306, between WD40-3 and the PHD, is designated as the linker region. B, FLAG-FANCL WD40 repeat deletions do not bind FANCA. FLAG-FANCL was immunoprecipitated with anti-FLAG M2 agarose from lysates of retrovirally transduced EUFA868 (FA-L) lymphoblasts. The immunoprecipitation was then analyzed by Western blot for FLAG-FANCL and FANCA. C, the linker, but not the PHD, is required for binding of FANCA and FANCG. FLAG-FANCL was immunoprecipitated and analyzed by Western blot. Asterisks indicate nonspecific bands. D, the WD40 repeats and PHD are required for mono-ubiquitination of FANCD2. Cells were treated with 0.48 μM (160 ng/ml) MMC for 24 h and then lysed for analysis by Western blot. Asterisks indicate nonspecific bands.

Netherlands) was subcloned into the retroviral vector pMMP-puro. pMMP-puro-FLAG-FANCL was generated by adding the FLAG tag (DYKDDDDK) at the amino terminus of FANCL. Production of pMMP retroviral supernatants was performed as previously described (24). FLAG-FANCL point mutants were generated through directed PCR mutagenesis (QuikChange Kit, Stratagene). Truncations and deletions were generated with standard PCR cloning techniques. Glutathione S-transferase (GST) fusions of residues 275–375 of FANCL (GST-PHD) were generated with pGEX-6p-1 vector (Amersham Biosciences).

Retroviral Infection—FA-L lymphoblasts underwent three rounds of infection with pMMP supernatants: each round lasted 24 h in the pres-
ence of 8 μg/ml polybrene (Sigma) and was followed by incubation for 24 h in regular RPMI (15% fetal bovine serum). After the final round, infected cells were washed free of viral supernatant and resuspended in growth media. After 48–72 h, the cells were transferred to media containing 1 μg/ml puromycin. Dead cells were removed over Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) cushion after 5 days, and the surviving cells were grown under continuous selection in puromycin.

**MMC Sensitivity and Chromosomal Breakage Assays**—MMC sensitivity assays for lymphoblasts were performed essentially as described previously (25) but with a CyQuant cell proliferation assay kit (Invitrogen). Chromosome breakage analysis was performed by the Cyto-
genetics Core Facility of the Dana-Farber Cancer Institute as described previously (26).

Antibodies—Antibodies against FANCA (N-terminal) (27), FANCD2 (E35) (28), FANCF (29), and FANCG (30) have been described previously. Anti-FLAG antibody (monoclonal M5 or rabbit polyclonal, Sigma) and anti-tubulin (monoclonal, Abcam) were purchased.

Immunoprecipitation—Whole-cell extracts were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100) supplemented with protease tablets (Roche Applied Science). Each lysate was normalized to contain 2 mg of total protein in 1 ml of lysis buffer, and FLAG-FANCL was immunoprecipitated with 50 μl of packed M2 anti-FLAG agarose (Sigma) for 24 h at 4 °C. The agarose was then washed three to four times with chilled lysis buffer. FLAG-FANCL was eluted with either 150 ng/ml FLAG peptide or with SDS sample buffer (Bio-Rad) containing 5% β-mercaptoethanol.

In Vitro Auto-ubiquitination Assay—We carried out in vitro auto-ubiquitination assays essentially as described previously (11, 31). The
25-μl reaction mixture contained E1 (25 ng, Boston Biochem), UBCH5a (250 ng, Boston Biochem), GST-PHD fusion protein containing residues 275–375 of FANCL (0.6–1.2 μg), His-ubiquitin (20 μM, Sigma), Tris-HCl (50 mM, pH 7.5, Sigma), ATP (2 mM, Sigma), MgCl₂ (5 mM, Sigma), dithiothreitol (0.5 mM, Sigma), and okadaic acid (10 nM, Sigma). The reaction was incubated for 90 min at room temperature and terminated with 2× Laemmli sample buffer (Bio-Rad).

