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Ubiquitylation and the Fanconi Anemia Pathway

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Abstract

The Fanconi anemia (FA) pathway maintains genome stability through co-ordination of DNA repair of interstrand crosslinks (ICLs). Disruption of the FA pathway yields hypersensitivity to interstrand crosslinking agents, bone marrow failure and cancer predisposition. Early steps in DNA damage dependent activation of the pathway are governed by monoubiquitylation of FANCD2 and FANCI by the intrinsic FA E3 ubiquitin ligase, FANCL. Downstream FA pathway components and associated factors such as FAN1 and SLX4 exhibit ubiquitin-binding motifs that are important for their DNA repair function, underscoring the importance of ubiquitylation in FA pathway mediated repair. Importantly, ubiquitylation provides the foundations for cross-talk between repair pathways, which in concert with the FA pathway, resolve interstrand crosslink damage and maintain genomic stability.

Keywords

Monoubiquitylation; FANCL; FANCD2; FANCI; SLX4; FAN1

First described as a signal for protein degradation [1] modification by ubiquitin functions extensively as a regulatory signal to control diverse biological pathways. As seen by the reviews in this issue, DNA damage is a fertile ground for identification of ubiquitin function. Here we concentrate on Fanconi anemia (FA), a rare but very instructive disease for ubiquitin signaling. FA patients show defects in DNA interstrand crosslink repair and have biallelic mutations in one of fourteen genes that code among others, for a ubiquitin ligase (FANCL), proteins modified by monoubiquitylation (FANCD2 and FANCI), and a protein with ubiquitin-binding domains (FANCP/SLX4). The functional implications of ubiquitylation and de-ubiquitylation within the FA pathway and the regulation of interstrand crosslink repair will be the focus of this review.

The Fanconi Anemia Pathway

Fanconi anemia (FA) is a pathologically diverse, recessively inherited disease that typically culminates in bone marrow failure in affected individuals. Patients also display profound genome instability that correlates with cancer predisposition and may have developmental defects [2]. On a molecular level, the FA pathway represents a facet of the cellular DNA repair strategy employed to resolve DNA damage, particularly interstrand crosslinks (ICLs), which covalently link the Watson and Crick strands of the DNA. ICLs particularly affect processes that inherently require DNA unwinding and strand separation such as DNA

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replication and transcription [3] (Figure 1A). Consequently, ICLs are particularly deleterious if unresolved. Besides being activated by exogenous damage inflicted by DNA crosslinking agents such as diepoxybutane (DEB), mitomycin C (MMC) or cisplatin, FA proteins are activated during normal S phase suggesting that they might participate in repair of replication errors [4, 5].

Identification of mutated genes in FA patients has paved the way for the identification of a diverse set of proteins necessary for crosslink repair. A ‘genuine FANC protein’ must be rooted in the human disorder. This is usually a consequence of mutation leading to loss of gene function. Currently biallelic mutations in any one of the 14 genes is responsible for FA with the 15th gene (RAD51C) being mutated in an FA-like syndrome [6] (Figure 1). The known patients with RAD51C mutations have not yet presented signs of bone marrow failure and as such RAD51C has not yet been assigned an official FANC name. A FANCO designation has been set aside if more evidence is gathered to firmly establish RAD51C as an FA gene. Eight proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM) form a complex commonly referred to as the core complex which monoubiquitylates both components of the ID complex, FANCD2 and FANCI [4, 7-9] (Figure 1B). Although FANCM has been first reported as an essential component of the core complex [10], it is now clear that FANCM is important for resistance to interstrand crosslinks but not at the step of monoubiquitylation of the ID complex [11]. Furthermore, unlike the other components of the core complex, FANCM has FA pathway independent function including ATR-mediated checkpoint signaling [12]. Monoubiquitylated ID complex interacts with FAN1 (Fanconi anemia Associated Nuclease 1), which *in vitro* has both endo- and exo-nuclease activity [13-16]. There are no known FA patients with mutations in FAN1. The downstream proteins in the pathway include FANCD1/BRCA2 and the BRCA2 interacting partner, FANCN/PALB2 [17-19], both of which are essential components of homologous recombination repair, and a helicase FANCJ /BRIP1/BACH1 [20-23]. FANCJ interacts with BRCA1 but this interaction is apparently dispensable in the context of FA pathway [24]. Depletion of BRCA1 results in exquisite sensitivity of cells to crosslink damage but no individuals with biallelic BRCA1 mutations have been found to date. The newest addition to the Fanconi anemia protein family is FANCP/SLX4 [25-27]. SLX4 acts as a scaffold that interacts with multiple proteins including nucleases XPF, MUS81 and SLX1 [28-30]. XPF and MUS81 have been previously implicated in crosslink repair [31, 32] and the SLX4-SLX1 interaction is responsible for Holliday junction resolution activity *in vitro*. It is unclear at this point which activity of SLX4 is essential for crosslink repair. Besides the genuine FANC proteins discussed above, numerous proteins have been demonstrated to be associated with FA pathway components and as such need to be studied in parallel. Technological advances including RNAi continue to facilitate the identification of proteins whose absence yields a genome instability phenotype analogous to that of FA and the next generation sequencing approaches should soon identify the remaining FANC genes in FA patients with thus far unidentified genetic mutations.

