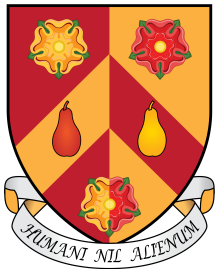
Characterisation of novel phosphorylation sites on FANCD2 and their role in the Fanconi anaemia pathway



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"There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after."

J.R.R. Tolkien, The Hobbit

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Abstract

Interstrand crosslinks (ICLs) are a highly deleterious form of DNA damage because they link the two strands of DNA, blocking replication, transcription and chromosome segregation. At the center of ICL repair is the FANCD2/FANCI complex, recruitment of which onto the ICL is a critical step for repair. Monoubiquitination of both FANCD2 and FANCI lead to their retention on chromatin and is essential for subsequent repair. However, recent evidence shows that FANCD2 monoubiquitination takes place only after recruitment to DNA (Liang et al., 2016). If the monoubiquitination is taking place after recruitment to DNA, the question of how the recruitment to DNA is regulated remains open. Our objective is to study the role of FANCD2 phosphorylation in regulating recruitment to DNA and repair.

We report a new cluster of phosphosites on FANCD2 whose phosphorylation by CK2 inhibits FANCD2 activation. Specifically, phosphorylation of FANCD2 in this cluster abrogates recruitment to ICLs and inhibits monoubiquitination *in vitro* and *in vivo.* Furthermore, phosphorylation of the cluster leads to a dramatic reduction in affinity of the FANCD2/FANCI complex towards DNA, which in turn blocks its monoubiquitination and the activation of the FA pathway. We describe a new regulatory mechanism where FANCD2 must be dephosphorylated prior to the loading of the FANCD2/FANCI complex onto DNA and activation of the FA pathway. This step could act as a safeguard against spurious activation of repair in the absence of damage.

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Abbreviations

53BP1	p53-binding protein 1
A-T	Ataxia telangiectasia
ALDH	Aldehyde dehydrogenase
ALT	Alternative lengthening of telomeres
AML	Acute myelogenous leukaemia
AP-site	Abasic site
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR-interacting protein
BER	Base excision repair
BLM	Bloom syndrome protein
BMF	Bone marrow failure
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
Cdc	Cell division control protein
CFS	Common fragile site
Chk	Chekpoint kinase
CK2	Casein kinase 2
CMG	Cdc45, Mcm2-7 and GINS
CPD	Cyclobutane pyrimidine dimer
CRISPR	Clustered regularly interspaced palindromic repeats
CS	Cockayne syndrome
DDR	DNA damage response

DNA-PKc	DNA-dependent protein kinase catalytic subunit
DSB	Double strand break
EM	Electron microscopy
FA	Fanconi anaemia
FAAP	Fanconi anaemia-associated protein
FAN1	Fanconi-associated nuclease 1
FANC	Fanconi anaemia complementation group
FHA	Forkhead-associated
HA	Human influenza hemagglutinin
HP1	Heterochromatin protein 1
HR	Homologous recombination
ICL	Interstrand crosslink
IP	Immunoprecipitation
IR	Ionizing radiation
MCM	Minichromosome maintenance protein
MDA	Malondialdehyde
MDC1	Mediator of DNA damage checkpoint protein 1
MHF	Histone-fold heterodimer
MMC	Mitomycin C
MMEJ	Microhomology-mediated end joining
MMR	Mismatch repair
MRN	MRE11/RAD50/NBS1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining

PAM	Protospacer adjacent motif
PCNA	Proliferating cell nuclear antigen
PICH	PLK1-interacting checkpoint helicase
PLK1	Polo-like kinase 1
PNKP	Polynucleotide kinase phosphatase
PPM	Metal dependent protein phosphatase
PPP	Phosphoprotein phosphatase
PRC	Polycomb repressive complex
PTM	Post-translational modification
RNF	Ring finger protein
ROS	Reactive oxygen species
RPA	Replication protein A
SIM	SUMO interacting motif
SLX	Structure-specific endonuclease
SSA	Single strand annealing
SSB	Single strand break
SUMO	Small ubiquitin-like modifier
TLS	Translesion synthesis
TMP	Trymethylpsoralen
TNFα	Tumour necrosis factor α
UDR	Ubiquitin-dependent recruitment domain
UHRF1	Ubiquitin-like, containing PHD and RING finger domains 1
USP7	Ubiquitin specific protein 7
UV	Ultraviolet

UV-DDB	UV-damaged DNA binding protein
UVSSA	UV-stimulated scaffold protein A
XP	Xeroderma Pigmentosum

Chapter 1. Introduction

1.1 DNA damage repair pathways

Our genomes are exposed to numerous damaging agents, both exogenous and endogenous. Cells count on a complex set of DNA repair pathways to counteract these different types of damage and avoid the inclusion of mutations into the genome that could lead to pathogenic outcomes. Different pathways have evolved for different types of damage: base excision repair (BER) for damaged bases and single strand breaks (SSBs); nucleotide excision repair (NER) for bulky lesions, adducts and intrastrand crosslinks; mismatch repair (MMR) for replication derived mismatches and insertions/deletions; homologous recombination (HR) and non-homologous end joining (NHEJ) for double strand breaks (DSBs) and, finally, the Fanconi anaemia (FA) pathway for interstrand crosslinks (ICL). The regulation of these repair events together with the necessary cellular responses that follow are called the DNA damage response (DDR). DDR events include regulation of the cell cycle through the DNA damage checkpoints, replisome stability, RNA transcription, chromatin remodeling and if repair fails, apoptosis (Jackson and Bartek, 2009).

1.1.2 BER, NER and MMR

BER is responsible for the repair of small lesions on bases arising from deamination, oxidation or methylation, generally. Firstly, the damaged base is removed by a specific DNA glycosylase, in mammals there are up to 11 different glycosylases, depending on the type of damage, which creates an abasic site (AP-site) (Jacobs and Schar, 2012). Following the excision, comes the incision

performed by an AP-endonuclease, APE1 in mammals, generating a nick 5' to the damaged site. Then, Pol β removes the remaining deoxyribonucleotide-phosphate through its lyase activity and fills in the generated gap through its polymerase activity. In fact, recent evidence shows that the lyase activity of Pol β plays an additional role in allowing Pol β to directly scan the DNA and bind to lesions (Howard et al., 2017). APE1 can also remove mismatched nucleotides resulting from Pol β activity due to its 3' end processing activity, recently identified (Whitaker and Freudenthal, 2018). Finally, the remaining 3' nick is sealed by DNA ligase I or III with the help of the scaffolding protein XRCC1. This type of BER is called short-patch because only a one-nucleotide opening is created. There is an alternative long-patch BER, where a bigger patch of around 10 nucleotides is opened. In this case, the replication machinery is involved including Pol δ/ε, PCNA (proliferating cell nuclear antigen), FEN1 and DNA ligase I (Krokan and Bjoras, 2013; Zharkov, 2008).

NER takes care of bulky lesions that distort the DNA double helix such as base adducts or intrastrand crosslinks generated through UV irradiation or chemical exposure. However, there is also growing evidence for NER playing a role in ICL repair. Currently, there are two main models of NER: global-genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER can be initiated at any lesion in the genome while TC-NER depends on the stalling of RNA polymerase II at a lesion to start (Figure 1-1).

In GG-NER, the lesion is recognized either by the XPC/RAD23B complex or the UV-DDB (UV-damaged DNA binding protein) complex. XPC/RAD23B can recognize bulky and distorting lesions although it does not directly bind to the lesion but to the distortion generated around it (Min and Pavletich, 2007). UV-

DDB, on the other hand, has a high affinity for less distorting lesions, which it can bind to directly, these are mainly UV related damage such as cyclobutane pyrimidine dimers (CPDs) (Yeh et al., 2012). UV-DDB has also been shown to interact with a ubiquitin E3 ligase triggering the ubiquitination of several proteins including XPC, which promotes its DNA binding and the transfer of the lesion to the XPC/RAD23B complex (Sugasawa et al., 2005). Then, the TFIIH complex is recruited; its XPB subunit opens the double helix while its XPD subunit moves along DNA until it finds the lesion in a verification process. The unloading of XPC/RAD23B follows due to further ubiquitination and SUMOylation events catalyzed by RNF111 (van Cuijk et al., 2015). Then XPA is recruited by TFIIH and together with RPA, it stabilizes the ssDNA formed (Sugitani et al., 2016). XPF/ERCC1 complex binds XPA and performs the 5' incision, repair synthesis by Pol δ, Pol ε or Pol κ starts and it is followed by the 3' incision by XPG, recruited through TFIIH. Once the repair synthesis is complete, the nick is sealed by XRCC1/LigIIIα or LigI (Figure 1-1) (Mu et al., 2018).

In TC-NER, RNA Pol II stalling at a lesion triggers repair (Wang et al., 2018). CSB (Cockayne syndrome B protein) is then quickly recruited and mediates CSA (Cockayne syndrome A protein) recruitment. CSB is thought to promote recruitment of other repair factors, some together with CSA, and it has ATPase and chromatin remodeling activities (Fousteri et al., 2006). CSA is part of an E3 ligase complex that mediates a ubiquitin signaling network at the site of damage. CSA can also recruit the UVSSA/USP7 complex (UV-stimulated scaffold protein A/Ubiquitin specific protein 7) that can deubiquitinate CSB regulating its degradation (Schwertman et al., 2013). UVSSA mediates the recruitment of the TFIIH complex, which performs the lesion verification process similarly to GG-

NER (Okuda et al., 2017). The final repair steps are thought to be the same to those described for GG-NER (Figure 1-1).

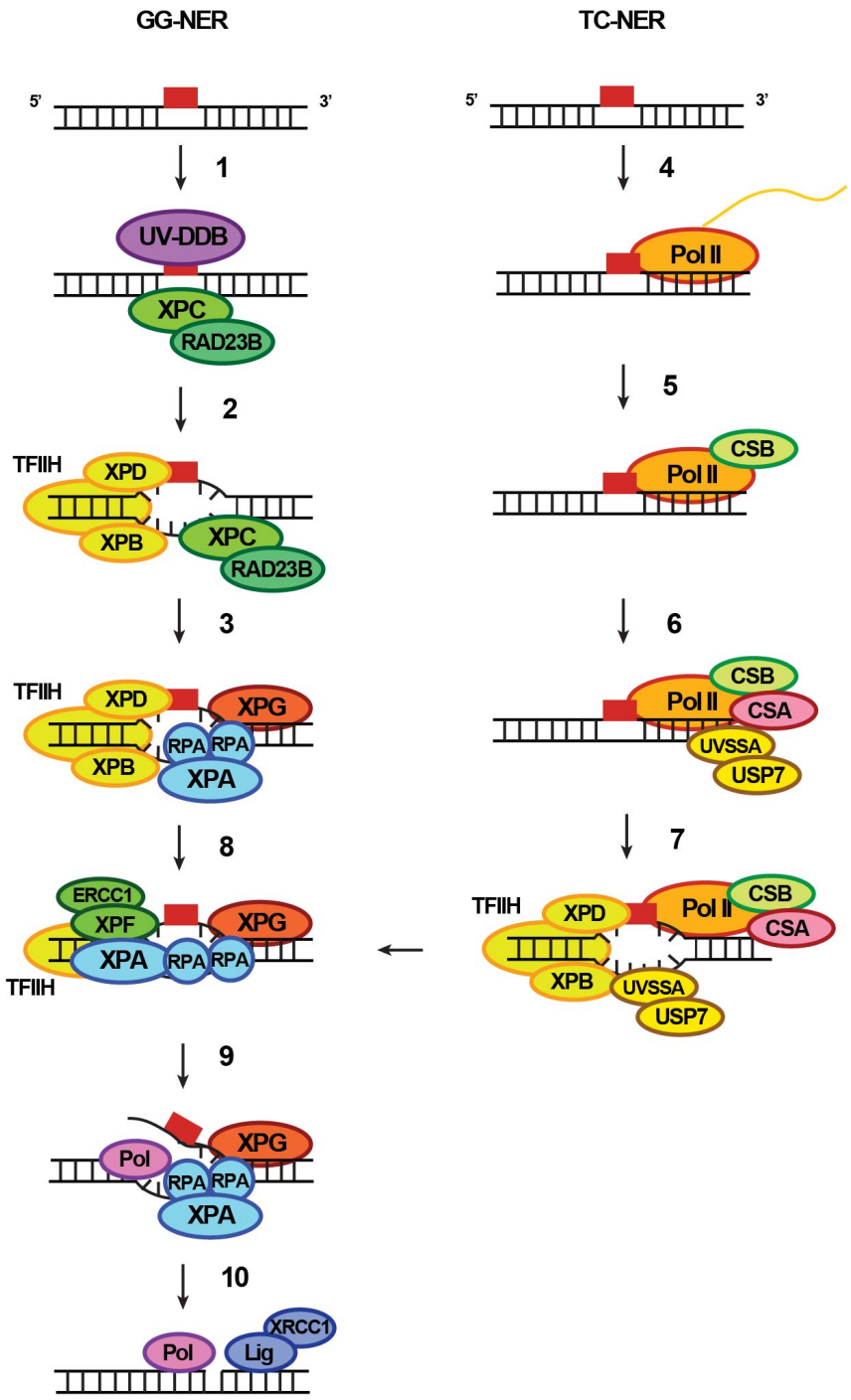


Figure 1-1. Schematic diagram of GG-NER and TC-NER. (1) In GG-NER, lesion is recognized by UV-DDB that then handles it to XPC/RAD23B or by the latter directly. (2) TFIIH complex is recruited to the lesion; XPB unwinds the DNA while XPD scans the DNA for the lesion. (3) The preincision complex (XPA/RPA/XPG) is recruited by TFIIH. (4) In TC-NER, RNA Pol II stalls at the lesion. (5) CSB is recruited by RNA Pol II. (6) CSA is recruited by CSB and promotes UVSSA/USP7 recruitment. (7) TFIIH is recruited by UVSSA. (8) XPF/ERCC1 is recruited by XPA and performs the 5' incision. (9) The replication machinery starts the repair synthesis and XPG cuts at 3'. (10) The nick is sealed by DNA Ligase I or III/XRCC1.

