The Fanconi Anemia DNA Repair Pathway: Structural and Functional Insights into a Complex Disorder

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Abstract

Mutations in any of at least sixteen FANC genes (FANCA–Q) cause Fanconi anemia, a disorder characterized by sensitivity to DNA interstrand crosslinking agents. The clinical features of cytopenia, developmental defects, and tumor predisposition are similar in each group, suggesting that the gene products participate in a common pathway. The Fanconi anemia DNA repair pathway consists of an anchor complex that recognizes damage caused by interstrand crosslinks, a multisubunit ubiquitin ligase that monoubiquitinates two substrates, and several downstream repair proteins including nucleases and homologous recombination enzymes. We review progress in the use of structural and biochemical approaches to understanding how each FANC protein functions in this pathway.

Contents

FANCONI ANEMIA

Three brothers who died of a "familial pernicious anemia" in early life were first described by Swiss pediatrician Guido Fanconi in 1927 (26). Today, their inherited disorder, now known as Fanconi anemia (FA), is the subject of intense research in many scientific disciplines. FA is held up as a model of "rare disease" research [the disease affects at minimum 1 in 100,000 individuals (95)], the outcome of which has multiple impacts on diverse areas of medical and scientific endeavor.

FA is a complex and chronic disorder that is often not correctly diagnosed until the onset of bone marrow failure in early adulthood. Hematologic presentations include aplastic anemia, myelodysplastic syndrome, acute myeloid leukemia (AML), single cytopenia, or pancytopenia. Other symptoms include hearing failure, endocrine and gastrointestinal abnormalities, congenital limb deformities, skin hyperpigmentation, and osteopenia; many of these were correctly identified by Guido Fanconi in his seminal description of the disease (3, 26). Almost all of these FA phenotypes, as well as an extremely high risk of developing leukemias and solid tumors, can be attributed to a cellular phenotype of genome instability (13, 29, 59, 93).

Currently, sixteen FANC genes associated with patient mutations (*FANCA–FANCQ*) have been described. The gene products collaborate in a pathway to repair DNA interstrand crosslinks (ICLs) that arise from exposure to chemicals such as cisplatin, diepoxybutane, mitomycin C, and, potentially, aldehydes (57). In cells from FA patients, elevated replicative stress and unrepaired DNA damage lead to levels of p53 and p21 that are higher than they are in cells from unaffected individuals. Specifically, elevated p53 and/or p21 levels are found in the bone marrow and circulating blood of FA patients, promoting a decrease in the hematopoietic stem/progenitor cell pool (13).

Direct evidence for an aldehyde-mediated cause of FA comes from *FANCD2−/− Aldh2−/−* mice, which show developmental defects, bone marrow failure in response to ethanol treatment, and a predisposition to leukemia (59). The increased levels of aplastic anemia in these mice have been linked to increased DNA damage, particularly in the hematopoietic stem and progenitor cell population (29). These studies have produced a mouse model that closely recapitulates FA symptoms and that paves the way for a fuller understanding of the clinical manifestations of FA.

Many excellent reviews cover both the clinical and biological understandings of Fanconi anemia (e.g., 5, 33, 51). In contrast, this review covers our current physical understanding of the 16 FANC gene products, as well as how they fit into a pathway that promotes DNA repair.

The FA pathway is required to repair ICLs as they covalently link the two strands of the DNA double helix, inhibiting replication and transcription. The FANCM-FAAP24-MHF1-MHF2 anchor complex recognizes the ICL. When activated, the anchor complex recruits a core complex that comprises FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FAAP20, and FAAP100. The core complex is required to attach a single ubiquitin molecule (monoubiquitination) to a specific site in each protein of the FANCI/FANCD2 (ID2) heterodimer. This monoubiquitination somehow signals nucleases, such as FANCP (SLX4) and FANCQ (XPF), and downstream repair factors that include FANCJ (BRIP), FANCN (PALB2), FANCD1 (BRCA2), and FANCO (RAD51C). **Figure 1** illustrates the assembly of these protein complexes and a simple model of how they fit into the FA pathway. Subsequent sections discuss structural and biochemical investigations of several components of this pathway on the basis of these groupings.

RECOGNITION OF DNA INTERSTRAND CROSSLINKS BY THE FANCM ANCHOR COMPLEX

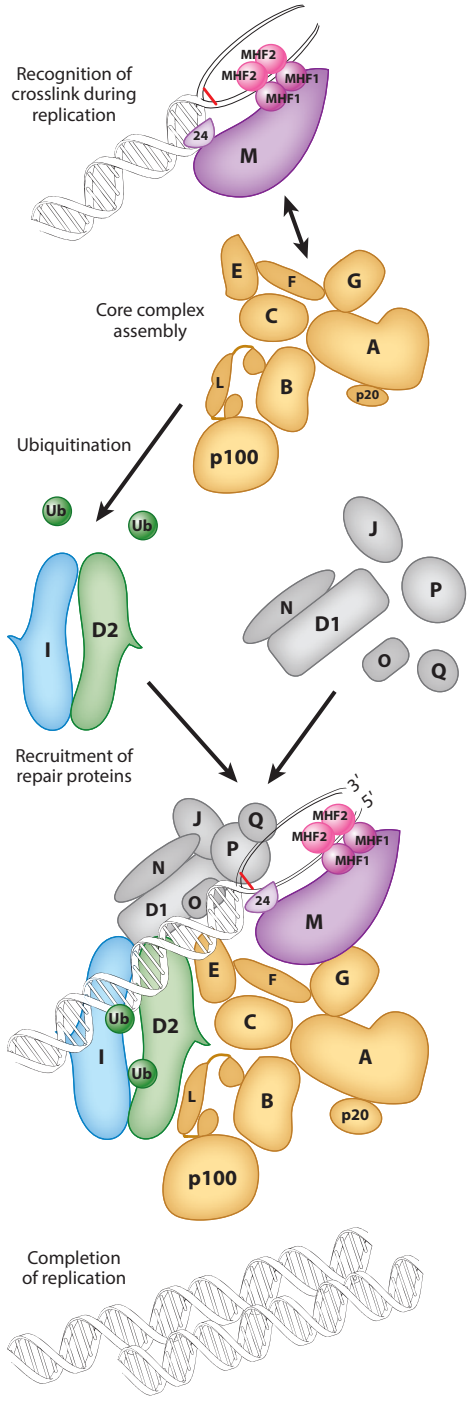
A longstanding problem posed by the nature of DNA ICLs is whether they provide a unique structure in double-stranded DNA (dsDNA) that can be recognized by the FA pathway. Distorting lesions such as those created by platinums and aldehydes can be detected by nucleotide excision repair or mismatch repair factors in all phases of the cell cycle (17, 28, 102), but these methods of repair are often futile because they cannot completely remove a crosslink (24, 77). Such crosslinks can only be repaired in synthesis (S)-phase, coordinated with replication (54). The FA pathway senses the ICL-mediated stabilization of a DNA intermediate in replication—the stalled replication fork.