Besides the major pathway of replication-dependent ICL repair, vertebrate cells also have a replication-independent repair pathway. This minor repair pathway is active during G0/G1 stages and is independent of replication and homologous recombination but dependent upon the nucleotide excision repair components XPC and XPA [33-37]. Many of the components of the FA pathway including the core complex, FANCI and FANCD2 seem to be required for this repair [38]. The key to understanding crosslink repair will be careful molecular and biochemical dissection of function of the proteins involved in repair including crystallographic studies of the pathway’s components. It is clear that characterization of the ubiquitin-driven events and nuclease regulation in the pathway will be key to understanding the Fanconi anemia pathway.

The E3 ubiquitin ligase activity of FANCL and the FA core complex

Ubiquitylation of FANCD2 and FANCI is absent in cells with mutations in the core complex components (except for FANCM). Prior to the identification of FANCL, the core complex was not shown to harbor proteins with any recognizable domains. At that time, other known ubiquitin ligases such as BRCA1 were under scrutiny in efforts to identify the components required for this central step in FA pathway regulation [7, 39]. It wasn't until the identification of FANCL that it was possible to attribute monoubiquitylation of FANCD2 as an intra-FA pathway step in the response to ICLs [40].

FANCL is considered a key part of the FA core complex and represents the E3 ligase necessary for ubiquitin conjugation to lysine 561 of human FANCD2 and lysine 523 of FANCI. Structurally FANCL has three distinct domains; an ELF, DRWD and RING domain [41]. Functionally, the DRWD domain coordinates substrate binding while the RING domain is required for E2 protein interactions. Yeast two hybrid experiments using full-length FANCL have identified two E2 proteins capable of interacting with FANCL, UBE2T and UBE2W [42]. The C-terminal RING domain is both necessary and sufficient for FANCL binding of UBE2T [43]. The importance of the RING domain is reflected in the evolutionary conservation of this domain in FANCL homologs [41]. UBE2T is considered to be the most functionally important E2 enzyme required for FANCL-mediated ubiquitylation of FANCD2 *in vivo* and is required for cellular resistance to ICLs [43]. *In vitro* data differ from *in vivo*-derived data as to the minimal requirements for FANCD2 monoubiquitylation, since E1, UBE2T, and FANCL are shown to be sufficient for FANCD2 monoubiquitylation in an *in vitro* context [42]. However, this reaction was quite inefficient and ubiquitylation occurred only on FANCD2, even when FANCI was also present in the reaction. Interestingly, presence of FANCI conferred specificity to the reaction so that K561 of FANCD2 was now correctly monoubiquitylated. This suggests that FANCI might direct the correct lysine residue to the core complex or perhaps mask alternative surface lysines. Reconstitution of the whole core complex might be necessary for robust *in vitro* ubiquitylation. In addition, since phosphorylation plays an important role in activating the pathway, using pre-activated FANCI (see below) might be required.

FANCD2 and FANCI are the only known substrates of the core complex. There remains a possibility that there will be other substrates for this complicated E3 ligase (Figure 1B and 1D). A hint of that might be seen in the result showing that monoubiquitylated FANCD2 on the chromatin is not sufficient for crosslink resistance in DT40 cells that lack the FA core complex [44]. Since FANCI monoubiquitylation in DT40 cells is almost noncontributory (see next section), it is likely that there will be other substrates of FANCL.

Regulation of ID complex monoubiquitylation

Monoubiquitylated ID complex is at the heart of the Fanconi anemia pathway. The monoubiquitylation of FANCD2 at lysine 561 is essential for the downstream function of the FA pathway in repair. Cells expressing K561R FANCD2 (which cannot be monoubiquitylated) as the only FANCD2 allele are as sensitive to crosslinking agents as null cells [7]. The contribution of monoubiquitylated FANCI to crosslink resistance seems to be less important than that of monoubiquitylated FANCD2 in human cells [4], and almost non-contributing in DT40 cells [45]. Monoubiquitylation of FANCD2 occurs following the localization of the core complex to sites of damage (Figure 1B). Core complex localization requires the combined activity of the FANCM and its interacting factor FAAP24 that facilitates the recruitment of the core complex to regions of damage [46, 47]. The recruitment of FANCM to sites of damage is enhanced further by the association of FANCM with additional FAAP proteins, MHF1 and MHF2 [48]. MHF1 and MHF2 contain

putative histone fold domains and form a dimeric complex with DNA binding affinity. MHF1 and 2 are found in complex with FANCM and FAAP24 in mammalian cells and MHF1 is critical for the integrity of complex. The MHF dimer complex augments the association of FANCM with chromatin and enhances FANCM dependent processing of branched DNA. Destabilization of the MHF1/2 dimer complex with FANCM by silencing of MHF1 expression leads to a decreased efficiency of FANCD2 monoubiquitylation in response to DNA damage and increased genome instability.