MMR handles base substitution mismatches or insertion/deletion mismatches that remain after faulty DNA replication. Both MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3) complexes can recognize mismatches (Jiricny, 2013). MutSα has also been shown to inhibit PCNA unloading, promoting post-replicative MMR (Kawasoe et al., 2016). Indeed, PCNA interacts and is essential for the activity of the next MMR factor: MutLα (MLH1-PMS2) complex (Genschel et al., 2017). MutLα contains endonuclease activity, leading to the introduction of nicks in the mismatched oligonucleotide that is then degraded by the exonuclease EXO1 (Hermans et al., 2016; Kadyrov et al., 2006). The repair synthesis is performed by Pol δ and PCNA, followed by ligation (Jiricny, 2013).

1.1.3 Double strand break repair

DSBs are repaired mainly through HR or NHEJ, though recently other alternatives such as single strand annealing (SSA) and alternative end joining (alt-EJ), also called microhomology-mediated end joining (MMEJ) have also been identified (Figure 1-2) (Jasin and Rothstein, 2013). Once a DSB is generated, one of these repair pathways is chosen depending on several factors. HR takes place mainly during S/G2 phase of the cell cycle since it requires a homologous template being available. However, NHEJ can occur both in G1 and in S/G2 phase of the cell cycle (Rothkamm et al., 2003). HR is dependent on 5' end resection catalyzed by the concerted nucleolytic activity of the MRN (MRE11/RAD50/NBS1) complex (endonuclease and 3' to 5' exonuclease) together with CtIP in mammals, which generates the 3' overhangs that once bound to RAD51 mediate the strand invasion of the homologous duplex (Anand et al., 2016; Shibata et al., 2014). Therefore, whether end resection takes place is

the first determinant of pathway choice. In NHEJ, the free ends are protected by the Ku proteins and this binding is stabilized by LIG4/XRCC4 (Pierce et al., 2001). Furthermore, BRCA1 promotes HR through enhanced end resection by interacting with phosphorylated CtIP while also excluding 53BP1 (p53-binding protein 1) and RIF1 from DSBs (Chapman et al., 2012; Escribano-Diaz et al., 2013). Another decision point is the formation of the RAD51 nucleofilament on DNA. This process requires the activity of BRCA2 to help displace RPA with RAD51 on DNA and it directs repair towards HR and away from SSA and potentially alt-EJ (Stark et al., 2004). The resolution of the Holliday junctions arising during HR are resolved either by the BLM (Bloom syndrome protein) complex or by the action of nucleases such as MUS81/EME1 or SLX1, that together with XPF/ERCC1 and the scaffolding protein SLX4 form a tri-nuclease SMX complex (Figure 1-2) (Wechsler et al., 2011; Wu and Hickson, 2003; Wyatt et al., 2017).

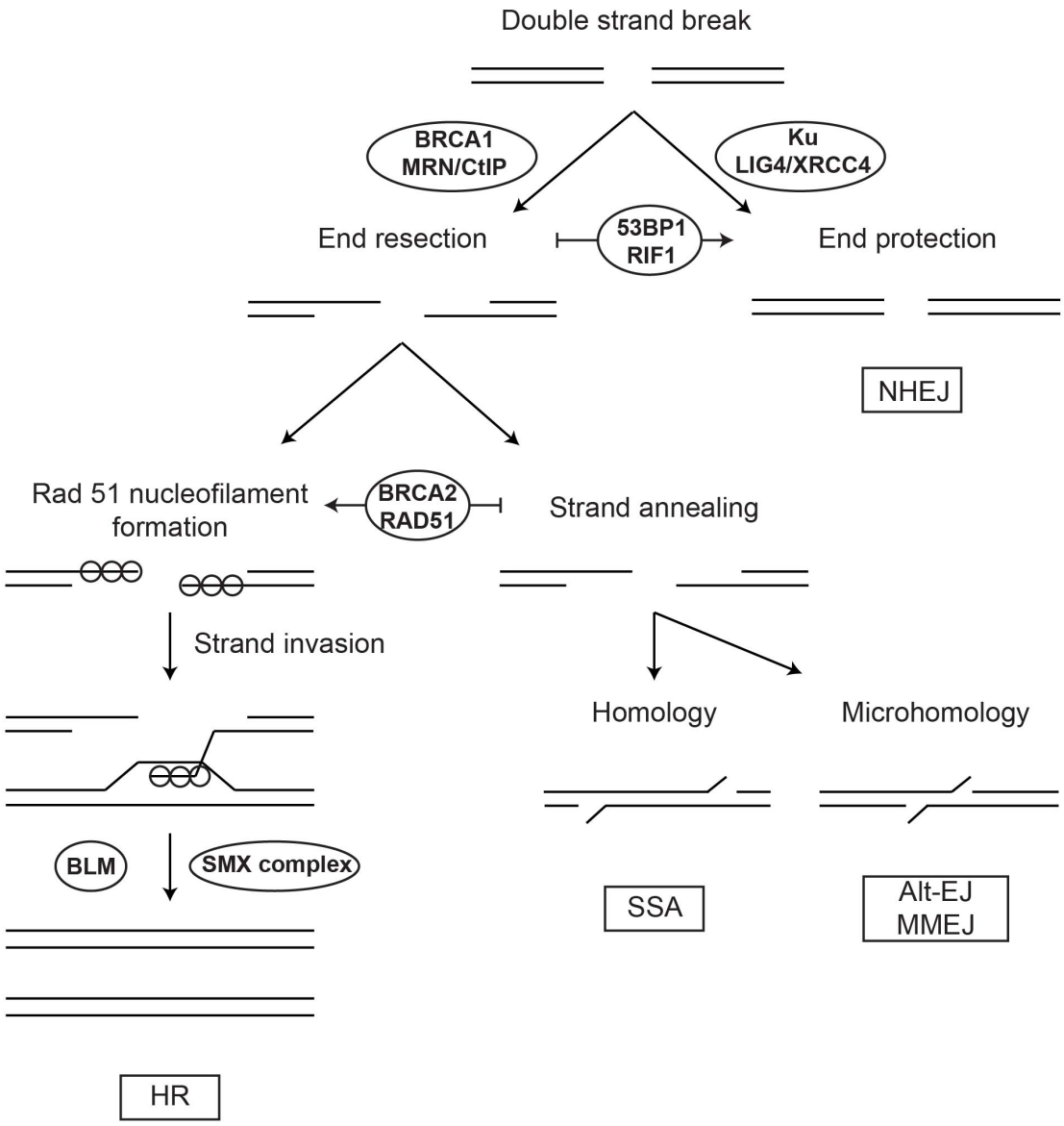


Figure 1-2. Diagram of the pathway choice in DSB repair. After the generation of a DSB, the free ends can be protected by Ku and LIG4/XRCC4 leading to NHEJ or end resection can take place leading to HR, SSA or Alt-EJ. End resection is promoted by BRCA1, MRN complex and CtIP while 53BP1 and RIF1 antagonize the resection. The amount of resection defines the pathway choice with limited resection leading to Alt-EJ and more extensive resection to HR or SSA. The formation of the RAD51 nucleofilament on the resulting 3' single strand is promoted by BRCA2 and leads to strand invasion and HR, away from SSA. HR is completed by resolution of the Holliday junctions either by BLM or SMX complex.

1.2 DNA damage response

Following the detection of any form of DNA damage a signaling cascade follows that leads to the repair of the damage as well as some cellular responses that help in the process, this is called DDR. At the heart of the DDR lie two structurally related kinases: ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related). ATM was initially identified as the defective gene causing a rare disease called A-T (ataxia telangiectasia) (Savitsky et al., 1995). In response to a DSB, ATM is recruited to DNA through NBS1 (a component of the MRN complex) (Falck et al., 2005). ATM can promote HR through the phosphorylation of CtIP, key in the DSB repair pathway choice (Shibata et al., 2011).

ATR, on the other hand, is recruited through ATRIP (ATR-interacting protein) to ssDNA coated by RPA generated by different forms of DNA damage such as replication fork stalling (Zou and Elledge, 2003). ATR has also been linked to the repair of ICLs and the FA pathway that, as will be discussed later, depend on the crosstalk among different repair pathways.

Another kinase, DNA-PKc (DNA-dependent protein kinase catalytic subunit), shares structural similarities with ATM and ATR, but plays a key role in the spatial DNA-end tethering during NHEJ (Graham et al., 2016).

1.3 Fanconi anaemia pathway

1.3.1 Origin of ICLs

ICLs are formed when the two strands of DNA are covalently bound through a crosslinking agent. The first crosslinking agents to be identified were the nitrogen mustards, later other compounds such as nitrosoureas, mitomycin C (MMC) and cisplatin were also found and they are routinely used as

chemotherapeutic agents (Deans and West, 2011). They can react with DNA and give rise to different kinds of products including DNA mono-adducts, intrastrand crosslinks and ICLs. They also differ in their sequence specificity for ICL formation and the disruption they generate in the DNA 3D structure. Naturally occurring crosslinking agents include psoralens, produced by certain plants, but also endogenous compounds such as reactive aldehydes (acetaldehyde, formaldehyde, malondialdehyde (MDA) and crotonaldehyde) and nitric oxide (Langevin et al., 2011; Pang and Andreassen, 2009). Some pollutants generated in the plastic industry like diepoxybutane are also crosslinking agents especially prone to generate ICLs (Wen et al., 2011; Zhang and Elfarra, 2006).

Nitrogen mustards, such as mechlorethamine, are bifunctional alkylating agents; their chloroethyl moieties can bind two bases on opposite strands of DNA. They bind two guanines in the sequence GNC (Figure 1-3A). Nitrogen mustards generate ICLs very inefficiently (around 5% of the products) and cause a small distortion of the double helix with an unwinding of 2-6° and a bend of 10° because of the shortening needed to accommodate the ICL (Dronkert and Kanaar, 2001; Rink and Hopkins, 1995).

MMC is a natural compound produced by *Streptomyces caespitosus*. MMC must be metabolically reduced before it can react with DNA, making it especially fit as a chemotherapeutic agent since the tumour micro-environment is generally hypoxic (Tomasz, 1995). MMC, then, reacts with two guanines in the sequence CG to form an ICL (Figure 1-3B) (Tomasz, 1995). ICLs only constitute around 15% of the products since MMC also generates monoadducts (50%) and intrastrand crosslinks (35%) (Suresh Kumar et al., 1997; Warren et al., 1998).

MMC binds to DNA through the minor groove, causing only a minor distortion (Norman et al., 1990).

Cisplatin (cis-diamminedichloroplatinum(II)) reacts with purine residues to form mainly intrastrand crosslinks at sequences GG and AG and ICLs are formed with a lower frequency at GC sites (Figure 1-3C) (Malinge et al., 1999). Cisplatin induced ICLs provoke a large distortion in the DNA double helix with the extrusion of two cytosines while the platinum locates itself in the minor groove. The double helix suffers an unwinding of 110° and a bent towards the minor groove of 47° (Figure 1-4B) (Coste et al., 1999; Malinge et al., 1999).

Psoralens are naturally occurring crosslinking agents generated by several families of plants (Scott et al., 1976). Their planar and hydrophobic nature allows them to intercalate the DNA bases, but they are unable to form ICLs unless irradiated with UVA (ultraviolet light). Psoralens react with thymines on the sequence TA (Figure 1-3D) and are very effective inducers of ICLs (40% of products). Trimethylpsoralen (TMP), a modified form of psoralen, can even form up to 90% ICLs (Huang et al., 2013). This is due to their inability to form intrastrand crosslinks once they intercalate in between opposing strands of DNA (Brendel and Ruhland, 1984). The ICLs generated do not bend the DNA double helix and only create a minor unwinding of 25° (Figure 1-4C) (Spielmann et al., 1995).

