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Regulation and Targeting of the FANCD2 Activation in DNA Repair

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Regulation and Targeting of the FANCD2 Activation in DNA Repair

by

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DEDICATION

I dedicate my work to Binka, Mima, my mom, my dad, my sister Cote, and Tom. Binka for being my furry study partner on those long nights studying and writing while everyone else in the house was asleep and for being my nap partner when I had the time to be lazy. To my lovely Mima for being the first person to encourage me to pursue the field of molecular biology and for being the best penpal I could have asked for; you are one of the strongest woman I know and you inspire me to work hard for what I want every day. To my parents for supporting me since I was little and always encouraging me to do my best. To my mom because even though she is far away she is one of the strongest supporters I have, and for always being available to talk on the phone for hours. To my dad for teaching me that I always have to give everything I did my 100%, for cooking for me when I was busy studying, and for always putting up with my moods. Thank you Dad. To my sister for being my confidant and counselor, for providing me with guidance when I was not sure where I was standing or where to go. And to Tom, even though you came in late in my Master's career, you have been such an inspiration and a supporter. You always know how to cheer me up and what to say so I can work for a bit longer even though all I want to do is nap.

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ABSTRACT

Fanconi anemia (FA) is a genome instability syndrome that is clinically manifested by bone marrow failure, congenital defects, and elevated cancer susceptibility. The FA pathway is known to regulate the repair of DNA interstrand crosslinks in part through DNA homologous recombination (HR) repair. Up to today 16 FA proteins have been discovered that may participate in the common pathway. Cells that have mutations in the FA genes are hypersensitive to DNA damaging agents and display chromosome instability. A key regulatory event in the FA pathway is monoubiquitination of FANCD2-FANCI heterodimer that is mediated by a multi-component E3 ubiquitin ligase complex called FA core complex. Current model suggests that once the FANCD2-FANCI heterodimer is monoubiquitinated it relocates to chromatin where it interacts with other key repair proteins to facilitate DNA repair. More than 90% of the FA cases are presumed to be associated with defects in the monoubiquitination reaction, suggesting the significance of the modification in the pathogenesis of the disease. Despite the significance, the molecular interplay between the FA core complex and the FANCD2-FANCI heterodimer remains enigmatic. We are interested in the assembly mechanism of the various FA subcomplexes into the core complex, and we are actively investigating how the FANCD2-FANCI heterodimer is

recruited to these putative subcomplexes. As the FA pathway is a crucial determinant for cellular resistance to DNA damaging agents, there have been hypotheses that disruption of this pathway may be beneficial in enhancing chemosensitivity of certain cancer cells. In collaboration with Dr. Cai's chemistry lab, we will develop a screen platform to identify a small molecules to interrupt the monoubiquitination reaction. Completion of these studies will enhance the much-needed knowledge of the key enzymatic reaction in the pathway, and perhaps the information can be used for development of novel chemotherapeutic strategies.

CHAPTER ONE: INTRODUCTION TO UBIQUITINATION

Ubiquitin as a Cellular Regulator

Ubiquitin is a small, globular protein that has 76 amino acids that gets conjugated to other proteins and regulates the cell's biological processes like transcription, DNA repair, protein degradation, endocytosis, inflammatory responses, and differentiation [1, 2]. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) that can be connected to each other in order to form a polymer. An ubiquitin polymer (polyubiquitin chain) is formed by the creation of an isopeptide bond in between the ε-amino group of an ubiquitin's lysine and glycine 76 of another ubiquitin. Monoubiquitin and polyubiquitin chains are conjugated to the substrate protein's lysine, specifically the ε-amino group, or to the protein's extreme N-terminus [3]. Proteins that interact and recognize ubiquitin have ubiquitin binding domains (UBDs) within their structures [4]. There are over 20 different UBD families, with over 200 proteins that have been discovered to have UBDs in their structures [5]. Ubiquitin is added to the substrate protein by an ATP-dependent hierarchical cascade of reactions: An ubiquitin is noncovalently adenylated to the C-terminus of the E1 activating enzyme as an ubiquitin donor, this ubiquitin is then transferred to a cysteine in the E1;

this second ubiquitin forms a thiol ester bond with the E1 [2]. E1 then transfers the ubiquitin to a cysteine residue of an E2 ubiquitin-conjugating enzyme, this bond is a thioester bond [2]. E2 enzymes have a UBC (ubiquitin conjugating) domain that is essential for the conjugation of ubiquitin [6]. Once the E2 is charged it interacts with an E3 protein ligase that is bound to the substrate to be ubiquitinated (figure 1).

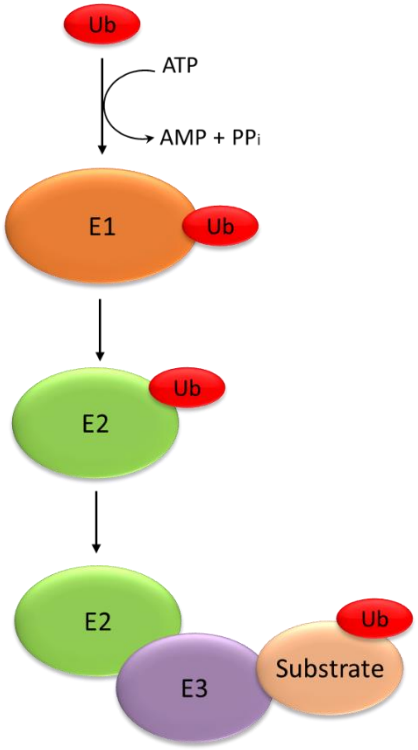


Figure 1. Overview of the E1, E2, and E3 interaction for the ubiquitination of a substrate. Ubiquitin is added to the E1 in an ATP dependent manner. The E1 then charges the E2, which interacts with the E3 and ubiquitinates the substrate (Adapted from Hicke et al., 2005 [1]).

E3 ligases can belong to two different families: RING (really interesting new gene) E3 enzymes and HECT (homologous to the E6-AP carboxyl terminus) domain E3 enzymes. The RING domain contains histidine and

cysteine residues in specific patterns with zinc ions bound by them. The HECT domain has two lobes formed of approximately 350 residues. E3s that are from the HECT family interact with the ubiquitin charged E2 through their N-terminus and their catalytic site is on the C-terminus [2, 7]. In the human genome there are two E1 activating enzymes, over 40 E2 UBC enzymes, and over 600 E3 ligases [6] (figure 2).

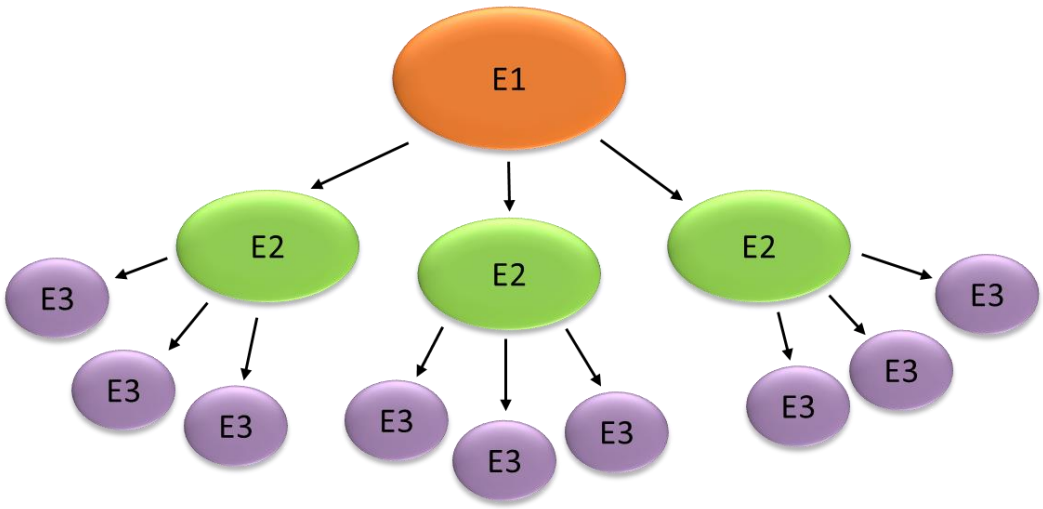


Figure 2. The hierarchy of the ubiquitin conjugating cascade. One E1 catalytic enzyme can interact and charge several E2 ubiquitin-conjugating enzymes. Each E2 enzyme can then interact with more E3 ligases (Adapted from Hicke et al., 2005 [1]).

Polyubiquitination vs. Monoubiquitination

A polyubiquitin chain of four ubiquitins or more on lysine 48 of a protein will lead to its degradation by the 26S proteasome [8]. The 26S proteasome contains 3 subunits: one 20S subunit and two 19S subunits. The 20S proteasome is a hollow cylinder composed of four rings that are stacked on top of each other, with the active site facing the inside of the tube. The proteins have to be denatured before entering the chamber through a

narrow opening on the ends of the 20S proteasome [9]. To each end of the 20S proteasome binds a 19S complex, this complex recognizes the polyubiquitination signal, unfolds the targeted protein, and activates the 20S proteasome for proteolysis [9] (Figure 3). Not every polyubiquitinated protein is destined to be broken down by the proteasome. It is all subject to the type of polyubiquitin chain, for instance polyubiquitin chains on lysine residue 63 can activate kinases [10].

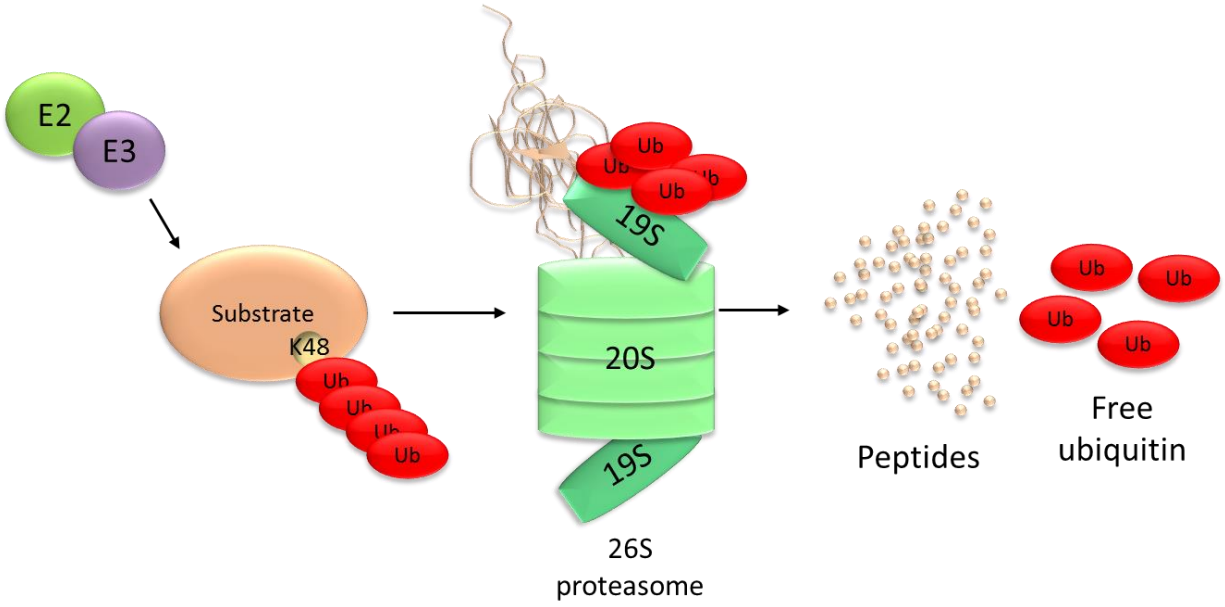


Figure 3. Protein proteolysis by the 26S proteasome after substrate polyubiquitination. One E1 catalytic enzyme can interact and charge several E2 ubiquitin-conjugating enzymes. Each E2 enzyme can then interact with more E3 ligases (Adapted from Hicke et al., 2005 [1]).

Monoubiquitination is the addition of only one ubiquitin per lysine residue to the protein. Some proteins can be monoubiquitinated on several sites. One example of how monoubiquitination regulates proteins is by the breakdown of plasma membrane proteins that can be triggered through

monoubiquitination, for example the monoubiquitination triggers the endocytosis of the protein, which later gets degraded in the lysosome [11]. Another example is the ubiquitination on lysine residue 119 of histone H2A, which can change the chromosome structure and the transcription of genes (i.e. gene silencing) [12]. During DNA damage repair the protein Proliferating Cell Nuclear Antigen (PCNA) gets monoubiquitinated by the E2 Rad6 and the RING E3 RAD18; this monoubiquitination leads to the recruitment of several DNA polymerases to the site of the lesion for the repair of the DNA [12]. Monoubiquitination of a protein can lead to different outcomes: changing the localization of the protein, assembly or disassembly of protein complexes, and altering the structure of the protein or the protein complex [4].

Deubiquitinating Enzymes

The addition of ubiquitin can be reversed and this process is dependent on deubiquitinating enzymes (DUBs). There are around 100 known DUBs in the human genome [12]. DUBs can be categorized into five different families (four thiol ester proteases and a metalloprotease): the ovarian tumor proteases (OTUs), ubiquitin-specific proteases (USPs), the Josephins, ubiquitin C-terminal hydrolases (UCHs), and the zinc-dependent metalloproteases [12]. The role of DUBs in the cell is almost as important as

that of the phosphatase. DUBs can inhibit proteolysis or promote it, they can change the localization of a specific protein, remodel a protein's structure, and even directly interact with an E3 ligase thus regulating it [13].

CHAPTER TWO: INTRODUCTION TO FANCONI ANEMIA AND THE FANCONI ANEMIA REPAIR PATHWAY

Clinical Aspects and Treatment of Fanconi Anemia

Fanconi anemia (FA) is an autosomal or X-linked genetic disorder that causes chromosomal instability in the cell which leads to several congenital defects and a high cancer incidence in FA patients. It has been estimated that one in 300 people are carriers of a FA mutation while the disorder itself is seen in one in 300,000 births [14]. Even though some patients have been diagnosed at 48 years old, the median age for the diagnosis of FA in girls is eight years and six point five in boys. In 2000, the median age for the death of FA patients was 30, compared to 19 in 1990. This number has probably increased due to medical advances which could have led to early diagnosis and better therapies once the disease has been confirmed [15]. A higher carrier frequency of 1:100 was found in Ashkenazi Jews, Afrikaaners, and the Romani people in Spain (gitanos) [16]. The most common congenital defect in FA patients is skin abnormalities (55%) followed by short stature (51%) even though 25% to 40% of FA patients do not have any physical abnormalities [15]. Bone marrow failure will usually appear during the

patient's first ten years of life. 90% of FA patients have bone marrow problems by the time they turn 40 years old, patients with FA-complementation group C (FANCC) mutated have the highest incidence of it. 28% of FA patients that are 40 years old develop solid tumors, with females having a higher incidence of them [17]. The most prevalent cancer in FA patients is acute myeloid leukemia which is seen in at least 20% of FA patients. The risk of a FA patient to develop acute myeloid leukemia is 800-fold higher compared to a healthy individual [18]. Other cancers that can affect FA patients are gynecological squamous cell carcinoma, neck and head squamous cell carcinoma, esophageal carcinoma. FA patients also develop tumors in the skin, brain, liver, and kidneys [19].