It is unclear how the ID complex localizes to chromatin before it is monoubiquitylated. It does posses intrinsic DNA-binding activity [49-51] with preference for branched structures, so it is possible that it gets to the sites of damage on its own via DNA binding. Once monoubiquitylated, it clearly resides at chromatin where it can be found in damage-induced foci [4, 7]. It has been previously proposed that a chromatin receptor for the monoubiquitylated ID complex might be present at the chromatin [44, 52, 53], although such a receptor has not yet been identified. In the light of FAN1 binding to the chromatin bound monoubiquitylated ID complex (see below), existence of such a receptor is less likely, although not impossible. If such a receptor does exist, understanding of the re-modeling of the ID complex architecture from interacting with a receptor via monoubiquitin to interacting with FAN1 and other effector proteins at chromatin will be very important.

Fanconi anemia proteins are regulated by DNA damage signaling involving ATM and ATR kinases. ATM is activated in response to double-strand breaks and in a manner that involves DNA resection while ATR signals replicative stress and is activated by binding of ATR/ATRIP to the RPA coated ssDNA [54, 55] (Figure 1A). Multiple components of the FA pathway are phosphorylated including FANCA, FANCG, FANCM, FANCD1 and both constituents of the ID complex [4, 5, 10, 45, 56-62]. The importance of many of the phosphorylation events is not entirely clear but some of them are important for crosslink resistance. In particular, phosphorylation of FANCI on SQ/TQ sites close to the monoubiquitylation site of FANCI is necessary for the monoubiquitylation and localization of both FANCI and FANCD2 to damage-induced foci [45]. FANCI carrying phosphomimic mutations of these sites showed constitutive activation of the pathway even in the absence of damage. Based on these findings, it has been proposed that FANCI phosphorylation functions as a molecular switch to turn on the FA pathway. Although it is unclear how this switch my work, one possibility is that phosphorylation of FANCI drives an interaction of the ID complex with a protein that interacts with the core complex or with a protein in the core complex itself. It will be important to understand how phosphorylation of FANCI triggers monoubiquitylation of FANCD2.

Ubiquitin mediated interaction of the ID complex and FAN1

The chromatin bound monoubiquitylated ID complex facilitates subsequent steps of ICL repair in ways that are still being elucidated. The ID complex and monoubiquitylation of FANCD2 is required for ICL unhooking and translesion DNA synthesis to facilitate bypass of the lesion [63, 64]. It is likely that monoubiquitylation of the ID complex forms a platform for at least some of these events and that the protein components involved in making the incisions and translesion synthesis may harbor ubiquitin-binding motifs themselves (Figure 1C). The newly identified nuclease required for ICL repair, FAN1, is one such protein. FAN1 has a UBZ domain at its N terminus. UBZ (ubiquitin binding zinc finger) domain is one of many ubiquitin recognition motifs (reviewed in [65]). Mutation of the UBZ domain precluded FAN1 from localizing to damage made by laser irradiation or by crosslinking damage [13-16]. The ID complex was also required for foci formation by FAN1. Moreover, cells with endogenous FANCD2 replaced with the K561R FANCD2 mutant lost FAN1 localization. Since FANCD2 and FAN1 were able to interact in

immunoprecipitation experiments, monoubiquitylated FANCD2 is considered a platform for FAN1 binding through the UBZ domain. Other regions of FAN1 protein might also be required for interaction with FANCD2 analogous to REV1 interacting not only with the monoubiquitin on PCNA but also with the PCNA itself through the BRCT motifs [66]. Since FAN1 displays both endonuclease and exonuclease activity this ubiquitin-mediated recruitment focuses the nuclease activity of FAN1 to the site of the ICL. Thus far, *in vitro* experiments have been performed on non-crosslinked substrates so it is unclear at which step of repair FAN1 acts. It may work in concert with the MUS81-EME1 and XPF-ERCC1 nuclease that have been implicated in crosslink unhooking [31, 32]. It might also act at the later stages of excision of the unhooked crosslink or even later during preparation for homologous recombination events. The concept of ubiquitin-mediated recruitment via the ID complex will undoubtedly be an important aspect of understanding the downstream processes of FA pathway-mediated DNA repair. It will be interesting to uncover how the ID complex co-ordinates the downstream effectors of the pathway, some of which may yet to be identified.