**Immunoblotting**—Cells were lysed with 2× sample buffer (Bio-Rad) containing 5% β-mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE in a 3–8% gradient gel with Tris acetate buffer (Invitrogen) for FANCD2 or a 4–12% gradient gel (Invitrogen) with MES buffer (Invitrogen) for FANCA, -F, -G, and -L. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes using a submerged transfer apparatus (Bio-Rad) filled with Transfer Buffer (Invitrogen). After blocking with 5% nonfat dried milk in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), the membrane was incubated with the primary antibody diluted in TBS-T (1:500 dilution for FANCA, -D2, -F, -G, and -L; 1:1000 for M5; and 1:250 for rabbit anti-FLAG), washed extensively, and incubated with the appropriate horseradish peroxidase-linked secondary antibody (Amersham Biosciences). Chemiluminescence was used for detection.

**Generation of DNA Damage**—For mitomycin C and hydroxyurea (Sigma) treatment, cells were continuously exposed to the drug for the indicated time.

**RESULTS**

**pMMP-FLAG-FANCL Functionally Corrects FA-L Lymphoblasts**—EUFA868 lymphoblasts transcribe FANCL cDNA with an insertion at a splice junction between intron 10 and exon 11, resulting in the removal of the PHD and part of the third WD40 repeat from FANCL (11). Therefore, no wild-type FANCL is detectable in EUFA868 lymphoblasts. We complemented these cells with retroviral pMMP-FLAG-FANCL or pMMP-vector only. To confirm that the cells were functionally corrected, we assayed for FANCD2 mono-ubiquitination and resistance to the DNA cross-linker, MMC. The upper band of FANCD2, representing the mono-ubiquitinated protein, was restored following treatment in TBS-T (1:500 dilution for FANCA, -D2, -F, -G, and -L: 1:1000 for M5; and 1:250 for rabbit anti-FLAG), washed extensively, and incubated with the appropriate horseradish peroxidase-linked secondary antibody (Amersham Biosciences). Chemiluminescence was used for detection.

**FIGURE 5.** Trp-341 is required for in vitro E3 ubiquitin ligase activity of the FANCL PHD. GST was fused to residues 275–375 of FANCL (containing the full PHD), purified from *Escherichia coli*, and incubated with E1 activating enzyme, E2 ubiquitin conjugating enzyme (UBCH5a), His-ubiquitin, and ATP. For negative controls, each component (except ATP) was omitted from the reaction, as indicated. The reactions were analyzed by Coomassie (upper panel) or Western blot against ubiquitin (middle panel) or GST (lower panel). GST-PHD and ubiquitinated species are indicated with arrows.
the FA complex was enhanced in cells treated with MMC. We saw no change in FANCA co-immunoprecipitation following DNA damage (data not shown). Furthermore, FANCL did not co-immunoprecipitate with its putative substrate, FANCD2 (data not shown), which is hyperphosphorylated following MMC-treatment (35).

**Trp-341 of FANCL Is Required for Efficient Mono-ubiquitination of FANCD2**

—Next, we investigated the function of the FANCL PHD. Based on sequence, structural, and functional similarity, it has been postulated that PHD E3 ubiquitin ligases are variants of RING finger E3s (20, 36). To determine whether these two types of E3 domains share sequence similarity, we carried out a sequence alignment comparing the PHD of FANCL, the RING variant (RINGv) domains of several E3 ligases, and the well characterized RING of c-CBL (Fig. 4A), an E3 that ubiquitinates receptor tyrosine kinases.

Sequence alignment revealed conservation of a tryptophan in PHD and RINGv E3 ligases, including FANCL, MEKK1, K3, and MIR1 (Fig. 4A) but not in canonical RING E3 ligases (17). In c-CBL, a tryptophan at this position interacts with UBCH7, an E2 enzyme (17). In FANCL, this tryptophan, Trp-341, is 100% conserved through fruit fly and mosquito, despite degeneracy in flanking sequences (Fig. 4B and Ref. 11).

Based on these alignments, we hypothesized that the PHD of FANCL plays a role similar to that of c-CBL by recruiting an E2 and that Trp-341 is involved in this interaction. To test this possibility, we expressed FLAG-FANCLW341G in EUFA868 lymphoblasts. As a control, we carried out a parallel infection of lymphoblasts with FLAG-FANCLC307A, which previously has been shown to be inactive and should be unable to coordinate a zinc ion, a key structural feature of PHD and RING fingers.

