The ubiquitin family meets the FANC proteins

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Abstract

Fanconi anaemia (FA) is a hereditary disorder that is characterized by a predisposition to cancer, developmental defects and chromosomal abnormalities. FA is caused by biallelic mutations that inactivate genes encoding proteins involved in the replication stress-associated DNA damage response. The 20 FANC proteins identified to date constitute the FANC pathway. A key event in this pathway involves the monoubiquitination of the FANCD2-FANCI heterodimer by the collective action of a group of proteins assembled in the FANC core complex, which consists of at least 10 different proteins. The FANC core complex-mediated monoubiquitination of FANCD2-FANCI is essential to assemble the heterodimer in subnuclear chromatin-associated foci and to regulate the process of DNA repair as well as the rescue of stalled replication forks. Several recent works have demonstrated that the activity of the FANC pathway is linked to several other protein post-translational modifications from the ubiquitin-like family, including SUMO and NEDD8. These modifications are related to DNA damage responses but may also affect other cellular functions potentially related to the clinical phenotypes of the syndrome. This review summarizes the interplay of ubiquitin and ubiquitin-like proteins with the proteins that constitute the surveillance system for genomic integrity called the FANC pathway and addresses the implications of these interactions in maintaining genome stability.

Introduction

It has been estimated that there are approximately 25,000 genes in the human genome. However, proteome diversity has been estimated to be greater by approximately three orders of magnitude [1]. Diversity can be explained not only by the different isoforms generated from alternative mRNA splicing but also by the enormous number of post-translational modifications (PTMs) on proteins that maintain cellular homeostasis. Indeed, proteins are generally modified by the covalent addition of several functional groups, such as phosphate groups (phosphorylation), carbonate groups (acetylation, methylation) or nitrate groups (nitrosylation). Proteins are also modified by more complex substrates, such as sugars (glycosylation) or small polypeptides, including ubiquitin and ubiquitin-like molecules (ubiquitination, NEDDylation, SUMOylation, etc.). PTMs have profound effects on protein behaviour by modifying their activity, subcellular localisation, interactions and stability.

The ubiquitin protein is a 76 amino acid polypeptide chain with a molecular weight of 8 kDa that is highly conserved among eukaryotes. This protein can be conjugated to the lysines (K) of target proteins *via* its C-terminal glycine (G). Ubiquitin, being rich in lysines *per se,* is also modified by itself, creating either several branched structures identified by the position of the modified K on the founder ubiquitin (K6, K11, K29, K48, K63) or mixed chains with different combinations [2]. The most described ubiquitin chain is that occurring on K48 of the ubiquitin, which is then added to a target protein and mediates its degradation via the proteasome complex [3]. A target protein can be modified by the addition of one or several simple and/or branched ubiquitin chains. Therefore, because a protein can undergo simultaneous or sequential addition of one or more mono- or branched-ubiquitin moieties, the regulation of its behaviour by the ubiquitination process is highly complex.

Ubiquitin is the most representative protein of the ubiquitin-like family of proteins that is composed of 9 members: ubiquitin itself and the 8 ubiquitin-like small proteins—NEDD8, ISG15, FAT10, SUMO1 to 4 and ATG12 (Figure 1A) [2,4–6]. Similarly to ubiquitin, these small proteins are conjugated to a lysine on target proteins. Notably, each member of the family can sequentially target the same lysine

on the same protein, adding an additional layer of complexity in the regulation of protein behaviour.

The conjugation process involves a cascade of enzymes that includes an ATP-dependent E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase (Figure 1A and 1B). Generally, each targeted K on a protein has its own specific E1, E2 and E3 triad. Finally, specific enzymes (DUBs, deubiquitinases) are devoted to the elimination of the added ubiquitin and ubiquitin-like peptides to fine-tune the regulation of the protein. Defects in protein modification by ubiquitin family members are the cause of or associated with severe human diseases, such as cancer [7,8] and neurodegeneration [9]. Germline mutations altering the ubiquitination pathways or modifying the ubiquitination of target proteins have also been reported in cases of rare genetic syndromes [10].

In this review, we will focus on the crosstalk between the FANC pathway, which is responsible for the human hereditary syndrome Fanconi anaemia (FA), and the ubiquitin and ubiquitin-like family of proteins.

Fanconi anaemia and the FANC genome surveillance pathway.

Fanconi anaemia is a rare recessive human genetic disorder first described by the Swiss paediatrician Guido Fanconi in 1927 [11]. The major clinical features of FA are bone marrow failure, developmental abnormalities and predisposition to cancer [12]. Cells from FA patients show cellular and chromosomal hypersensitivity to DNA interstrand crosslinking (ICL) agents, such as mitomycin C, diepoxybutane, cisplatin and photoactivated psoralens [13]. Abnormalities in cell cycle progression [14], reactive oxygen species metabolism [15,16], inflammatory cytokine production/responses [17–25] and chromosomal instability at common fragile sites (CSF) [26] are also considered hallmarks of the syndrome [12,27,28]. The FA phenotype is highly heterogeneous; it has been estimated that developmental abnormalities occur in 70% of patients, while 40% develop skin pigmentation defects [29]. Several other rare abnormalities, such as kidney or urogenital defects, have been reported in less than 10% of patients [30].

An enormous heterogeneity is also found at the genetic level [27]. Indeed, inactivating mutations in one of at least 20 genes (named *FANCA* to *FANCU*, Table 1) are responsible for FA. In the majority of cases, the FA patients have biallelic mutations, one inherited from each parent. One gene, *FANCB*, is located on the X-

chromosome [31]. In the case of *FANCR/RAD51,* only one FA patient has been identified to date with one wild type allele and one mutant allele encoding a protein with dominant-negative activity, suggesting that the mutation appeared during parental gametogenesis [32]. So far, only one patient has been described with FANCM mutations [33], but since he also harbours an heterozygote compound mutation on FANCA, FANCM was exclude as a causative FA gene [34].

It is widely accepted that the master function of the proteins involved in FA is to respond to DNA damage and replication stress, helping the cell to both rescue stalled replication forks during S phase, favouring homologous recombination (HR) instead of non-homologous end joining (NHEJ) [35–38], and to correctly manage misreplicated or incompletely replicated regions during mitosis to avoid the transmission of DNA breaks and gross chromosomal aberrations to daughter cells [39,40]. In the DNA damage response (DDR), the FANC proteins function in a common linear and finely regulated pathway, called the FA, FANC/BRCA or FANC pathway, which safeguards the genetic information of the cell (Figure 2).