Deubiquitylation and FA pathway regulation

Ubiquitylation of the ID complex is an activating event necessary for the repair of ICLs. Equally important is the event of turning that signal off once the repair is completed. Failure to de-ubiquitylate the ID complex results, somewhat counter intuitively, in sensitivity to crosslinking agents [67, 68]. This implies that the cycling of the ID complex between ubiquitylated and non-ubiquitylated forms is essential for the activity of the pathway. USP1 is the deubiquitylating enzyme that interacts with FANCD2 in chromatin and removes its monoubiquitin [69] (Figure 2). USP1 is regulated on many levels. USP1 levels are tightly regulated at a transcriptional level to peak during S-phase, presumably when its activity is most required. It is also regulated by polyubiquitylation and proteasome-dependent degradation at the end of S phase once it is no longer needed. USP1 levels during S phase however are fairly constant [69]. This implies that there will be as of yet unidentified regulatory steps in the pathway, possibly at the chromatin level, which will allow deubiquitylation of FANCD2 and resetting of the pathway. Interestingly, upon damage USP1 transcription is shut off with a subsequent rapid decline of USP1 protein [70]. It is not known what happens to the chromatin-associated USP1 pool after DNA damage and it is this fraction of USP1 that is of particular interest. It is unlikely that it disappears; rather its activity is probably regulated in some way to allow for deubiquitylation of the ID complex only after the damage is repaired. USP1 activity is greatly enhanced by its partner, a WD40 domain containing protein, USP1 Associated Factor 1 (UAF1) [70]. Future work should concentrate on regulation of USP1 since our appreciation of the mechanisms of deactivation, lags behind our understanding of the activation of the Fanconi anemia pathway. Interestingly, USP1 is also a deubiquitylating enzyme for PCNA (Figure 2), suggesting that regulation of deubiquitylation of PCNA and the ID complex might be co-regulated. Clearly there will also be separate regulatory networks, for example ELG1 interacts with USP1-UAF1 and affects PCNA deubiquitylation, but not FANCD2 ubiquitylation status [71].

The Ubiquitin binding motifs of FANCP/SLX4

FANCP/SLX4 represents the newest member of the FA pathway to date and constitutes the 14th Fanconi anemia complementation group FA-P [26, 27]. The SLX4 protein is a large scaffold with multiple recognizable domains. At the N terminus, it has two UBZ domains followed by an MLR (MUS312/MEI9 interaction like region), BTB domain, SAP domain, and finally a helix-turn-helix motif at the C terminus [28-30]. Human SLX4 interacts with multiple proteins including XPF-ERCC1, MUS81-EME1, SLX1, MSH2, MSH3, TRF2, RAP1, and PLK1 among others. It appears that most if not all of the cellular pool of MUS81

and SLX1 interact with SLX4 while only a small portion of the cellular XPF pool interacts with SLX4 [30]. *In vitro* SLX4 is able to enhance the activity of the associated nucleases. Cells derived from FA-P patients display normal monoubiquitylation of FANCD2 following DNA damage but genomic instability and sensitivity to inter-strand crosslinking agents characteristic of an FA phenotype. The early steps of ICL repair that requires the core components of the FA pathway are unaffected by a loss of SLX4 suggesting that it is a downstream effector of the pathway [26, 27]. Obvious candidates for the essential components of SLX4 in the setting of FA are XPF and MUS81 but the exact role of SLX4 in FA pathway still needs to be determined. Patient mutations suggest an important role for the UBZ domains. Two of the four families with SLX4 mutations have a very interesting in-frame internal deletion of amino acids 317 to 387, corresponding to half of the first UBZ domain and the whole second part of UBZ domain. Although the level of expression of this allele was lower than wild type, it was able to interact with XPF and MUS81.

The UBZ domains of SLX4 interact efficiently with K63-linked ubiquitin chains *in vitro* in a manner that requires intact UBZ domains [26]. Mutation of the two cysteines in both of the UBZ domains resulted in abrogation of ubiquitin chain binding. These data led to a proposal that FANCP might localize to sites of damage via ubiquitin mediated interactions. The substrates of such interactions are unknown at this time (Figure 1C). It is still possible that SLX4 interacts with monoubiquitylated ID complex as suggested for the chicken FANCP in DT40 cells [72]. However, the presence of tandem UBZ domains would be consistent with SLX4 binding to a target that is endowed with ubiquitin chains. Identity of ubiquitylated substrates is a hot subject and identification of these in the context of the FA pathway will be truly exciting. It is appealing to think that different nucleases will arrive at the sites of a crosslink via different, albeit ubiquitin-dependent interactions. FAN1 would arrive via monoubiquitylated ID complex, SLX4/XPF/MUS81 complex, via a different, unknown interactor. This would give an opportunity to regulate these nucleases separately and make sure that the incisions are made only at the correct sites and at the right time.

Translesion polymerases in the ICL repair

Translesion synthesis (TLS) polymerases are specialized enzymes that are able to insert a nucleotide across a damaged site in DNA. Multiple translesion polymerases have been implicated in the FA pathway. Knockout of REV1, and components of POLζ (REV3, REV7) in chicken DT40 cells leads to exquisite sensitivity to crosslinking agents [73-77]. Moreover REV1 and REV3 are epistatic with FANCC for crosslink sensitivity suggesting that they are in the same pathway [73, 75]. The current model for ICL repair based on the repair of a crosslinked plasmid in *Xenopus* egg extract proposes that translesion polymerases are essential for the bypass of the crosslink that was freed, commonly referred to as “unhooked”, by endonucleolytic incisions [63, 64]. It has been proposed that REV1 inserts one nucleotide against the unhooked crosslink, which is further extended by the REV3 catalytic subunit of POLζ [63, 64]. Depletion of FANCD2 affected the translesion step as well as the “unhooking” step. It is not yet known whether both of these events are direct consequences of FANCD2 depletion. It is possible that both of these activities are co-regulated by the ID complex or that the ID complex regulates only one of them and that the second depends upon the initiation (or even completion) of the first. Interestingly, REV1 focus formation due to ICLs is dependent on the functional FA core complex but does not seem to involve FANCD2 or RAD18-dependent PCNA monoubiquitylation [78] (Figure 1D). How the translesion polymerases get to the ICL is an exciting topic. Many others polymerases might also be involved in ICL repair including POLη, POLκ, POLι, POLθ, and POLν The involvement of these polymerase was recently extensively reviewed in [79]. Of particular interest to the FA pathway is POLν (POLN), which is an A type DNA polymerase. Depletion of POLN in human cells results in DNA crosslink sensitivity with