One of the diagnostic tests for Fanconi anemia is the chromosomal breakage test done with an interstrand crosslinking (ICL) agent, usually mitomycin C (MMC) or diepoxybutane (DEB). Cells from FA patients are hypersensitive to ICL agents and accrue DNA damage very fast leading to breaks in the chromosomes [20, 21]. Another diagnostic test for FA that is done in conjunction with the DEB test is the assay of the monoubiquitination of FANCD2 in the patient's primary lymphocytes. If the monoubiquitination of FANCD2 does not occur in these cells, the transfer of FA genes by retroviral infection is performed, if the transcription of any of the FA proteins rescues the monoubiquitination of FANCD2 then the diagnosis of FA can be made [22].

Patients with FA receive androgen therapy in order to treat the bone marrow failure but hematopoietic stem cell transplant is the main treatment for bone marrow failure in FA patients, even though it does not treat the high cancer incidence of FA patients. The chemoradiation used in the HSCT can be detrimental for the FA patients due to the defective DNA repair in their cells so a non-irradiation immunosuppressive technique is preferred for the conditioning portion of the treatment [18]. Since FA patients cannot undergo any type of chemotherapy or radiation for the treatment of cancer they have to get regular check-ups in order to preclude the cancer before it can develop [18].

Fanconi Anemia Pathway

The Fanconi anemia pathway integrates different repair pathways, nucleotide excision repair (NER), translesion synthesis (TLS), and homologous recombination (HR), in order to repair and resolve these lesions in the cell's DNA [23]. Up to this date there has been 16 proteins that have been discovered to have mutations in FA patients (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, and FANCQ). Out of the 16 FA proteins, eight of them make up the FA core complex, which is activated upon DNA damage [24]. An overview of the proteins of the FA core complex, FANCD2, FANCI, and other key proteins of the FA pathway are discussed below (table 1).

Table 1. Summary table of the proteins involved in the Fanconi anemia pathway. (Adapted from Crossan et al., 2005).

Protein	Other names	Function in repair	Source
FANCA		Core complex; A-G-20 Subcomplex	[25]
FANCB		Core complex; B-L-100 Subcomplex	[25]
FANCC		Core complex; C-E-F Subcomplex	[25]
FANCD1	BRCA2	RAD51 interaction; homology search in HR	[26]
FANCD2		Core complex substrate; Recruits downstream repair factors	[24, 25]
FANCE		Core complex; C-E-F Subcomplex; interacts with FANCD2	[25, 27]
FANCF		Core complex; C-E-F Subcomplex	[25]
FANCG		Core complex; A-G-20 Subcomplex	[25]
FANCI		Core complex substrate	[24, 25]
FANCJ	BRIP1	5' to 3' DNA helicase	[28]
FANCL		Core complex; E3 Ring; B-L-100 Subcomplex	[25]
UBE2T		E2 of the FA pathway, monoubiquitinates FANCD2	[29]
FANCN	PALB2	Interacts/stabilizes BRCA2	[30]
FANCM		Core complex; Recruits FA core complex to site of damage	[25, 31]
FAAP24		Core complex; Recruits FA core complex to site of damage	[25, 31]
FANCO	RAD51C	RAD51 homologue	[32]
FANCP	SLX4	Nuclease scaffold for XPF-ERCC1 and MUS81-EME1	[33]
ATR		Phosphorylates FANCD2 and FANCI. Activates FA pathway	[34]
ATM		Phosphorylates FANCD2 after IR	[35]
BRCA1		Recruits FANCD2 to γH2AX	[36]
RAD51		Searches for homology on the sister chromatid	[30]
CtIP		Stabilizes FANCD2; interacts with MRN complex and BRCA1	[37]
PCNA		Necessary for FANCD2 monoubiquitination	[38]
FAN1		5' flap endonuclease and 5' to 3' exonuclease	[35]
XPF-ERCC1		3' flap endonuclease; unhooks crosslinker	[39]
MUS81-EME1		Processes ICLs to DSBs	[40]
MRN Complex		Stabilizes FANCD2 and localizes it to DNA damage site	[41]

The Fanconi Anemia Core Complex

As stated above, eight of the 16 FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) and four Fanconi anemia associated proteins (FAAPs – FAAP16, FAAP20, FAAP24, FAAP100) form a core complex [25]. This core complex acts as a multi-subunit E3 ligase, with FANCL acting as the E3 catalytic ligase subunit. Even though the functions of many of the FA core complex proteins are still unknown, studies have suggested that there are sub-complexes within the FA core complex: FANCB/FANCL/FAAP100 (B-L-100) which contains the E3 ligase unit FANCL as previously stated, FAAP100 a 100kDa polypeptide which is thought to stimulate FANCL [25, 42], and FANCB whose gene resides in the X chromosome and gives the X-inheritance pattern to the disorder and it is suspected to stabilize FANCM [31, 43]; FANCA/FANCG/FAAP20 (A-G-20) which has been shown to bind to bind to chromatin and DNA through the UBZ domain of FAAP20 and through the TPR repeats of FANCG, and FANCA which has been found in 66% of all of FA patients [44]; FANCC/FANCE/FANCF (C-E-F) which is suggested to be the sub-complex necessary for the physical interaction in between the FA core complex and FANCD2-FANCI. FANCE has been shown to be the FA core complex substrate adaptor and it interacts directly with FANCD2 [27], FANCC has been shown to be mutated in 12% of all FA patients [44] , and the N-terminus of FANCF has been proven to interact with the MM1 (FANCM motif 1) motif of FANCM

and it is suspected to act as a mediator that can interact with the other sub-complexes making it the central connector of the FA core complex [45].

FANCM and FAAP24

As stated previously, FANCM is also a protein of the FA core complex. FANCM has DNA binding activity, it specifically binds to stalled replication forks and as of this moment it is the only known protein in the FA core complex with this capability, it has been shown that FANCM can bind double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) *in vitro* [45]. FANCM is homologous to the Hef protein (helicase-associated endonuclease for fork-structured DNA) and has a Superfamily 2 (SF2) helicase domain that is a DEAH helicase domain at its N-terminus and with FAAP24 part of the XPF endonuclease family. On the C-terminus FANCM contains an excision repair cross complementation group 4 (ERCC4) endonuclease domain [46]. The DEAH helicase domain is the domain that contains the ATPase activity and DNA binding properties of FANCM [47]. The C-terminus' ERCC4 endonuclease domain is actually inactive due to some amino acid variations in the catalytic section of FANCM's ERCC4 compared to other proteins' active ERCC4 nuclease domains [48]. It is through the C-terminus that FANCM interacts with FAAP24. FAAP24 (Fanconi anemia-associated protein of 24kDa) also contains an inactive ERCC4 domain that has affinity for ssDNA [31]. It is speculated that together, FANCM and FAAP24, sense and

recognize stalled replication forks and direct the FA core complex to the site of DNA damage during S-phase [49]. Both of them, FANCM and FAAP24, contain an HhH (helix-hairpin-helix) motif in their C-terminus, this region being essential for the binding to DNA in a sequence-nonspecific manner [49].

FANCM has been shown to be phosphorylated by ATR after DNA damage. The phosphorylation of FANCM intensifies its affinity to chromatin [50]. On the other hand, hyperphosphorylation of FANCM during mitosis that is induced by Plk1 leads to its inactivation, dissociation from the FA core complex, and degradation, which explains why there is no active FA pathway during mitosis [51]. After FANCM's hyperphosphorylation event, β-TRCP (part of the SCF E3 ligase that is active during mitosis) recognizes FANCM and the serine residues of its DSGYNS sequence and triggers the degradation of FANCM [51].

Interestingly, FANCM links Bloom's Syndrome (BS) and FA by interacting with RM1 and topoisomerase IIIα of the Bloom complex [45]. It has been shown that FANCM interacts with these proteins through its MM2 motif, and that this motif is indispensable for its interaction with the FA core complex, through FANCF as it was stated previously, and the monoubiquitination of FANCD2 [45].

FANCL and UBE2T

FANCL is another protein of the FA core complex that has a specific activity within the complex. FANCL acts as the E3 ubiquitin ligase of the FA pathway [52]. FANCL contains a RING domain at its C–terminus, it is through this domain that it interacts with UBE2T [6] (figure 4). The RING

Figure 4. Crystal structure of UBE2T and the RING domain of FANCL. Ribbon model of the crystal structure of UBE2T in blue and the RING domain of FANCL in purple which interacts with UBE2T. Their termini are labeled for clarification. PDB file downloaded from: http://www.rcsb.org/pdb/explore.do?structureId=4CCG - 3D image created with Swiss-PdbViewer 4.1.0.

domain of FANCL contains a slight different amino acid sequence that is seen in most RING domains; FANCL's RING domain has two zinc atoms that are in between four cysteine residues, one histidine residue, and three cysteine residues (compared to three cys, one his, and four cys)[6]. On the N-

terminus FANCL contains three WD-40 domains that are arranged in a RWD-like domain. The WD-40 domains have been shown to stimulate the activity of FANCD2 and to increase the monoubiquitination of FANCD2 [53]. UBE2T is the only known E2 of the FA pathway, it contains a UBC-homology domain that catalyzes the ubiquitination event, and its active site is Cys86 where E1 transfers a ubiquitin molecule to [29]. UBE2T has also been shown to become monoubiquitinated at Lys91 this monoubiquitination serves as a negative auto-regulation event that inactivates UBE2T and it is enhanced by FANCL [29].

FANCD2 and FANCI Structure

FANCD2 is the key protein the FA pathway and its monoubiquitination is the main event of the FA pathway. 5% of FA patients have a FANCD2 mutated and the incidence of a FANCI mutation is even lower [21]. Most studies use the monoubiquitination of FANCD2 in order to study whether the FA core complex is functional or not. Monoubiquitination of FANCD2 occurs during S-phase and after the cell has been exposed to DNA damaging agents like MMC, cisplatin, radiation (UV), and ionizing radiation [44]. FANCD2 and FANCI form a heterodimer complex that is phosphorylated by ATR (ataxia telangiectasia and Rad3-related) and monoubiquitinated by the FA core complex [34]. The crystal structure of the FANCD2-FANCI complex has been purified (figure 5).

FANCD2 has only four domains that have been discovered up to date. Montes de Oca et al. demonstrated that FANCD2 has a span of 24 amino acids at its C-terminus that is necessary for ICL repair but not monoubiquitination or chromatin localization after DNA damage. This segment is conserved in rodents and humans and it has been named EDGE due to the amino acid sequence within it [54]. FANCD2 also contains a PIP-motif (PCNA-interacting protein) in its middle section [38]. It is through this motif that it physically interacts with PCNA, they both co-localize to the nucleus after DNA replication has been inhibited [38]. The PIP-motif and the interaction in between FANCD2 and PCNA are both necessary for the monoubiquitination of FANCD2 following DNA damage but not for FANCD2 localization to chromatin, its phosphorylation by ATR, or its interaction with FANCE [38]. In its N-terminus FANCD2 has a CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation) UBD [55]. This domain has been shown to be necessary for the interaction in between FANCD2 and FANCI and their co-localization to chromatin [55]. FANCD2's CUE UBD interacts with ubiquitin on lysine523 of FANCI in a noncovalent way. This interaction is dispensable for the monoubiquitination of FANCD2 but it somehow does stabilize it and prevents it from being degraded by the proteasome [55]. Further down the N-terminus FANCD2 has a NLS (nuclear localization signal) that is necessary for the monoubiquitination of FANCD2,

FANCD2 and FANCI movement into the nucleus, and for the repair of ICL on the cell's DNA [56] (figure 6).

FANCD2 has been shown to have affinity for dsDNA ends and Holliday junctions, one of the only known biochemical properties FANCD2 has [55]. FANCD2 and FANCI interact with each other through a ~560 amino acid region along the middle of both proteins in an antiparallel manner with their ends remaining free [34] (figure 5). FANCD2 monoubiquitination site is lysine 561 and FANCI monoubiquitination site is lysine 523, interestingly these sites are in the interface of the two proteins but their side chains are still solvent exposed even though the channels are too small for UBE2T interaction [34]. FANCD2 and FANCI both are comprised of mainly alpha-helices that are organized in pairs antiparallel to each other that form α-solenoids [34]. FANCD2 and FANCI do not share homology in their whole structure but mainly through the solenoid segments. Solenoid 2 of both proteins share the most homology, this is also the segment where both proteins have their monoubiquitination sites [34]. It is known that the FA core complex and the monoubiquitination of FANCD2 are necessary for the monoubiquitination of FANCI but functional and monoubiquitinated FANCI is not necessary for FANCD2 monoubiquitination or essential for DNA repair [57]. What is still not known about these two proteins is whether they come as a heterodimer complex to the FA core complex or as separate proteins. It is also not known what the monoubiquitination of the proteins does to the

complex, it might separate or bring together the proteins, or just change the complex conformation, this issue is discussed in more detail further down.

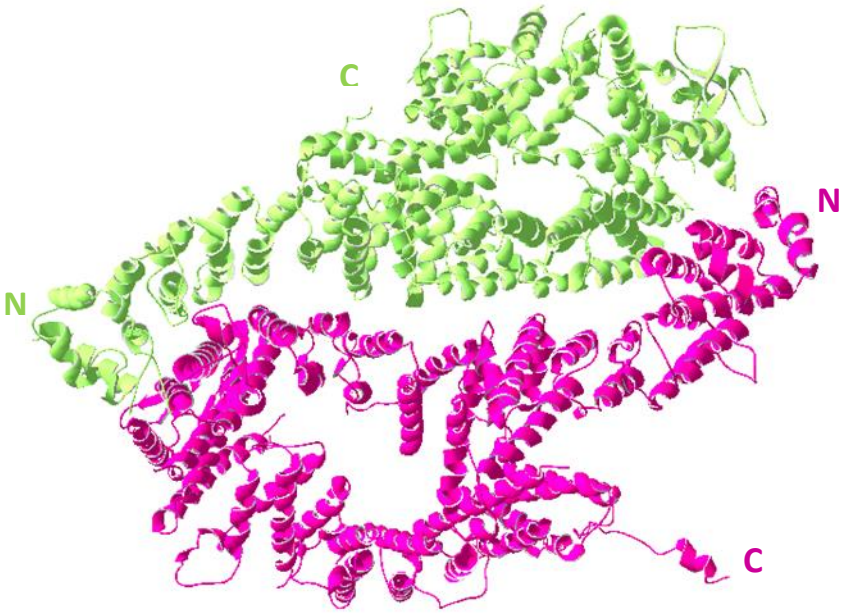


Figure 5. Crystal structure of the FANCD2 and FANCI complex. Ribbon model of the crystal structure of FANCD2 in green and the FANCI in pink. Their termini are labeled for clarification. PDB file downloaded from: http://www.rcsb.org/pdb/explore.do?structureId=3s4w - 3D image created with Swiss-PdbViewer 4.1.0.