FLAG-FANCLW341G had significantly reduced activity in comparison to the wild-type protein. In the absence of DNA damage, there was a lower level of mono-ubiquitinated FANCD2 compared with cells corrected with wild-type FANCL (Fig. 4C, compare lane 3 with lane 7). This modification could not be further activated with MMC treatment.
FIGURE 7. Model of FANCD2 ubiquitination by FA complex. FANCL interacts with the FA complex via its WD40 repeat region. The PHD is not required for this interaction but is required for mono-ubiquitination of FANCD2, likely through recruitment of an E2 ubiquitin-conjugating enzyme. FANCL does not interact with its substrate, FANCD2, and therefore, the substrate may be recruited by a different subunit of the complex. Based on past studies (41, 43, 49), FANCE is a plausible candidate for FANCD2 recruitment.

(Fig. 4C, compare lane 7 with lane 8). In comparison, FLAG-FANCLW341G did not restore the FANCD2 upper band.

To confirm that this mutation did not lead to gross structural instability that disrupts the association of FANCL with the FA complex, we immunoprecipitated FLAG-FANCLW341G and wild-type FLAG-FANCL. For both proteins, we observed normal binding to FANCA, FANCF, and FANCG, as compared with wild-type protein (Fig. 4D). Furthermore, both proteins stabilized the subunits of the FA complex to a similar extent (Fig. 4D). Tubulin is shown as a loading control for the input and as a negative control for binding. These data confirm that Trp-341 is necessary for full FANCL activity and are consistent with the hypothesis that the PHD plays a role independent of FA complex binding.

Trp-341 Is Required for in Vitro Auto-ubiquitination of the FANCL PHD—To test whether reduced FANCD2 mono-ubiquitination in FA-L lymphoblasts expressing FLAG-FANCLW341G is due to a reduction in E3 ligase activity, we carried out in vitro ubiquitination with GST fusions of the FANCL PHD (GST-PHD). After incubation of GST-PHDWT with E1, E2, His-ubiquitin, and ATP, we observed an increase in higher molecular weight species by Coomassie stain (Fig. 5, top panel, compare lane 1 with lanes 2–5). These bands were immunoreactive with anti-GST antibody (Fig. 5, lower panel) and anti-ubiquitin antibody (Fig. 5, middle panel), indicating auto-ubiquitination of GST-PHDWT.

The negative control, GST-PHD*C307A, was inactive (Fig. 5, lane 6), indicating that auto-ubiquitination is mediated by an intact PHD. GST-PHDW341G was also inactive (Fig. 5, lane 7). This result is consistent with the hypothesis that Trp-341 is required for full E3 ubiquitin ligase activity of FANCL and mono-ubiquitination of FANCD2.

FLAG-FANCLW341G Partially Rescues MMC Sensitivity and Chromosomal Breakage in EULFA868 Lymphoblasts—We tested several mutants for their functional effects downstream of FANCD2 ubiquitination. FLAG-FANCL constructs individually lacking each WD40 repeat displayed levels of MMC sensitivity similar to that of vector controls (Fig. 6A). Lymphoblasts corrected with FLAG-FANCLW341G, however, exhibited an intermediate phenotype. Unlike the other mutants presented in the graph, FLAG-FANCLW341G corrects the MMC sensitivity of the FA-L lymphoblasts at low doses of MMC. At greater doses of MMC, we observed a statistically significant difference between FLAG-FANCLW341G and wild-type protein. At intermediate doses of MMC, FLAG-FANCLW341G results in intermediate sensitivity, and at high doses, the mutant behaved similarly to the vector-only cells. In contrast, cells complemented with FLAG-FANCL1–306 were similar to the vector control, indicating that this partial resistance is dependent on the PHD domain. Similarly, at a low dose of MMC (60 nM), FA-L lymphoblasts corrected with FLAG-FANCLW341G exhibited lower levels of radial chromosomes, while FLAG-FANCL1–306 was comparable with vector alone. These results are consistent with the observation that FLAG-FANCLW341G partially restores FANCD2 mono-ubiquitination.