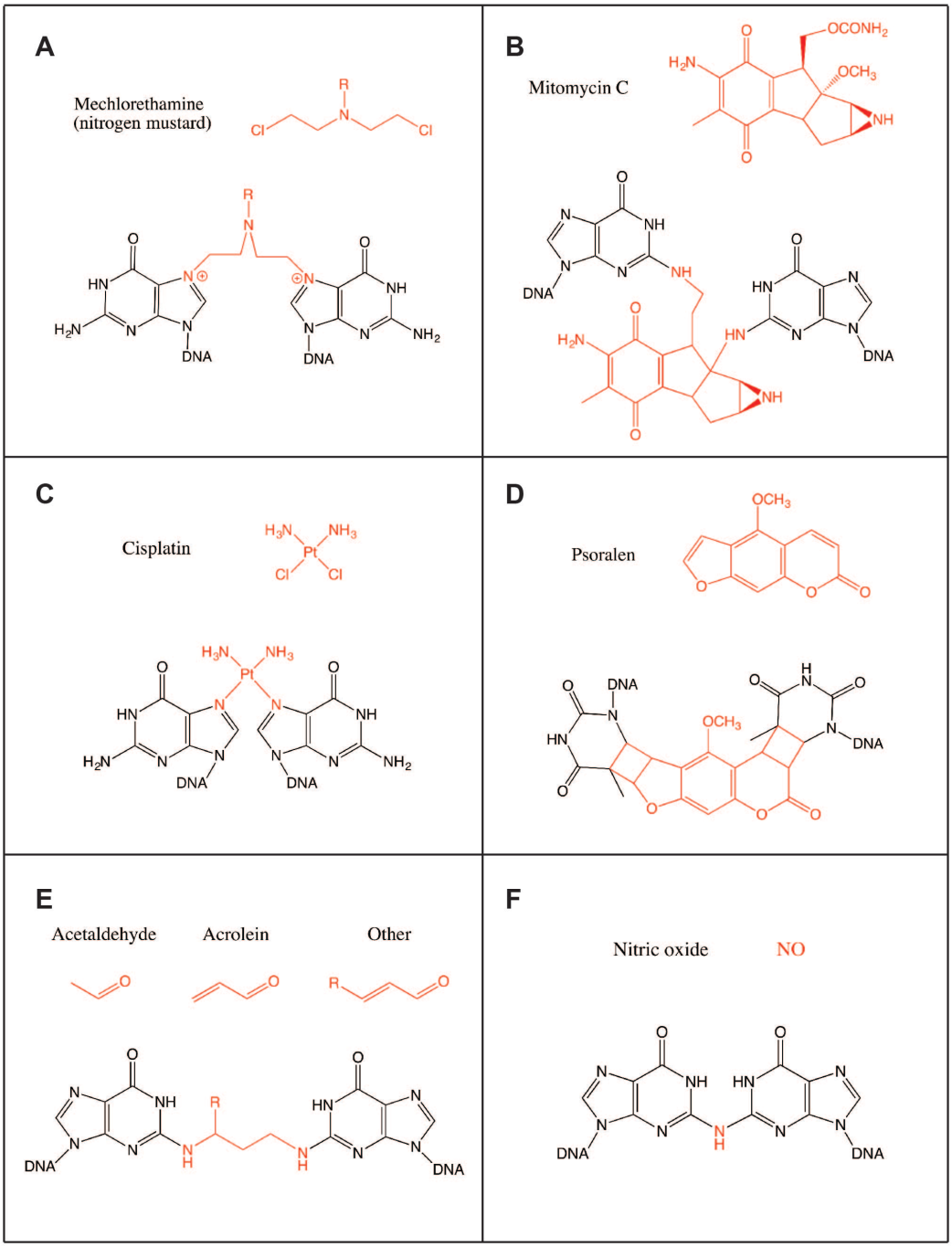


Figure 1-3. Schematic representation of the chemical structure of the main crosslinking agents and the ICLs they form. (A) Mechlorethamine (nitrogen mustard). (B) Mitomycin C. (C) Cisplatin. (D) Psoralen. (E) Aldehydes (acetaldehyde, acrolein and crotonaldehyde (R = CH_3)). (F) Nitric oxide. Crosslinking agents are shown in red. Adapted from (Lopez-Martinez et al., 2016).

The main endogenous sources of ICLs described so far include acetaldehyde (a product of ethanol metabolism), formaldehyde, products of lipid peroxidation like MDA, crotonaldehyde and acrolein and also nitric oxide.

Aldehydes can react with guanine and generate monoadducts, but two aldehydes need to combine in order to generate ICLs or protein-DNA crosslinks, since two reactive groups are necessary for these (Brooks and Theruvathu, 2005; Lorenti Garcia et al., 2009). Aldehyde induced ICLs generally occur on GC sequences and they bind DNA through the minor groove without distorting the double helix (Figure 1-3E and Figure 1-4D) (Cho et al., 2007; Kozekov et al., 2003; Stone et al., 2008). Acetaldehyde has been proposed as a main source of endogenous ICLs, though it is usually quickly processed by aldehyde dehydrogenases (ALDH) in cells. Formaldehyde, however, has been shown to be abundant in the plasma and an important metabolite in the synthesis of purines and amino acids (Ridpath et al., 2007). Actually, cells deficient in the FA pathway show more sensitivity to formaldehyde than to acetaldehyde treatment, pointing to a more relevant role *in vivo* (Rosado et al., 2011). Lipid peroxidation results from oxidative stress and it is enhanced by a high fat diet in mice (Folmer et al., 2003), so it could also constitute an important source of ICLs in humans. Lipid peroxidation leads to the formation of MDA, as well as unsaturated aldehydes such as crotonaldehyde and acrolein, which can then react with guanine both through the carbonyl group and the double bond (Niedernhofer et al., 2003; Stone et al., 2008). Nitric oxide can form ICLs joining two guanines in the sequence CG, forming a monoatomic bridge, and this reaction is promoted by the presence of methylated cytosines nearby (Figure 1-3F) (Caulfield et al., 2003).

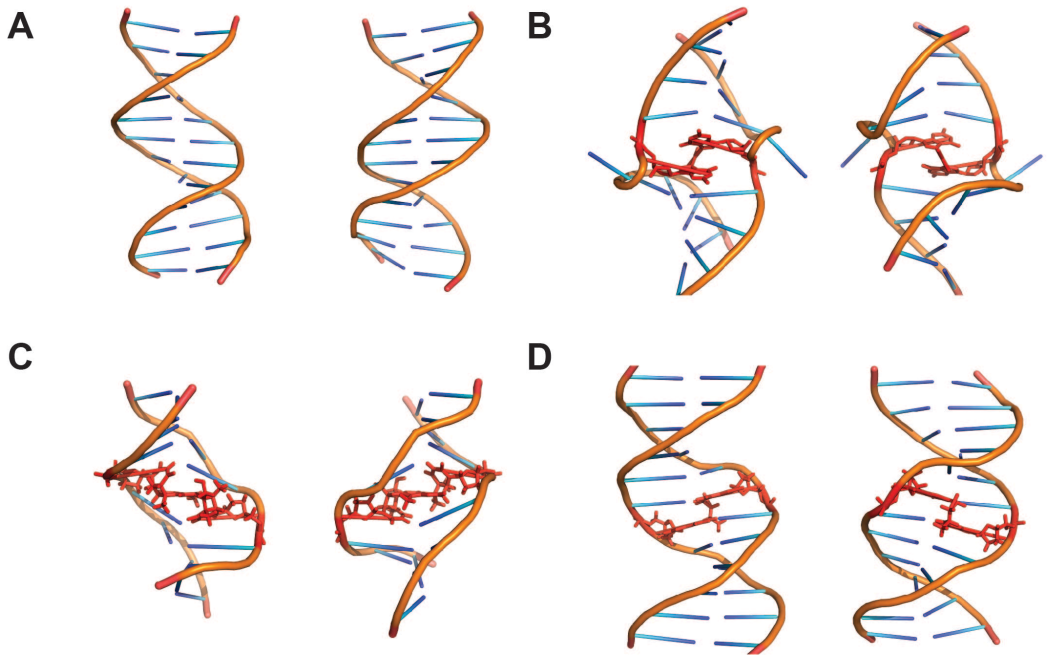


Figure 1-4. Structures of various ICLs. (A) B-DNA. (B) Cisplatin ICL. (C) Psoralen ICL. (D) Acetaldehyde and crotonaldehyde ICL. Structures viewed from the major groove (left) or the minor groove (right). The crosslinked bases and the crosslinking agents are shown as red sticks. Structures taken from PDB, accession numbers: B-DNA (1-BNA) (Drew et al., 1981), cisplatin (1A2E) (Malinge et al., 1999), psoralen (204D) (Spielmann et al., 1995), acetaldehyde (2HMD) (Cho et al., 2007). Adapted from (Lopez-Martinez et al., 2016).

1.3.2 Fanconi anaemia and the repair of ICLs

FA is a rare genetic disorder, affecting around 1 in 100.000 people in the USA, which leads to developmental abnormalities and early bone marrow failure (BMF) (Nalepa and Clapp, 2018). FA patients are especially prone to develop tumours, particularly acute myelogenous leukaemia (AML). These patients were found to be extremely sensitive to chemotherapeutic agents, that is, to crosslinking agents and it was established that FA proteins are responsible for ICL repair (Nalepa and Clapp, 2018). Therefore, it was initially proposed that this defect in ICL repair would be responsible for the phenotype of FA patients including BMF. In fact, hematopoietic stem cells are sensitive to endogenous

aldehydes in the absence of the FA pathway and the relevant detoxifying catabolic pathway (Garaycoechea et al., 2012; Garaycoechea et al., 2018; Pontel et al., 2015). However, except for a small group of FA patients in Japan that carry mutations in the *ALDH2* gene (Hira et al., 2013), most FA patients do not carry defects in the metabolic pathways that process aldehydes, shedding doubts on aldehyde induced ICLs being the primary cause of FA phenotypes. Furthermore, even if the toxicity associated with aldehydes is responsible for FA, other forms of damage apart from ICLs could be the main drivers such as DNA-protein crosslinks (Duxin and Walter, 2015).

On the other hand, it has also been proposed that an exacerbated inflammatory response mediated by tumour necrosis factor α (TNFα), interferon γ and reactive oxygen species (ROS) leading to apoptosis of the hematopoietic stem cells could cause the BMF in FA (Sejas et al., 2007). Both models are not necessarily in contradiction since the inflammation signalling pathways could be triggered by defects in DNA damage repair (Garaycoechea and Patel, 2014), maybe through the release of DNA fragments into the cytosol and the activation of the cGAS-STING pathway (Bregnard et al., 2016). Moreover, new functions for the different FA proteins are continuously being identified such as replication fork protection, ultra-fine bridges association, spindle assembly and S-phase checkpoints regulation as well as roles in other DDR pathways (Ceccaldi et al., 2016). In conclusion, the traditional equation between Fanconi anaemia and ICL repair might not hold as true as it was once thought.

Nevertheless, ICL repair is still considered part of the FA pathway, though there is also crosstalk with other DDR pathways, and FA proteins perform functions outside of ICL repair. Up to date 22 FA proteins have been described

(Table 1-1), that together with FA-associated proteins (FAAP) and non-FA proteins process ICLs (Nalepa and Clapp, 2018).

Table 1-1. FA proteins, their synonyms, sizes and functions. *FANCM is traditionally included as an FA gene. However, there is some controversy because the first FA patient identified with biallelic mutations in *FANCM* also had alterations on *FANCA* (Singh et al., 2009) and also the fact that individuals with homozygous loss of function of FANCM did not display FA symptoms (Lim et al., 2014). Even if *FANCM* alterations do not cause FA, they can lead to infertility and early onset cancer (Bogliolo et al., 2018; Fouquet et al., 2017; Yin et al., 2018).

FA protein	Synonym	Size (aa)	Function
FANCA	-	1,455	FA core complex
FANCB	-	859	FA core complex
FANCC	-	558	FA core complex
FANCD1	BRCA2	3,418	Homologous recombination
FANCD2	-	1,451	Recruitment of downstream effector proteins
FANCE	-	536	FA core complex
FANCF	-	374	FA core complex
FANCG	XRCC9	622	FA core complex
FANCI	-	1,328	Recruitment of downstream effector proteins
FANCJ	BRIP1, BACH1	1,249	Homologous recombination, helicase
FANCL	-	380	FA core complex, E3 ubiquitin ligase
FANCM*	-	2,048	FA core complex, DNA translocase
FANCN	PALB2	1,186	Homologous recombination, BRCA2 partner
FANCO	RAD51C	376	Homologous recombination
FANCP	SLX4	1,834	Scaffolding protein for nucleases
FANCQ	ERCC4, XPF	916	ERCC1 partner, nuclease
FANCR	RAD51	340	Homologous recombination
FANCS	BRCA1	1,863	Homologous recombination, removes CMG
FANCT	UBE2T	197	FANCL partner, E2 conjugating enzyme
FANCU	XRCC2	280	Homologous recombination
FANCV	REV7	211	Translesion synthesis
FANCW	RFWD3	774	E3 ligase, homologous recombination

The FA pathway can be divided in three main parts: first, detection of the ICL and recruitment of the FA core complex (FANCA, -B, -C, -E, -F, -G, -L, -M, FAAP20, FAAP24 and FAAP100); second, recruitment of FANCD2/FANCI complex leading to their monoubiquitination and, finally, repair of the ICL by

effector proteins including nucleases, polymerases, HR and TLS-related (translesion synthesis) components (Figure 1-5) (Lopez-Martinez et al., 2016).

The detection of the ICL was initially ascribed to a member of the FA core complex, FANCM, which was found to bind DNA and have translocase activity (Meetei et al., 2005). FANCM was then shown to interact with FAAP24, which is essential for FANCM loading on chromatin together with the core complex (Ciccia et al., 2007; Kim et al., 2008). FANCM also interacts with histone-fold heterodimers 1 and 2 (MHF1/2), the resulting complex is recruited to replication forks stalled by ICLs where they have been proposed to promote processing of the branched DNA structures, protect the stalled replication fork and even to mediate the replication fork traverse through the ICL (Blackford et al., 2012; Huang et al., 2013; Yan et al., 2010; Zhao et al., 2014). Despite the important functions of FANCM at stalled replication forks, it remains unclear whether FANCM is able to detect ICLs specifically and independently of replication.

The detection of ICLs has also been linked to proteins involved in MMR and this pathway has been more widely associated with ICL repair. MutSβ was found to recognise and promote the processing of psoralen induced ICLs *in vitro* and in cells, and FANCJ and FANCD2 interact with MMR components: MutLα and MSH2/MLH1, respectively (Peng et al., 2007; Williams et al., 2011; Zhang et al., 2002; Zhao et al., 2009). Although initially discarded, MutSα has recently been shown to bind highly distorting ICLs in cell free extracts from *Xenopus* eggs and it was proposed to mediate repair independently of FA proteins and replication or transcription (Kato et al., 2017). However, whether MutSα is an ICL sensor and not just indirectly binding to the ICL remains unclear.