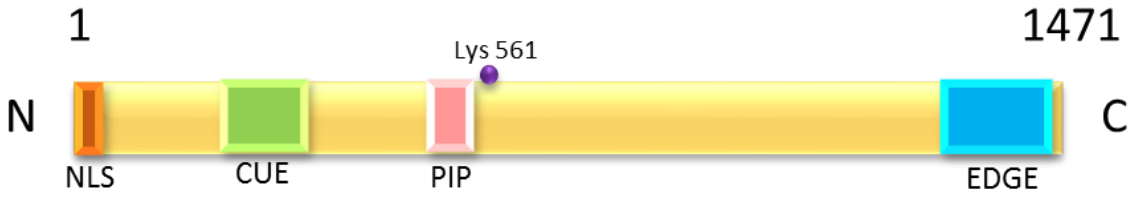


Figure 6. Schematic depiction of the domains of FANCD2. FANCD2's NLS is at the N-terminus, followed by the CUE domain around amino acid 222 and the PIP box, lysine residue 561 that undergoes monoubiquitination is highlighted, and at the C-terminus is the EDGE domain (Adapted from Boisvert et al., 2014 [56]).

FANCD2 Regulation and Interacting Proteins

On top of being monoubiquitinated, FANCD2 and FANCI are also phosphorylated. As stated before the phosphorylation of both is dependent on ATR [34]. FANCI contains six S/TQ motifs that are phosphorylated; two of the most important sites are residues serine 558 and serine 561, both of them are close to lysine 523 where FANCI gets monoubiquitinated [57]. The phosphorylation of FANCI is necessary for the monoubiquitination of FANCD2 and its own monoubiquitination, making it a key step in activating the FA pathway [58]. Another key step in activating the FA pathway through FANCD2 monoubiquitination is the phosphorylation of FANCD2 by ATR on threonine 691 and serine 717 [56]. Sareen et al. have demonstrated that the phosphorylation of FANCI leads to the dissociation of FANCD2 and FANCI and that without it FANCD2 does not become monoubiquitinated nor does it localize to chromatin [59]. The results of Sareen's work suggest that the FANCD2-FANCI complex is the inactive form of the proteins and that they can perform their functions in the DNA damage repair only after they have dissociated from each other. This model proposed by Sareen et al. would justify the size of the small channels that are around the ubiquitination sites when FANCD2 and FANCI are in a complex since the phosphorylation event and the dissociation of the proteins would leave the necessary lysine residues readily available for monoubiquitination by UBE2T [56].

In addition, FANCD2 can get phosphorylated by ATM (ataxia telangiectasia mutated) on serine 222 in response to ionizing radiation (IR) in order to activate the S-phase checkpoint and it is not dependent on FANCD2 monoubiquitination [35]. ATM phosphorylation of FANCD2 is not necessary for its monoubiquitination like phosphorylation by ATR is. Foci formation, and MMC sensitivity are also not dependent on ATM phosphorylation since ATM (-/-) cells still see an increase of FANCD2 monoubiquitination, FANCD2 foci formation, and MMC resistance [35, 54].

After FANCD2 gets monoubiquitinated by the FA core complex and UBE2T, it co-localizes to chromatin [54] where it interacts with several proteins. It is speculated that the role of FANCD2 in the DNA damage response is to act as a regulator and a landing pad for other downstream proteins. Garcia-Higuera et al. have seen co-localization of monoubiquitinated FANCD2 and BRCA1 (breast cancer associated 1) after DNA damage during S-phase [36]. The interaction of BRCA1 with monoubiquitinated FANCD2 is an indispensable step for the recruitment of FANCD2 to γH2AX [60]. H2AX gets phosphorylated on serine 139 by ATR when stalled replication forks form after UV damage and as a result of this, monoubiquitinated FANCD2 relocates to the site of damage on the chromatin [60].

BRCA2 (breast cancer associated 2), known as FANCD1, also interacts with FANCD2 [26]. BRCA2 and FANCD2 interact through the C-terminus of

BRCA2, and this interaction is necessary for the co-localization of FANCD2 and BRCA2 to chromatin, but not for the foci formation of the two proteins [26]. It is important to point out that the monoubiquitination of FANCD2 is necessary for these interactions since the interaction of the two happens once FANCD2 has been targeted to chromatin [26]. After BRCA2 has been loaded onto the chromatin with the help of FANCD2 it stabilizes the replication fork and it mediates RAD51 foci formation on ssDNA after DNA damage [61, 62]. RAD51 is a homologue of RecA that is required for homology directed repair (HDR), it co-localizes with BRCA2 after the cell has been exposed to IR and during S-phase, and with FANCD2 during S-phase [63]. RAD51 binds to processed ssDNA forming a nucleoprotein filament and searches for homology in the sister chromatid in order to repair the damaged DNA [30].

As stated before another protein that interacts with FANCD2 is PCNA, the details of the interaction were discussed in the previous section. It is important to point out though that the monoubiquitination of PCNA by RAD18-RAD6 on lysine 164 is necessary for the monoubiquitination of FANCD2, making RAD16-RAD6 another ubiquitin ligase that regulates FANCD2, even though in this case is in an indirect manner [56].
CtIP (CtBP-interacting protein) is another protein that has been shown to interact with FANCD2 in a BRCA1 dependent manner by Yeo et al. CtIP has been reported to work in a DNA damage independent manner to stabilize

FANCD2 [37]. CtIP has also been known to interact BRCA1 in a complex and with MRE11 from the MRN (MRE11/RAD50/NBS1) complex to moderate DNA end resection during HR and to promote HR repair [37]. FANCD2 interacts with CtIP on stalled replication forks and together they suppress new replication origins from starting and they promote the restart of the replication fork [37]. MRE11/RAD50/NBS1 have also been shown to interact with FANCD2 during S-phase, this interaction stabilizes FANCD2 and MRE11 is crucial in the localization of FANCD2 to the DSBs [41].

Fanconi Anemia Downstream Factors

FANCJ, also known as BRIP1 (BRCA1 interacting protein 1) is a DNA helicase, with 5’ to 3’ polarity, that interacts with BRCA1 during the repair of DSBs and it co-localizes with BRCA2 and RPA in the DNA repair structures. In addition, FANCJ has been shown to be a tumor suppressor protein that is regulated by the E2F/Rb pathway [28]. FANCJ has been shown to be functional in cells that lack a functional FANCD2 demonstrating that FANCJ acts either downstream or parallel to FANCD2 but its role in the repair of ICLs is not known yet.

FANCN also termed PALB2 (partner and localizer of BRCA2) is a protein that interacts with BRCA2 in order to localize it to the nuclear matrix, stabilize it in its nuclear repair structure, and promote its activity in the nucleus for the repair of damaged DNA [30].

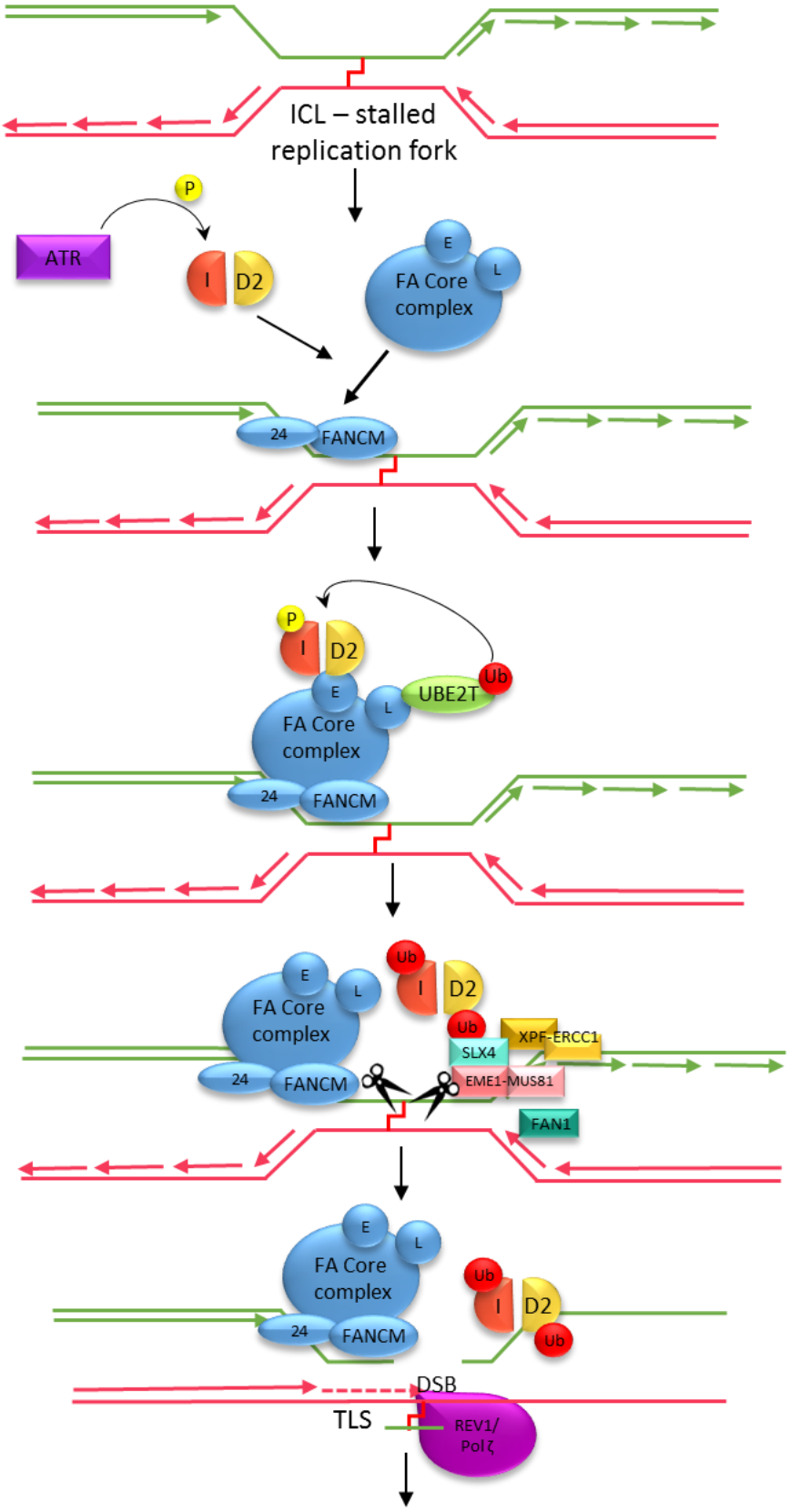
Fanconi Anemia Deubiquitinating Complex

In order to inactivate FANCD2 and FANCI and to terminate the DNA repair FANCD2 and FANCI need to be deubiquitinated. This task is performed by the DUB complex USP1-UAF1 (ubiquitin-specific protease 1 and USP1-associated protein 1) [56]. UAF1 is an activator of USP1 and it interacts with the SLIM (SUMO-like domain-interacting motif) of FANCI through its C-terminus where it has two SUMO-like domains, it is believed that this interaction is the one that connects the USP1-UAF1 complex to FANCD2 and FANCI [56].

Fanconi Anemia Pathway Mechanism

Homologous recombination is one of the many mechanisms that a cell has in order to ensure that there is no DNA damage. HR is employed by the cell in order to fix DSBs, damaging deletions, and ICLs. HR is also activated by the cell to maintain telomeres, preserve replication forks, and during meiosis I chromosome segregation [64]. Since the FA pathway is used to repair ICLs, the focus will be on the use of mainly HR for the repair of ICLs. If the DNA of the cell is damaged during S-phase, FANCM and FAAP24 together recruit the FA core complex to the site of damage [33]. The FA core complex and UBE2T monoubiquitinate FANCD2 and FANCI, leading to their

activation and localization to the chromatin, most likely they localize independently of each other [21]. Once activated, FANCD2 interacts with PCNA, BRCA2, and RAD51 as stated previously. Activated FANCD2 also recruits FAN1 (Fanconi anemia-associated nuclease 1), a 5’ flap endonuclease and an exonuclease with a 5’-3’ polarity, to the site of damage [35]. Another protein that is recruited by FANCD2 is FANCP (also known as SLX4). FANCP itself acts like a nuclease scaffold that interacts with the endonucleases XPF-ERCC1 and MUS81-EME1 [65]. XPF-ERCC1 is a 3’ flap endonuclease that unhooks the ICL from one of the strands of the replicating DNA strand [39]. MUS81-EME1 is a structure specific endonuclease that processes stalled replication forks due to ICLs into DSBs [40]. Once these endonucleases are recruited to the site of the ICL they unhook the cross-linker from the template DNA strand. While the complimentary strand gets repaired through TLS by REV1 and Pol ζ, the leading strand, now with a DSB, gets repaired in a RAD51-dependent manner through HR [33]. The cross-linker on the complimentary strand is removed by NER. In order to inactivate the pathway and terminate the repair the complex USP1-UAF1 deubiquitinates FANCD2 and FANCI [33] (figure 7).


ICL – stalled replication fork
P
ATR
I
D2
E
L
FA Core complex
24
FANCM
P
I
D2
E
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UBE2T
Ub
FA Core complex
24
FANCM
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I
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FA Core complex
24
FANCM
Ub
XPF-ERCC1
SLX4
EME1-MUS81
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REV1/ Polζ

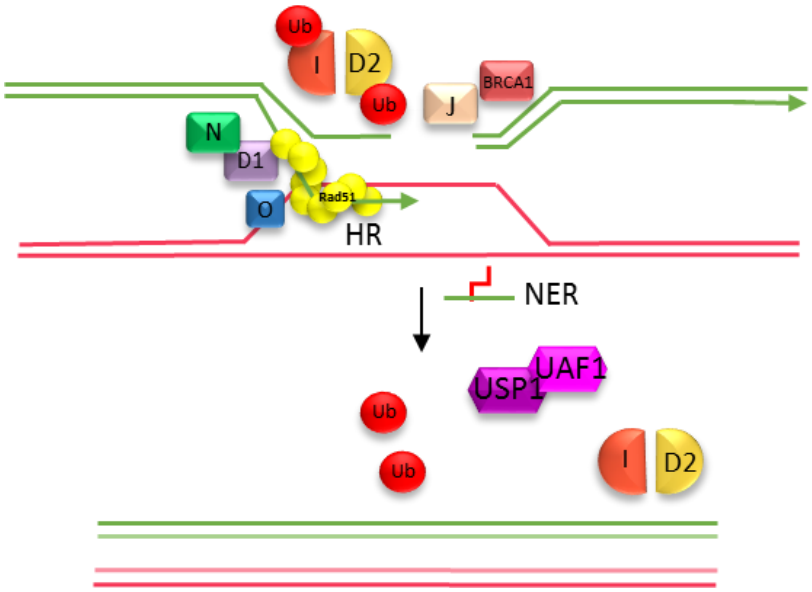


Figure 7. Depiction of the removal of an ICL in a replication fork by the Fanconi anemia DNA repair pathway. Once a cross-linker creates the ICL FANCM and FAAP24 recognize the lesion and recruit the FA core complex, at the same time the damage activates ATR to phosphorylate FANCI. Once it is recruited the FA core complex and UBE2T monoubiquitinate FANCD2 and FANCI. The two proteins then localize to the site of damage where FANCD2 recruits several endonucleases and exonucleases to remove and unhook the cross-linker. One of the strands is repaired by TLS polymerases. The other strand now has a DSB which is repaired through HR by RAD51/FANCD1 and other FA proteins (Adapted from Kim et al., 2012 [33]).