defects in homologous recombination [80]. POLN was found to interact with monoubiquitylated FANCD2 on chromatin after mitomycin C treatment, although it is unclear if monoubiquitylation of FANCD2 is required for the interaction, Furthermore, the POLN dependent requirements for this interaction are not yet known.

RAD18 and FA pathway activation

Although RAD18 does not seem to be involved in REV1 localization to ICLs, lack of RAD18 has been reported by several laboratories to be involved in activation of the FA pathway since depletion or lack of RAD18 led to slower kinetics of FANCD2 and FANCI monoubiquitylation [81-84]. The mechanism of how RAD18 might affect ID complex monoubiquitylation is not clear at this time since the published data from different groups do not agree with each other. RAD18 is an E3 ligase and together with RAD6 it has been shown to catalyze monoubiquitylation of PCNA on lysine 164 and this modification promotes error-prone translesion bypass [85, 86]. According to two papers [82, 83], PCNA ubiquitination on lysine 164 is required for activation of the FA pathway upon cisplatin or benzo[a]pyrenedihydrodiol epoxide (BPDE) damage and that ubiquitylated PCNA recruits the core complex which in turn ubiquitylates the ID complex. Findings of Williams *et. al* [81] contradict those conclusions. They show that RAD18 co-immunoprecipitates with FANCD2 and that the RING domain of RAD18 is necessary for this interaction. However, they show that FANCD2 ubiquitylation is normal after mitomycin C treatment of cells containing an ubiquitylation-resistant form of PCNA, and that the chromatin loading of FA core complex proteins appears normal in RAD18-knockout cells. The authors suggest that RAD18 could be responsible for modifying the core complex itself or other unidentified DNA repair proteins upstream of the ID complex. These discrepancies need to be addressed by the labs that reported these findings. There were some obvious differences in cell lines and in damaging agents used between all the reports, including the use of BPDE and cisplatin in reports describing PCNA monoubiquitylation-dependent mechanism, and mitomycin C in the report that did not. Regardless of the mechanism, RAD18 is certainly a factor that regulates the FA pathway.

Ubiquitin binding motif of SNM1A

S.cerevisiae SNM1 (sensitivity to nitrogen mustard), also known as PSO2 (sensitivity to psoralen), has been identified in screens for selective sensitivity to ICLs [87, 88]. The 5’ to 3’ exonuclease activity of Pso2p is thought to be important for processing of incisions made by nucleotide excision repair in yeast. In vertebrates, there are three orthologs of SNM1; SNM1A, SNM1B/Apollo, and SNM1C/Artemis (for review see [89]) SNM1A and SNM1B both seem to participate in ICL repair, and the double knockout in DT40 cells is more sensitive to cisplatin than either of the single knockouts, suggesting that they function in separate pathways or that they are redundant for cisplatin resistance [90]. SNM1A is also not epistatic in crosslink resistance with FANCC, RAD18 or XRCC3 (homologous recombination pathway) in DT40 cells. SNMA1A, but not SNM1B was able to complement crosslink sensitivity of the yeast Pso2 mutant [91] suggesting that SNM1 is a functional ortholog of Pso2. Recent work led to the identification of a PIP box motif in vertebrate SNM1 required for binding to PCNA irrespective of DNA damage [71]. A newly identified UBZ domain in the N-terminus of SNM1A, which is conserved even in S.cerevisiae Pso2, was required for damage induced foci formation by a GFP-tagged SNM1 in human cells (Figure 1C). SNM1A foci formation after DNA damage was dependent on RAD18 [71] though the functional consequences of these interactions are not yet clear. It remains to be determined whether SNM1B functions in crosslink repair in human cells.