Finally, a non-FA protein, UHRF1 (Ubiquitin-like containing PHD and RING finger domains 1), has been shown to recognise psoralen ICLs *in vitro* and in cells and it has been proposed to promote ICL repair through recruitment of FANCD2/FANCI complex as well as ERCC1 and MUS81 (Figure 1-5) (Liang et al., 2015; Tian et al., 2015).

The next key event in the pathway is the recruitment of the FA core complex, maybe through FANCM/FAAP24, as previously described, or through other components. An alternative model for the recruitment of the core complex involves FAAP20 recognising histone H2A polyubiquitination by RNF8/UBC13 in response to DNA damage (Yan et al., 2012). The main known function of the FA core complex is to catalyse the monoubiquitination of both FANCD2 and FANCI, by FANCL (E3 ubiquitin ligase) and FANCT (UBE2T, E2 conjugating enzyme) (Longerich et al., 2009; Machida et al., 2006; Meetei et al., 2003). However, it is unclear what are the exact roles of the other components of the core complex, even though more than 85% of FA patients carry mutations in either FANCA, FANCC or FANCG (Wang and Smogorzewska, 2015). FANCA, for example, has been shown to play a role in homologous recombination and to catalyse single strand annealing and strand exchange, in combination with FANCG (Benitez et al., 2018; Nakanishi et al., 2005; Yang et al., 2005). Whether these newly discovered roles of FANCA and FANCG are related to ICL repair and their FA phenotypes remains unknown.

Most of what we know about the downstream mechanism of ICL repair comes from studies done *in vitro* with cell free extracts from *Xenopus* eggs (Figure 1-5) (Knipscheer et al., 2012). The repair of ICLs has mainly been studied in a replication dependent manner (Raschle et al., 2008). In this system, it was

found that two replication forks need to converge on an ICL in order for the CMG (Cdc45, Mcm2-7 and GINS) helicase to be unloaded, a process dependent on BRCA1 (Long et al., 2014; Zhang et al., 2015). Recently, it was shown that the CMG unloading leads to fork reversal on one side of the ICL, though the exact importance of this step in later repair is unclear (Amunugama et al., 2018). This, in turn, allows the recruitment of FANCD2/FANCI and their monoubiquitination on DNA by the FA core complex (Knipscheer et al., 2009; Liang et al., 2016). Monoubiquitinated FANCD2/FANCI, then, recruit nucleases such as XPF/ERCC1 together with the scaffolding protein SLX4, which catalyse incisions around the ICL leading to its unhooking (Klein Douwel et al., 2014; Klein Douwel et al., 2017; Kuraoka et al., 2000; Yamamoto et al., 2011). TLS, then, proceeds through the recruitment of Rev1-Polζ by the FA core complex, which fill the gap opposing the unhooked ICL through their ability to accommodate bulky DNA (Budzowska et al., 2015). CtIP and, potentially, the MRN complex are recruited through FANCD2 and resect the DSB generated in the process (Murina et al., 2014; Nakanishi et al., 2002; Roques et al., 2009; Unno et al., 2014). RAD51 mediated strand invasion follows while MUS81 and SLX1 help resolve the junctions generated and the repair is complete through NER and the elimination of the damaged base (Figure 1-5) (Long et al., 2011; Mouw and D'Andrea, 2014; Wood, 2010; Wyatt et al., 2013).

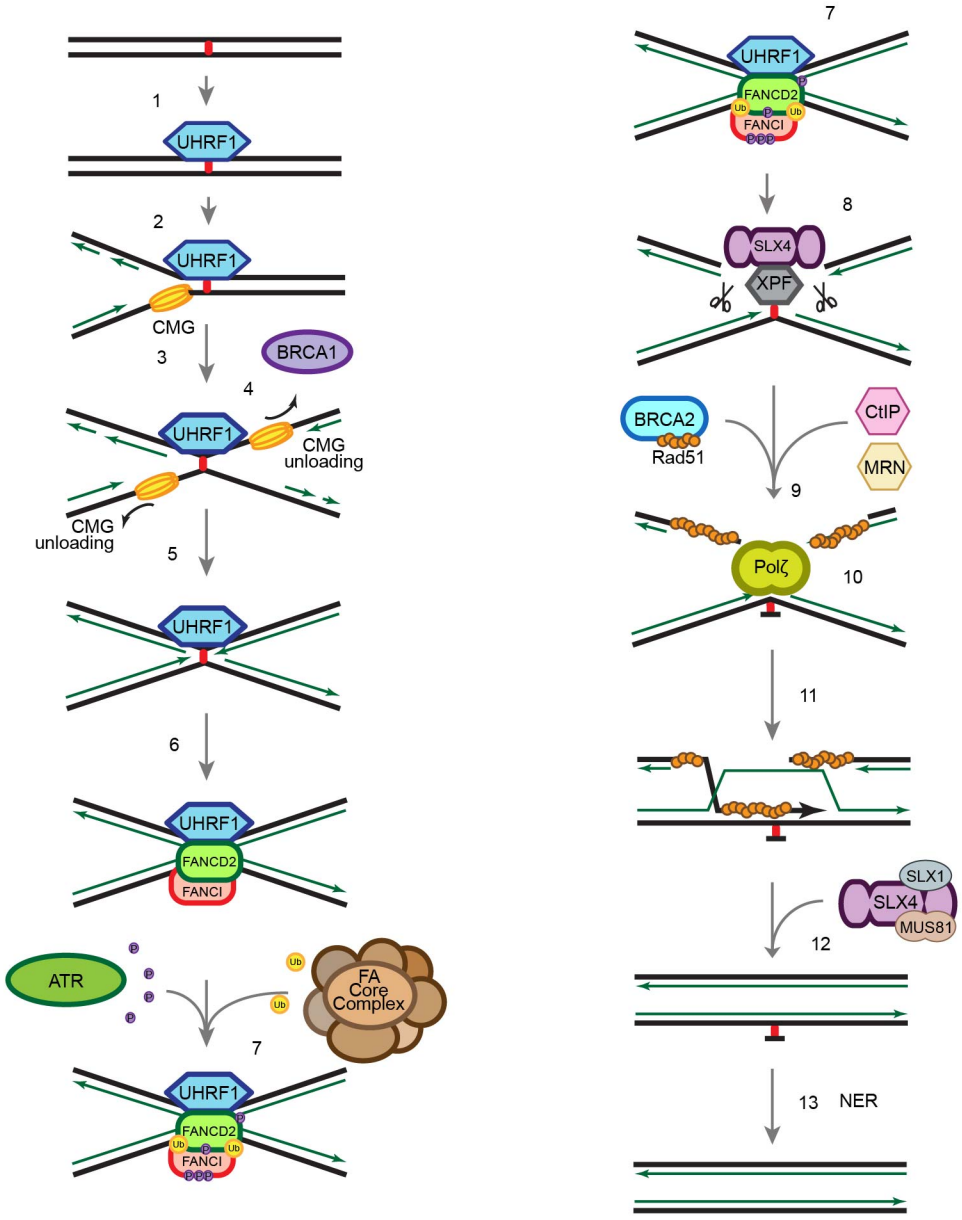


Figure 1-5. Diagram of replication coupled ICL repair. (1) UHRF1 is quickly recruited to ICLs. (2) Single replication fork arrives at the ICL. (3) A second fork converges at the ICL. (4) BRCA1 (FANCS) facilitates the unloading of the CMG helicase complex. (5) The replicative polymerase proceeds to the -1 position of the ICL, generating an X-shape structure. (6) FANCD2/FANCI is recruited to the ICL at the replication fork. (7) ATR phosphorylates FANCD2/FANCI and FA core complex monoubiquitinates FANCD2/FANCI. (8) Ubiquitinated FANCD2/FANCI complex recruits SLX4/XPF to ICL to unhook the ICL. (9) CtIP and the MRN complex resect the double strand break ends generated by the unhooking, and BRCA2 facilitates Rad51 filament formation on the ssDNA generated by the resection. (10) Polζ polymerizes new strand of DNA through the unhooked ICL. (11) Rad51 facilitates the strand invasion, which allows extension of the other strand. (12) SLX4/SLX1/MUS81 resolve the double Holliday junction. (13) The damaged nucleotide is removed by NER. Adapted from (Lopez-Martinez et al., 2016).

There is growing evidence for a replication independent repair of ICLs that would take place in G1 phase and could be especially important for cells in G0 such as neurons. The main mechanism proposed so far is a repair dependent on NER and TLS; some key components include XPC, ERCC1/XPF, Polζ/Rev1 and Polκ (Muniandy et al., 2009; Sarkar et al., 2006; Wang et al., 2001; Williams et al., 2012). It has also been suggested that in G1, repair could depend on transcription, since ICL can also block transcription progression leading to a transcription coupled-NER (Enoiu et al., 2012). Although ERCC1/XPF has been involved in both replication dependent and independent ICL repair, it has been suggested that it may play different roles in each type of repair with a NER-independent function during S phase and NER-dependent in G1 (Seol et al., 2018).

1.3.3 Regulation of the Fanconi anaemia pathway

The repair of ICLs is a complex process that may vary depending on the organism, the phase of the cell cycle or the origin of the ICL. Therefore, an intricate regulation is in place during ICL repair involving several modifications such as phosphorylation, ubiquitination and SUMOylation (small ubiquitin-like modifier). The keystone of these modifications is the monoubiquitination of FANCD2/FANCI, which leads to their activation and locking on chromatin, and it is essential for the repair to happen. Many other modifications in FANCD2/FANCI or other proteins revolve around this monoubiquitination step (Figure 1-6).

One of the early events in ICL repair is the recruitment of FANCM. FANCM is hyperphosphorylated in *Xenopus* during S phase by ATR or ATM, which helps

recruitment to chromatin (Sobeck et al., 2009). Similarly, in human cells FANCM is phosphorylated at S1045 by ATR, promoting its chromatin localization (Singh et al., 2013). On the other hand, once the cell enters mitosis, FANCM is phosphorylated by PLK1 (polo-like kinase 1) leading to its degradation and timely shutdown of repair (Kee et al., 2009). It has also been proposed that ATR helps physically to recruit FANCM/FAAP24 to ICL stalled replication forks through a common binding partner, HCLK2 (TEL2: telomere length regulation protein 2) (Collis et al., 2008). This, in turn, could help recruit the FA core complex. Several other members of the core complex are themselves regulated by phosphorylation. For instance, CDK1 phosphorylates FANCG during mitosis at S383 and S387 promoting the dissociation of the core complex from chromatin, probably after the repair is complete (Mi et al., 2004; Qiao et al., 2001). FANCA is phosphorylated on S1449 specifically after DNA damage (not in S phase) by ATR and, even though the mechanism remains unknown, this event is critical for MMC resistance (Collins et al., 2009). Through the study of a clinical case, it was found that FANCA is also SUMOylated by UBC9 and then polyubiquitinated by RNF4, which leads to its degradation and might regulate the core complex presence on DNA (Xie et al., 2015). FANCE is phosphorylated after DNA damage by Chk1 at T346 and S374, which promotes FANCE degradation and potentially shutdown of the pathway (Wang et al., 2007).

As already mentioned, the monoubiquitination of the FANCD2/FANCI complex by FANCL is an essential step in the FA pathway that activates the complex by locking it on chromatin where it can mediate the recruitment of downstream effectors (Liang et al., 2016). This monoubiquitination step is promoted by ATR, and also Chk1, phosphorylation of both FANCD2 (S331, T691

and S717) and FANCI (S556, S559, S565, S596, S617, S629) (Andreassen et al., 2004; Cheung et al., 2017; Ho et al., 2006; Ishiai et al., 2008; Zhi et al., 2009). ATR has also been shown to promote FANCD2/FANCI monoubiquitination through RAD9/RAD17 (Guervilly et al., 2008). Recently, it has been described that a subpopulation of FANCD2/FANCI is subject to SUMOylation by PIAS1/4 and UBC9 once on chromatin that is reversible by SENP6. The SUMOylated complex is thought to be polyubiquitinated by RNF4, promoting its dissociation from chromatin and regulating its dosage on DNA (Gibbs-Seymour et al., 2015). The deubiquitination of FANCD2/FANCI by USP1/UAF1 is also essential for ICL repair, pointing to the need for the complex to dissociate in order to complete the repair (Figure 1-6) (Cohn et al., 2007; Oestergaard et al., 2007).