Taking into account both biochemical and functional criteria, the FANC proteins have been subdivided into three groups [41]. The proteins of the first group were shown to be in a complex, referred to as the FANC core complex, by immunoprecipitation (IP) [42]. FANCA, B, C, E, F, G and L, together with FA-associated protein 20 and 100 (FAAP20 and FAAP100), respond to genotoxic stress and are, in turn, recruited to the chromatin by FANCM, which is associated with FAAP24 and FANCM-interacting Histone-Fold protein 1 and 2 (MHF1 and MHF2) [43]. Although no FA patient has been identified thus far with mutations in FAAP20, FAAP24, FAAP100, MHF1 or MHF2, their inactivation in model organisms and/or cells resulted in a FA-like cellular phenotype, supporting their inclusion in the FANC group of proteins [44–49]. Assembled in the nucleus, the FANC core complex interacts with UBE2T, one of the most recently identified FANC gene (*FANCT*) [50,51]. FANCT/UBE2T is the E2 that participates in the monoubiquitination of FANCD2 and FANCI, together with the E3 FANCL [52].

FANCD2 and FANCI, the two proteins in group II, are the only known targets of the ubiquitin ligase activity of the FANC core complex. Indeed, in the absence of any of the FANC core proteins, monoubiquitination of FANCD2 and FANCI is defective [53]. FANCD2 and FANCI monoubiquitination is necessary and sufficient for their subsequent translocation to the chromatin-associated, detergent-resistant

subnuclear foci, where they colocalize with several known proteins involved in the replication, repair and recombination of damaged DNA. USP1, a DUB also involved in PCNA de-ubiquitination, acts on monoubiquitinated FANCD2/FANCI to deubiquitinate the complex, and loss of USP1 results in the constitutive monoubiquitination of both FANCD2 and FANCI (as well as PCNA) and a FA-like phenotype in mice [54,55].

Group III harbours proteins whose inactivation does not lead to major defects in FANCD2 and FANCI ubiquitination. In group III, there are several proteins that are associated with the homologous recombination (HR) pathway, such as FANCD1/BRCA2 [56], FANCJ/BRIP1 [57], FANCN/PALB2 [58], FANCO/RAD51C [59], FANCR/RAD51 [32], FANCS/BRCA1 [60], FANCU/XRCC2 [61,62] and proteins participating in two different structure-specific endonuclease complexes, FANCP/SLX4 (which interacts with SLX1) [63,64] and FANCQ/XPF (which interacts with ERCC1) [65,66]. Both heterodimers, FANCP (SLX4)/SLX1 and FANCQ (XPF)/ERCC1, can be found in a supramolecular complex with another structure-specific endonuclease, the EME1/MUS81 heterodimer. Inactivation of several of the proteins belonging to this third group has been associated with a predisposition to breast and ovarian cancer. Notably, XP-F inactivation or malfunction, due to mutations in different domains of the protein, has been associated with cancer predisposition and developmental syndromes: xeroderma pigmentosum, FA, Cockayne syndrome, XFE progeria and the cerebro-oculo-facio-skeletal syndrome [67].

Yeast two-hybrid assays, cellular analysis by immunofluorescence and/or immunoprecipitation and western blots with nuclear/cytoplasmic fractions were used to assess interactions and localization of the the FANC core complex in unstressed conditions. The proteins forming the soluble part of the complex were principally localized to the cytoplasm in different modules or sub-complexes and were found massively in the nucleus only in presence of DNA damage or stalled replication forks [68]. Thus, FANCA and FANCG associate with FAAP20; FANCB and FANCL with FAAP100; and FANCC and FANCE with FANCF. FANCL and its direct partners, FAAP100 and FANCB, represent the catalytic subunit of the FANC core complex. FANCA, FANCG and FAAP20 are important to anchor the complex to the chromatin, while FANCC, FANCE and FANCF act as a scaffold, which is important for the complex stability [53,69]. However, it is still unclear how these modules move from

the cytoplasm to the nucleus, how they work together, which key functions they have inside the FANC core complex and what their function(s) are outside the core complex.

Recent work from Levine's group shows that the FANC pathway might have additional role outside its DNA damage surveillance function. Indeed, in a screen to identify protein involved in autophagy and mitophagy, the authors identified FANCC, FANCF and FANCF as a candidate protein [70]. They finally validate that the entire pathway is required to address the Parkin protein to damaged mitochondria and consequently mediate their degradations [71].

Ubiquitin and the FANC pathway.

The cloning of *FANCD2* [72] increased the understanding of the main, and so far only, biochemical function of the FANC core complex, which is spatially and temporally regulated. FANCD2 is a 160 kDa protein that can be monoubiquitinated on lysine 561 (K561) [73]. Consistent with the concept of the "unity of the FA phenotype", the first reports supported the hypothesis that the inactivation of any FANC core protein leads to the same defect in FANCD2 monoubiquitination. However, only FANCL harbours the RING finger domain that characterizes an E3 ubiquitin ligase, and some punctual mutations of the RING domain impair the ubiquitination of FANCD2 without perturbing the formation of the FANC core complex [74]. Indeed, recent work showed that the essential module for FANCD2/FANCI ubiquitination is formed by FANCL, FANCB and FAAP100, to which FANCT/UBE2T must be added to reconstitute the E2-E3 module. In contrast, disruption of any other FANC core protein leads to a general destabilization of the complex and to an important decrease in the FANCD2/FANCI ubiquitination level without completely abrogating it, challenging the canonical model of unity of the phenotype associated with the FANC core complex inactivation [53]. Interestingly, in DT40 cells, double inactivating mutations in *FANCC* and *USP1* restored the ubiquitination of FANCD2 without restoring the cellular MMC sensitivity but instead aggravating it. These results suggest that the FANC core complex (and its individual components or subgroups as well) may have important roles in the DNA damage response as well as other functions that do not involve regulation of FANCD2/FANCI ubiquitination [53]. The FANC core complex also monoubiquitinates the FANCI protein on lysine 523 (K523) [75].

Optimal monoubiquitination of FANCD2 and FANCI also requires several other proteins as well as PTMs. In particular, it has been reported that ATM-, ATR- and/or CHK1-mediated phosphorylation of FANCD2 and FANCI, as well as several of the FANC core complex proteins, is important for the optimal monoubiquitination of FANCD2 and FANCI [76–78]. FANCD2/FANCI monoubiquitination is also partially dependent on Rad18 activity [79]. Moreover, Rad18 and phosphorylated H2AX (γ-H2AX) are required for the association of FANCD2 and FANCI with subnuclear foci [79,80].