CHAPTER THREE: CO-RECRUITMENT OF THE FANCD2-FANCI HETERODIMER TO THE FA E3 LIGASE COMPLEX PROMOTES DNA REPAIR

Rationale

FANCD2 is a 1471 amino acid molecule that is involved in the key regulatory step of the DNA repair pathway Fanconi anemia. The FA pathway repairs ICLs after the cell has been exposed to cross-linking agents and during S-phase [55]. The activation of the FA pathway occurs when the FA core complex, made up of eight FA proteins and four FA associated proteins (FANCA, -B, -C, -E, -F, -G, -L, -M, FAAP16, -20, -24, and -100), and the E2 enzyme UBE2T monoubiquitinates FANCD2 and FANCI; FANCD2 and FANCI form a heterodimer [27]. . A central regulatory event in the FA pathway is monoubiquitination of FANCD2 and FANCI. Monoubiquitinated FANCD2 is thought to trigger its association with other DNA repair proteins such as RAD51, BRCA1, BRCA2, FAN1, and FANCP [55]. The monoubiquitination of FANCD2 and FANCI is thought to be defective in more than 90% of all of FA cases, which shows how important this key step is in the maintenance of the cell's genome through the FA pathway [27]. There is also evidence that

FANCD2 has monoubiquitin-independent functions, such as recruitment of BLM proteins to chromatin [66, 67]. FANCD2 also localizes to fragile sites of damaged chromosome during mitosis, although its exact function there is unclear [68]. Therefore the interplay between FANCD2 and the FA E3 ligase complex (the FA core complex) must be precisely regulated in order to dictate the differential functions of FANCD2. Ours and previous reports suggested that FANCE is the essential component of the FA core complex and for the FANCD2 and FANCI monoubiquitination [27]. The precise biochemical reactions that involve FANCE, the rest of the FA core complex, and FANCD2-FANCI heterodimer is yet to be elucidated. Better and precise understanding of this key E3-substrate reaction in the DNA repair pathway will contribute to the ubiquitin biology in general, as well as designing potential chemosensitizing strategies that can target the FA pathway in tumors. FANCD2 has been shown to interact with the FA core complex through FANCE which is part of the C-E-F subcomplex of the FA core complex [25, 27]. Due to the important role of FANCD2 in the repair of ICLs we investigated the FANCE-interaction region of FANCD2. A better understanding of how FANCD2 interacts with the FA core complex to be monoubiquitinated will help in finding the cure for FA. In addition, gaining insight into the regulation of the FA pathway will aid in the design of therapeutic treatments for cancers that have increased DNA repair pathways.

Experimental Design and Methods

Cell Survival Assay

The patient derived PD-20 cell line (a FANCD2-deficient cell line) and the complemented PD-20-Flag-FANCD2 cell line were seeded in triplicates in 12-well plates. MMC was added at a final concentration ranging from 0nM to 20nM. After the addition of MMC the cells were incubated for 7 days, and the surviving cells were then fixed with fixation solution (10% methanol + 10% glacial acetic acid) for 15 minutes. After fixation the cells were stained with staining solution (5 g of crystal violet dissolved in 500 mL of methanol) at room temperature for 10 minutes. The staining solution was then rinsed with deionized water and the plates were left to dry overnight. Once the plates were dry 300 µL of Sorenson's buffer (14.705 g of 0.1 M sodium citrate, pH 4.2 + 250 mL of 50% ethanol + 200 mL of nanopure water) were added to each well and the plates were placed on the shaker for 15 minutes. 150 µL from each well was then transferred to a 96-well microplate. The amount of purple in each well was read with a Biotek plate reader at a wavelength of 590 nm.

Yeast Two-hybrid Analysis

Human FANCD2 cDNA and FANCI were cloned into Matchmaker™ GAL4 Two-Hybrid System 3 vector PGBKT7 (Clontech) which contains the DNA-

binding domain (DBD). Human FANCE cDNA and human FANCI cDNA were cloned into Matchmaker™ GAL4 Two-Hybrid System 3 vector PGADT7 (Clontech) which contains the activation domain (AD). Site-directed mutagenesis of FANCD2 was achieved using pGBKT7-FANCD2 as a template (except the F48P-L51P double mutant, the pGBKT7-F48P-FANCD2 mutant was used as a template in this case) and the following primers for each point mutant: D19P: 5'-T AAA GAG AGC CTG ACA GAA CCT GCC TCC AAA ACC AGG AAG-3', F48P: 5'-GAA AAT GAC AGC ATC CCT GTA AAG CTT CTT AAG-3', V49P: 5'-GAA AAT GAC AGC ATC TTT CCA AAG CTT CTT AAG ATA TCA GG-3', L51P: 5'-GAC AGC ATC TTT GTA AAG CCT CTT AAG ATA TCA GG-3', L51A: 5'-GAC AGC ATC TTT GTA AAG GCT CTT AAG ATA TCA GG-3', and F48P-L51P: 5'-GAC AGC ATC CCT GTA AAG CCT CTT AAG ATA TCA GG-3'. The constructs were then transformed into the yeast strain AH109 by following the Small-Scale LiAc Yeast Transformation Procedure in the Clontech Yeast Protocols Handbook (PT3024-1). In order to select for transformation the transformants were plated on selection media lacking leucine (pGADT7) and/or tryptophan (pGBKT7), depending on which plasmid they were transformed with. For the interaction plates the transformants were spotted in triplicates in 10-fold serial dilutions on media that was lacking leucine, tryptophan, and histidine; this media also contained 3 mM of 30AT and 20 μg/mL of X-α-gal. In order to check for protein expression the proteins were extracted from the yeast by using the TCA method described

in the Clontech Yeast Protocols Handbook (PT3024-1). The protein extracts were then run in their appropriate percentage SDS-gel and blotted with either DBD GAL4 antibody or AD GAL4 antibody (Santa Cruz). FANCE protein expression western blots were blotted with FANCE antibody courtesy of the Fanconi anemia Foundation.

FANCD2 INTERACTS WITH FANCE THROUGH THE N-TERMINUS OF FANCD2

To identify which region of FANCD2 is necessary for the interaction with FANCE we used the crystal structure of FANCD2 and a disorder probability software to plan the different truncations that would be tested in our yeast-two hybrid assay (figure 8). The crystal structure was taken into consideration in order to not disrupt a helix or a beta-sheet and to pick an amino acid that was conserved in several species.

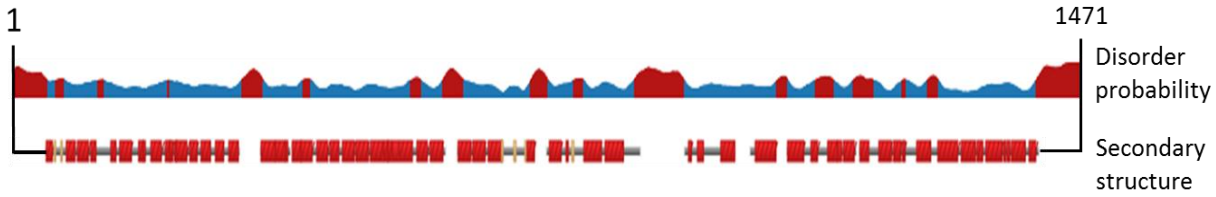


Figure 8. Disorder probability diagram of FANCD2 and its secondary structure. The disorder probability diagram shows the disordered areas in red while the orders areas are in blue. The secondary structure diagram shows helices in red, coils in a gray line, and beta strands as yellow. Disorder probability and secondary structure diagrams were created in the RCSB PDB website: http://www.rcsb.org/pdb/protein/Q80V62

We first cloned FANCE into the pGADT7 vector and transformed it into our AH109 yeast strain; the expression of FANCE was then confirmed by

western blotting (anti-FANCE). Then we cloned full length wild-type FANCD2 into the pGBKT7 vector and transformed it into the AD-FANCE yeast transformant, its expression was also confirmed by western blotting (anti-DBD). After we confirmed that our yeast-two hybrid worked (figure 9) we cloned a FANCD2 truncation that was missing the last 279 amino acids (FANCD2-ΔC279) into the pGBKT7 vector. This truncated FANCD2 interacted with FANCE in our yeast-two hybrid assay thus suggesting that the C-terminus of FANCD2 is dispensable for the interaction with FANCE (figure 9). In order to narrow down our search we created several more truncations that were cloned into the pGBKT7 vector and then transformed into the AD-FANCE yeast transformant. Their interactions were then tested in the yeast-two hybrid assay (figure 10).

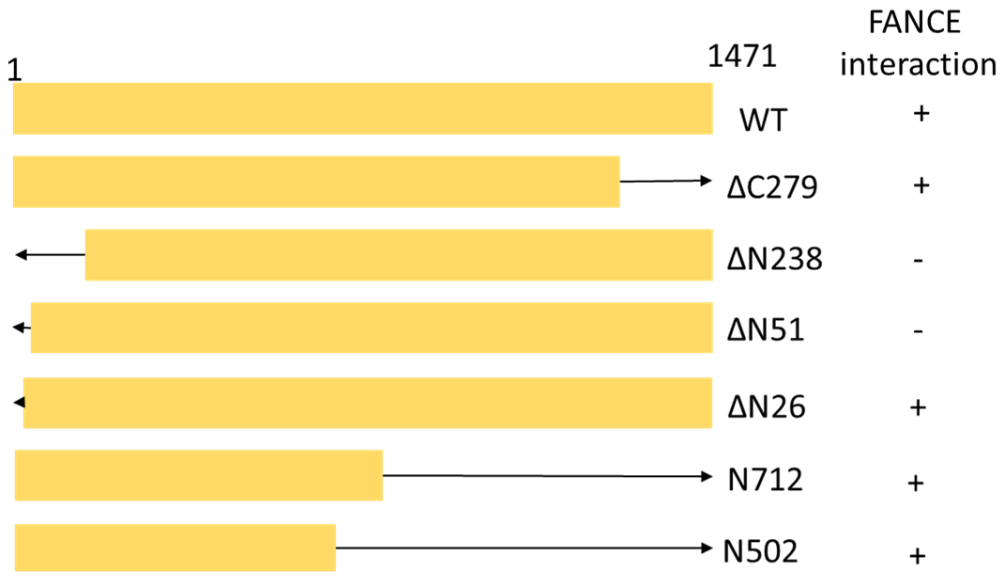


Figure 9. Summary of FANCD2 truncations. These truncations were engineered in order to test the interaction in between FANCD2 and FANCE the yeast-two hybrid assay.

From the results from the yeast-two hybrid assay we could determine that it was the N-terminus of FANCD2 that was necessary for the interaction with FANCE since FANCD2-ΔN51 failed to interact with FANCE and FANCD2-ΔN26 interacted with FANCE (figure 10).

Disordered Region of FANCD2 is Not Required for the FANCD2 Interaction with FANCE

Once we determined that the N-terminus of FANCD2 was necessary for the interaction of FANCD2 with FANCE we decided to change Asp19 for a proline residue in order to create the FANCD2-D19P point mutant. We chose to replace the selected amino acids with proline instead of other amino acids because proline is usually excluded from alpha helices and beta sheets due to the bulkiness of its side chain [69, 70]. We picked this specific residue based on a helical propensity analysis done on the disordered N-terminal region of FANCD2 (figure 11) [71, 72]. The logarithm that was used to predict the helical propensity of the region predicts three regions where a helix could form. Since we were interested in the disordered region Dr. Schmidt suggested we mutate Asp19 since it was the residue that would disrupt the most probable helix in the disordered region (figure 11). We then performed point-mutagenesis on the full wild-type FANCD2 that we had previously cloned into our pGBKT7 vector and cloned it into the AD-FANCE expressing yeast that we had previously transformed. FANCD2-D19P did

interact with FANCE in our yeast two hybrid system (figure 12). These results led us to the conclusion that FANCD2-D19P does not disrupt the interaction of FANCD2 with FANCE (figure 12).

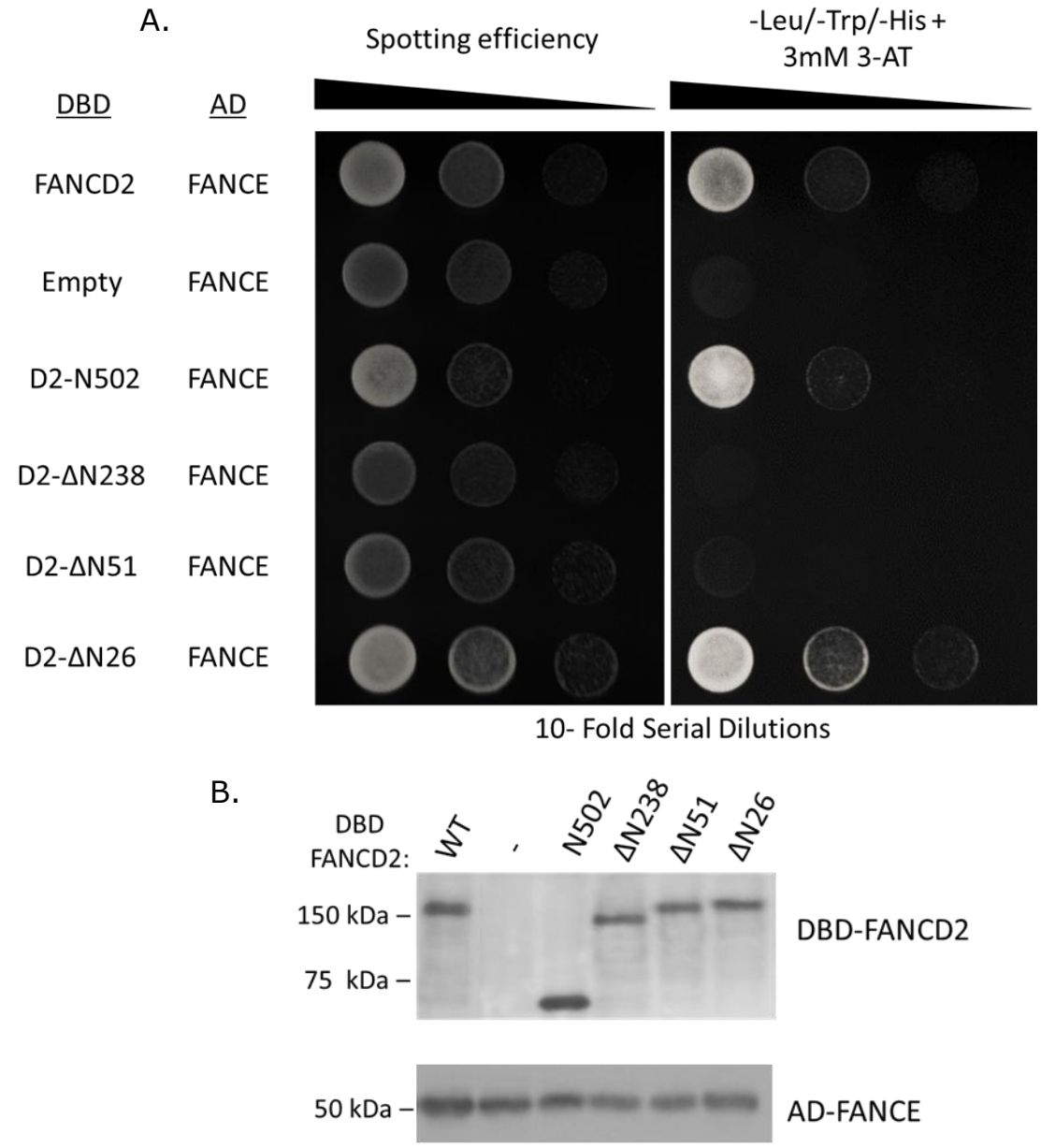


Figure 10. The N-terminus of FANCD2 contains the epitope for its interaction with FANCE. A. FANCD2-ΔN26 and FANCD2-N502 interact with FANCD2, while FANCD2-ΔN51, FANCD2-ΔN238 do not in our yeast-two hybrid analysis. B. Expression of proteins in the transformants for the yeast-two hybrid analysis.