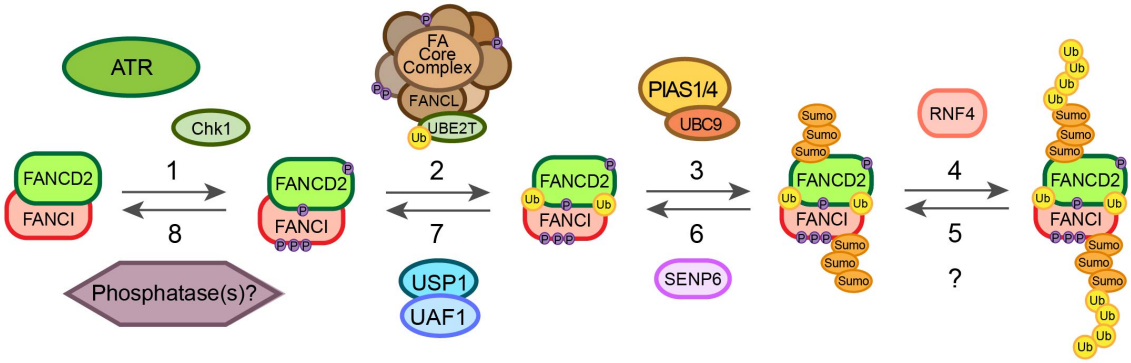


Figure 1-6. Diagram of the main regulatory events around FANCD2/FANCI in response to ICLs. Once the ICL is detected it triggers the ATR/Chk1 pathway leading to the phosphorylation of several components of the FA core complex. ATR and Chk1 phosphorylate FANCI and FANCD2 (1) priming the complex for its monoubiquitination. These phosphorylation events lead then to the monoubiquitination of the FANCD2/FANCI complex by FANCL and UBE2T (2), which locks the complex on chromatin and promotes the recruitment of effector proteins. On the other hand, the dosage of the FANCD2/FANCI complex on chromatin can be regulated through SUMOylation-dependent polyubiquitination mediated by PIAS1/4, UBC9 and RNF4 (3,4). Finally, these events can be reversed by the action of SENP6 (6), the deubiquitinase complex USP1-UAF1 (7) as well as putative phosphatases (8) still unidentified. Adapted from (Lopez-Martinez et al., 2016).

The regulation of downstream effectors has been studied mainly in the context of their roles in DSB repair, but these modifications may also be present in ICL repair. BRCA1 phosphorylation by ATM was one of the first regulatory events identified in DSB repair (Cortez et al., 1999). Later, kinases such as PLK1 and CDK1 have been shown to promote BRCA1 foci formation (Chabalier-Taste et al., 2016; Johnson et al., 2009). Ubiquitination also plays a role regulating BRCA1 activity. PALB2 interaction with BRCA1 is essential for HR progression, but PALB2 ubiquitination can abrogate this interaction (Orthwein et al., 2015). BRCA1 can also interact with polyubiquitinated FANCG, a process that could be key for the HR steps of ICL repair (Zhu et al., 2015). Finally, SUMOylation of BRCA1 at its BRCT and RING domains seems to play an important role in regulating the loading of other repair factors through their SUMO-interacting motifs (SIMs) (Morris et al., 2009; Vialter et al., 2011). SUMOylation could play a double role in DDR, on the one hand promoting recruitment of SIM containing factors to SUMOylated complexes such as BRCA1, FANCD2/FANCI, etc., synergistically (Psakhye and Jentsch, 2012), and, on the other hand, leading to SUMO-dependent polyubiquitination and degradation, shutting down the repair.

Chapter 2. Identification of new phosphosites on FANCD2 and their function in human cells

2.1 Introduction

At the centre of the FA pathway is the heterodimer formed by FANCD2 and FANCI. FANCD2 was the first of the two to be identified (Timmers et al., 2001), and it was found to become active through its monoubiquitination that led to its localisation to nuclear foci after DNA damage (Garcia-Higuera et al., 2001). FANCI was later identified as a key binding partner of FANCD2 that localised to DNA damage and was also monoubiquitinated after DNA damage (Sims et al., 2007; Smogorzewska et al., 2007). Interestingly, this double monoubiquitination step is essential for the FA pathway and the repair to happen, mutant forms of FANCD2/FANCI that lack monoubiquitination are extremely sensitive to ICLs (Smogorzewska et al., 2007). The function of FANCD2/FANCI in ICL repair is not fully understood, but they were early found to bind DNA (Park et al., 2005; Yuan et al., 2009) and also to interact to downstream repair factors such as BRCA1 (Garcia-Higuera et al., 2001), MRN complex (Nakanishi et al., 2002; Roques et al., 2009), FAN1 (Kratz et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010), SLX4 (Yamamoto et al., 2011), Pol eta (Fu et al., 2013) and CtIP (Murina et al., 2014; Unno et al., 2014). Therefore, the FANCD2/FANCI complex is thought to bridge the early events in ICL repair (detection, FA core complex recruitment) with the downstream repair proteins.

Given their important role, it is not surprising that FANCD2 and FANCI are part of a complex regulatory network, ensuring a correct timing of repair and avoiding spurious events happening on DNA. A link between ATM/ATR and FANCD2 was early identified (Taniguchi et al., 2002b), and lack of ATR was found to affect FANCD2 monoubiquitination in response to DNA damage (Andreassen et al., 2004). Although this effect could be indirect, it was later found that T691 and S717 on FANCD2 are phosphorylated by ATR and needed for ICL repair, though not for FANCD2 monoubiquitination (Ho et al., 2006). A downstream target of ATR is the kinase Chk1, which has also been linked to FANCD2 phosphorylation (Guervilly et al., 2008). Specifically, Chk1 phosphorylates S331 on FANCD2 and this event is needed for both ICL repair and FANCD2 monoubiquitination (Zhi et al., 2009). ATR was also found to phosphorylate FANCI on at least 6 different sites, and this was thought to promote FANCI and FANCD2 monoubiquitination and function (Ishiai et al., 2008). However, recent evidence showed a more complex picture where some sites are phosphorylated prior to monoubiquitination (S556) while others are phosphorylated after monoubiquitination (S559 and S565) and inhibit FANCD2 deubiquitination (Cheung et al., 2017).

We, thus, decided to study whether there are other phosphorylation sites on FANCD2, which kinases and phosphatases are implicated and how they affect FANCD2 function and the regulation of the FA pathway and ICL repair.

2.2 Identification of new phosphorylation sites on FANCD2

First, we decided to generate a FANCD2$^{-/-}$ HeLa cell line that will allow us to study the effect of the different phosphosites by reintroducing mutant forms

of the sites that either cannot be phosphorylated (alanine mutants) or that act as phosphomimetics (aspartic acid mutants). The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) system made it possible to introduce DSBs at specific sites as long as they are followed by a PAM (protospacer adjacent motif) sequence of the form NGG (Doudna and Charpentier, 2014; Mojica et al., 2009). In order to generate a knock-out cell line a target sequence followed by a PAM sequence must be found in an exon of the gene of interest so that repair of the DSB introduced in the exon through NHEJ can lead to short insertions or deletions that will cause a frameshift and an early truncation of the protein. Using the algorithm developed by MIT (http://crispr.mit.edu/) we found a high scored target sequence in exon 4 of *FANCD2* (Figure 2-1A). Clones were screened using Western blot to assess the lack of FANCD2 expression (Figure 2-1B) and the successful clone was checked through quantitative Western blot (Figure 2-1C). The resulting genomic sequence around exon 4 was deconvoluted with TIDE (https://tide.nki.nl/), an online software able to extract mixed sequences from one sequencing reaction. The sequence was found to contain a one nucleotide deletion in one case and a one nucleotide insertion in the other (Figure 2-1D), which explains the lack of FANCD2 expression through a frameshift and early stop codon in the engineered *FANCD2* gene.

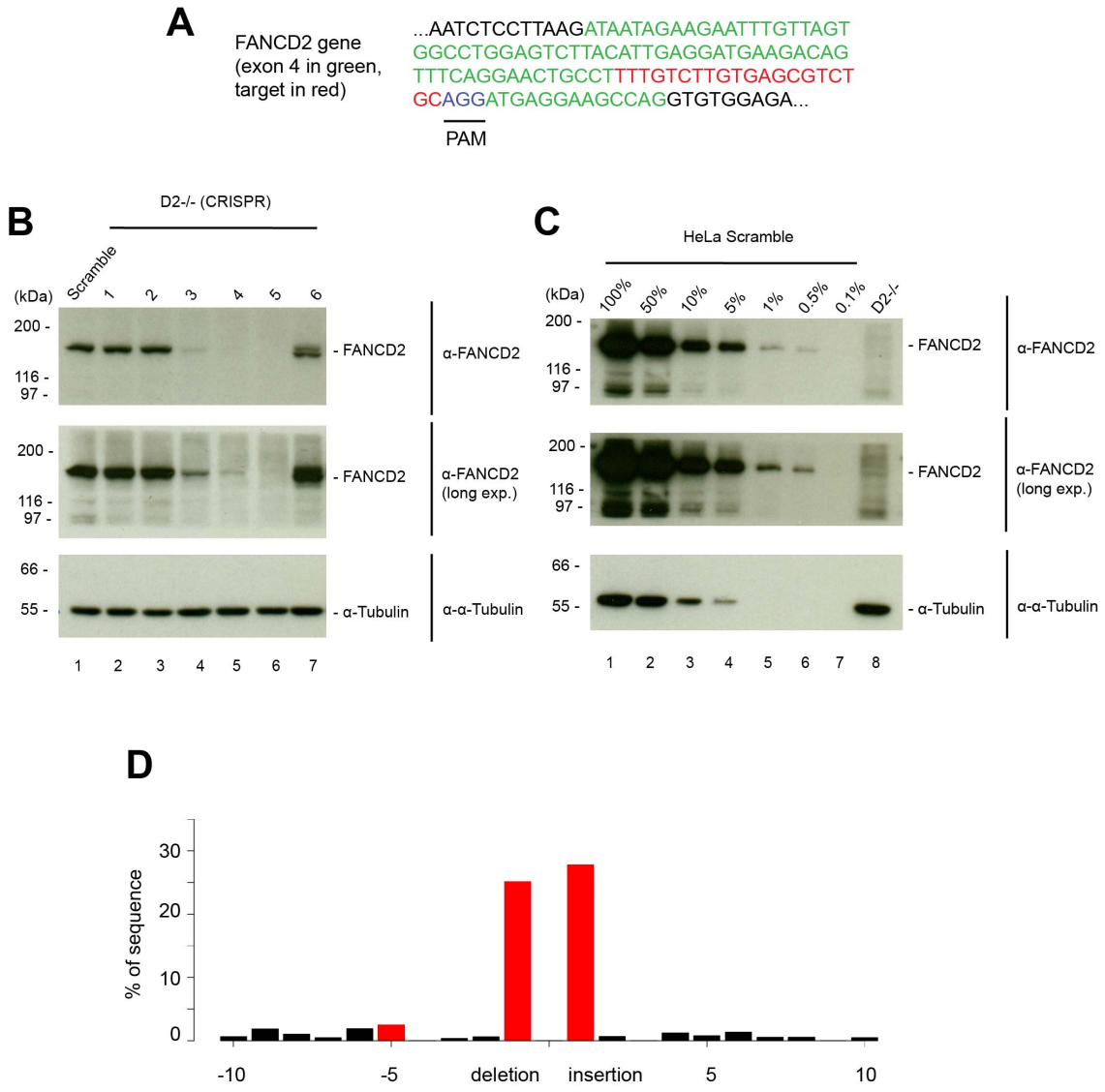


Figure 2-1. Generation of a FANCD2$^{-/-}$ HeLa cell line. (A) CRISPR/Cas9 targeted sequence in the *FANCD2* gene to generate the knock-out *FANCD2*: exon 4 is in green, the CRISPR target is red and the PAM sequence in blue. (B) Western blot showing the screening for FANCD2$^{-/-}$ clones, HeLa Scramble cell were used as a control. Clone 5 was used for further testing. (C) Quantitative Western blot confirming the absence of FANCD2 protein in the FANCD2$^{-/-}$ clone 5. The detection limit of the Western Blot is lower than 0.5% of the endogenous FANCD2 protein. (D) Sequence trace decomposition analysis (TIDE) of the resulting sequence in exon 4 in FANCD2$^{-/-}$ clone 5. Two mutations were detected: one nucleotide deletion and one nucleotide insertion, which lead to a frameshift and early truncation of the protein.

In order to study phosphorylation events in FANCD2, we introduced a Flag-HA-tag into the *FANCD2* gene in HeLa S3 cells using CRISPR/Cas9, creating an N-terminal tagged fusion protein (Figure 2-2A). The generation of such a knock-in cell line is similar to what was described for the knock-out, with the exception that a template sequence must be provided together with the CRISPR/Cas9 coding sequences so that the generation of the DSB can be repaired through HR and the desired sequence introduced into the endogenous gene. The efficiency of this event is lower than that of the knock-out though, but the concomitant introduction of selection markers can improve the result. Another difference is that the target sequence should be inside the intron near exon 1 to avoid the generation of knock-out alleles if HR fails (Figure 2-2B). We used Western blot to confirm the presence of the Flag-HA-FANCD2 and found a positive cell line, though it still contained untagged FANCD2 due to the lower efficiency of this process in the absence of selection markers (Figure 2-2C).