The key player in this detection is FANCM—a 2,048–amino acid (230-kDa) anchor for activation of the FA pathway in S-phase (80). Electron microscopy with branched DNA molecules localizes recombinant FANCM to an α-structure junction that resembles a stalled replication fork (32), and two junction-specific DNA binding domains exist in FANCM (**Figure 2*a***). The N-terminal DEAH domain with ATPase activity is stimulated specifically by branched oligonucleotides (21) or by large, circular, single-stranded DNA (ssDNA) molecules that are predicted to contain secondary structures (125). Measured by electrophoretic mobility shift assay (EMSA), this domain of FANCM also shows binding specificity for three-way and four-way (Holliday) junction DNA (76, 125). In a fluorescence polarization assay at physiological salt concentrations, however, the specificity is only approximately twofold over that of dsDNA (21).

The FANCM DEAH domain contains the archetypal Walker A and B motifs of an SF2 family translocase: namely, a motor domain required for movement along DNA without the unwinding ability of a helicase. A K117R mutation in the Walker A motif increases cellular sensitivity to crosslinking, whereas a D203A mutation in the Walker B motif does not (94, 125). The mutants could differ in retention of ATP binding, although this potential difference is yet to be explored. Cellular experiments show that the ATPase activity is not required for FANCD2

Figure 1

Schematic of the complexes in the Fanconi anemia pathway. The FANCI/FANCD2 (ID2) complex is depicted in blue/green. The core complex is shown in gold, and the anchor complex shown in pink/purple. Downstream repair factors are in gray. Each complex is thought to exist separately but converge at sites of DNA interstrand crosslinks (*red lines*). Abbreviation: Ub, ubiquitin.



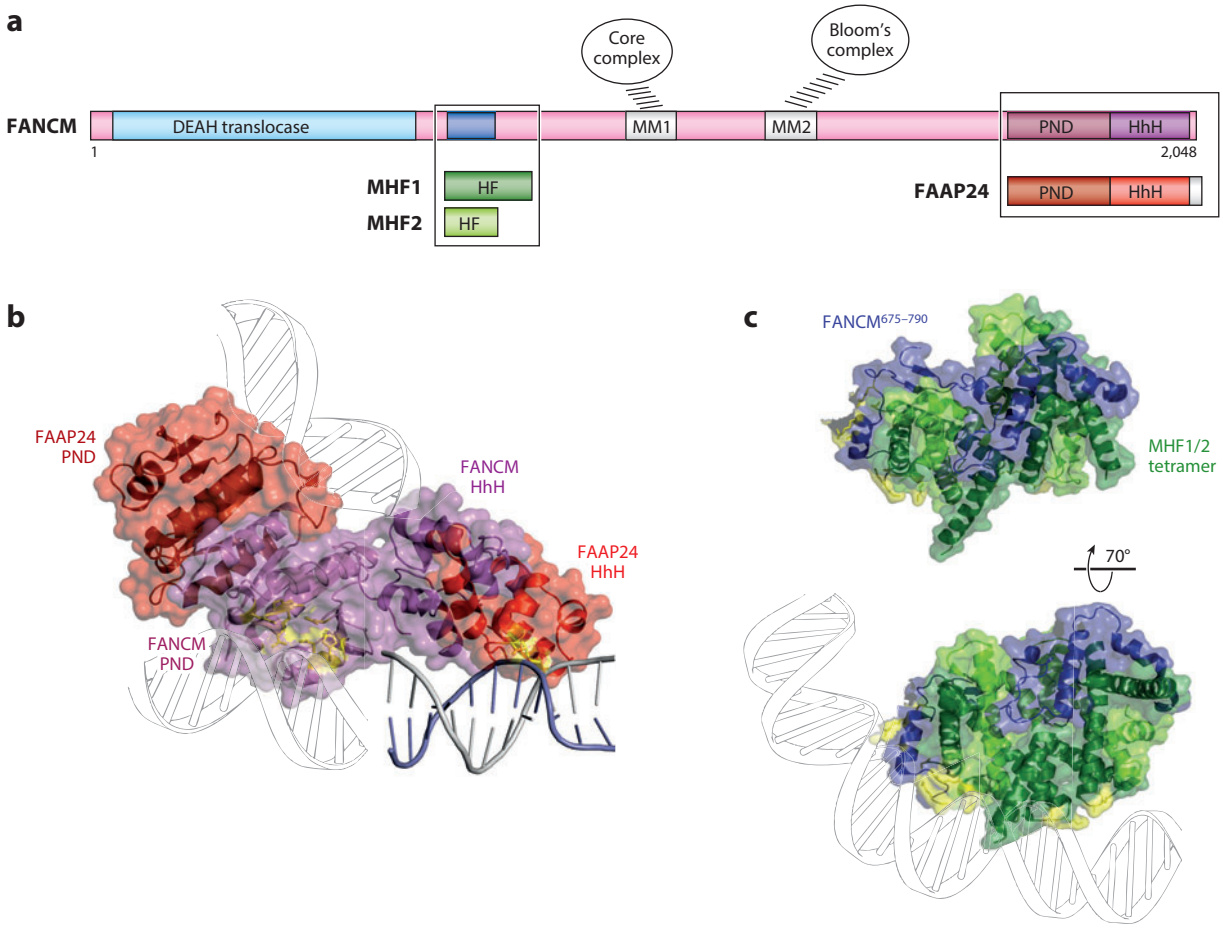


Figure 2

Structural insights into DNA binding by the Fanconi anemia (FA) anchor complex. (*a*) The overall domain architecture of the anchor complex components: FANCM, FAAP24, MHF1, and MHF2. Boxes indicate known protein interaction regions that also coincide with the published crystal structures shown below in panels *b* and *c*. (*b*) FANCM$^{1799–2048}$:FAAP24:11mer DNA structure is represented by surface and ribbons, with domains colored as per labels in panel *a*. (*c*) FANCM$^{675–790}$ (*dark blue*):MHF1 (*dark green*):MHF2 (*lime green*) complex is represented by surface and ribbons. In panels *b* and *c*, residues that experimentally contact DNA are shown in yellow—the DNA helices shown are putative paths. Abbreviations: HF, histone fold; HhH, helix-hairpin-helix; PND, pseudonuclease domain.

monoubiquitination (20, 125). However, after the core complex activates ubiquitination (discussed below), the ATPase activity of FANCM is likely required for a subsequent step in the repair reaction and for the activation of cell-cycle arrest (20, 31). This cell-cycle checkpoint role appears to be independent of the presence of the other FANC proteins and may be related to an enzymatic role of FANCM in protecting stalled replication forks (8, 20).