In addition to the spatial distribution of the FANC core proteins, the cell cycle phase is crucial for FANCD2/FANCI monoubiquitination. Indeed, although their protein levels are generally constant through the mitotic cycle, the FANC core complex is considered active only during S phase, as assessed by FANCD2 monoubiquitination, foci formation or relocalisation to locally irradiated nuclear regions [80,81]. The presence of FANCD2 foci directly on chromatids was also reported in cells in G2 and M phases [39,82,83].

In response to genotoxic stress, in S/G2 cells, FANCD2 and FANCI are monoubiquitinated and, in turn, localize to the chromatin, probably in the proximity of a lesion or a stalled replication fork. These two proteins form a chromatin-associated heterodimeric complex, and formation of the complex requires the monoubiquitination of both partners [84]. Overexpression of a FANCD2 protein monoubiquitinated on its C-terminal ameliorated the FA phenotype in DT40 cells, while a mutant of ubiquitin did not [85]. Finally, a fusion between FANCD2 and histone H2B, which constitutionally targets FANCD2 to the chromatin, also restored the resistance to MMC, confirming that the ubiquitination is involved in chromatin localisation and anchoring [85], which is necessary to protect against DNA damage. To date, FANCD2 and FANCI are only known direct targets of the FANC core complex ubiquitin ligase activity [86].

Regulation of the FANCD2-FANCI modifications has been described. The FANC core complex acts as the E3 ubiquitin ligase with UBE2T as the E2 enzyme, which was identified by a yeast two-hybrid screening with a peptide of FANCL [87]. *In vitro*, UBE2T undergoes its own monoubiquitination on lysine K91, inactivating itself. This activity is still not completely clear *in vivo*, although modification of this lysine has been detected *in vivo* by several large proteomic screens [88,89]. Similar

to FANCM, UBE2T is constitutively present in the nucleus, unlike the other proteins of the FANC core complex. Recently, patients harbouring biallelic *UBE2T* mutations resulting in a FA phenotype have been identified, and UBE2T was classified as a new member of the FANC proteins, FANCT [50,51,90]. Another E2 enzyme, UBE2W, has been reported to regulate the monoubiquitination of FANCD2/FANCI specifically in response to ultraviolet (UV) irradiation stress [91], although this is likely a minor role [53]. Ubiquitination is necessary to anchor the dimer to the chromatin. Nevertheless, for an effective response to induced DNA damage, both proteins must undergo ubiquitination/deubiquitination. A siRNA screening identified USP1 as the deubiquitinating enzyme [55], which acts together with UAF1 [92]. The USP1-UAF1 complex directly assembles on FANCI and its SIM domain for deubiquitination of both FANCI and FANCD2. This deubiquitination is essential for an efficient DDR, since USP1 inhibition results in a FA-like cell phenotype showing hypersensitivity to treatment with DNA crosslinking agents [93]. USP1 also deubiquitinates PCNA [94], another key player in the rescue of stalled replication forks, as well as in DNA crosslink repair, recruiting the gap-filling DNA polymerases after the removal of the replication-blocking DNA lesion or during translesion synthesis. Deubiquitination appears to signal the completion of the repair process, allowing replication to restart. Another layer of regulation for FANCD2/FANCI comes from the chromatin itself. *In vitro* studies showed that the structure of the stalled replication forks stimulate FANCI-dependent FANCD2 ubiquitination [95]. It has been also reported that histone H2AX phosphorylation at DNA double strand breaks associated to collapsed replication forks is necessary for the chromatin association of FANCD2 in nuclear foci after a MMC or UVC stress despite a normal level of ubiquitination [80].

Finally, on the basis of a pronounced deficiency in FANCD2 monoubiquitination, two other E3 ubiquitin ligases have been reported to be involved in the regulation of FANCD2 ubiquitination. The first one is BRCA1, recently identified as FANCS, which acts in an unknown manner because biochemical analysis revealed that it did not function as a *bona fide* direct E3 ligase of the FANCD2-FANCI complex [96]. The second one, RAD18, interacts with FANCD2 and is necessary for FANCD2 chromatin localisation [79,97]. Whether altered FANCD2 assembling in the subnuclear foci in RAD18-deficient cells is due to impaired monoubiquitination or impaired relocalisation affecting monoubiquitination has not

yet been established. A better understanding of how these E3 ligases regulate the FANCD2/FANCI complex is an exciting challenge for the future.

Identification of the ubiquitination site on the FANCD2 protein prompted further analysis of the function of the protein in untreated cells, as well as in cells under genotoxic stress. Recent studies have shown that FANCD2 can act independently of its ubiquitination as a transcription factor for NF-κB, particularly at the TNF-alpha promoter [98]. A study from D'Andrea and colleagues suggests that this activity is modified under stress conditions and that the expression of FANCD2-regulated genes are altered after UV-induced DNA damage. This particular pathway also involves the SLX4/FANCP protein, whose localisation is dependent on FANCD2 ubiquitination [99]. Of note, there is no evidence that FANCI can also act as a transcription factor in the cell.

The role(s) of the ubiquitinated FANCD2/FANCI is still under intense investigation. One major function of the ubiquitinated heterodimer is to transport proteins to the right place at the right time. Indeed, this seems to be the case for FAN1 and Tip60 [37,100–104]. Thus, FAN1 would be recruited to chromatin in response to DNA damage to unhook the crosslinks and/or to disentangle replicated DNA, while the FANCD2-mediated chromatin localisation of Tip60 would be required to acetylate histone H4K16, promoting repair of stalled replication fork-associated double-strand breaks (DSBs) by HR. FAN1 and Tip60 are both required to restart replication. Finally, SLX4 is also recruited to DNA damage sites by FANCD2. However, FANCD2 ubiquitination may not be required, despite the fact that the ubiquitin-binding domain of SLX4 appears to be essential for its chromatin localisation [105,106].