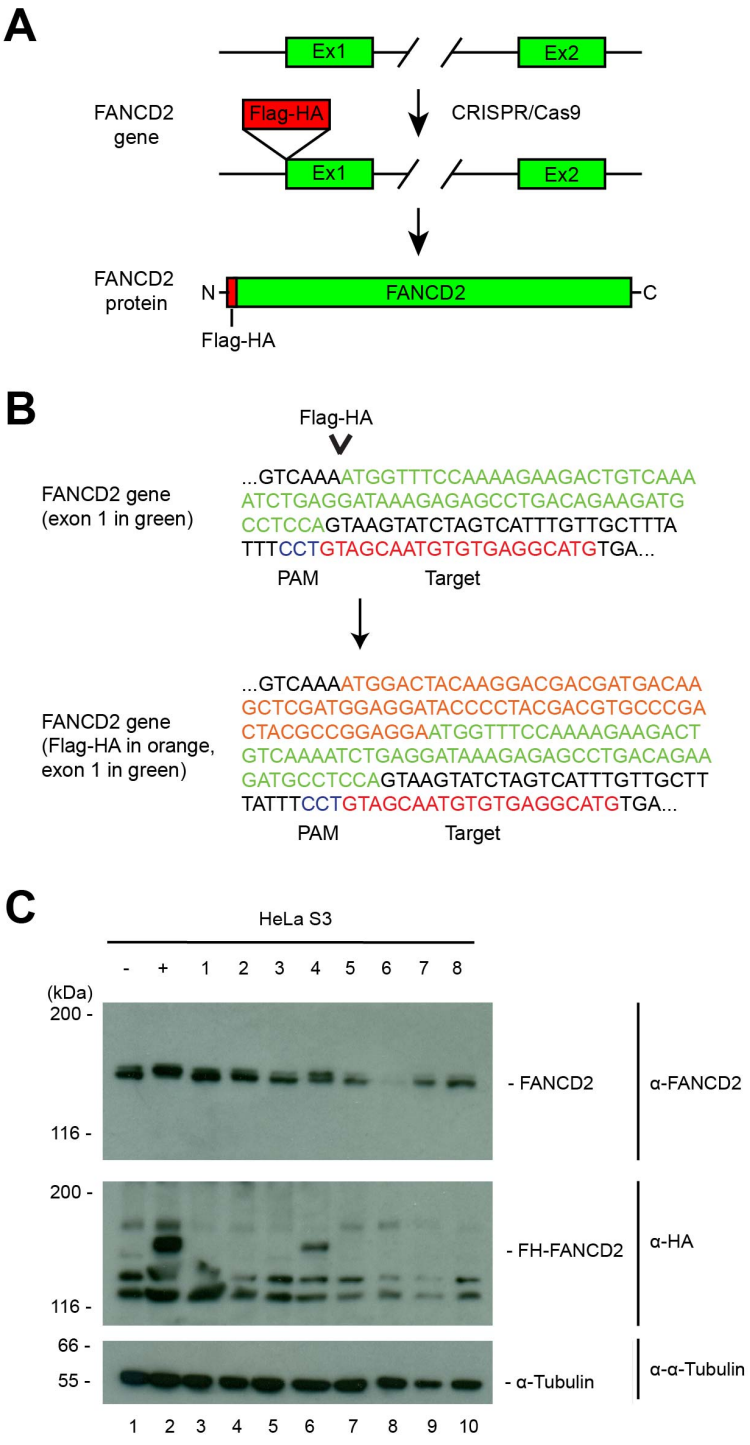


Figure 2-2. Generation of Flag-HA-FANCD2 knock-in HeLa S3 cells. (A) Schematic representation of the CRISPR/Cas9 strategy to introduce the Flag-HA sequence into the 5' end of exon 1 of *FANCD2* gene. Exons in green, Flag-HA tag in red. (B) Sequence around exon 1 (green) of the *FANCD2* gene showing the CRISPR target sequence in red and the PAM sequence in blue. The knock-in Flag-HA tag sequence is shown in orange. (C) Western blot of the screening for Flag-HA-FANCD2 knock-in clone of HeLa S3. Clone 4 showed the presence of Flag-HA-FANCD2. HeLa S3 is used as negative control (lane 1) and HeLa S3 shFANCD2 + Flag-HA-FANCD2 is used as positive control (lane 2).

We then introduced ICLs in the cells and purified Flag-HA-FANCD2 (Figure 2-3A). Induction of monoubiquitinated FANCD2 was robust compared to untreated control cells (Figure 2-3B). The 4 bands were sent for MS in order to study the dynamics of phosphorylation of FANCD2 before and after damage, but also in non-ubiquitinated and ubiquitinated FANCD2. MS/MS analysis of the purified protein revealed phosphorylation of multiple residues, including residues previously reported: S1257, S1401, S1404, S1407 (Taniguchi et al., 2002b) underscoring the validity of the experiment (Table 2-1). Several of them are predicted to be phosphorylated by kinases already linked to the FA pathway such as ATM/ATR, but some new kinases were also found.

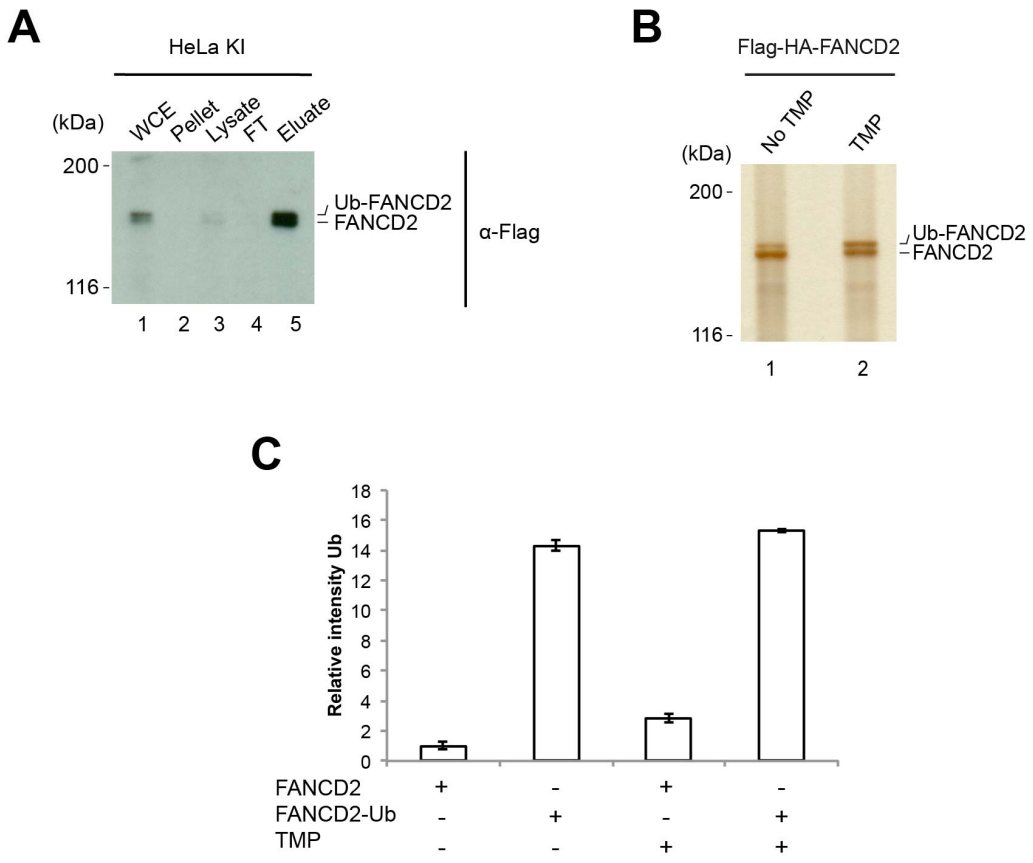


Figure 2-3. Purification of Flag-HA-FANCD2 and MS/MS ubiquitination control. (A) Flag purification of both FANCD2 and Ub-FANCD2 from the knock-in HeLa S3 cell line. Western blot showing whole cell extract (WCE), pellet, lysate, flow-trough (FT) and elution of the purification. (B) Silver stain showing the four elution products used for MS/MS in unperturbed conditions (no TMP) and after the induction of ICLs with TMP (TMP). (C) Relative intensity of ubiquitinated peptides to unmodified peptides as identified in MS/MS in four samples: no ubiquitination/no TMP (1), ubiquitination/no TMP (2), no ubiquitination/TMP (3), ubiquitination/TMP (4). Mean ± SD in n=2 independent experiments.

Table 2-1. Phosphorylation sites identified on FANCD2 through MS/MS. Frequency indicates whether the sites were identified in one or two independent experiments and the predicted kinase was found with NetPhos 3.1.

Sites	Frequency	Kinase
S8	1	PKA
S10	1	CK2
T61	2	PKC/GSK3
S64	2	DNA-PK
S126	1	GSK3/cdc2
S319	2	DNA-PK/ATM
S592	2	RSK
S594	1	GSK3
S880	1	CK1
S881	2	CK2
S882	1	CK2
T884	2	CK2
S886	2	PKC/CK2
S891	2	CK2
S1214	1	GSK3/cdc2
T1253	1	GSK3
S1257	2	ATM/DNA-PK
S1401	1	DNA-PK/ATM
S1404	2	DNA-PK/ATM
S1407	2	CK2
T1408	2	CK2
S1412	2	CK2
S1435	1	CK2/CK1

Mass spectrometry can sometimes provide insight into dynamics of posttranslational modifications, although quantitative determination is not always possible (Presler et al., 2017). To test whether our data could give such information, we first assessed the degree of monoubiquitination in the four samples analysed (FANCD2 and Ub-FANCD2, either before and after introduction of ICLs). Indeed, a clear increase in monoubiquitination could be observed in the samples containing Ub-FANCD2 compared to the other samples (Figure 2-3C).

We also discovered the existence of a phosphorylation cluster, spanning amino acids 882-898. We identified 6 phosphorylated amino acids in the cluster,

several of which are well conserved (Figures 2-4A). Some acidic residues in the cluster are also well conserved. Based on the amino acid sequence, the predicted kinase responsible for phosphorylation of this cluster is CK2 (Pinna, 2002). More importantly, the target pattern of CK2 is a serine or threonine followed by an acidic residue or another phosphorylated serine or threonine at position +1 or +3, and this pattern is also well conserved and supports the hypothesis of a functionally relevant phosphorylation cluster. We then assessed whether changes in the phosphorylation status of the 882-898 cluster could be observed. A decrease in phosphorylation of the cluster was observed in the monoubiquitinated FANCD2 protein after introduction of ICLs (Figure 2-4B).

These data suggest that the phosphorylated form is inactive, non-ubiquitinated, whereas the dephosphorylated form is active, monoubiquitinated. However, the data is not significant due to the limitations of the MS in detecting phosphorylated peptides in a cluster were many possible peptides might exist in the phosphorylated form (combinations of 1, 2, 3, etc. phosphosites) but only one dephosphorylated peptide, leading to an underestimation of the degree of phosphorylation.

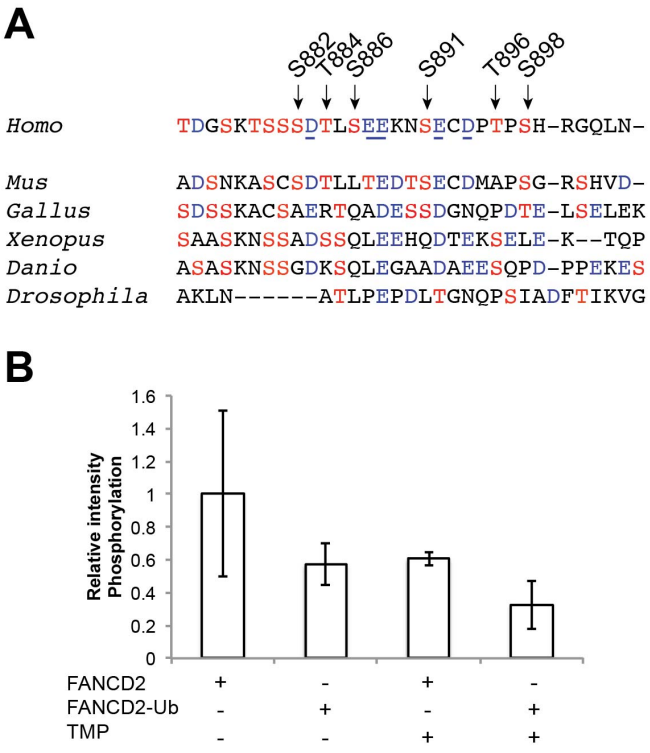


Figure 2-4. Identification of new phosphosites on FANCD2 through MS/MS. (A) Alignment of residues 874-904 of human FANCD2 protein to those in mouse (*Mus*), chicken (*Gallus*), toad (*Xenopus*), zebrafish (*Danio*) and fruit fly (*Drosophila*). Serine and threonine residues are in red and aspartic and glutamic acid residues are in blue (alignments done with ClustalW2). (B) Relative intensity of phosphorylated peptides to unmodified peptides (containing residues 882, 884, 886, 891, 896 and 898) as identified in MS/MS in four samples: no ubiquitination/no TMP (1), ubiquitination/no TMP (2), no ubiquitination/TMP (3), ubiquitination/TMP (4). Mean ± SD in n=2 independent experiments.

Examination of the crystal structure of the mouse FANCD2/FANCI complex (Joo et al., 2011) and the cryo-EM structure of the human FANCD2/FANCI complex (Liang et al., 2016), suggest that the 882-898 phosphorylation cluster is located on the surface of the inner cavity of the heterodimer, potentially in proximity with DNA (Figure 2-5). Taken together, our data suggest that phosphorylation of the 882-898 cluster is associated with the complex in its inactive form, while the dephosphorylated state defines the complex in its active form.