The C-terminal ERCC4:helix-hairpin-helix (HhH) domain of FANCM (**Figure 2*a***) shows lower affinity for DNA than does the DEAH domain, but EMSA reveals that it has an order of magnitude higher specificity for branched DNA molecules (16). A study using fluorescence polarization found that a heterodimer of FANCM$^{1799–2084}$ and its constitutive partner FAAP24 also shows eightfold higher specificity for branched DNA over dsDNA (21). Recombinant FAAP24 alone binds to crosslinked DNA species from cellular extracts but only weakly to noncrosslinked

DNA (44). This binding may be responsible for a FANCM-independent (and FA pathway–independent) role for FAAP24 in crosslink repair that is not associated with replication (124). It may also merely reflect the presence of ssDNA in the crosslinked substrate, for which FAAP24 also has high affinity (16, 21).

A crystal structure of FANCM$^{1799-2084}$:FAAP24 reveals contact between FAAP24-HhH domains and a cocrystallized 9–base pair (bp) duplex (21). The HhH domains of FANCM are buried within the complex, where they are unlikely to bind DNA. The FANCM-ERCC4 and HhH domains are enveloped by FAAP24, forming a rigid pseudosymmetric interaction reminiscent of the evolutionarily related Mus81-EME1 or the archaeal XPF heterodimer (14, 82). Comparatively, the orientation of the ERCC4 domain packing relative to the HhH domains is increased by 100° compared with that of Mus81 and by 40° compared with that of XPF. This means that the FAAP24-HhH domains directly feed duplex into the pocket equivalent to the archaeal XPF catalytic center (**Figure 2*b***). The catalytic center contains at least one divalent metal binding site even though neither FANCM nor FAAP24 retain the essential residues required for nuclease activity in XPF and Mus81 (25, 75). As nuclease activity has not been detected in purified FANCM (16, 75, 125), it is hypothesized that the metal binding site may have been retained to aid facilitated diffusion of the protein on DNA. In this manner, the ERCC4:HhH domains of FANCM:FAAP24 have been co-opted for a purely targeting function. Potential routes of branched DNA molecules through this channel are proposed in **Figure 2*b***.

Evidence for cooperation between the DEAH and ERCC4 domains of FANCM in targeting branched DNA molecules comes from the three-dimensional electron microscopy reconstruction of full-length FANCM (21). The domains colocalize in space, suggesting that they may bind the same DNA structure. A region of FANCM, the MM1 domain, interacts with FANCF in the core complex (23). As the MM1 domain is between the DEAH and ERCC4 domains, a conformational change may be generated, creating the hypothetical landing pad for the FA core complex (120). Deletion of the MM1 domain does not alter DNA binding activity of FANCM, but it does prevent the recruitment of the FA core complex to facilitate FANCD2 ubiquitination (23). Biochemical studies with HEF, the archaeal homolog of FANCM, also show an interaction between the conserved DEAH and ERCC4 domains (55). It remains to be seen how these two domains cooperate in the activation of the FA pathway and in the downstream processing steps carried out by FANCM.

A Role for MHF1 and MHF2 in the Chromatin Association of FANCM

FANCM localizes constitutively to chromatin through its interaction with the histone-fold containing kinetochore proteins MHF1 and MHF2 (also known as CENP-S and CENP-X or FAAP16 and FAAP10) (101, 127). Knockdown of these proteins in HeLa cells, or deletion in the chicken DT40 cell line, leads to reduced chromatin localization of the FA core complex and to reduced FANCD2 and FANCI monoubiquitination. MHF1 or MHF2 knockdown also results in crosslinker sensitivity (101, 127). These proteins are among the few in the FA pathway that are conserved outside of vertebrates: Homologs with a role in DNA repair have been described in nematodes, plants, and yeast (22, 71, 127).

In human cells, both MHF1 and MHF2 relocalize to the sites of psoralen-induced ICLs in the nucleus (127), as well as to the centromere in undamaged cells (110). Structurally, MHF1 and MHF2 form a head-to-tail heterotetramer similar to the $(H3/H4)_2$ histone heterotetramer. MHF1 forms the tetramer interface, which is composed of hydrophobic and electrostatic interactions and a critical hydrogen-bond network (83). FANCM$_{661-800}$ interacts extensively, through three helices and an irregular coil, in a cocrystal with MHF1/2 (**Figure 2*c***) (110). These elements wrap

around both MHF proteins to create a buried surface with an area of more than 4,000 $Å^2$, greatly increasing the DNA binding affinity of full-length FANCM and stimulating its branch migration activity (110, 127). These changes probably occur because the interface between MHF1/2 and $FANCM_{686–693}$ creates a new DNA binding site (110). Size exclusion chromatography or small angle X-ray scattering of the MHF1:MHF2:$FANCM_{686–693}$ complex also suggests that a higher-order complex containing an $(MHF1:MHF2)_4FANCM_2$ stoichiometry is possible (122).

Other Mechanisms and Facilitators of Targeting to DNA Damage Sites

The FANCM:FAAP24:MHF complex plays a major role in targeting the FA complex to sites of DNA damage. However, in contrast to deletion of other components of the core complex, FANCM, FAAP24, or MHF1 knockout does not result in a 100% loss of downstream ubiquitination activity (6, 16, 127). Several other proteins have been described to play an alternative or stabilizing role in localization of this activity. Two potential facilitators or stimulators of FA core complex localization to DNA damage are FAAP20 and the MSH2:MSH6 heterodimer, MutS. The contributions of these proteins to physiological activation of the FA pathway are unclear, as their importance only becomes obvious in the absence of FANCM and at very high doses of crosslink damage (6).

MONOUBIQUITINATION BY THE FANCONI ANEMIA CORE COMPLEX

Downstream of the anchor complex, the FA core complex is critical for the addition of a single ubiquitin to both FANCD2 and FANCI during S-phase and in response to DNA damage (46). Ligation of ubiquitin, a highly conserved 8.5-kDa protein, is a common signaling mechanism in DNA repair; this process leads to signal amplification and repair protein recruitment (118). Although the nine-protein FA core complex is commonly referred to as a ubiquitin ligase, the FANCL component can monoubiquitinate FANCD2 or FANCI in vitro, in the absence of any other core complex protein (2, 65, 98). As the other components lack any obvious structural or functional domains, and as little is known about the stoichiometry or regulation of the FA core complex proteins, there is much speculation about their biological function (**Figure 3**).

Importantly, the majority of patient mutations associated with FA are found in the components of the core complex. *FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, and *FANCL* are bona fide FA genes with hundreds of unique mutations described in the Fanconi Anemia Mutation Database (**http://www.rockefeller.edu/fanconi/**). FAAP20 and FAAP100 are required for core complex assembly and function, but no disease-associated mutations have been found in these proteins as yet (1, 63). Subcomplexes and subcellular localization of FA proteins have been extensively and recently reviewed (41, 49), so this section focuses instead on the individual proteins and our current molecular and physical understanding of them.