In addition to FANCD2 and FANCI, FANCA and FANCG are also modified by ubiquitin. FANCA is ubiquitinated for degradation by the proteasome system, which likely negatively regulates its activity [107] (see below). In contrast, FANCG has K63-branched polyubiquitin chains on potentially three different K residues. Notably, FANCG modification is not essential for the monoubiquitination of FANCD2 and FANCI, but it is required for the interaction of FANCG with the RAP80-BRCA1 complex [108] via a SIM motif in RAP80. The FANCG-RAP80-BRCA1 interaction may be involved in the FANC pathway regulation of HR/NHEJ repair of DSBs

associated with collapsed replication forks in response to DNA crosslinking agents [35–38].

NEDDylation and the FANC pathway.

The monoubiquitination of FANCD2 and FANCI requires an effective FANC core complex and both direct and indirect participation of several other proteins, including proteins associated with the ATR signalling pathway. The FANC core complex does not appear especially efficient: more than 10 proteins work together to monoubiquitinate only two targets, FANCD2 and FANCI. Thus, it has been suggested that the FANC core complex may have other targets whose monoubiquitination deficiency could contribute to explain the FA phenotype [86]. By taking advantage of recent purification methods of ubiquitinated proteins with the anti K—GG antibody, that recognise the moiety remaining at ubiquitin sites after trypsin digestion [88,109–111], we wanted to determine whether there are other possible targets of the FANC core complex and tried to identify them using a comparative mass spectrometry (MS) analysis [112]. FA-A and FA-C cells, after treatment with hydroxyurea a DNA damaging agent. Unexpectedly, in our analysis, only the polypeptides containing the FANCD2 and FANCI monoubiquitinated lysines, K561 and K523, respectively, were found to show significantly reduced modification in both the FA-A and FA-C cells, demonstrating that our approach was able to isolate *bona fide* FANC core complex targets. However, our results invalidated our starting hypothesis and indicated that FANCD2 and FANCI are the only targets of the FANC core complex activity [112].

Nevertheless, we identified several other proteins with a significantly reduced modification in either FA-A or FA-C cells. A first surprise was that several of the proteins identified as differentially modified in absence of FANCA belong to the "membrane receptor" or "membrane-associated protein" categories, which was unexpected for a protein believed to function specifically inside the nucleus during DDR. Our observations represent a further argument in favour of the existence of non-canonical functions unrelated to the FANC pathway for each individual FANC protein [113,114].

As a proof of concept that a FANC core protein could specifically affect the behaviour of proteins or pathways independently of the other FANC core complex partners, we demonstrated that FANCA is involved in the NEDDylation of CXCR5, a

membrane receptor responsible for B-cell migration and germinative centre organisation in response to infection [112]. We demonstrated that a defect in CXCR5 NEDDylation at K339 affects the receptor relocalisation at the cytoplasmic membrane and alters cell migration ability (Figure 3A). To our knowledge, it was the first time that FANCA protein was shown to be involved in a biological process outside the known complex functions. The precise biochemical role of FANCA in this process is still unknown. The impact of a defect in CXCR5 NEDDylation in FA patients has not yet been evaluated. However, in light of the described role of CXCR5 in B-cell migration during infection, it is possible to speculate that a deficiency in NEDDylation could affect the sensitivity to infections reported in FA patients, which has been ascribed to a deficiency in NK cells, a decreased B and T cell response and/or defects in immunoglobulin maturation [115–117].

Interestingly, recent reports described a connection between the FANC pathway and NEDD8, with the latter playing a potential role in FANCD2 regulation. A study reported that inhibition of the NEDDylation cascade by a chemical inhibitor (MLN4924) that targets NAE1, the only NEDD8-specific E1 enzyme, leads to the loss of FANCD2 monoubiquitination in response to treatment with several DNA damaging agents. Consequently, defects in its chromatin relocalisation were observed, ultimately leading to cellular hypersensitivity to MMC. Inhibition of the NEDD8-specific E2 enzyme UBE2M, but not UBE2F, led to a similar phenotype [118]. The authors proposed that a yet unidentified protein modified by NEDD8 plays an important role in FANCD2 ubiquitination, (Figure 3B). However, it is noteworthy that an accurate analysis of the data presented in this paper reveals that NEDDylation inhibition increased the basal, uninduced level of FANCD2 monoubiquitination. Thus, the NEDD8-FANCD2 connection appears to be more complex that originally suspected. Moreover, another group reported that MLN4924 sensitizes tumours to the DNA crosslinking agent cisplatin without impairing FANCD2 ubiquitination [119]. The authors proposed a model in which a defect in the NEDDylation of CUL3, an E3 ubiquitin ligase, resulted in this sensitivity. Indeed, CUL3 targets multiple substrates involved in cell survival, such as proteins in the NF-κB pathway, BCL-2 or BCL-XL. Other in-depth studies of the precise mechanisms leading to sensitivity to crosslinking agents will be required to better understand the role of the FANC pathway in response to NEDD8 inhibition. Nevertheless, these

findings may support the use of both cisplatin and NEDD8 inhibitors in the treatment of cancer to better sensitize the cancer cells to the cytotoxic effects of the chemotherapy.

The fact that the FANC pathway regulates NEDDylation and that, in turn (although at a different level), NEDD8 could regulate the FANC pathway in response to DNA damage must be put into perspective with the fact that NEDD8 is the only ubiquitin-like protein, in addition to ubiquitin itself, that can be observed in the DNA-damage associated nuclear foci [120]. The accumulation of NEDD8 at the DNA damage-associated foci appears to be due to histone H4 modifications mediated by the E2 UBE2M, together with the E3 RNF111/ARKADIA. This NEDDylation step promotes the recruitment of the E3 ubiquitin ligase RNF168, which is downstream of MDC1, as an alternative to the canonical H2A polyubiquitination-dependent pathway [10]. RNF168 activity is required to recruit DNA repair factors, such as BRCA1 and 53BP1. Moreover, the RNF168 protein is itself activated by NEDD8 [121]. Recent studies from our group have shown that the FANC pathway regulates 53BP1 and BRCA1 localisation by acting on histone H4 acetylation by TIP60 [37]. Whether the FANC pathway deficiency also results in altered H4 NEDDylation has yet to be determined. Altogether these results show that a deficiency within the FANC pathway leads to a defect in homologous recombination and an increase of non-homologous end-joining repair. This defect appears to be counteracted by inhibition of the TGFbeta, elevated in FA cells [122].

SUMOylation and the FANC pathway.