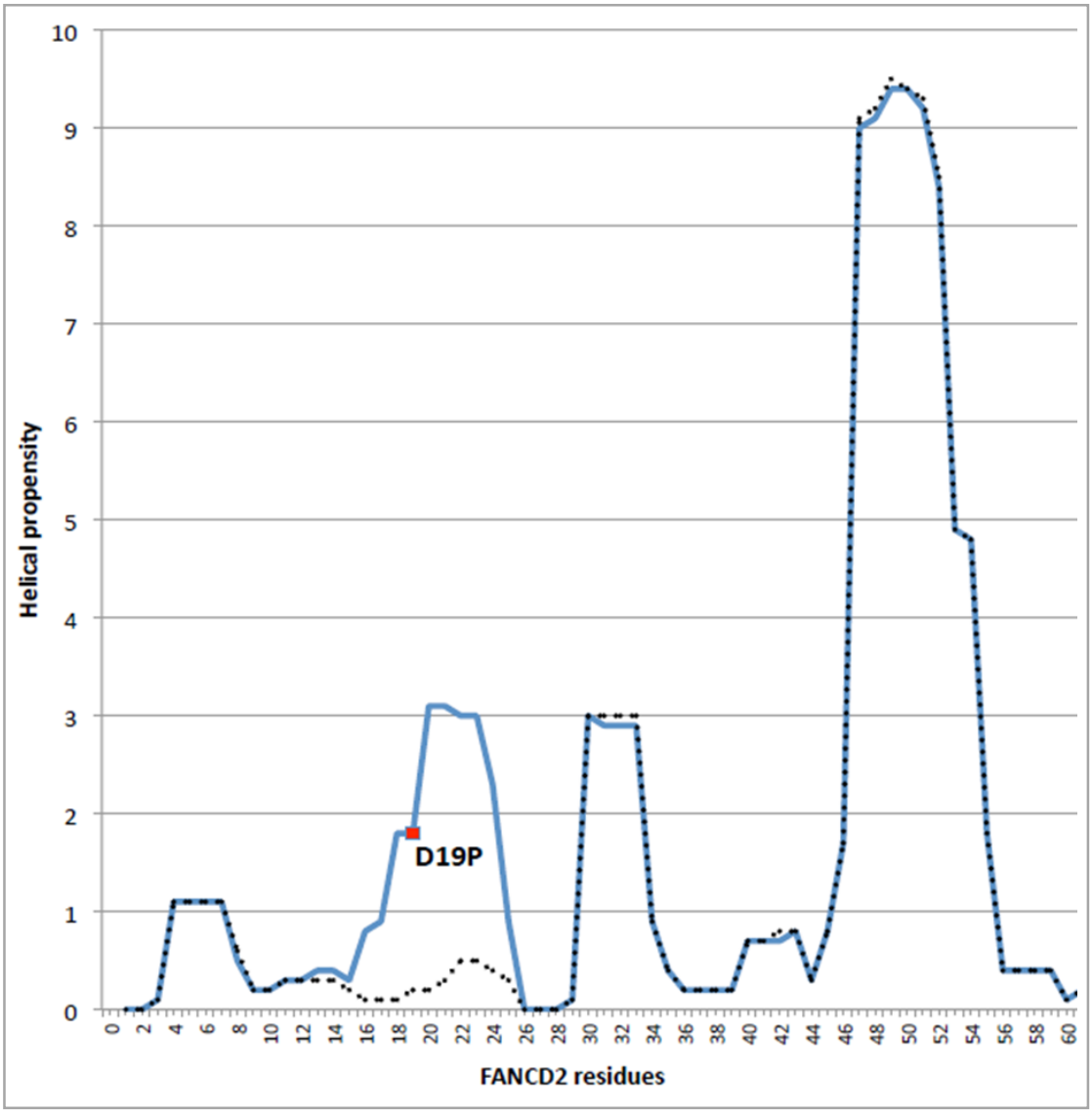


Figure 11. Helical propensity prediction of the first 100 residue FANCD2. Graph depicts the helical propensity of the first 100 amino acids of FANCD2 [71, 72]. Amino acid Asp19 resides in the disorder region of FANCD2 and performing point mutagenesis and changing it to proline would disrupt this probable helix. Analysis provided by Dr. Kristina Schmidt, USF.

FANCD2 Leucine 51 is Required for the Interaction of FANCD2 and FANCE

In order to find more amino acids that could be potentially necessary for the interaction of FANCD2 with FANCE we provided Dr. Daughdrill from CMMB with the crystal structure and the amino acid sequence of FANCD2. Dr. Daughdrill suggested that we try several amino acids in the N-terminus of FANCD2. From those that he suggested we selected three to mutate and test how they affected their interaction of FANCD2 with FANCE (figure 13). Just like before, the selected amino acids were changed to proline residues: phenylalanine 48, valine 49, and leucine 51. Since FANCD2-F48P was the

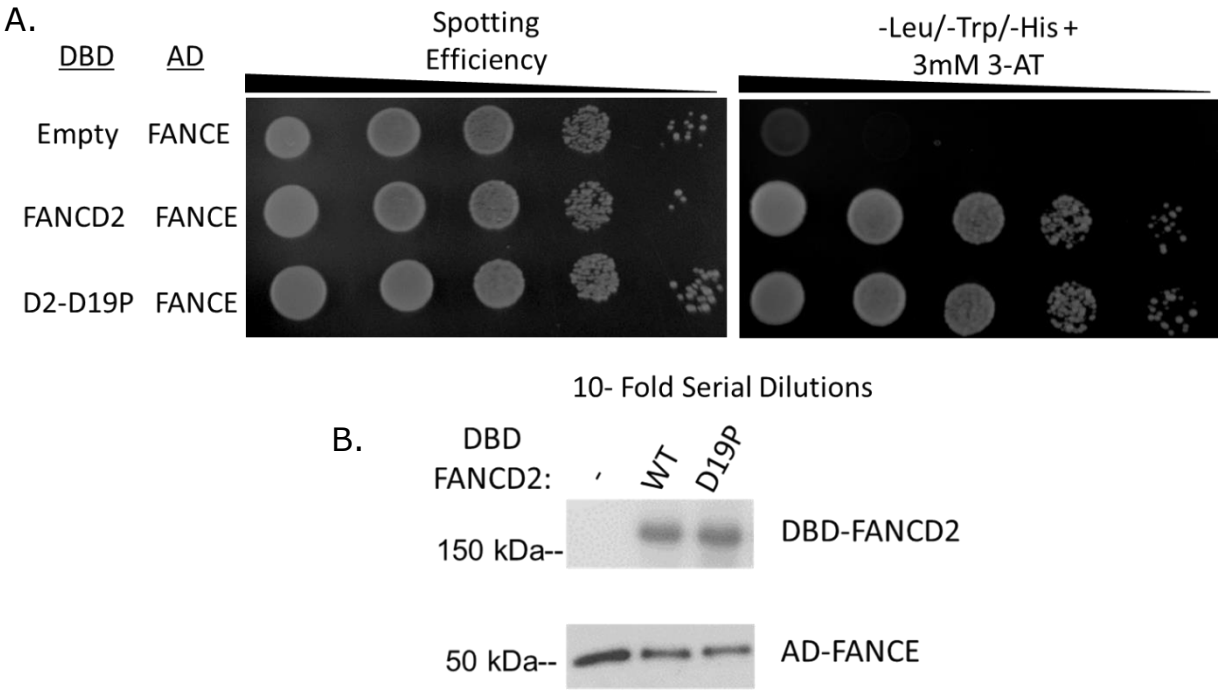


Figure 12. FANCD2-D19P does not disrupt the interaction with FANCE. A. FANCD2-D19P does not disrupt the interaction with FANCE in our two-hybrid yeast assay. FANCD2-D19P grew just like WT-FANCD2 in the selection media. B. Expression FANCD2-D19P and AD-FANCE in the transformants for the yeast-two hybrid analysis.

first point mutant to be cloned into the pGBKT7 plasmid, we decided to also try a double-point mutant that was created using FANCD2-F48P as a template. The second residue that was mutated in the FANCD2-F48P construct was leucine 51. Once we confirmed the point mutations by DNA sequencing we transformed each one of them into the AD-FANCE yeast that we had previously transformed. FANCD2-F48P and FANCD2-V49P did not disrupt the interaction of FANCD2 with FANCE. However, FANCD2-L51P and FANCD2-L51P-F48P did disrupt this interaction (figure 14). These results led us to the conclusion that FANCD2's leucine 51 is an indispensable amino acid for the interaction with FANCE.

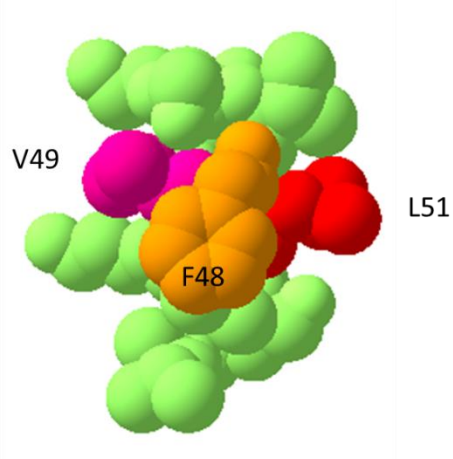


Figure 13. Selected amino acids for FANCD2 point-mutagenesis. Some of the amino acids that Dr. Daughdrill suggested to mutate. The amino acids can be seen in the N-terminal helix of FANCD2 with the N-terminus being at the top and the growing peptide at the bottom of the figure. PDB downloaded from: http://www.rcsb.org/pdb/explore.do?structureId=3s4w - 3D image created with Swiss-PdbViewer 4.1.0.

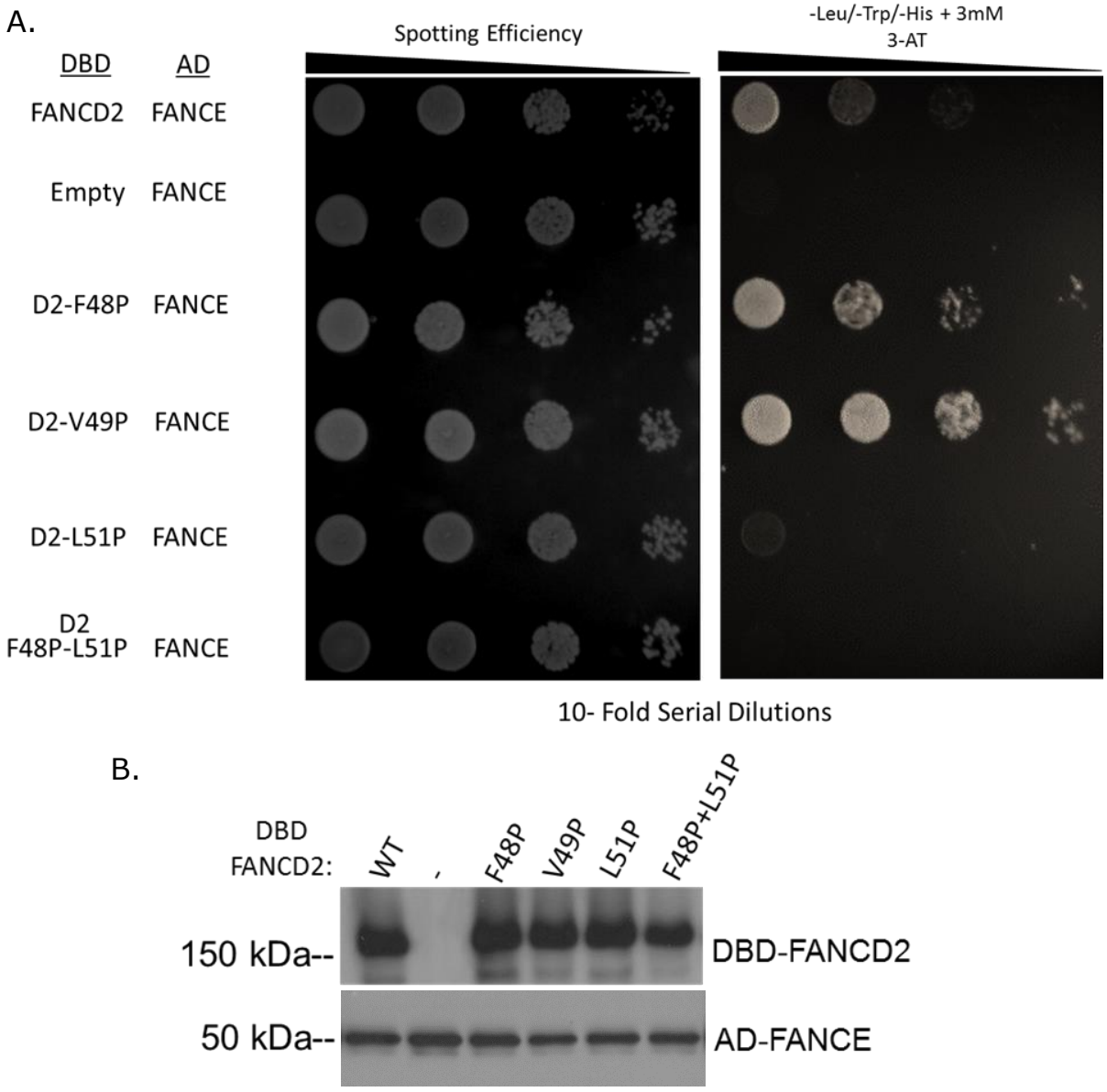


Figure 14. FANCD2-L51P and FANCD2-L51P-F48P disrupt the interaction with FANCE. A. FANCD2-F48P and FANCD2-V49P do not disrupt the interaction of FANCD2 with FANCE. FANCD2-L51P and FANCD2-F48P-L51P disrupt the interaction of FANCD2 with FANCE B. Expression FANCD2-F48P, V49P, L51P, F48P-L51P and AD-FANCE in the transformants for the yeast-two hybrid analysis.