DISCUSSION

Many WD40 proteins have been shown to mediate protein-protein interactions (32–34). Similarly, our study demonstrates that FANCL binds and stabilizes the FA complex through its three WD40 repeats and linker region. This interaction does not change with DNA damage, suggesting that the complex is constitutively assembled, an observation that is in agreement with previous studies (27, 30, 37).

Of note, β-propeller structures formed by WD40 domains typically contain seven repeats, while FANCL possesses only three. FANCL may contain cryptic repeats between the identifiable WD40 repeats that cannot be detected by current algorithms. There are enough residues between each repeat for such a possibility. Similarly, BUB3p, a protein that mediates the spindle checkpoint, was thought to possess only four WD40 repeats until a crystal structure revealed seven authentic repeats (38). Alternatively, FANCL may oligomerize to complete the full set of repeats.

Surprisingly, the PHD domain is not required for complex assembly, indicating a role distinct from FA complex stabilization. Sequence alignment reveals similarity of E3 PHD and RING variant domains to the canonical RING finger of c-CBL, an E3 ubiquitin ligase. This observation is in agreement with other published studies concluding that PHD domains of E3 ligases are, in fact, RING finger variants (20, 36).

In particular, we observed conservation of a tryptophan that, in c-CBL, was shown in a crystal structure to interact with its E2, UBC7 (17). Mutation of this tryptophan in the c-CBL RING abrogates in vitro E2 binding as well as in vitro and in vivo ubiquitin ligase activity (39). Similarly, mutation of a conserved tryptophan, Trp-341, in the FANCL PHD disrupts its activity both in vitro and in vitro. This mutation does not, however, affect the assembly of the FA complex in FA-L lymphoblasts. Based on these observations, we predict that the FANCL PHD is structurally similar to RING finger E3 ligases and recruits an unidentified E2 UBC.

These results also suggest that the E2 is not required for complex
stabilization. Interestingly, the FA core complex is intact in cells belonging to the FA-I complementation group despite the absence of FANCD2 mono-ubiquitination (40). The FANCI gene, which has not yet been cloned, could, in principle, encode for the E2 UBC of the FA pathway.

Surprisingly, we did not observe an interaction between FANCL and the FA substrate, FANCD2. It is possible that the recruitment of FANCD2 is carried out by another member of the complex. A rough interaction map of the FA complex has emerged through numerous co-immunoprecipitation and yeast two-hybrid studies. FANCA and FANCC interact and each requires the other for stability (37). Other interacting FA pairs include FANCF and FANCG (22, 37), FANCC and FANCC (22, 41), and FANCB and FANCL (12). Yeast two-hybrid studies have also characterized FANCE interactions with both FANCC and the N-terminal of FANCD2 (42). A C-terminal truncation of FANCE interacts with FANCC but not with FANCD2 (42, 43). These data suggest that the C-terminal one-third of FANCE recruits FANCD2 to the FA complex, while the N-terminal two-thirds interact with the FA complex.

Based on the data presented here, we propose a model in which the binding of the substrate and the E2 is mediated by different subunits (Fig. 7). This mechanism is similar to that of multisubunit E3s, such as SCF ligases (44). In this model, FANCL is functionally analogous to the RING-box protein (Rbx) and recruits an E2, while FANCE may be analogous to the F-box protein that binds both the substrate, conferring substrate specificity, and the multisubunit SCF scaffold.

Consistent with this hypothesis, we were unable to ubiquitinate FANCD2 by FANCL alone in vitro (data not shown). It is possible that multiple FA complex subunits, perhaps even the entire complex, will be necessary for FANCD2 ubiquitination in vitro. Similarly, omission of any one of the three core components of SCF 

The structure/function analysis carried out by this study raises new questions. What is the identity of the E2 in the FA pathway? Does this UBC regulate cellular processes other than DNA damage and, if so, what is their relation to FA? Which mechanistic features dictate transfer of mono-ubiquitin, rather than poly-ubiquitin, to FANCD2? Finally, what is the identity of the other substrates of the FA pathway? The answer to these questions may shed further light on the mechanism of the FA pathway in DNA cross-link repair.

Acknowledgments—We thank Hans Joenje for the EUIFA868 (FA-L) lymphoblasts and Weidong Wang for the FANCI cDNA and antibody. We also thank members of the D’Andrea laboratory for helpful discussions.

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