The first core complex protein to be structurally characterized in its entirety was FANCL (18). FANCL is a 375–amino acid (42-kDa) protein that is represented by very few FA patient mutations. It is, however, the minimal unit required for ubiquitination, and invertebrates, which lack an identifiable core complex, still express a FANCL homolog (114). A crystal structure of *Drosophila melanogaster* FANCL revealed that it is a RING-domain-containing E3 ubiquitin ligase, with an N-terminal E2-like fold (ELF) domain and a double RWD (DRWD) domain (18) (**Figure 4*a***). The role of the ELF domain remains unknown, whereas the DRWD domain directly interacts with FANCD2 and FANCI in vitro (18, 40). The RING domain, found in a large number of

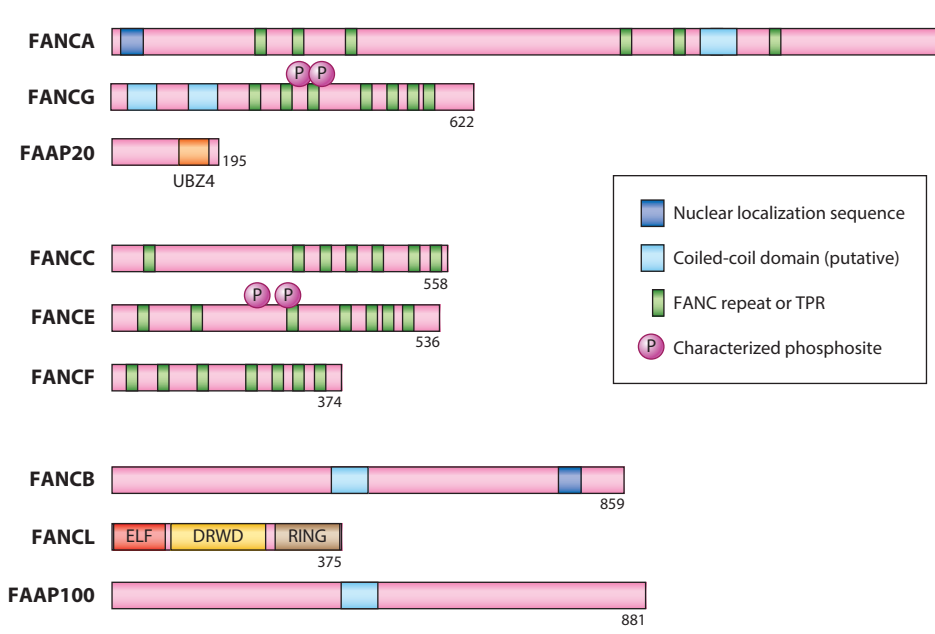


Figure 3

Domain structures of the Fanconi anemia core complex proteins. Proteins are grouped loosely into an order that recognizes potential subcomplexes. Only phosphorylation sites that have been experimentally determined to affect core complex assembly are shown, although others exist in public databases. Abbreviations: DRWD, double RWD motif; ELF, E2-like fold; RING, really interesting new gene; TPR, tetratricopeptide repeat. UBZ4, ubiquitin binding zinc finger 4.

E3 ubiquitin ligases, interacts with UBE2T, an E2 enzyme that is also essential for in vivo ID2 ubiquitination (2, 40, 66). Although the FANCL structure revealed substrate and E2 binding sites, it has been difficult to predict from this structure how FANCL associates with the remainder of the core complex.

The core complex may play a role in the localization of the FANCL catalytic unit to the sites of DNA damage recognized by the anchor complex. As discussed earlier, the FANCF N-terminus brings the core complex into contact with the FANCM-anchor complex at DNA damage sites (23). The 374–amino acid FANCF protein is predicted to be entirely helical. This prediction was verified by the determination of the structure of a C-terminal portion of human FANCF (residues 156–357) (56) (**Figure 4*b***). This crystal structure reveals a hydrophobic surface required for coimmunoprecipitation of FANCA, FANCC, and FANCE, making FANCF a potential bridge between the putative FANCA:FANCG and FANCC:FANCE subcomplexes (56, 61) and the FANCM anchor.

Another feature of the FANCF protein is a series of repeats that resemble the so-called HEAT and ARM repeats of proteins in other large protein complexes. These structural repeats of two α-helices stack via hydrophobic interactions to form a continuous α-α superhelix, also known as a solenoid. The crystal structure of a C-terminal portion of the 536–amino acid (59-kDa) FANCE protein also contains many of these repeated helical hairpins (84), which were named FANC repeats. Seven FANC repeats found in this half of FANCE fold into a continuous, right-handed solenoidal pattern (**Figure 4*c***) that is structurally similar to CAND1, a component of the anaphase promoting complex (APC) ubiquitin ligase. It is highly likely, given the leucine-rich nature of most core complex proteins, that the FANC repeat is a common feature. Indeed, similar repeats are predicted in the 622–amino acid (68-kDa) FANCG protein; these repeats align with the

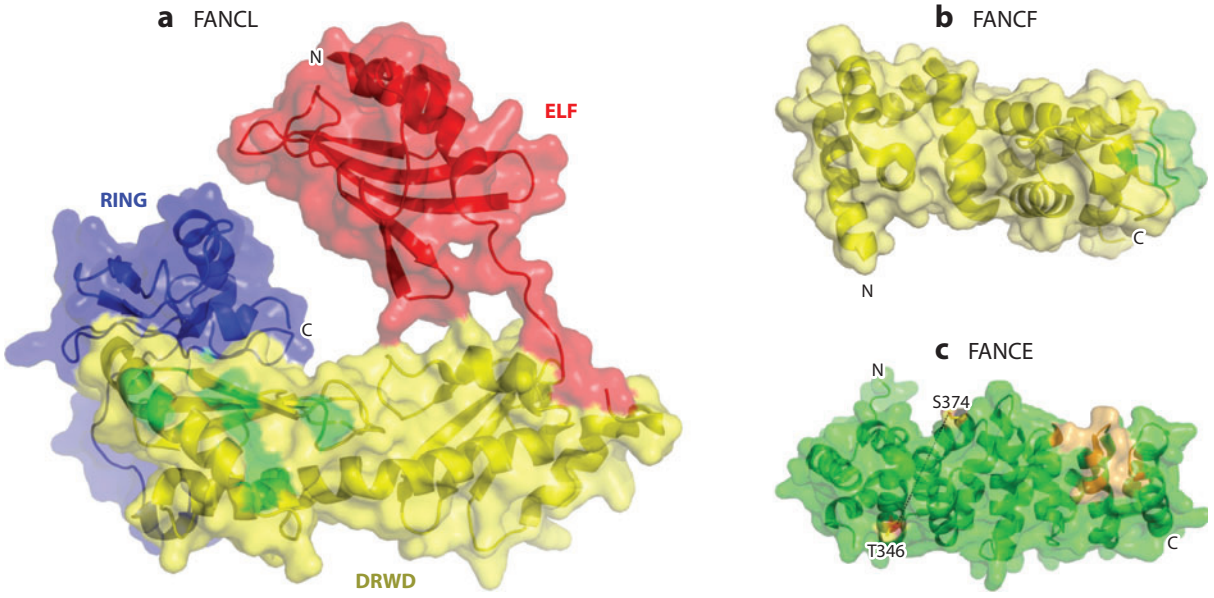


Figure 4

Structural insights into the Fanconi anemia core complex. (*a*) The structure of FANCL is represented by surfaces and ribbons; individual domains are colored and labeled. The patch that binds substrates is shown in green. (*b*) The structure of the C-terminus of FANCF is represented as both surface and ribbons. The hydrophobic surface that mediates FANCA, FANCC, and FANCE immunoprecipitation is shown in green. (*c*) The structure of the C-terminal portion of FANCE is represented as surface and cartoon. The dotted line between the Chk1 phosphorylation sites (S374 and T346) shows the distance between them. The surface patch required for interaction with FANCD2 is shown in orange. Abbreviations: DRWD, double RWD motif; ELF, E2-like fold; RING, really interesting new gene.