Evolutionary conservation of proteins in the Fanconi anemia pathway

The most conserved of the FA proteins are FANCM, SLX4 and BRCA2, orthologs of which are present from yeast to human [28-30, 92-94]. Such an exquisite conservation; however, may reflect functions of these proteins outside of ICL repair. Vertebrates, flies, frogs, worms, plants and even a slime mold *D. discoideum*, have recognizable orthologs of some of the components of the core complex, importantly FANCL [41, 95-98]. Conservation of the components of the core complex, other than FANCL, fluctuate throughout evolution with some components lost or gained in different species (reviewed in [98]). All organisms that have FANCL also have FANCD2 and FANCI, which have been shown to be monoubiquitylated in some [64, 95, 97, 98]. *S. cerevisiae* and *S.pombe* have no core complex nor ID complex components. However, *S. pombe* does have a FAN1 ortholog whose function is unknown. *S. pombe* Fan1 has a well-conserved nuclease domain and a SAP domain but no recognizable ubiquitin binding domain [16]. SAP (SAF-A/B, Acinus and PIAS) domains have been postulated to be modules involved in targeting proteins to chromatin [99]. As such, it is possible that the SAP domain in the *S.pombe* FAN1 might be involved in the localization of FAN1 to sites of damage.

Both *S. cerevisiae* and *S. pombe* have Slx4 orthologs. Indeed, Slx4 and Slx1 were first identified in yeast in a screen for factors required for the viability of *S. cerevisiae* lacking RecQ helicase Sgs1 [100]. There are clear similarities between the yeast and human orthologs of Slx4 in that they both interact with XPF-ERCC1 and SLX1 orthologs [101]. SLX4 from yeast to human also contains SAP domains and the C-terminal Helix-turn-helix domains. The Slx4 orthologs in yeast do not have the UBZ domains, BTB domains nor do they interact with MUS81. These differences suggest that there has been an evolutionary expansion of SLX4 function, but also adoption of MUS81 into the SLX4 complex, offering an opportunity for coordination of multiple nuclease activities through the SLX4 protein.

Proteolysis and DNA damage signaling

Ubiquitylation clearly plays pan-cellular roles that are critical for signaling and, as discussed here, crucial for FA pathway regulation and DNA repair. Monoubiquitylation and polyubiquitin chains may be of primary importance in signaling events, as platforms for complex interactions. However, the importance of proteolytic degradation in response to polyubiquitylation and proteasome targeting cannot be underestimated in terms of its importance for DNA repair. Many of the regulatory factors discussed here may be targeted for proteasomal degradation as is the case for the FA regulatory deubiquitylating enzyme USP1 (discussed above, Figure 2) and FANCM, which is degraded during M phase, releasing the core complex from chromatin [102]. It has also been shown that proteasome function is in fact required for monoubiquitylation and foci formation of FANCD2 [103]. The proteasome has also been linked to various aspects of DSB repair and homologous recombination [104, 105]. Understanding how the proteasome balances the accumulation of repair factors with their degradation will involve a more thorough understanding of polyubiquitylation targets. In turn, how the proteasome is localized at sites of damage and its regulation is an important aspect of DNA damage signaling and repair.

Perspectives

There is a lot to be learned about the role of ubiquitylation in the Fanconi anemia pathway. Many of the remaining issues were noted in their respective sections above. Here we highlight a few, including the activation of ubiquitylation of the ID complex, role of ubiquitin in regulation of nucleases, and a potential role of other, ubiquitin-like modifiers in the FA pathway.

Turning on ubiquitylation

Phosphorylation of FANCI turns on the FA pathway in response to damage. Understanding how this takes place is an important concept in how ubiquitylation events occur in response to challenges such as DNA damage. Are ubiquitin ligase complexes constitutively active such that the recruitment of substrates to the ligase complex is the regulated step? Are ubiquitin ligase complexes themselves activated by post-translational modification? In the case of damage-induced ubiquitylation of the ID complex it may be that phosphorylation of both the core complex and the ID complex regulate the 'activation' of ID complex ubiquitylation. In this respect phospo-binding motifs may be the chromatin 'receptors' that localize core complex with substrate leading to ubiquitylation.

Targets of FANCL monoubiquitylation

Thus far only FANCD2 and FANCI have been identified as targets of FANCL monoubiquitylation. It is compelling to think that there may be alternative targets for FANCL monoubiquitylation. This hypothesis is supported by the observation that the core complex is not dispensable for crosslink repair even when FANCD2 monoubiquitylation has been satisfied [44]. The missing monoubiquitylated factor in that study could have been FANCI, however, FANCI monoubiquitylation appears not to be very important in the DT40 cells. In light of these findings, there still might be some missing substrates of the core complex. Experimentally, finding alternative targets of FANCL monoubiquitylation may not be simple. Such a modification is not represented in abundance and dissecting monoubiquitylated targets from polyubiquitylated targets may prove challenging. Additionally the interdependence of posttranslational modification (like the phosphorylation switch of FANCI for the ID complex ubiquitylation) may complicate efforts towards examining this hypothesis.

Co-ordination of nucleases

Interstrand crosslink repair involves number of coordinated nucleolytic events. There is a plethora of nucleases implicated as being important in the repair of crosslink lesions. To advance our understanding of how these events are regulated requires dissection of the step-wise order of events that guides repair through unhooking events, translesion synthesis and recombination events. Since there is likely to be redundancy between the functions of these nuclease complexes, dissecting these events both *in vivo* may not allow us to gain an accurate picture of how the nucleolytic repair events are regulated. In this instance *in vitro* recapitulation of these reactions may be the only way to know the absolute requirements for, and order of, events at an interstrand crosslink lesion. Implicit in gaining an understanding of nuclease regulation is the identification of how nucleases are organized at sites of repair. The identification of FANCP/SLX4 shed some light on the recruitment of nucleases to the sites of damage. The docking sites for these recruitment factors will provide important insight into dynamics of these processes and understanding of the ubiquitin binding motifs in these proteins will be essential.