After the identification and characterization of the hypomorphic and separation-of-function mutant FANCA I939S [107], it was shown that FANCA undergoes a UBC9/PIAS1-dependent SUMOylation at K921. This modification is a pre-requisite for subsequent RNF4-mediated polyubiquitination of FANCA, which promotes the proteasome-dependent degradation of DNA-damage activated FANCA. Indeed, the FANCA degradation following the UBC9/PIAS1/RNF4 pathway is part of the DNA damage and stalled fork repair process. In other words, FANCA proteasomal degradation is required to turn off the FANC core complex at the damaged chromatin to optimally complete DNA repair and restart halted replication. Accordingly, in siRNA-silencing RNF4 cells, FANCA is not cleared from the chromatin, and the cells show MMC hypersensitivity.

Two groups have recently reported that FANCD2 and FANCI are also modified by SUMO. Indeed, in a proteomics screening of SUMOylated proteins in response to ionizing and UV irradiation, the authors identified FANCI as over-SUMOylated after genotoxic stress [123,124]. Work from Mailand's group showed that FANCD2 and FANCI are both modified by SUMO1/2 in an ATR-dependent manner. This SUMOylation is mediated by PIAS1/4 and promotes the polyubiquitination of the FANCD2-FANCI complex by RNF4. After this modification, FANCD2 and FANCI are targeted by the DVC1-p97 segregase to be released from the chromatin and subsequently degraded by the proteasome or recycled. The ATR-dependent SUMOylation appears to be antagonized by SENP6. Finally, the authors proposed a model in which ubiquitination is required for the FANCD2-FANCI complex to recruit nucleases during crosslink repair, and SUMOylation leads to the unloading of the complex from the chromatin (Figure 3B). This tight regulation avoids a prolonged dangerous localisation of the nucleases to the chromatin. This hypothesis explains why USP1 deficiency, which maintains FANCD2/FANCI monoubiquitination, results in DNA ICL hypersensitivity.

Among the group III proteins, SLX4 is a scaffold endonuclease recruited by ubiquitinated FANCD2 to cooperate with the endonucleases MUS81-EME1, SLX1 and XPF/FANCQ-ERCC1 to remove ICLs from DNA [125] and to resolve untangled/underreplicated DNA at mitosis [40]. SLX4 harbours a SIM domain (SUMO interacting motifs), which is important for the recruitment of the protein to DNA damage sites. This enzyme also has E3 SUMO ligase activity and can therefore modify itself and at list one of its partner: XPF/FANCQ [40,126]. This activity does not appear to be involved in removing DNA interstrand crosslinks but to have a key role in suppressing mitotic defects. The potential E3 SUMO ligase activity of SLX4 is important because even if thousands of proteins are modified by SUMO just few proteins are known to mediate such modification, suggesting that they have a key regulatory role in cell behaviour. Additionally, it has been reported that SLX4-SUMO promotes the localisation of SLX4 to telomeres via an interaction with the TRF2 proteins. The discovery of a SUMO E3 ligase associated with SLX4 is appealing in light of the hypothesis of Mailand's group: FANCD2/FANCI monoubiquitination is required to target endonucleases at the right place and time to resolve the ICL. Thus, SUMOylation by a member of the endonuclease complex to

limit endonucleolytic action as a negative feedback mechanism would be an optimal strategy.

Finally, a DNA damage-induced SUMO modification has also been reported for BRCA1 [127]. The SUMO modification increased the ubiquitin ligase activity of the BRCA1/BARD1 heterodimer involved in the DNA damage response.

Atypical ubiquitin-like modifiers.

UBL5 is an atypical member of the ubiquitin-like family. Indeed, UBL5 is unable to be conjugated to other proteins [128]. However, UBL5 has been shown to be involved in the regulation of the FANC pathway [129]. UBL5 is implicated in the splicing of the FANCI mRNA. Moreover, via a direct interaction with a small fraction of FANCI, UBL5 promotes increased stabilisation of FANCI and, consequently, of FANCD2.

Future directions

At least 85% of FA patients have mutations in one of the FANC core complex genes (60 to 70% are mutated in FANCA), leading to disruption or severe impairment of the E3 ubiquitin ligase activity of the FANC core complex, which is necessary for the monoubiquitination of FANCD2 and FANCI [21]. Apart from the major established role of the FANC core complex in the regulation of the activity of the FANC pathway during DDR, recent studies have suggested that at least some of FANC core complex proteins could also have alternative functions. FANCC seems to be important for the level of STAT1 activation controlling apoptosis [114,130]. It is also appears that the FANC Core complex, independently of its role in response to genotoxic stress, controls the transcription factor activity of FANCD2 [98,99].

It has been shown that several of these functions are associated with regulation via PTMs on proteins in and outside the nuclear compartment. Indeed, several membrane or membrane-associated proteins appear to be post-translationally modified in a FANCA- or FANCC-dependent manner [112]. Moreover, beyond the role of the FANC core complex in the monoubiquitination of FANCD2 and FANCI, the FANC proteins appear to be connected with other ubiquitin-like proteins, such as NEDD8 and SUMO. In light of the many reported abnormalities in both the production and responses of interferons and other pro-inflammatory cytokines in FA, it can be hypothesized that some of these alterations may be associated with

ubiquitin-like proteins, such as FAT10 and ISG15. It should be of interest to study how these small proteins are expressed in FA cells and if some of their targets are modified in FA in a manner that would affect the DDR. Indeed, it has been shown that PCNA is not only modified by ubiquitin and SUMO but also by ISG15 to regulate the recruitment of DNA polymerases [131]. Finally, FA is not only a disease with aberrant DNA repair systems and chromosomal abnormalities but also a pathology involving dysfunction in cell migration, cytokine production, and specific site cancer development. How the defects in the FANC pathway and the PTMs associated with the regulation of this pathway are involved in the development of the complex FA phenotype remains largely unknown.

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Figures Legends

Table 1. The FANC proteins. The alternative name is indicated, if any, as well as the molecular weight of the protein (kilodaltons, kDa), the chromosome location in the human genome and the main function of the proteins. The last column indicates the modifications of the ubl family conjugated to the FANC proteins. The indicated modifications have been reported and validated in the literature. An exhaustive list of potentially modified sites on FANC proteins, i.e., identified in high-throughput proteomics screens, can be obtained by consulting Phosphosite at http://www.phosphosite.org.