FANCD2 Point Mutants Do Not Disrupt the Interaction in Between FANCD2 and FANCI

We wanted to make sure that the FANCD2 point mutants did not interrupt the interaction in between FANCD2 and FANCI. In order to do this we cloned FANCI in to the pGADT7 vector and transformed into the AH109 yeast; we tested the expression of AD-FANCI by western blot (anti-AD). We then transformed the AD-FANCI expressing yeast with either wild-type FANCD2 or the FANCD2-F48P and FAND2-L51P point mutations. Neither FANCD2-F48P nor FANCD2-L51P disrupted the interaction in between FANCI and FANCD2 in our yeast-two hybrid assay (figure 15). These results suggest that leucine 51 is not necessary for the interaction in between FANCD2 and FANCI.

Conclusion and Future Directions

The physical and functional interplay among FANCD2, FANCI, and the FA core complex is still not clearly understood. Our results suggest that FANCD2 interacts with FANCE of the FA core complex through its N-terminus and that leucine 51 is critical in this interaction (figure 16 and figure 17). FANCD2's leucine 51 is also conserved across species (figure 18).

The interaction assay in yeast used here must be validated in either human cells using co-immunoprecipitation assay, or using purified proteins.

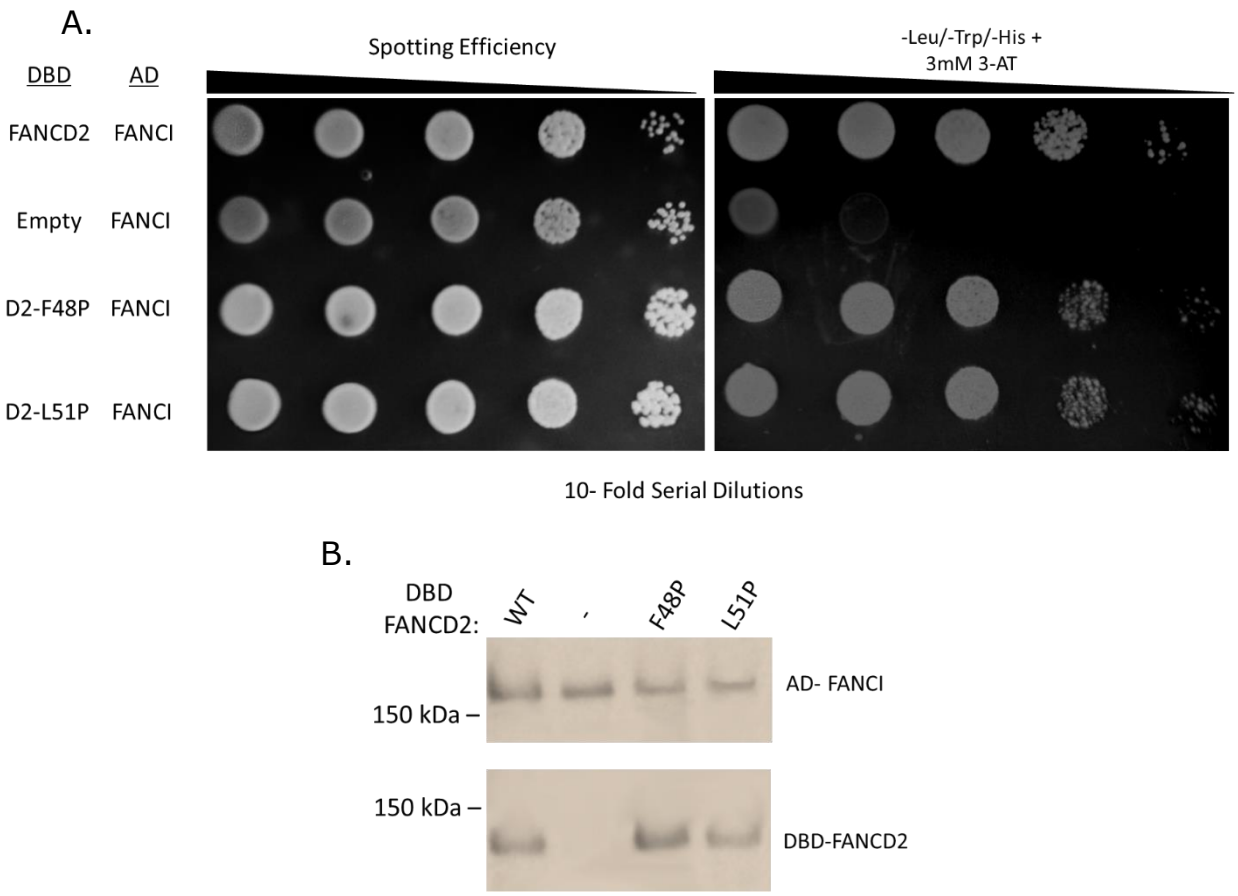


Figure 15. FANCD2-L51P and F48P do not disrupt the interaction with FANCI. A. FANCD2-F48P and FANCD2-L51P do not disrupt the interaction of FANCD2 with FANCI in this yeast-two hybrid assay. B. Expression FANCD2-F48P, L51P, and AD-FANCI in the transformants for the yeast-two hybrid analysis. FANCD2 runs smaller in this blot because FANCD2-ΔC279 was used for this yeast-two hybrid assay.

We are currently making attempts to generate purified FANCD2-FANCI complex, using Bac-to-Bac insect cell system. Our current work is to determine the effect of the mutation we found (e.g. L51P) in a relevant cell culture system. We are currently working to generate a FANCD2-null patient-derived PD-20 cell line that stably expresses wild-type and the mutant counterpart of FANCD2 using a lentiviral system.

This complementation system can be used for validating the effects of mutations on FANCD2-FANCI monoubiquitination, foci formation, chromosome breakage rate, and cellular sensitivity to Cisplatin or MMC (figure 19). We hypothesize that the L51P mutant will behave as, or close to, FANCD2-null, based on the lack of interaction with FANCE. However there is a possibility that it is not the case; L51P mutant may rescue the FANCD2 null phenotype only to a certain degree. This outcome would suggest that FANCD2 clearly has functions independently of its interaction with FANCE and possibly with the FA core complex. Indeed, recruitment of BLM protein to chromatin is mediated by FANCD2, but it may be independent of its monoubiquitination status [66, 67]. In addition, a series of three papers recently suggested that FANCD2 has a role in recruiting CtIP, an exonuclease that plays essential role in DNA double strand end resection during the HR repair; however, there appears to be a conflicting information as to whether monoubiquitination is essential for this process. Our complementation system may be able to dissect the functions of the FANCD2 monoubiquitination that contribute to the field.

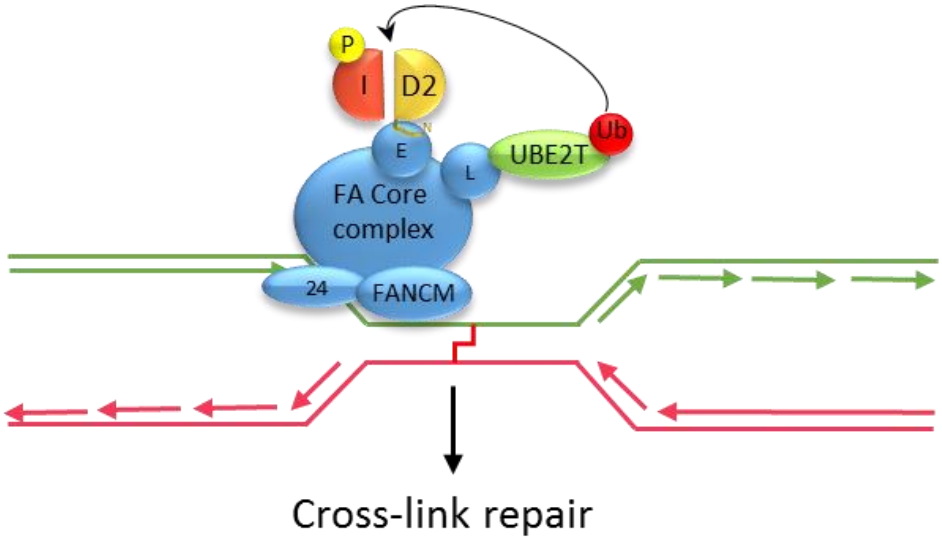
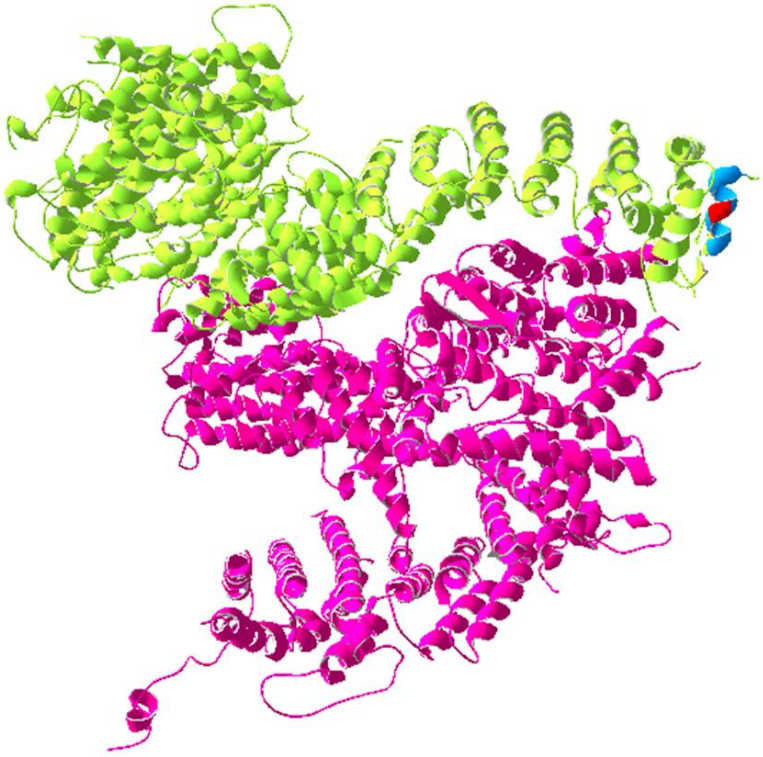


Figure 16. Proposed model of the interaction in between FANCD2 and FANCE of the FA core complex. FANCD2 interacts with FANCE through its N-terminus and residue leucine 51 is critical for this interaction. This interaction then leads to the monoubiquitination of FANCD2 by UBE2T and the recruitment of downstream DNA repair factors.

A.



B.

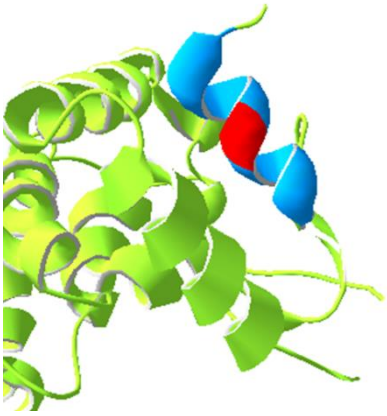


Figure 17. Leucine 51 of FANCD2 in the crystal structure of the FANCD2-FANCI complex. A. Crystal structure of FANCD2 in pink and FANCI in pink. The N-terminus of FANCD2 can be seen in blue with leucine 51 highlighted in red. B. A close-up of the structure of the N-terminus of FANCD2 with leucine 51 highlighted in red. PDB downloaded from: http://www.rcsb.org/pdb/explore.do?structureId=3s4w - 3D image created with Swiss-PdbViewer 4.1.0.

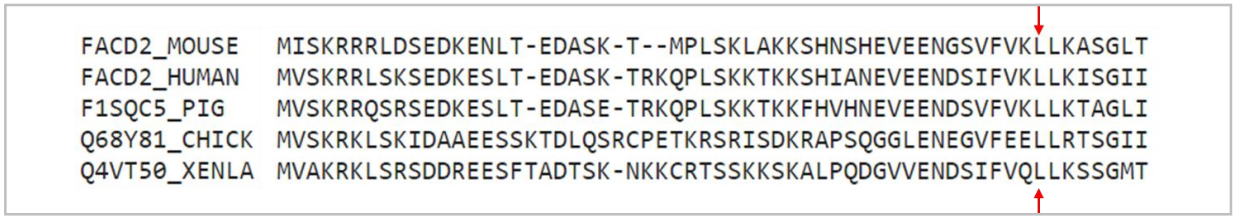
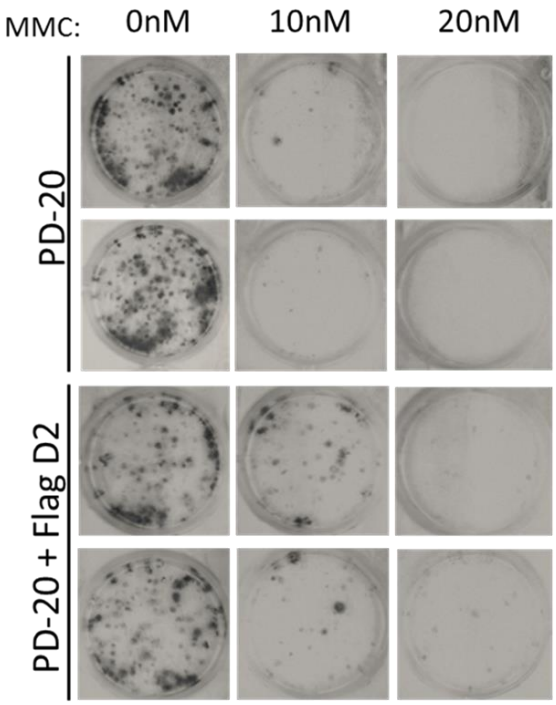


Figure 18. Leucine 51 is conserved across species. Aligned sequences of the first 60 amino acids of FANCD2 of Homo sapiens (human), Mus musculus (mouse), Sus scrofa (pig), Gallus gallus (chicken), and Xenopus laevis (frog). Leucine 51 is marked by two red arrows at the top and at the bottom. Sequences aligned using Clustal Omega http://www.ebi.ac.uk/Tools/msa/clustalo/.

A.



B.