tetratricopeptide repeat (TPR) superfamily (9, 45). TPR folds are common to scaffolding and adaptor proteins, suggesting a potential role for FANCG, FANCF, and FANCE as scaffolds in the core complex.

Core Complex Proteins for which There Is No Structural Information

One of the largest gaps in our knowledge is a definitive role for FANCC and FANCA. Together, their gene mutations account for over 75% of cases of FA. FANCC was the first FA gene to be cloned (106). As such, many studies have investigated FANCC function, proposing roles for this protein in apoptosis, redox regulation, and cytokine signaling, in addition to its role in DNA repair (36, 81, 87). No structural analysis has been performed on the 558–amino acid (63-kDa) protein; however, FANCC is predicted to be entirely helical and made up of 15% leucine residues. FANCC is reported to be predominantly cytoplasmic (39), and it forms different complexes depending on its subcellular location (111). This protein also forms a ternary complex involving FANCF, FANCE, and FANCD2 (35, 85).

Because *FANCA* mutations are diverse, including point mutations, insertions, deletions, and truncations, they add little clue about the function of the protein (12). This 1,455–amino acid (163-kDa) protein is also predicted to be almost entirely helical; its only characterized features are a nuclear localization signal (NLS) at the N-terminus (62) (**Figure 3**) and an essential ATR-phosphorylated serine at the extreme C-terminus (position 1,449) (19). Despite the absence of

known DNA binding motifs in FANCA, a recent study reported that purified human FANCA has intrinsic DNA binding activity with a preference for single-stranded DNA and RNA (130). Interestingly, the DNA binding activity appears to reside in the C-terminal half of FANCA, which is where most of the patient mutations reside.

A C-terminal region of FANCA (residues 1,095–1,200) is also required for interaction with FAAP20, a small core complex protein that contains a ubiquitin binding zinc finger 4 (UBZ4) domain (1, 60). The UBZ4 zinc finger, beginning at cysteine-147, binds preferentially to K63-linked polyubiquitin chains at sites of DNA damage (1, 52) and recruits monoubiquitinated REV1 of the REV–DNA polymerase zeta complex, potentially linking the FA and translesion synthesis (TLS) pathways (discussed below) (13). The UBZ4 domain of FAAP20 is necessary for normal FANCD2 monoubiquitination, but it is not involved in binding to ubiquitinated FANCD2 (60). Instead, FAAP20 may be particularly important for the correct localization of monoubiquitinated FANCD2 at DNA damage sites, although the importance of FAAP20 in this context appears to vary depending on which cell type is analyzed (128).

Finally, we know the least about the FANCB and FAAP100 subunits. *FANCB* encodes an 859–amino acid (98-kDa) protein essential for ubiquitination by FANCL in vivo and causes FA in ~2% of patients. Interestingly, *FANCB* mutations are also causative in a more severe disorder—X-linked VACTERL with hydrocephalus syndrome (70). *FANCB* differs from the other complementation groups in that it is subject to X-inactivation and is thus present as a single active copy (74). The protein encoded by this gene is predicted to have both alpha and beta secondary structure, as well as an N-terminal bipartite NLS (74). FANCB has been shown to interact with FANCL and FAAP100 (63, 73). FAAP100, an 881–amino acid (100-kDa) protein, is also predicted to contain alpha and beta secondary structure, but it has not been characterized further (63).

What Do the Structures of Core Complex Proteins Tell Us about Core Complex Function?

Despite a great deal of research effort, we do not yet understand the role of the core complex in higher organisms. Is it simply a scaffold to support FANCL? Do individual components regulate the activity of FANCL or the proximity of FANCD2? For example, the structural analysis of FANCE reveals a patch of surface residues that mediate its interaction with FANCD2 (84, 85) (**Figure 4*c***). These residues are essential for FANCD2 ubiquitination in vivo, but we know from in vitro work that FANCL can also interact directly with FANCD2 to allow ubiquitination (40).

What are the molecular consequences of regulating individual subunits through phosphorylation? For example, FANCE phosphorylation by Chk1 is necessary for in vivo ubiquitination (123), but stuctural analysis suggests that these residues are nowhere near the FANCE:FANCD2 interface (84) (**Figure 4*c***). Do they and other phosphorylations therefore regulate core complex assembly? Are there additional posttranslational modifications that regulate the core complex composition or localization? In addition to these questions, there may be more members of the core complex. Certainly there are cryptic cases of FA that are not assigned to complementation groups, new FA genes are being identified, and some cases of FA go undiagnosed (10, 89). Thus, future research will likely uncover novel roles, substrates, and regulatory aspects of the FA core complex.

FANCD2 AND FANCI: THE TARGETS OF MONOUBIQUITINATION IN THE FANCONI ANEMIA PATHWAY

As a result of a major breakthrough in determining the structures of the FANCD2 and FANCI proteins by the Pavletich laboratory, we know much more about the substrates of the

monoubiquitination reaction than we do about the core complex (48). Human FANCD2 is a 1,451–amino acid (164-kDa) protein that is conserved across species ranging from slime mold to humans (72). Mammalian homologs share ~75% sequence identity, and invertebrates share ~20% identity. The crystal structure of mouse Fancd2 reveals an elongated helical structure composed of multiple antiparallel pairs of helices arranged into α-solenoids (48). The large deletions and truncations that constitute most of the FANCD2 patient mutations would clearly lead to poorly folded proteins, but, interestingly, pathogenic point mutations also arise in key parts of the hydrophobic core, resulting in destabilization (48) (**Figure 5*a***). In addition, several of the pathogenic mutations line the path of a channel predicted to mediate DNA binding (48).

The 1,323–amino acid (149-kDa) FANCI protein is the second substrate for monoubiquitination by the FANCL-containing core complex. It appears that FANCD2 and FANCI proteins have coevolved; all organisms with a FANCD2 homolog also contain FANCI (72, 104). The crystal structure of mouse Fanci (81% identical to human FANCI) reveals a solenoidal structure that is surprisingly similar to that of FANCD2 despite having only 14% overall identity. When refined to 3.1 Å, the structure shows that patient mutations map primarily to regions involved in maintaining the stability of the overall solenoid structures, with the exception of R1285Q, which appears to be on the surface of Fanci (**Figure 5*b***).