Ubiquitin-like modifications

Ubiquitin modification modulates molecular interactions and localization of substrates. Other small molecule modifications have been identified that like ubiquitin are conjugated onto target substrates and can play a role in the regulation of cellular processes such as cellular signal transduction, cell cycle regulation and DNA repair. SUMO (small ubiquitin-like modifier) modification dynamics are similar to that of ubiquitylation in that they involve an enzymatic cascade to conjugate SUMO to target substrates. This equilibrium is maintained by desumoylating enzymes. In yeast PCNA is a platform for both ubiquitination

and SUMOylation [106]. SUMOylation of PCNA takes place during S-phase creating a binding site for down-stream effectors and can suppress homologous recombination. SUMOylation motifs are well characterised and various protein components of the FA pathway harbor putative SUMOylation sites. It will be interesting to see if and how SUMOylation and indeed other ubiquitin-like modifications may be involved in FA pathway function.

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Highlights

- The Fanconi anemia (FA) is a bone marrow and cancer predisposition syndrome
- The FA pathway is responsible for repair of interstrand DNA crosslinks
- FA core complex ubiquitylates FANCI and FANCD2 which localize to chromatin and direct repair
- Ubiquitin interaction motifs are used to recruit nucleases to sites of damage
- Deubiquitylation plays an important role in terminating DNA damage signaling

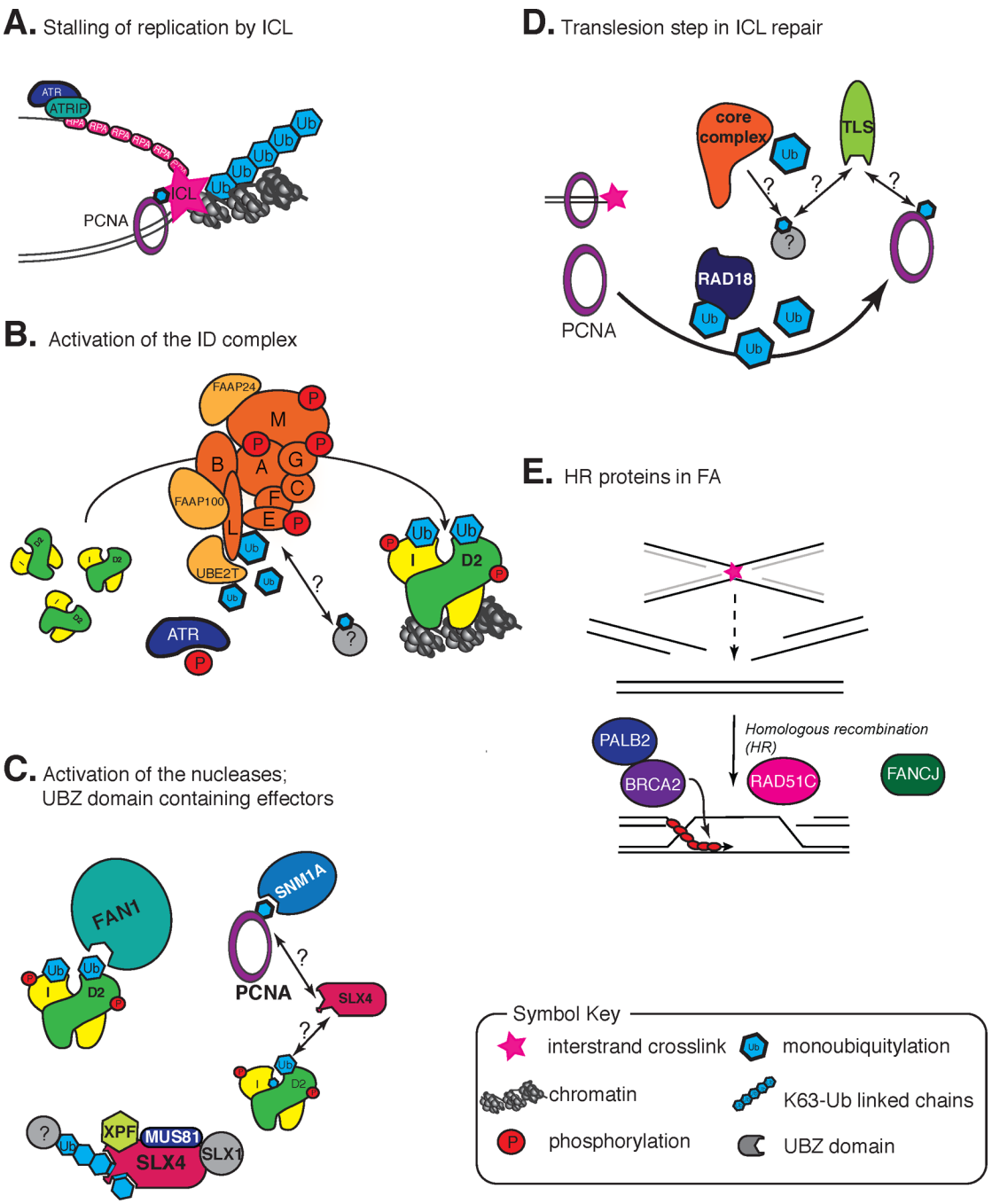

Figure 1.