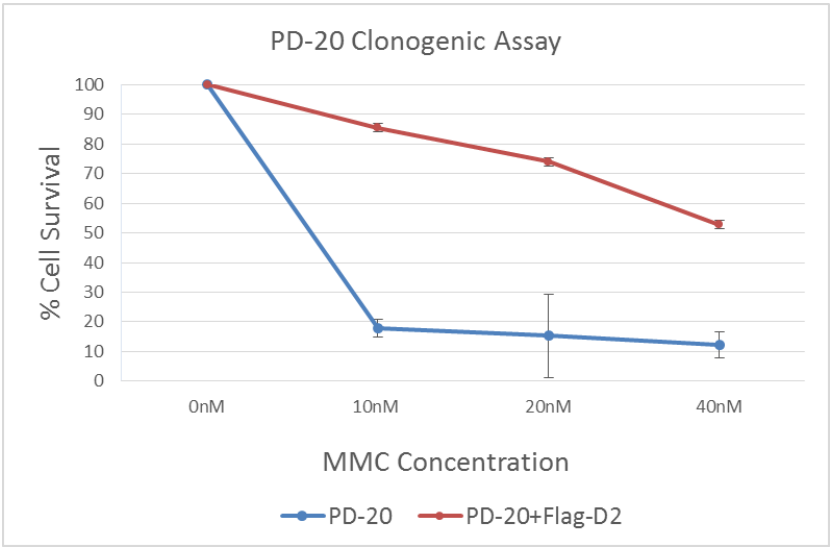


Figure 19. PD-20 cell line is sensitive to the cross-linker MMC while PD-20 complemented with WT FANCD2 is not. A. Colony formation on the plate after PD-20 and PD-20+FANCD2 have been treated with MMC for 7 days. Colonies form and grow in the well that have PD-20+FANCD2 but not in the well with PD-20 cells. B. Graph depicting the percentage of the cells that survived the MMC treatment. PD-20 cell survival drops at 10 nM of MMC.

CHAPTER FOUR: INHIBITION OF UBE2T BY SMALL PEPTIDES

Rationale

The FA pathway regulates DNA repair in order to maintain genome stability. The FA pathway is seen to be highly active in rapidly proliferating tissue. 11 proteins from the FA pathway have increased mRNA levels in brain tumors when compared to normal brain tissue [73]. Even though the FA pathway has an important role in the cell's genome integrity and it has been seen to be upregulated in cancer cells, no specific inhibitor of the FA pathway has been reported yet. An inhibitor of CDK1, which phosphorylates BRCA1, has been shown to inhibit HR and make cells sensitive to the chemotherapy treatment; this CDK1 inhibitor is still not specific enough in order to be utilized as a therapeutic agent for cancer patients [74]. Cisplatin is a commonly used chemotherapy drug, many cancers will become resistance to cisplatin treatment. This resistance could be in part due to upregulation the FA pathway, which is required to repair cross-links in the cell and is highly active in cancer cells (figure 7, p.24-25) [75].

UBE2T is the E2 conjugating enzyme of the FA pathway and it binds to FANCL, the E3 ligase unit of the FA core complex [53]. Cells that do not

express UBE2T are sensitive to DNA damaging agents, which leads to chromosomal breaks and genome instability [29]. The monoubiquitination of FANCD2 by UBE2T is the key regulatory step of the FA pathway, if this crucial step can be inhibited cells can become sensitive to cross-linking agents like cisplatin [29]. We are currently studying the inhibition of UBE2T by small peptides and its effect on the monoubiquitination of FANCD2. Dr. Cai from the USF Chemistry department has previously found a γ-AApeptide that inhibits STAT3's interaction with DNA in cultured cells at a concentration of 100 μM [76]. Using the same approach Dr. Cai provided us with peptides that were designed to mimic the interphase in between UBE2T and FANCL and in addition, he provided us peptides that have been picked from a library and have been shown to interact with UBE2T in their initial UBE2T interaction screen.

Experimental Design and Methods

GST Protein Purification

BL21 bacteria that had been transformed with pGEX-6P-1 expressing the desired proteins (either FANCL-RING, UBE2T, or no protein) (GE Healthcare) was inoculated in 50mL of media overnight at 37°C. The next morning 250 mL of media was added to the culture. The culture was left to incubate for two hours at 37°C. IPTG was then added to the culture to a final concentration of 300μM and the culture was incubated for two more hours.

The culture was spun at 5,000 rpm for seven minutes. The supernatant was discarded. The pellet was re-suspended in GST buffer (150 mM NaCl, 20 mM Tris pH 7.4, 0.5% triton, 0.1% EDTA) and sonicated (01, 01, 50%) for one minute, the homogenate was then spun down at 20,000 rpm for 40 minutes. The supernatant was incubated with 100 µL of glutathione beads (Sigma Aldrich) overnight. The beads were washed with GST buffer four times and the purified proteins were then ran in a SDS page gel and stained with coomassie dye. In the case of UBE2T, the GST tag was then cleaved by washing the beads with cleavage buffer (1 M tris pH 8, 5 M NaCl, 500 mM EDTA, 1 M DTT, and 10% NP-40) and then incubating PreScission Protease (GE Healthcare) overnight. The beads were then spun down and the supernatant was collected. The purified UBE2T was then confirmed by running it on a SDS gel and staining with coomassie. The amount of UBE2T was then assessed by constructing a standard curve.

Screening of Small Peptides

Dr. Cai's lab from the Chemistry Department at USF has synthesized and provided to us several γ-AApeptides, which contain N-acylates-N-aminoethyl amino acid units (figure 20) and are resistant to proteolytic degradation [76]. Dr. Cai acquired a peptide library which was incubated with purified UBE2T that we provided for him. After this incubation, the beads were incubated with UBE2T antibody (Bethyl labs). Next the beads

were incubated with Alexa Fluor 594 secondary antibody. Fluorescent beads were then selected under a microscope. The peptides were cleaved off from the beads, sequenced, and synthesized. Once we acquired the peptides we dissolved in water to a final concentration of 6 mM.

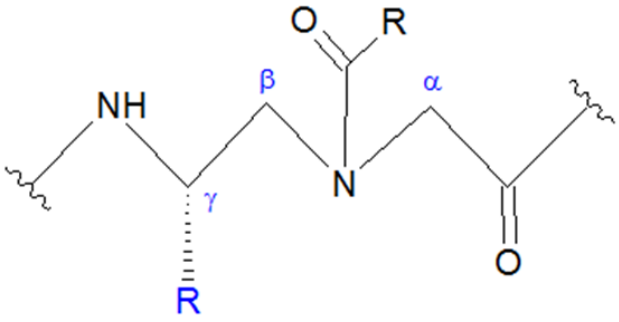


Figure 20. Structure of a γ-AA peptide. (Adapted from Teng, et al., 2014 (73).

GST Pulldown

Once the GST proteins were purified (see method above), purified UBE2T was incubated with either GST-FANCL-RING or GST in binding buffer (50 mM NaCl, 10 mM Tris pH 7, and 0.25% NP-40) for four hours. The beads were then washed with the binding buffer four times. 2X Laemmli buffer was then added to the beads and they were boiled for 3 minutes. The supernatant was run in a SDS gel. The membranes were then blotted with UBE2T antibody (Bethyl labs).

In Vitro E2 Charging Assay

The E2 charging assay was performed by adding 500 nM of E1 (BostonBiochem), 10 μM of His-ubiquitin (Sigma Aldrich), and 0.8μM of

UBE2T in reaction buffer (10 mM NaCl, 2 mM ATP, 10 mM tris pH 7.5, 1 mM $MgCl_2$, 0.1 mM DTT). This reaction was then incubated at room temperature for 45 minutes. The tubes then got added either 2X Laemmli buffer without β-mercaptoethanol or Laemmli buffer with β-mercaptoethanol. The tubes that contained β-mercaptoethanol were boiled for 3 minutes. The reaction was then ran on a SDS gel. The membranes were blotted with ubiquitin antibody (Millipore) and UBE2T antibody (Bethyl labs).

In Vivo FANCD2 monoubiquitination assay

HeLa cells were plated in a 6-well plate, once the cells reached a 70% confluency the peptide inhibitors were added in different concentrations. The next morning the media was removed and the cells were treated with 30 μJ ▪ m^{-2} UV irradiation. The media was added back on and the cells were harvested five hours later. The cell extracts were ran on a SDS gel and blotted for FANCD2 (Santa Cruz).

GST-FANCL-RING Pulls Down UBE2T *In Vitro*

We purified UBE2T by GST purification and PreScission protease cleavage and the RING structure of FANCL by GST purification (figure 21). GST-FANCL-RING and GST were incubated separately with UBE2T in order to establish the interaction in between GST-FANCL-RING and UBE2T.

We did see an enrichment in the interaction in between UBE2T and GST-FANCL-RING *in vitro* but not in between GST and UBE2T (figure 22). These results show us that just the RING domain of FANCL is necessary for the interaction with UBE2T *in vitro* and it gives us a good *in vitro* assay to test the interaction of the proteins.

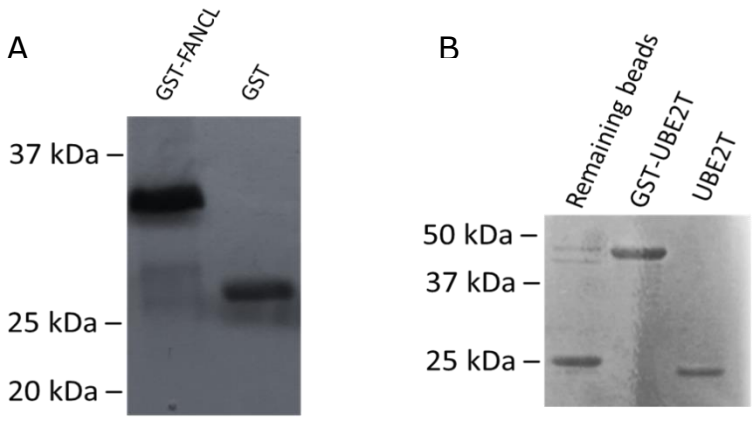


Figure 21. Purified GST, GST-FANCL-RING, and UBE2T. A. Coomassie stained gel shows the purified GST and GST-FANCL-RING. B. Coomassie stained gel shows the purified GST-UBE2T and the GST cleaved UBE2T.

Two Peptides Inhibit FANCD2 Monoubiquitination *In Vivo*

We wanted to test whether the peptides that we acquired from Dr. Cai's lab inhibited the monoubiquitination of FANCD2 *in vivo*. HeLa cells were treated with the peptides for 16-17 hours. Afterwards the cells were damaged with UV irradiation. Inhibition of the monoubiquitination of FANCD2 was seen with peptide 1 (i-1) and peptide 2 (i-2), but not with the others (figure 23, panel A, lanes 5 and 8). It is important to note that these two

peptides are the same molecular formula, peptides 2 (i-2) is the circular conformation of peptide 1 (i-1) (figure 24).

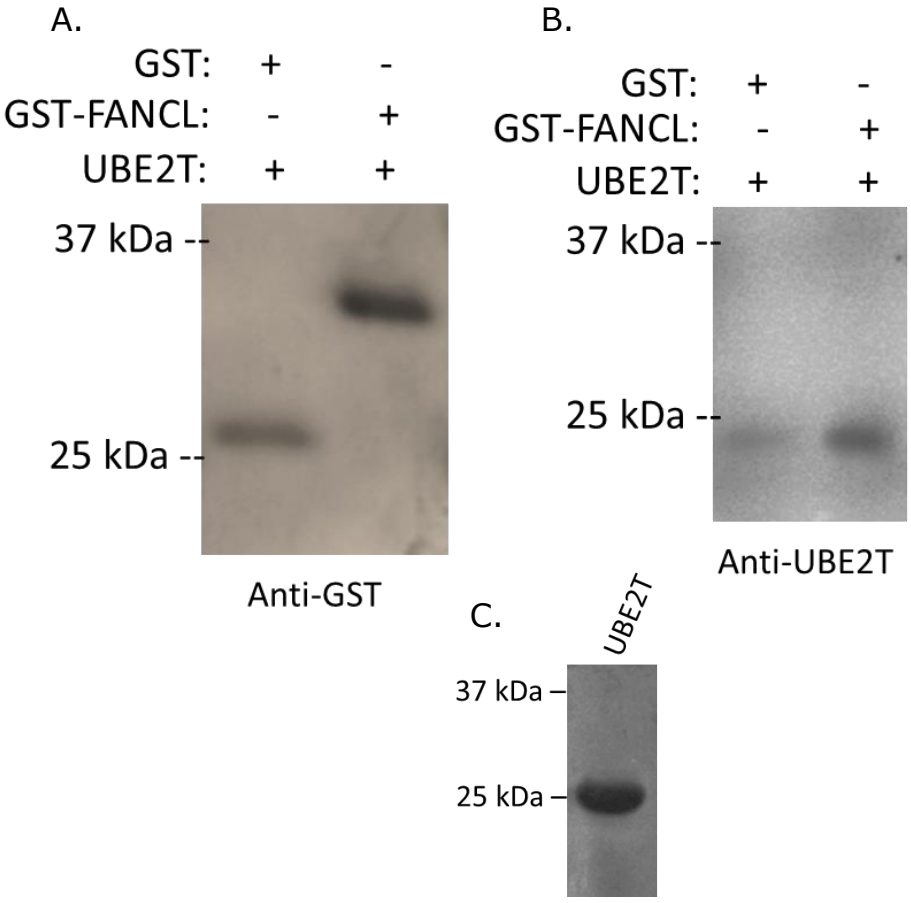


Figure 22. UBE2T interacts with GST-FANCL-RING *in vitro*. A. GST blot showing GST and GST-FANCL-RING purified proteins in the GST pull down reactions B. UBE2T protein is enriched in the reaction incubated with GST-FANCL-RING. C. Coomassie stained gel of UBE2T that was added to the pull down reactions.