FANCI and FANCD2 heterodimerize to form the ID2 complex. A crystal structure of the mouse ID2 complex reveals an extensive interface between the two proteins that runs the length of both (48). In vitro, FANCL can interact directly with isolated FANCI or FANCD2 (18, 40), and monoubiquitination occurrs at K561 or K523, respectively (85, 104). Intriguingly, the sites of monoubiquitination in each protein are embedded within the heterodimer interface, prompting several questions regarding the mechanism of ubiquitination. How does the UBE2T-FANCL (E2-E3) pair ubiquitinate each protein (**Figure 5*c***)? Does this ubiquitination occur in the complex? Or is dissociation required prior to ubiquitination, and, if so, how is this achieved? What signals prompt the formation or dissociation of the complex? A number of studies are beginning to shed light on these questions.

Functional Implications of ID2 Complex Monoubiquitination

It has been known since 2001 that monoubiquitination of FANCD2 is crucial for ICL repair (113). A point mutation that converts the key lysine residue (K561) into a nonubiquitinatable arginine residue results in increased sensitivity of cells to ionizing radiation and ICLs in human cells and in chicken DT40 cells (30, 69). Indeed, the monoubiquitin signal is so important that fusion of ubiquitin to the C-terminus of FANCD2 can partially rescue function in FANCD2-deficient cells (69). In contrast, ubiquitination of FANCI seems to be largely dispensable for efficient ICL repair in DT40 cells (46) and of less importance than FANCD2 ubiquitination in human cells (104).

In vitro ubiquitination reactions with FANCL and UBE2T show enhanced activity when FANCI is included in the reaction. Furthermore, FANCI promotes the restriction of monoubiquitination to K561 in FANCD2 (2). However, under these experimental conditions, the level of FANCD2 ubiquitination was still very low, making it challenging to probe the mechanistic details of the reaction. In 2012, several key findings from Kurumizaka's group (98) showed that the in vitro monoubiquitination of FANCD2 could be dramatically enhanced. First, FANCD2 ubiquitination is increased in the presence of FANCI, suggesting that ubiquitination occurs in the context of the heterodimer. Second, the heterodimer binds DNA more tightly than the constituent subunits do. Finally, the addition of splayed DNA structures (which contain dsDNA:ssDNA junctions) to the assay greatly enhances FANCD2 ubiquitination to levels similar to those found in cells. This enhancement is absolutely dependent on the presence of FANCI. These findings together suggest

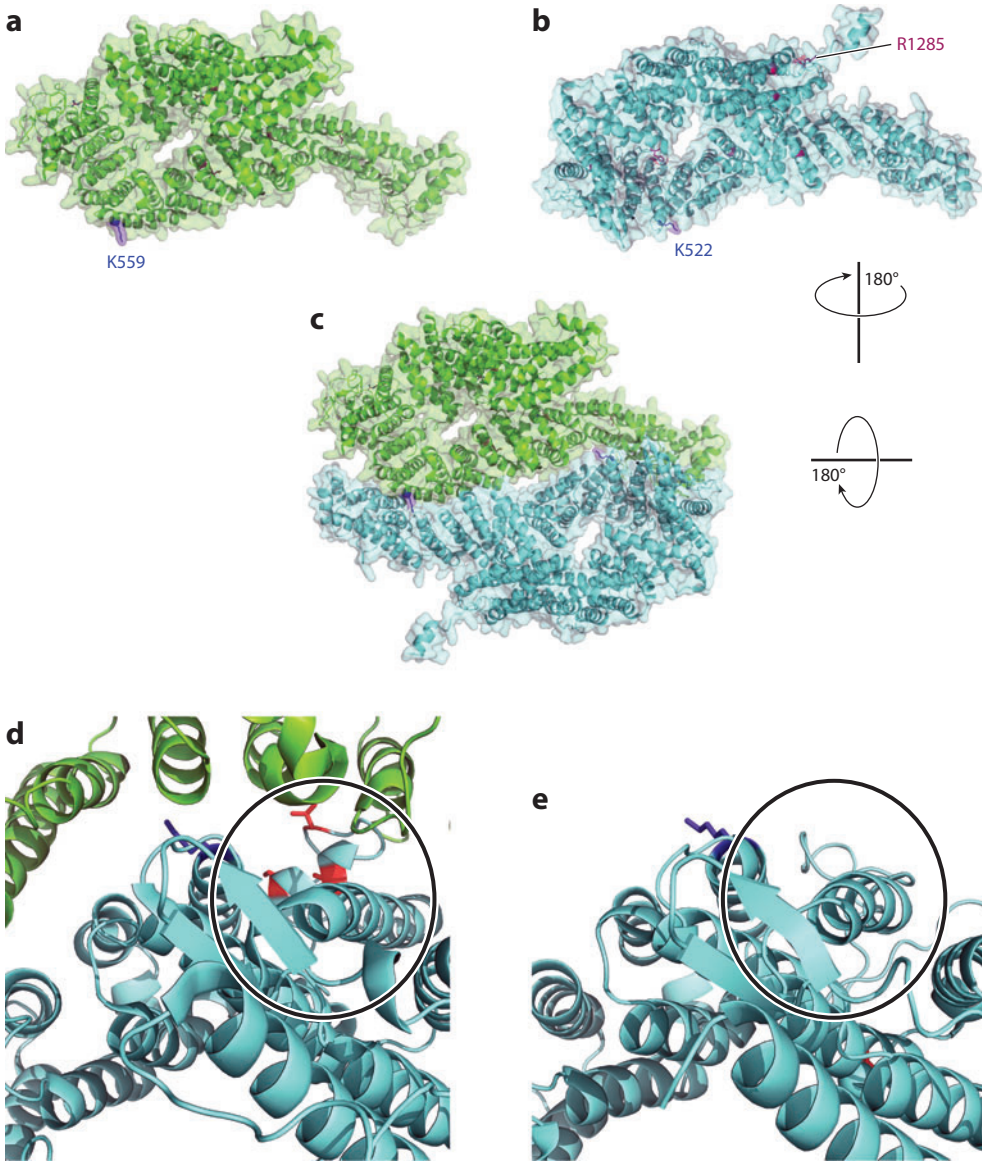


Figure 5

The mouse Fanci-Fancd2 complex. (*a*) The structure of mouse Fancd2 is represented as both surface and ribbons, showing a solenoid helical structure. Sites of patient mutations are indicated in brown, and the ubiquitination site is in dark blue. (*b*) The structure of mouse Fanci is represented as surface and ribbons, revealing a similar architecture to Fancd2. Patient mutations are shown in magenta (site R1285 is highlighted), and the monoubiquitination site K522 is in blue. (*c*) The Fanci/Fancd2 (ID2) heterodimer. Fancd2 is in green, in the same orientation as in panel *a*. Fanci is in cyan, rotated 180° about both the *x*- and *y*-axes. The monoubiquitination sites (*dark blue*) are located at the interface. (*d*) A close-up view of the phosphorylation sites in FANCI as part of the ID2 heterodimer (*red*). K522 of Fanci is shown in dark blue. (*e*) The same region of Fanci depicted in panel *d*, showing the changes in secondary structure content when Fanci is isolated. The phosphorylation sites are no longer located at what would be the interface; instead, they are buried in secondary structure.

that the true substrate for the FA pathway is the ID2 complex bound to DNA. Thus, DNA binding by the complex is hypothesized to induce some local conformational change that allows greater access to the acceptor lysines in the heterodimer interface.