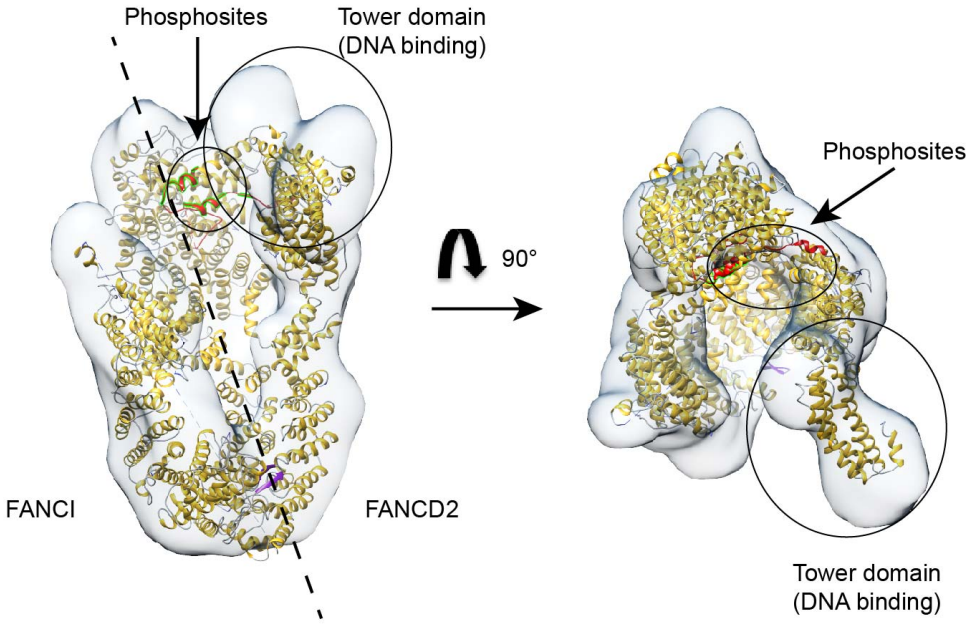


Figure 2-5. Approximate location of the 882-898 cluster on FANCD2. Crystal structure of the mouse FANCD2/FANCI complex (marked in yellow, PDB: 3S4W) docked into the Cryo-EM structure of the human FANCD2/FANCI complex (in grey) showing the approximate location of the 882-898 cluster (marked in red) as well as the C terminal Tower domain of FANCD2 where DNA binding residues have been found.

2.3 Role of the phosphorylation of the 882-898 cluster on FANCD2

We decided to test whether the 882-898 phosphorylation cluster is functionally important for the activity and regulation of the FANCD2/FANCI complex. HeLa FANCD2$^{-/-}$ cells were complemented with either EGFP-FANCD2, or mutant derivatives where the six phosphorylation sites in the cluster have been mutated to either alanines to prevent phosphorylation, or to aspartic acid residues to mimic a constitutive phosphorylation, EGFP-FANCD2-6A and EGFP-FANCD2-6D, respectively. Importantly, all three proteins were expressed to the same level as endogenous FANCD2 (Figure 2-6A). The resulting cell lines were then subjected to a clonogenic survival assay under increasing concentrations of the ICL-inducing drug MMC. As expected, HeLa FANCD2 -/- cells were extremely sensitive (Figure 2-6B). EGFP-FANCD2 and EGFP-FANCD2-6A restored

resistance to MMC, whereas EGFP-FANCD2-6D was significantly less functional, suggesting that phosphorylation of the cluster leads to an inactive complex.

We then assessed the abilities of the two versions of FANCD2 to be monoubiquitinated *in vivo*. EGFP-FANCD2-6A was monoubiquitinated more strongly than the WT form, and both complemented monoubiquitination of endogenous FANCI (Figure 2-6C). On the contrary, EGFP-FANCD2-6D was not monoubiquitinated, and did not complement monoubiquitination of FANCI. This shows that FANCD2-6A is hyperactive compared to WT while FANCD2-6D is defective.

Whether some residues within the cluster could play a more preeminent role in this phenotype remains unclear. However, some preliminary experiments comparing triple mutants point to an additive effect of the different residues within the cluster and no clear predominance of the phosphomimetic mutants.

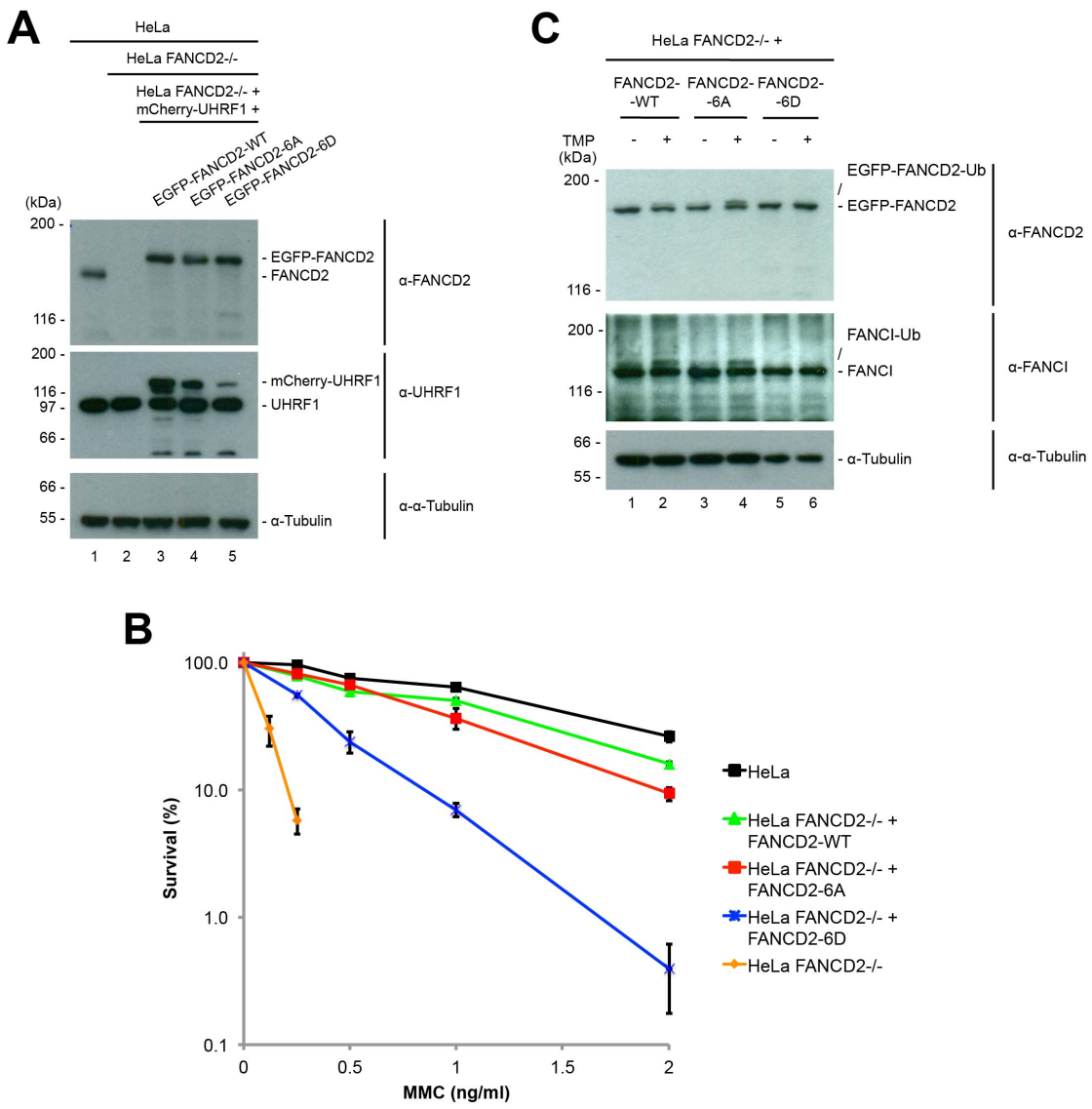


Figure 2-6. Cells expressing phosphomimetic FANCD2 (6D) show increased sensitivity to MMC and reduced FANCD2 and FANCI monoubiquitination. (A) Western blot showing the expression levels of FANCD2 and UHRF1 in the cell lines used. (B) Survival assay to the crosslinking agent mitomycin C (MMC) added at the indicated concentrations and left for 2 weeks. Survival is assessed as the number of colonies formed after 2 weeks. HeLa cells were used, FANCD2 was knocked out with CRISPR/Cas9 and the HeLa FANCD2$^{-/-}$ were stably complemented with either EGFP-FANCD2, EGFP-FANCD2-6A or EGFP-FANCD2-6D (Mean ± SEM, n=3). (C) Western blot of the HeLa FANCD2$^{-/-}$ complemented with EGFP-FANCD2, EGFP-FANCD2-6A or EGFP-FANCD2-6D before and after induction of ICLs through treatment with TMP (2µg/ml) and UVA (50 mJ/cm^2).

We speculated that EGFP-FANCD2-6D was not recruited to chromatin, a defect that could lead to the observed phenotype of defect in monoubiquitination. Therefore, we biochemically fractionated cells after the induction of ICLs, and assessed the localization of the non-modified and the monoubiquitinated forms of FANCD2. ICLs were introduced by treating the cells with TMP, a chemical that intercalates DNA but only generates ICLs after irradiation with UVA. While EGFP-FANCD2 and EGFP-FANCD2-6A were correctly localized in the chromatin fraction, EGFP-FANCD2-6D failed to localize properly (Figure 2-7A). Similarly, endogenous FANCI also failed to localize to chromatin in the EGFP-FANCD2-6D expressing cells (Figure 2-7A).

To further confirm these observations we decided to assess the recruitment of the proteins to ICLs in real time in live cells. mCherry-UHRF1 was co-expressed in the three HeLa cell lines described as a control for the introduction of ICLs. Since the introduction of mCherry-UHRF1 followed the generation of the clonal cell lines expressing equal levels of EGFP-FANCD2, the levels of mCherry-UHRF1 differ among cell lines but they do not pass that of endogenous UHRF1 (Figure 2-6A). ICLs were then introduced by microirradiation after treatment with TMP, and the recruitment of FANCD2 and UHRF1 monitored over time. EGFP-FANCD2 and EGFP-FANCD2-6A were recruited normally, while EGFP-FANCD2-6D was completely defective in recruitment to ICLs (Figure 2-7B). mCherry-UHRF1 was recruited equally in all three cell lines. Held together, the phosphomimetic FANCD2 protein is dysfunctional in complementation assays, is not monoubiquitinated, and is not recruited to ICLs in chromatin.

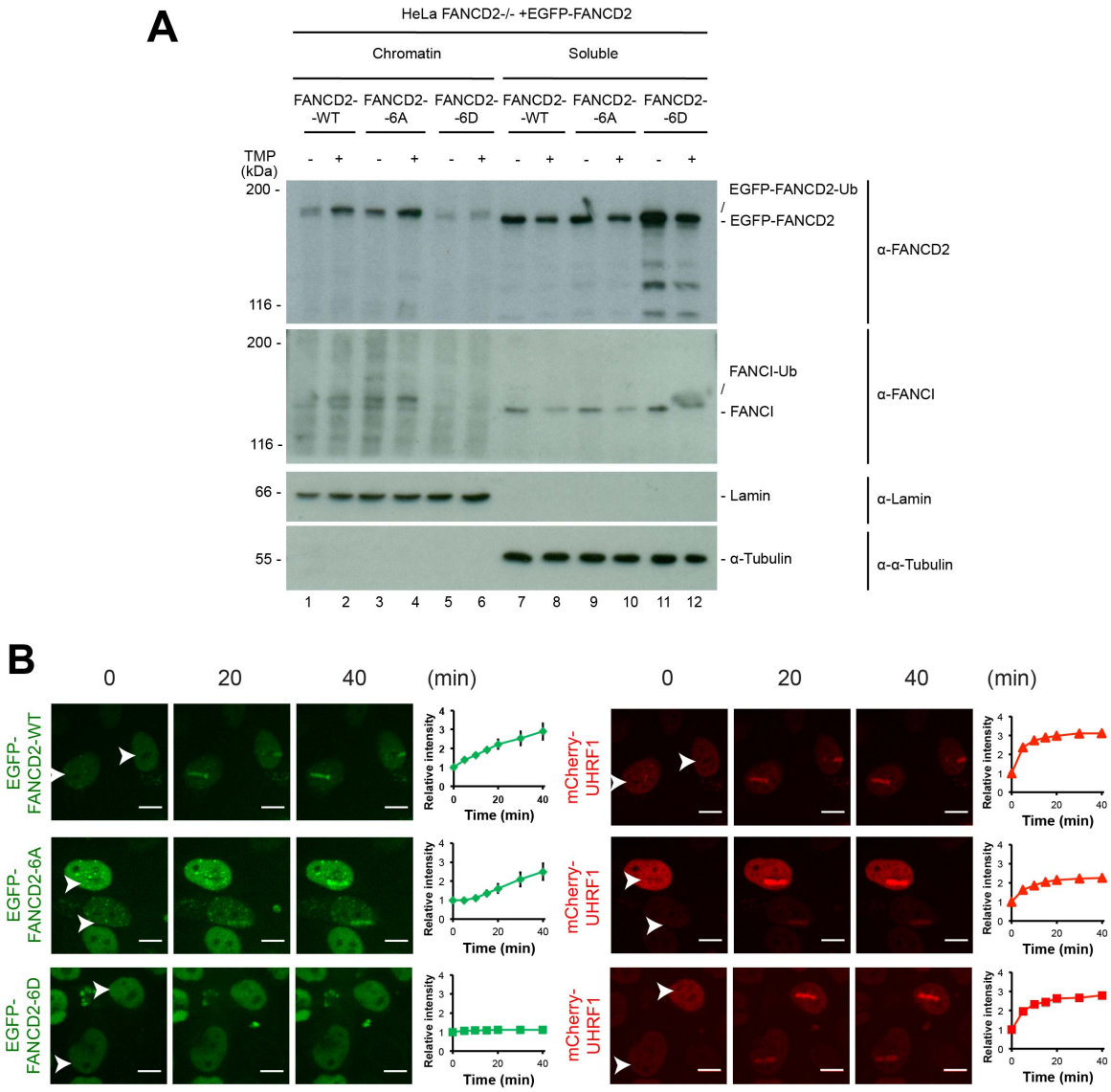


Figure 2-7. Recruitment to chromatin is reduced in the phosphomimetic FANCD2 (6D). (A) Western blot of the HeLa FANCD2$^{-/-}$ complemented with EGFP-FANCD2, EGFP-FANCD2-6A or EGFP-FANCD2-6D before and after treatment with TMP (2μg/ml) and UVA (50 mJ/cm^2). Samples were fractionated with CSK buffer so chromatin bound and soluble fractions could be separated. (B) Live cell imaging of HeLa FANCD2$^{-/-}$ complemented with EGFP-FANCD2, EGFP-FANCD2-6A or EGFP-FANCD2-6D. Cells were treated with TMP (20μg/ml) and microirradiated at the indicated areas (white arrows) and followed for the indicated times (stripe intensity quantified as mean ± SEM, n=5) (scale bar = 10μm).