UBE2T is Ubiquitinated *In Vitro* but Not Inhibited by the Peptides

Once we established which inhibitors inhibited the monoubiquitination of FANCD2 *in vivo,* we wanted to see if it was the UBE2T ubiquitin charging step that was being inhibited by the two peptides that showed inhibition of FANCD2 in our *in vivo* assay. We first set up the *in vitro* UBE2T charging

assay and made sure that it worked properly (figure 25). Human purified E1 and UBE2T were incubated in the presence of ATP and ubiquitin for 45 minutes at room temperature. The results showed that both E1 and UBE2T

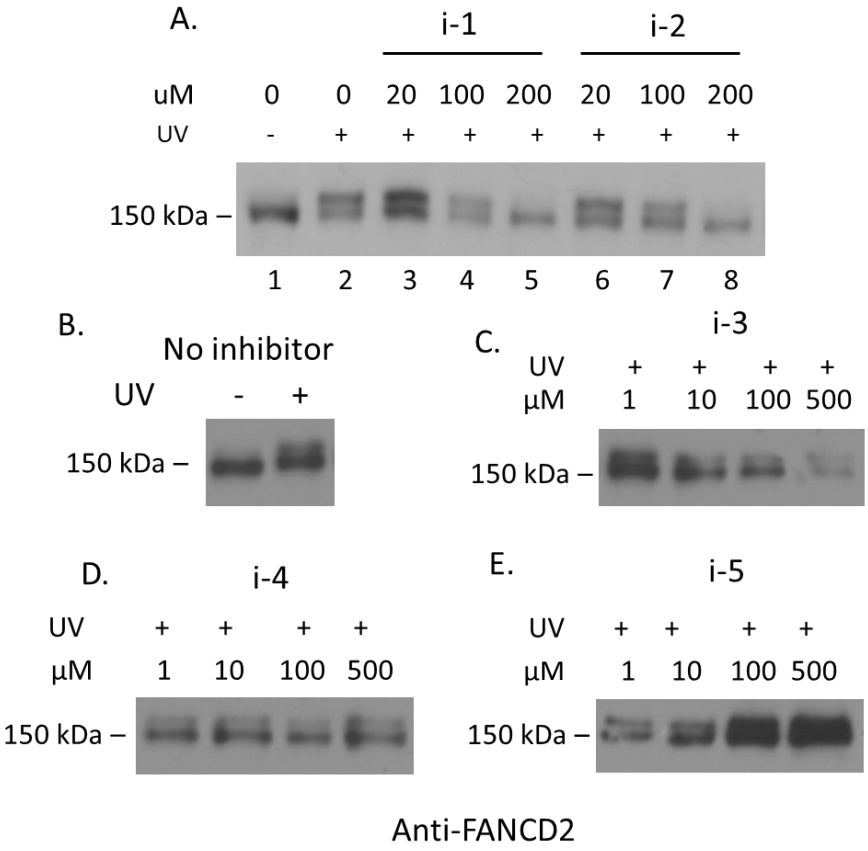


Figure 23. i-1 and i-2 peptides inhibit the monoubiquitination of FANCD2 *in vivo*. A. The monoubiquitination of FANCD2 is only inhibited by i-1 and i-2 but not by the rest of the provided inhibitors. B. FANCD2 monoubiquitination with and without damage without any inhibitor. C, E, D. Inhibitors 3 through 5 do not inhibit FANCD2

were ubiquitinated *in vitro*. Reducing the reactions by the addition of 2X laemmli buffer with β-mercaptoethanol and 3 minutes of boiling removed the ubiquitin that is bound on the E1 by a thiol ester bond (figure 26). Once the E2 charging assay was confirmed to work by repeating, we repeated the reactions but added different concentrations (10 µM, 50 µM, 100 µM, or 150 µM) of i-1 or i-2. Neither i-1 nor i-2 inhibited the ubiquitin charging of

UBE2T. These results lead us to the conclusion that these two inhibitors do not disrupt the first step of the FA ubiquitin cascade.

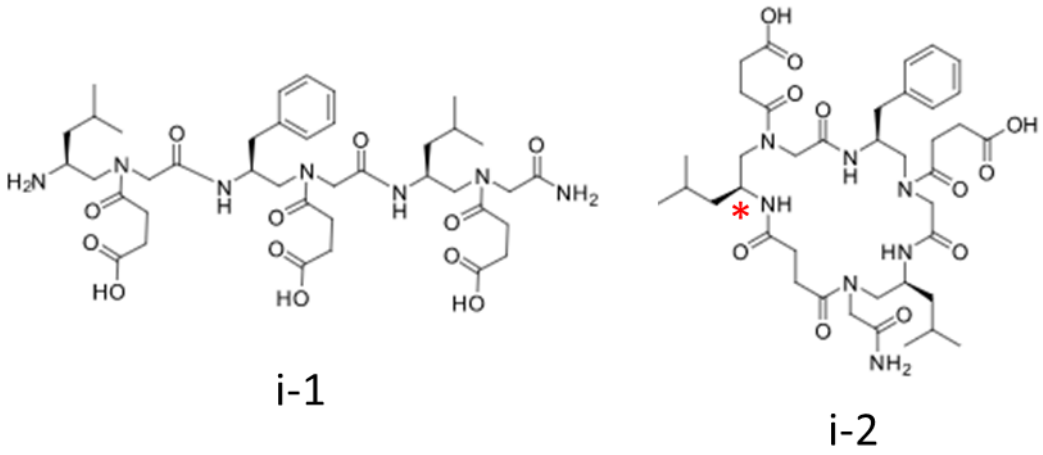


Figure 24. Molecular structure of i-1 and i-2 peptides. The red asterisk in i-2 depicts where the molecule was linearized.

Conclusion and Future Directions

UBE2T is located in 1q32.1, a region of chromosome one that has been shown to be amplified in many cancers. Since UBE2T is essential for the monoubiquitination of FANCD2 its inhibition in some cancers would, in theory, be a valid combination-therapy for tumors that have acquired cisplatin resistance. Despite the recognition of the FA pathway as a therapeutic target, no specific FA pathway inhibitors have been developed up to date [75].

Our study was conceptualized and designed with the goal that isolating a specific inhibitor of the UBE2T-FANCL interaction would: 1) provide a proof-of-principle that the critical E2-E3 reaction in the FA pathway can be a

valid therapeutic target, 2) provide a useful pharmacologic tool that allows 'acute' inhibition of the FA pathway. For the latter point, current methods of disrupting the FA pathway in cultured cells rely on RNAi-mediated knockdown or, more recently, genome-editing knockout technology, which often hampers researchers to investigate more direct functions of the FA proteins that are independent of other changes associated with when the gene is depleted (e.g. cell cycle).

Although our current effort so far was unsuccessful in identifying an inhibitory peptide, we will continue to screen potential peptido-mimetics that can specifically disrupt the UBE2T-FANCL interaction. We will need to further optimize our assay condition that measures the interaction between UBE2T and FANCL, in order to test the efficacy of these inhibitors in vitro. We may further expand our efforts to screen for peptide inhibitors of the FANCE-FANCD2 interaction, once we are able to purify large amounts of these recombinant proteins that can be used for the peptide-selection procedures. Our interaction analysis of FANCE-FANCD2 suggests that the FANCE-binding surface of FANCD2 may not be an extensive flat-surface, but rather a small hot spot within a helical area that may be sufficient for binding. This possibility must be more rigorously tested with structural analysis of the two purified proteins together; however, this can be a daunting task due to the very low affinity between the two proteins.

In order for the peptide-mimetic based inhibitor approach to be successful, a few technical improvements have to be made; at this point we have no way of determining that these peptide-mimetics are successfully entering the cytoplasm and nucleus of the cultured cells, where they can interact with their target proteins. Lipid-based delivery system can be adapted for more efficient delivery of these peptides.

Consistent with previous reports we have established that UBE2T and the RING domain of FANCL interact *in vitro* and that UBE2T gets charged with ubiquitin *in vitro* [29]. Our *in* vitro ubiquitination and GST pulldown assay will allow us to evaluate the efficacy of the small peptides selected in Dr. Cai's laboratory. We found that two of them, i-1 and i-2, inhibit the monoubiquitination of FANCD2 *in vivo*. We are currently trying to set up and optimize the GST-FANCL-RING pulldown assay in order to determine whether the inhibitors disrupt the interaction between UBE2T and the RING domain of FANCL (figure 27) *in vitro*.

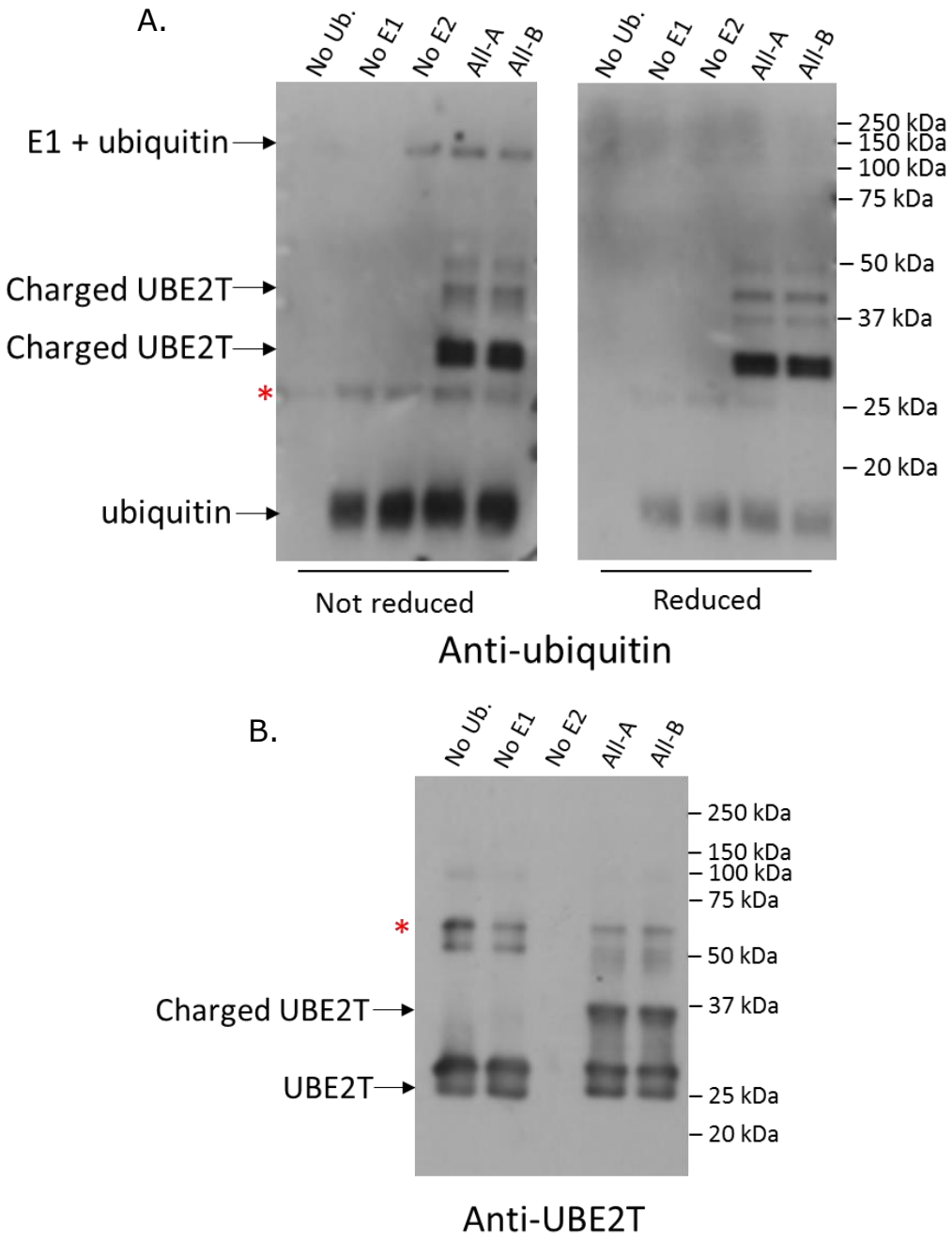


Figure 25. UBE2T gets ubiquitinated *in vitro*. A. Anti-ubiquitin blot showing E1 ubiquitination in not reduced samples. The ubiquitination of UBE2T can be seen in both figures. B. Ubiquitination of UBE2T can be seen by UBE2T blotting in the reactions that contain all the needed components. Both red asterisks represent non-specific bands.

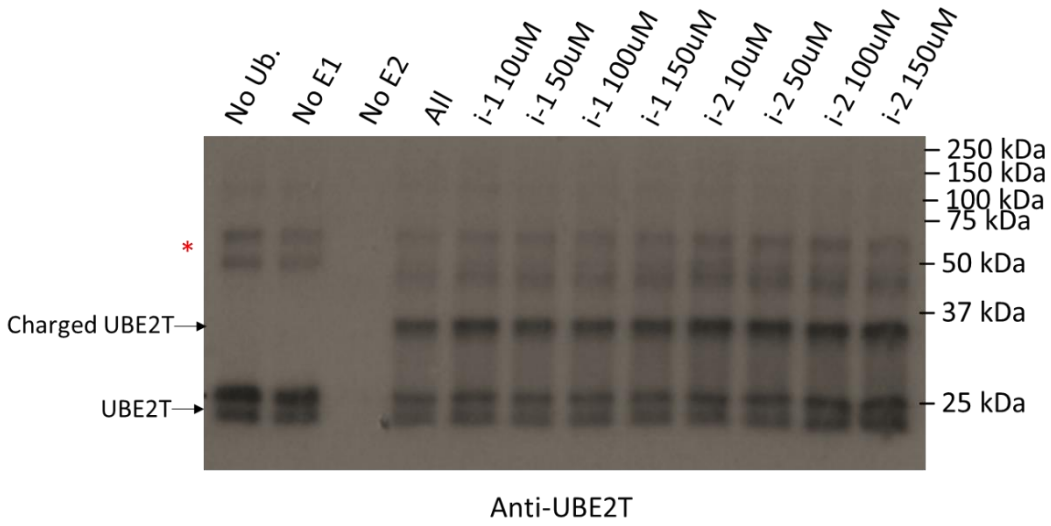


Figure 26. Inhibitors do not disrupt UBE2T charging. Anti-UBE2T blot shows that the inhibitors do not disrupt the ubiquitination of UBE2T *in vitro*. Red asterisk indicates a non-specific band.

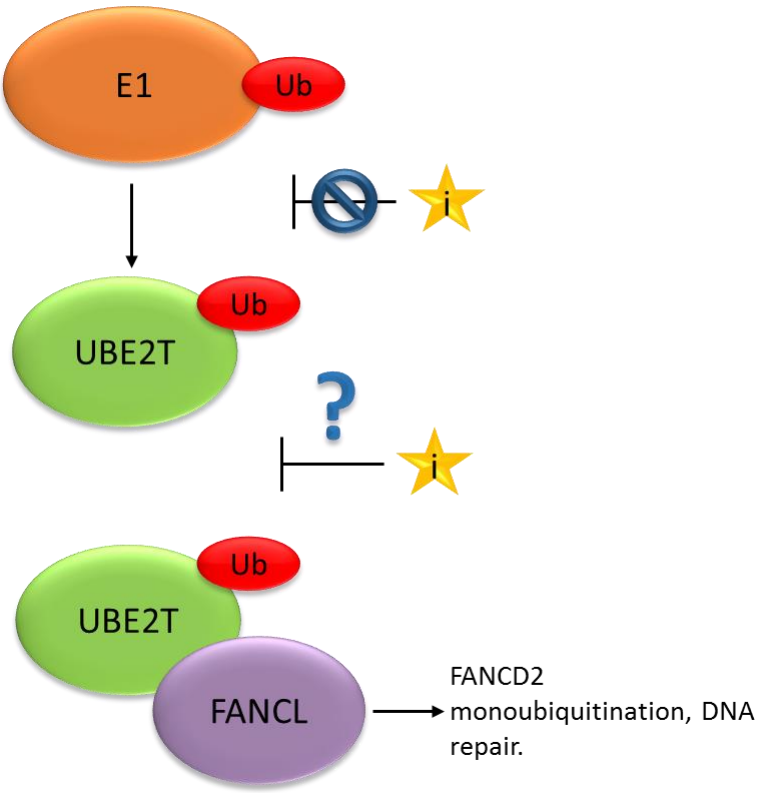


Figure 27. i-1 and i-2 might inhibit UBE2T-FANCL interaction. The inhibitors provided by Dr. Cai might inhibit the interaction in between FANCL and UBE2T which leads to the inhibition of the monoubiquitination of FANCD2 *in vivo.*

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