Interestingly, the R1285Q FA-associated mutation in FANCI is unable to heterodimerize with FANCD2 (129). The equivalent chicken FANCI mutation (R1288Q) supports DNA binding to the same extent as wild-type FANCI, but it fails to support DNA-stimulated ubiquitination of FANCD2 (98). The location of R1285 in the crystal itself is packed against symmetry-related mates, suggesting that this residue plays a role in stabilizing FANCI structure, potentially anchoring the remaining amino acids in the FANCD2 interface (48).

A model using *Xenopus laevis* egg extracts shows that throughout S-phase, as much as 80% of FANCI and FANCD2 exists as a heterodimer (97). In vitro, the ID2 complex affinity is sufficient for it to stay intact during crystallization, coimmunoprecipiation, and native gel electrophoresis but not during gel filtration (48, 97). It will be useful to physically measure the dissociation constant between the proteins in different states in order to better understand the dynamics of the complex. Some work has, however, been undertaken to show that neither the ATR kinase nor the FA core complex is required for heterodimer stability (97). Another unresolved issue is whether ubiquitination increases or decreases the affinity of the heterodimer.

One recently proposed scenario suggests that ubiquitination disrupts the heterodimer (97). A study by the Sobeck lab (97) showed that immunodepletion of FANCI leads to codepletion of unmodified, but not monoubiquitinated, FANCD2. The free FANCD2-ubiquitin may then be exposed to recruit the potential downstream factors in the pathway (discussed below). This model also allows FANCI to regulate the level of FANCD2 ubiquitination such that the conformation of FANCD2 might be restricted to a particular DNA-bound conformation. High-resolution structures of either the individual proteins or the heterodimer bound to DNA are needed to establish which structural rearrangements occur upon DNA binding and how these rearrangements influence K561 monoubiquitination. A structural understanding of a ubiquitinated ID2 complex will also be informative.

Phosphorylation of FANCI and FANCD2

There are currently nine experimentally determined phosphorylation sites in FANCD2, several of which are validated targets of the ATM or ATR kinases (68, 79, 109). However, most of these sites are not conserved between species. The exceptions are S222, which is required for the intra-S-phase checkpoint (109), and S1418. FANCI also contains a number of conserved SQ and TQ sites, which may be recognition sites for the ATM or ATR kinases. S556 and S559 are evolutionarily conserved and have been experimentally determined to be kinase substrates (68, 104). Four additional sites in chicken FANCI, T567, S598, S619, and S631 (S565, S596, S617, and S629, respectively, in human FANCI) have been shown to activate the FA pathway (46). All six sites cluster at or near the FANCD2 interface (48) and are thus potential mediators of the heterodimerization (**Figure 5*d***). Expression of FANCI^{6D} (a phosphomimic for which each serine is mutated to an aspartate) in cells increases basal FANCD2 ubiquitination and decreases damage-induced FANCD2 ubiquitination. But in vitro, FANCI^{6D} appears to have wild-type binding affinity for FANCD2 (46), as well as wild-type levels of DNA binding (98). The only in vitro discriminator characterized to date is a reduced level of DNA-stimulated FANCD2 ubiquitination by FANCI^{6D} compared with FANCI (98).

Looking at the structure of the ID2 complex, FANCI S556 and S559 are in a helical arrangement at the interface of FANCD2 binding. In the structure of free FANCI, they are disordered, and there is substantial local conformational change (48) (**Figure 5*d*,*e***), but both sites would be

readily accessible for phosphorylation. Although the combined results of these studies suggest that FANCI phosphorylation may stabilize the ID2 complex and support its DNA binding and subsequent D2-ubiquitination, it is important to note that FANCD2 binding by phosphorylated FANCI has not been directly tested. As such, experiments in cellular extracts provide some counterevidence to the hypothesis. Sareen et al. (97) found that FANCI^{6D} is unable to coimmunoprecipitate FANCD2 after DNA damage and propose that phosphorylation of FANCI promotes disassembly of the ID2 complex. It is challenging to visualize a model of the order of events that accounts for all of the currently available data. A major point of contention in the field thus appears to be whether ubiquitination of either or both components occurs in the context of the heterodimer. It will be important to understand ID2 complex association or dissociation, which can be accomplished through measurement of the complex formation and mutation of key interface residues. It will also be interesting to determine whether experimentally phosphorylated FANCI, as opposed to an artificial phosphomimic, stabilizes or destabilizes the complex. Such experiments may uncover why phosphorylation of FANCI is required to activate the FA pathway in cells and in cell lysates.

RECOGNITION OF THE MONOUBIQUITIN SIGNAL AND INTERSTRAND CROSSLINK UNHOOKING

The 10 *FANC* genes upstream of and including their ubiquitination substrates account for over 95% of all cases of Fanconi anemia. The absence of a single monoubiquitination reaction associated with severe clinical symptoms in these patients highlights the important nature of this signal in repairing ICL damage. Conversely, the relatively low number of mutations in the remaining *FANC* subgroups hints that these genes may be even more essential, as evolution is less tolerant of deviant alleles. It is currently accepted that the products of these genes act downstream of the ID2 complex.

At a cellular level, the ID2 complex and monoubiquitination of FANCD2 are required for unhooking of the ICL, as well as for bypassing the lesion via translesion DNA synthesis (54, 91). The monoubiquitin signal on FANCD2 has long been assumed to act as a platform for some unknown DNA repair factor(s). Identification of such candidates, all of which contain ubiquitin binding domains, has been a recent breakthrough. In 2010, four groups (58, 64, 67, 103) reported the identification of FAN1 (Fanconi anemia associated nuclease 1), which contains a UBZ domain. This domain is required for FAN1 localization to sites of DNA damage and for coimmunoprecipitation with the ID2 complex. Patients with homozygous loss of FAN1 expression have been identified. Surprisingly, however, these patients are not particularly sensitive to ICL damage, nor do they display features of FA (116). In contrast, dual knockdown of FANCA and/or FAN1 in human tumor cells causes a similar sensitivity to ICLs, suggesting the two genes act in the same pathway, at least with respect to DNA damage sensitivity (67). In support of this hypothesis, the FA pathway is essential for FAN1 activity, and cells expressing a ubiquitination-incompetent form of FANCD2 (K561R) fail to localize FAN1 to chromatin foci (58, 64, 67, 103). The physical details of this interaction and the regulation of FAN1 recruitment and potentially other DNA repair factors are a rich area for future study.