Figure 1. The ubiquitin family and the conjugation process. A. A table describing the ubiquitin-like family. The percentage identity compared to ubiquitin is indicated. The number or the name of E1 activating, E2 conjugating, E3 ligase and deubiquitinase (DUB) enzymes is also indicated. **B.** The ubiquitin conjugation process involves activation of the ubiquitin by the E1 activating enzyme, which is dependent on ATP, leading to the transfer of the ubiquitin to the E2 conjugating enzymes. In association with the E3 ubiquitin ligase, the ubiquitin is linked to the substrate at specific lysine sites. Ubiquitin itself can undergo the same process, leading to the formation of long ubiquitin chains. This process is reversible by the action of specific deubiquitinase enzymes. (Ub: ubiquitin, ATP: adenosine triphosphate, AMP: adenosine monophosphate, PPi: pyrophosphate, K: lysine).

Figure 2. The FANC core complex is an E3 ubiquitin ligase targeting FANCD2 and FANCI to coordinate DNA repair. In response to stalled replication forks by DNA lesions (here a crosslink), the FANC core complex is recruited by FANCM-FAAP24 and MHF1 and MHF2. In association with the E2 enzyme UBE2T, the FANC core complex modifies FANCD2 and FANCI with a single ubiquitin, followed by localisation to the lesion and coordination of the repair. After repair, the deubiquitinase enzymes will remove the ubiquitin proteins. The modules forming the FANC core complex are represented in different colours—see text for more details. Sizes of proteins are proportional to their molecular weight.

Figure 3. Crosstalk between ubiquitin-like proteins and the FANC pathway. A. In the cytoplasm, the FANCA protein promotes the NEDDylation of CXCR5 and its traffic to the membrane. **B**. In the nucleus, the NEDDylation cascade involving NAE1 and UBE2M is believed to modify an unknown protein involved in the regulation of FANCD2 ubiquitination and, consequently, the chromatin localisation. FANCD2 and FANCI are modified by SUMO and polyubiquitin chains after DNA repair, promoting release from the chromatin and recycling or degradation of the proteins.

A

	% ID	E1	E2	E3	DUB
Ubiquitin	100	UBE1 - UBA6	~40	>600	~100
NEDD8	58	NAE1	UBE2M - UBE2F	~10	
ISG15	32-33	UBE1L	UBCH6-UBCH8	HERC5	USP18
FAT10	27-36	UBA6	USE1		
Sumo1	14	SAE1-SAE2	UBC9	~15	SENP1-2
Sumo2	13	SAE1-SAE2	UBC9	~15	SENP1-3-5-7
Sumo3	13	SAE1-SAE2	UBC9	~15	SENP1-3-5-7
Sumo4	12	SAE1-SAE2	UBC9		
ATG12	12	ATG7	ATG10		

B

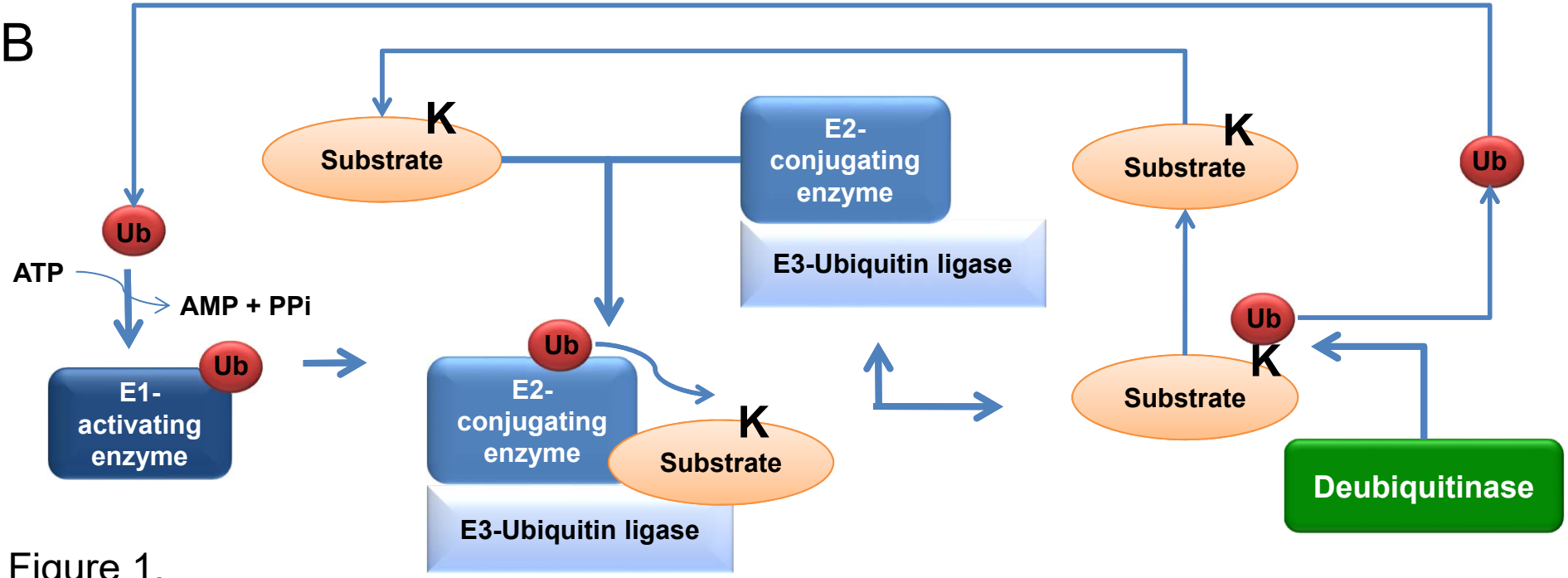


Figure 1.

A

	% ID	E1	E2	E3	DUB
Ubiquitin	100	UBE1 - UBA6	~40	>600	~100
NEDD8	58	NAE1	UBE2M - UBE2F	~10	
ISG15	32-33	UBE1L	UBCH6-UBCH8	HERC5	USP18
FAT10	27-36	UBA6	USE1		
Sumo1	14	SAE1-SAE2	UBC9	~15	SENP1-2
Sumo2	13	SAE1-SAE2	UBC9	~15	SENP1-3-5-7
Sumo3	13	SAE1-SAE2	UBC9	~15	SENP1-3-5-7
Sumo4	12	SAE1-SAE2	UBC9		
ATG12	12	ATG7	ATG10		