Ubiquitylation in response to interstrand crosslinks, highlighting Fanconi anemia proteins and the associated factors necessary for repair. Particular attention is drawn to some of the unknown factors in interstrand crosslink repair. **A.** Interstrand crosslinks prevent separation of the DNA helix. During replication this can lead to replication fork collapse when forks encounter lesions from one or both directions. For a full model of DNA transactions, see [63, 64]. This leads to RPA-ssDNA binding and activation of the ATR and the downstream DNA damage response **B.** Activation of the ID complex. ATR phosphorylates many FA proteins including FANCI. FANCI phosphorylation acts as a molecular switch in the FA

pathway. The FA core complex is recruited to chromatin at sites of damage where it functions as an E3 ubiquitin ligase to monoubiquitylate the ID complex at the damage site. It is currently unknown if there are other substrates for ubiquitylation by the FA core complex. **C.** UBZ domain containing effectors. Downstream effectors FAN1, SLX4, and SNM1A harbor ubiquitin-bindingmotifs that are thought to target them to the site of damage through ubiquitin mediated interactions. FAN1 binds the monoubiquitylated FANCD2. SLX4 displays binding affinity for ubiquitin chains though the docking sites for SLX4 are not currently known. SLX4 could potentially interact with ubiquitylated FANCD2 or PCNA. Interestingly all three proteins harbor nuclease activity or are associated with nucleases. Thus, control of ubiquitylation events regulates the localization of nucleases at the site of damage. One of the remaining questions is why there are so many nucleases at the repair sites. **D.** Translesion synthesis step. Chromatin bound PCNA is monoubiquitylated by RAD18 in response to interstrand crosslinking damage and coordinates translesion synthesis steps during interstrand crosslink repair. Additional, non-PCNA and non-RAD18-dependent events are likely to allow translesion synthesis to occur. **D.** Homologous recombination (HR) concludes the repair process. FA proteins BRCA2, PALB2, and the Fanconi anemia-like syndrome protein RAD51C are involved in this step. FANCJ is also important for HR but in the context of FA, it clearly has other functions, which are not yet fully explored.

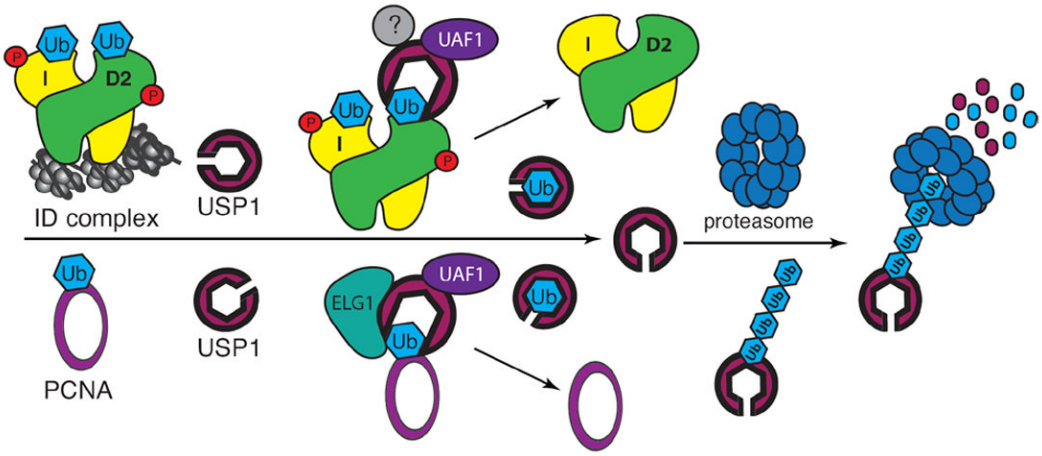

Figure 2.

Deactivation of the Fanconi anemia pathway by deubiquitylation

The signaling that takes place in the activation of DNA repair must be attenuated when DNA repair has concluded. The ID complex is deubiquitylated by USP1 whose activity is enhanced by interaction with UAF1. USP1 has a parallel role in the deubiquitylation of PCNA. USP1/UAF1 substrate affinity is regulated by auxiliary factors such as ELG1, which is required for the deubiquitylation of PCNA specifically. The DUBs are themselves subject to regulation on both a transcriptional and post-transcriptional level. Importantly, USP1 is polyubiquitylated as DNA replication concludes. Polyubiquitylated USP1 is targeted for proteasomal degradation.