Two additional nucleases that cause FA when mutated are also in the FA pathway. FANCP (SLX4) and FANCQ (XPF) are flap endonucleases that are conserved further back in evolution than the rest of the FA pathway. These two nucleases are part of a complex that also contains their respective heterodimeric partners, SLX1 and ERCC1 (107). Key details about how nuclease activity is coordinated at the sites of DNA damage are slowly being uncovered (for an excellent recent review, the reader is referred to Reference 15), but, interestingly, the XPF protein is also required during nucleotide excision repair (NER). However, the so-called FANCQ mutations

in XPF do not alter NER function; they only interfere with the repair of ICLs (10). As such, FANCQ (XPF) could be thought of as a dual-purpose nuclease, which cuts DNA during the repair of crosslinks or other damage. The crystal structures of the C-termini of FANCQ and ERCC1 are similar to that of FANCM-FAAP24, a dual dimerization interface between a central domain and tandem HhH domains that bind DNA (117). The HhH domains of ERCC1 mediate ssDNA binding (115), whereas the nuclease activity is found within the FANCQ subunit (7). More detailed structural investigation using a DNA-bound heterodimer may be required to resolve the controversy surrounding the role of FANCQ in cutting DNA adjacent to ICLs.

No structural investigation of FANCP has been performed, but the protein contains several highly conserved domains (4, 27, 53, 78, 107). Similar to FAAP20, FANCP possesses a UBZ4 domain that may interact with ubiquitinated FANCD2 (126). The protein also contains an MEI9 interaction-like region (MLR), a SAP domain, and a SLX1-binding domain (SBD) that are all essential for the repair of ICLs (53). SLXΔMLR is particularly sensitive to ICLs, and this domain mediates direct protein binding of the FANCQ-ERCC1 heterodimer (53). As such, it is assumed that a combination of nuclease activities, including those of FAN1, FANCP, and FANCQ, as well as MUS81 and possibly even FANCD2 itself, is utilized in ICL repair (24, 53, 86).

LINKS BETWEEN THE FANCONI ANEMIA PATHWAY, UNHOOKING, AND TRANSLESION SYNTHESIS

Unhooking by the FA pathway is linked with the initiation of TLS, an error-prone DNA repair process required to bypass a stalled replication intermediate. TLS is achieved by replacing the replicative DNA polymerases with dedicated translesion polymerases that can accommodate DNA lesions in a larger active site (50, 96). FANCD2 monoubiquitination is linked directly to the monoubiquitination of the master regulator of TLS, proliferating cell nuclear antigen (PCNA), at Lys164 by RAD18 (42, 50). FANCL and RAD18 are epistatic with respect to ICL sensitivity (88). At the protein level, FANCL binds PCNA via the DRWD domain (34) and promotes the monoubiquitination of FANCD2.

A second link with translesion synthesis occurs downstream of PCNA monoubiquitination, through direct cross talk with Y-family DNA polymerases. FANCC and Y-family polymerases DNA polymerase zeta and REV1 are epistatic with respect to ICL sensitivity and repair (37, 81). FAAP20 also links the core complex with REV1–Pol z (52). The molecular mechanisms underlying the cross talk between FA and TLS in ICL repair have been reviewed recently (38) and are an exciting area for future study.

DOWNSTREAM REPAIR FACTORS—LINKS TO HOMOLOGOUS RECOMBINATION

FANCD1 (BRCA2), FANCJ (BRIP1), FANCN (PALB2), and FANCO (RAD51C) are referred to as downstream components in the FA pathway, as they act in a process that is activated by the monoubiquitination step. Compared with upstream genes, homozygous mutations in downstream genes are more rare, and the resulting phenotypes in patients are more severe, particularly with respect to the onset of cancer (43). The downstream genes also share an additional association with elevated breast and ovarian cancer risk when a single mutant allele is inherited. For this reason, the FA pathway is often referred to as the FA/BRCA pathway (121).

The components downstream of FANCD2 ubiquitination likely coordinate homologous recombination after the nuclease-unhooking step. Important players in this process include FANCJ [a helicase (99)] and FANCO [a RAD51-paralog recombinase (105)]. Mutation of another RAD51

paralog, XRCC2, has also been described in an FA patient in Saudi Arabia (100). The most important link between FA and homologous recombination came with the identification of BRCA2 mutations in the FANCD1 subgroup. BRCA2 is a large (3,418–amino acid) protein that binds to ssDNA and recruits the recombinase Rad51 to double-strand breaks (47). The functions of BRCA2 are stimulated by FANCN, which is also called partner and localizer of BRCA2 (PALB2) (90, 92, 108). FANCN, FANCJ, and FANCO (RAD51C) interact with BRCA1, the most commonly mutated breast-cancer predisposition gene. Strikingly, in BRCA1-deficient breast tumor cell lines, FANCD2 is ubiquitinated normally, but it does not form characteristic nuclear foci (119), suggesting that BRCA1 and the other downstream components may also provide feedback to stabilize the localization of ubiquitinated FANCD2 at DNA damage sites.

There is a large gap in our knowledge of this final step in the pathway. This is especially true with respect to its biochemical mechanism and the biophysical characterization of these proteins, owing to their large size and complex interaction network. But interesting results with electron microscopy analysis of the very large PALB2, BRCA2, and FANCM proteins suggests that plenty of insight can be gained in the near future (11, 21, 112).

FUTURE CHALLENGES

Much has been learned about FA since it was first described 87 years ago, including many important clinical steps that have improved quality of life among FA patients. Nonetheless, Fanconi anemia has many devastating symptoms and places a great psychological toll on afflicted individuals and their families. The *FANC* genes are also involved in cancer prevention and treatment in non-FA patients. In less than two decades of discovery, genetic and cell-based assays have revealed the basics of how 16 *FANC* genes fit into a DNA repair pathway. However, the only FANC proteins in the pathway that have been observed in their entirety at the molecular level are FANCL, FANCD2, and FANCI. Other partial structures give a glimpse of the mechanisms by which the remaining FANC proteins regulate ubiquitination and downstream repair. However, further biochemical and biophysical investigation of the FA pathway will certainly reveal its hidden links, points of weakness, and necessity. Such work will pave the way for approaches to molecular targeting of this pathway in the treatment of FA and cancer in general.

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RELATED RESOURCES:

Fanconi Anemia Antibody Project (**http://www.ohsu.edu/research/fanconi-anemia/index.cfm**): antibodies against FANC proteins, provided to the FA research community.

Fanconi Anemia Mutation Database (**http://www.rockefeller.edu/fanconi/**): resource providing known FA-associated patient mutations.

Fanconi Anemia Research Fund (**http://www.fanconi.org**): FA-related information for patients, clinicians, and researchers.

Genome Stability lab Twitter account (**http://twitter.com/GenomeStability**): updates on FA-related research.