B

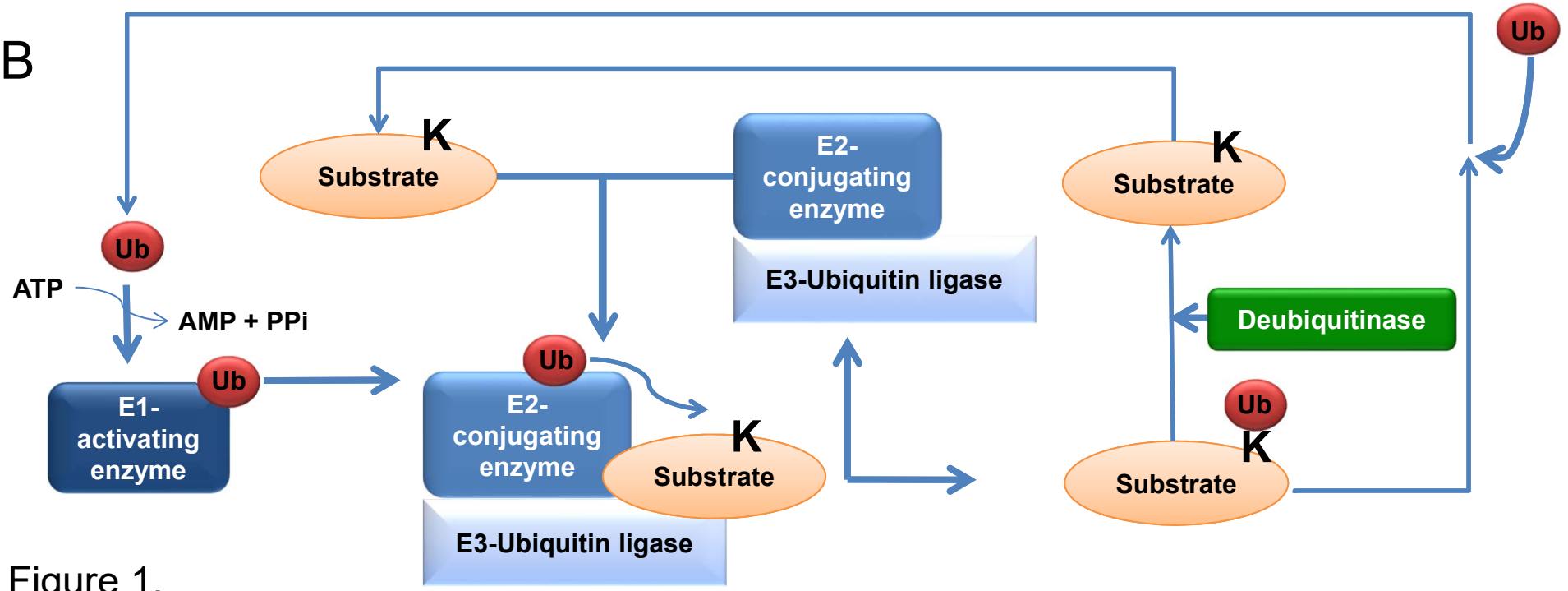
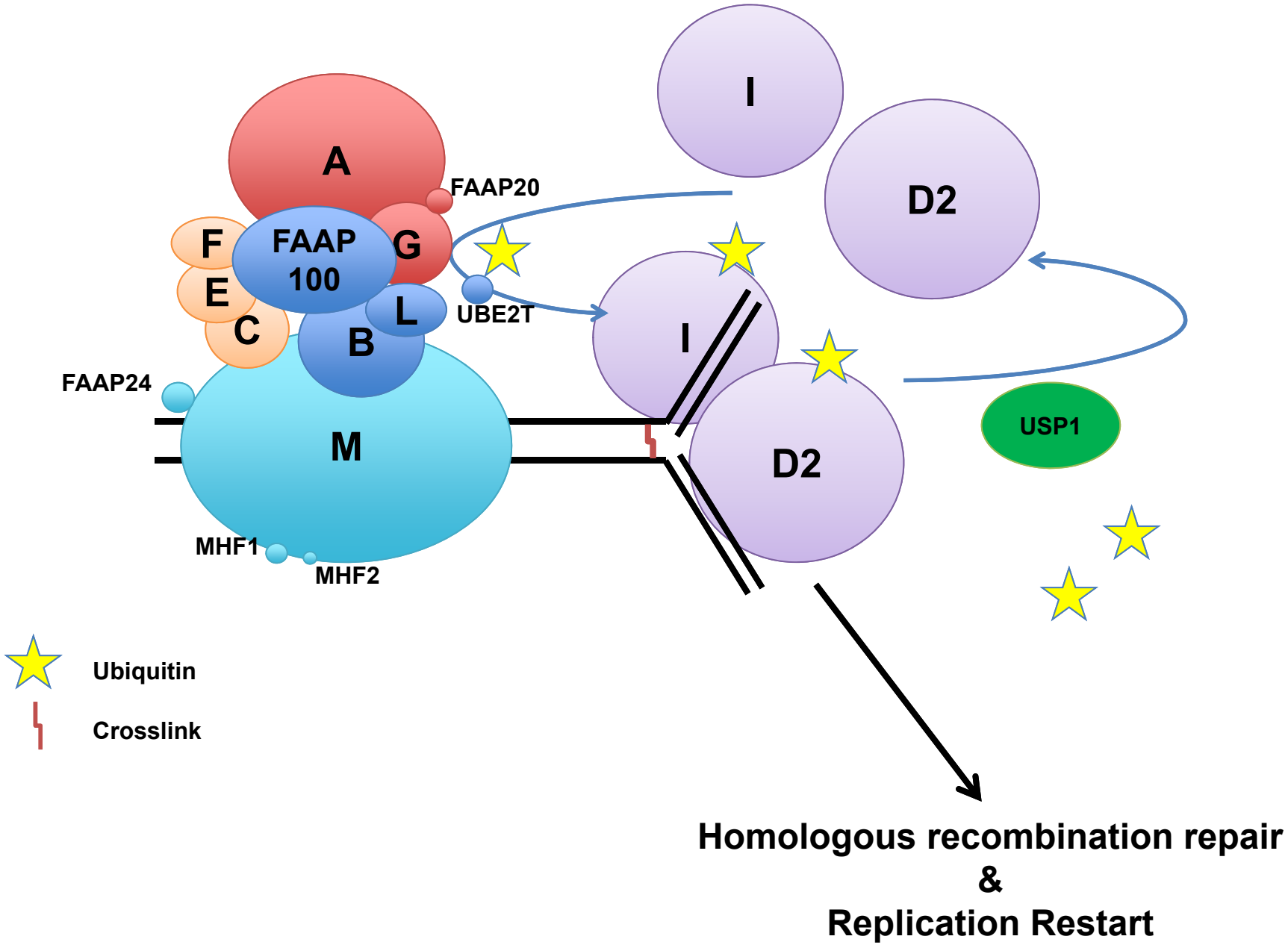


Figure 1.


A
FAAP20
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FAAP24
M
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D2
I
D2
USP1
Ubiquitin
Crosslink
Homologous recombination repair
&
Replication Restart

Figure 2.

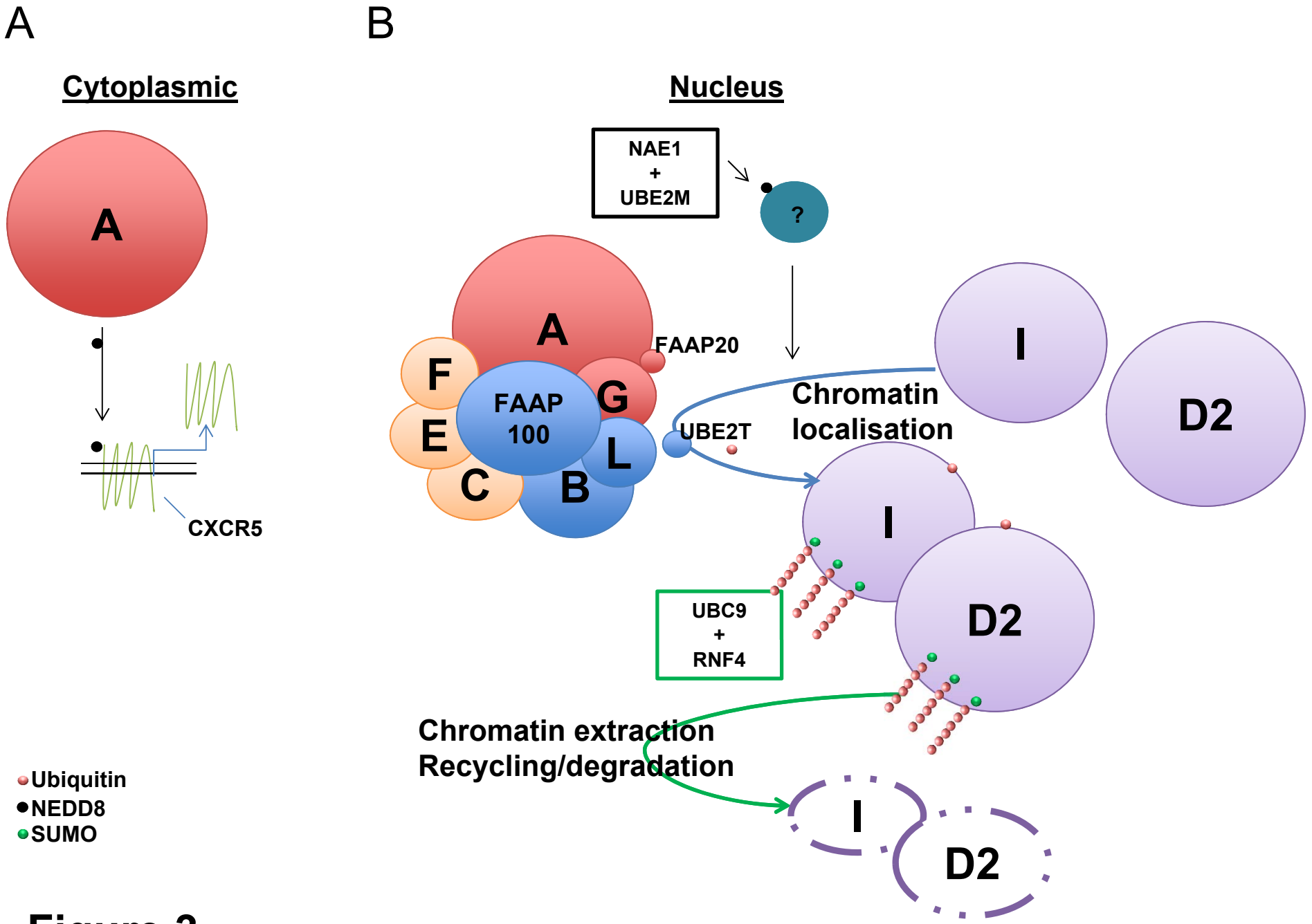
A
B
Cytoplasmic
A
CXCR5
Nucleus
NAE1
+
UBE2M
?
A
FAAP20
F
G
FAAP
100
E
L
C
B
UBE2T
I
D2
Chromatin
localisation
I
D2
UBC9
+
RNF4
Chromatin extraction
Recycling/degradation
I
D2
Ubiquitin
NEDD8
SUMO

Figure 3.

FANC proteins	Molecular Weight (kDa)	Chromosome localisation	Function	Modification
FANCA	163	16q24.3	FANC Core	Ubiquitin SUMO
FANCB	95	Xp22.31	FANC Core	-
FANCC	63	9q22.3	FANC Core	-
FANCD1/BRCA2	380	13q12.13	Homologous Recombination	-
FANCD2	162	3p25.3	DNA damages signalling Replication forks Transcription factor	Ubiquitin SUMO
FANCE	60	6p21.22	FANC Core	-
FANCF	42	11p15	FANC Core	-
FANCG	68	9p13	FANC Core	Ubiquitin
FANCI	140	15q25.16	DNA damages signalling Replication forks	Ubiquitin SUMO UBL5
FANCJ/BRIP1	140	17q22-q24	Helicase	-
FANCL	43	2p16.1	FANC Core E3 ubiquitin ligase	-
FANCM	250	14q21.3	Anchor the FANC Core to chromatin translocase	-
FANCN/PALB2	130	16p12.1	Homologous Recombination	-
FANCO/RAD51C	42	17q25.1	Homologous Recombination	-
FANCP/SLX4	200	16p13.3	Endonuclease Transcription factor Potential E3 SUMO ligase	SUMO
FANCQ/XPF	101	16p13.12	Endonuclease	SUMO
FANCR/RAD51	37	15q15.1	Homologous Recombination	-
FANCS/BRCA1	207	17q21.31	E3 Ubiquitin ligase Homologous recombination	SUMO
FANCT/UBE2T	23	1q32.1	E2 conjugating Enzyme	Ubiquitin
FANCU/XRCC2	32	7q36.1	Homologous Recombination	-