2.4 Effect of phosphatase inhibition on FANCD2 recruitment to chromatin

Since phosphomimetic FANCD2 (6D) is defective in ICL repair, monoubiquitination and recruitment to sites of damage, we speculate that phosphorylation of FANCD2 shuts down the FA pathway and thus dephosphorylation must precede the recruitment of FANCD2 and promote activation of the pathway. In order to find the phosphatase responsible for this event we decided to test family wide inhibitors and assess the reduction in FANCD2 recruitment to sites of damage. Serine/threonine phosphatases are divided into 3 families: PPP (phosphoprotein phosphatases), PPM or PP2C (metal-dependent protein phosphatases) and aspartate based phosphatases (mainly RNA polymerase II CTD phosphatases). The third family has a very specific role so we discarded them. Within the PPP superfamily there are several families including: PP1, PP2A-like (PP2A, PP4 and PP6), PP2B, PP5 and PP7 (Shi, 2009).

We tested 4 family wide inhibitors: okadaic acid, calyculin A, cyclosporine A and sanguinarine. Okadaic acid and calyculin A affect mainly PP1 and PP2A-like phosphatases, cyclosporine A the PP2B while sanguinarine affects the PPM or PP2C superfamily (Aburai et al., 2010). However, there is also evidence that sanguinarine can inhibit the dual-specificity phosphatase 1 (DUSP1/MKP1) (Vogt et al., 2005). The concentrations used have been described in the literature previously and were tested for their lethality on HeLa cells (Aburai et al., 2010; Chowdhury et al., 2005; Ishihara et al., 1989; Nacev and Liu, 2011). We found that while okadaic acid and cyclosporine A did not affect the recruitment

of EGFP-FANCD2 to sites of damage, calyculin A led to a significant reduction (p=0.046, t=40 min) and sanguinarine completely abrogated recruitment (p=0.022, t=40 min) (Figure 2-8).

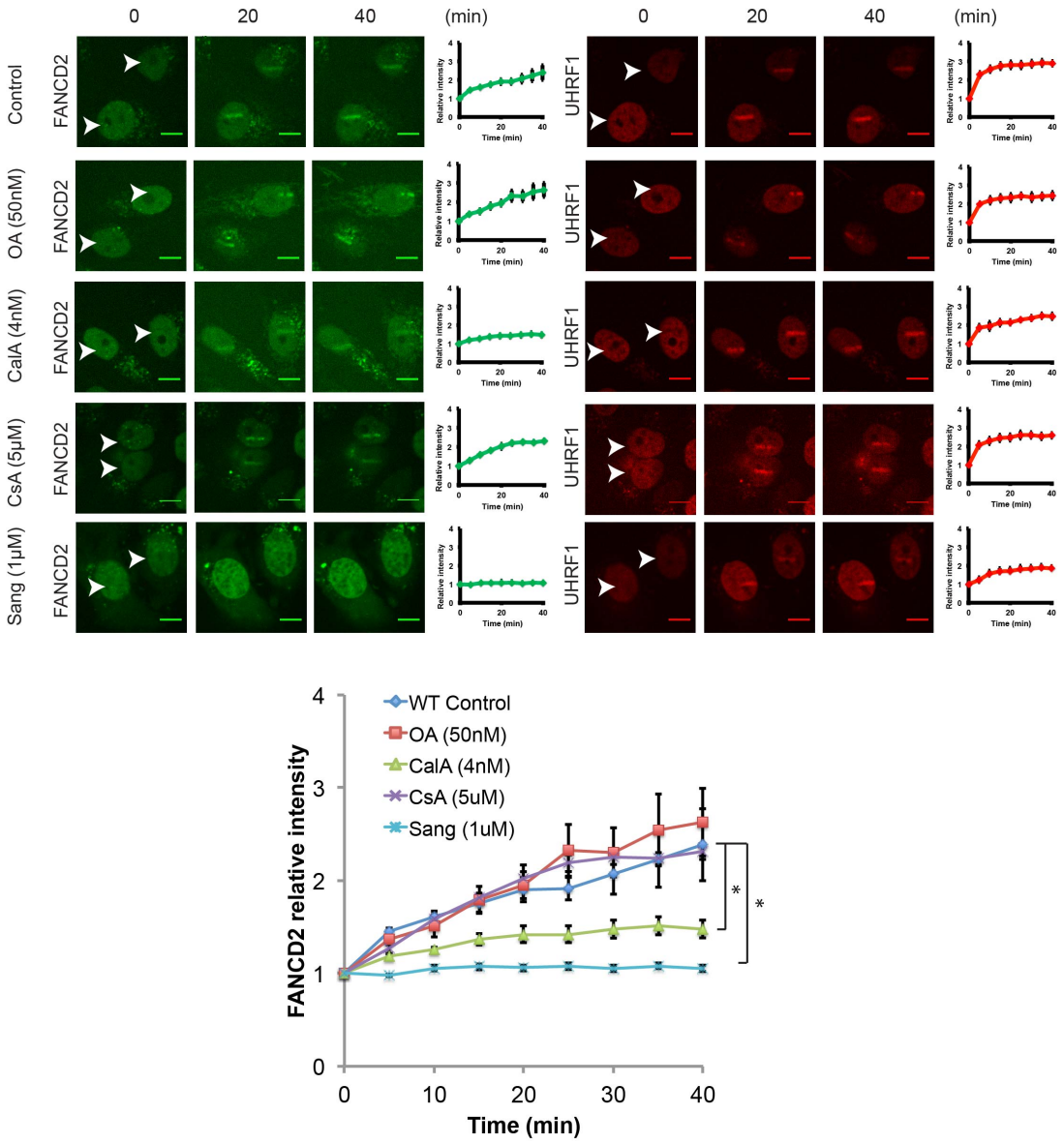


Figure 2-8. Treatment with Sanguinarine (PP2C family wide inhibitor) and Calyculin A (PP1 and PP2A family inhibitor) affects FANCD2 recruitment. Live cell imaging of HeLa FANCD2$^{-/-}$ complemented with EGFP-FANCD2. Cells were treated with phosphatase inhibitors: okadaic acid (50nM), Calyculin A (4nM), Cyclosporin A (5μM) or Sanguinarine (1 μM). ICLs were induced through treatment with TMP (20μg/ml) and microirradiated at the indicated areas (white arrows) and followed for the indicated times (stripe intensity quantified as mean ± SEM, n=5, * $p<0.05$) (scale bar = 10μm).

Since sanguinarine inhibition had the highest effect on FANCD2 recruitment, we decided to test whether this effect was really due to dephosphorylation of the 882-898 cluster and not due to other targets of the PPM family. We could see a partial immunity of the FANCD2-6A recruitment to sanguinarine. FANCD2-6A recruitment was higher than the WT after sanguinarine treatment (p=0.0036, t=40min), but it was also less recruited than the untreated control (p=0.06, t=40min) (Figure 2-9). In summary, these experiments show that the dephosphorylation of the 882-898 cluster on FANCD2 could be catalysed by a member of the PPM (PP2C) or the PP2A-like family while the PP2B family can be discarded.

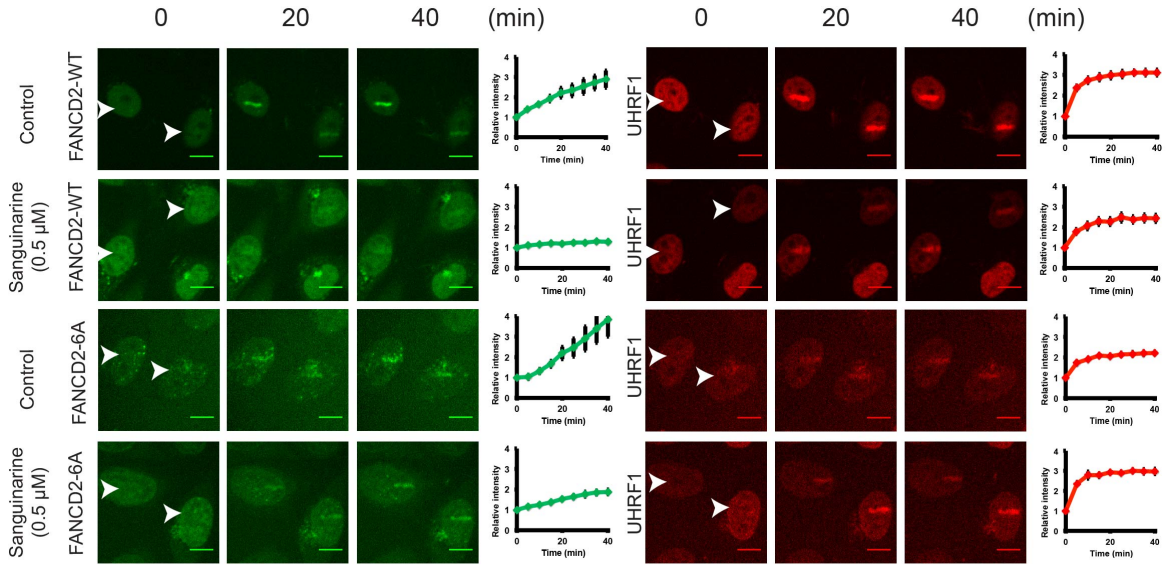


Figure 2-9. Recruitment to chromatin is less affected in the non-phosphorylable FANCD2 (6A) than in the WT form. Live cell imaging of HeLa FANCD2$^{-/-}$ complemented with EGFP-FANCD2 or EGFP-FANCD2-6A. Cells were treated with PP2C phosphatase inhibitor Sanguinarine (0.5 µM). ICLs were induced through treatment with TMP (20µg/ml) and microirradiated at the indicated areas (white arrows) and followed for the indicated times (stripe intensity quantified as mean ± SEM, n=5) (scale bar = 10µm).

2.5 Discussion

The regulation of ICL repair in the cell is more complex than it was initially thought. The monoubiquitination of FANCD2 and FANCI was thought to be the main switch in the pathway but new insights into phosphorylation and other modifications are changing this view. The master DNA damage response kinases ATM and ATR have been linked to many proteins implicated in DNA repair (Blackford and Jackson, 2017). The FA pathway has been mainly linked to ATR, through its phosphorylation of FANCA, FANCM, FANCD2 and FANCI (Cheung et al., 2017; Collins et al., 2009; Ishiai et al., 2008; Singh et al., 2013; Sobeck et al., 2009; Taniguchi et al., 2002a). The complex formed by FANCD2/FANCI links the early events of detection and activation of the repair with the downstream effector proteins, including nucleases and polymerases.

Therefore, it is reasonable that FANCD2/FANCI are subject to a strict regulation. Our MS results show a complex set of phosphorylation events happening on FANCD2, potentially implicating several kinases such as ATR or ATM, DNA-PK, Cdk1 (Cdc2), PKA, PKC, GSK3, CK1 and CK2. Some of the sites are forming clusters, which make them especially interesting since it constitutes a more robust form of regulation. The CK2 targeted cluster between 882-898 residues of FANCD2 showed increased sensitivity to ICLs when mutated into its phosphomimetic form. This result was especially interesting since it is the first instance of an inhibitory role of phosphorylation on FANCD2 and the FA pathway. We could confirm that phosphomimetic form of FANCD2 could not localise properly to chromatin after damage and this probably causes its defect in monoubiquitination and activation of the FA pathway. We, thus, hypothesised that CK2 constitutively phosphorylates FANCD2 keeping it in an inactive state

and that dephosphorylation must take place prior to monoubiquitination and activation.

The early screening for the phosphatase using family wide inhibitors has showed that the putative phosphatase is probably a member of the PPM (PP2C) or PP2A-like families. Some members of these families have already been linked to DNA repair. For instance, Wip1 or PPM1D counteracts CK2 phosphorylation of LSD1, thereby promoting its interaction with the E3 ligase RNF168 and the ubiquitination and recruitment of 53BP1 to sites of DNA damage (Peng et al., 2015). PP4, a member of the PP2A-like family, dephosphorylates several proteins implicated in DNA repair such as RPA2 (Lee et al., 2010), 53BP1 (Lee et al., 2014), KAP-1 (KRAB-domain-associated protein 1) (Lee et al., 2012) and γH2AX (Chowdhury et al., 2008). Further work is still needed, however, to identify the precise phosphatase responsible for dephosphorylating FANCD2 on the cluster 882-898, leading to its activation and promotion of the FA pathway.

Chapter 3. Mechanistic insights into the phosphorylation of FANCD2

3.1 Introduction

The *in vivo* setting has been very useful in characterizing the phenotype of the 882-898 cluster on FANCD2 through the introduction of mutant forms of the protein into human cells. However, the precise mechanism behind this inhibitory role of phosphorylation of FANCD2 is difficult to address in this way. Therefore, we decided to move to an *in vitro* setting where the monoubiquitination as well as the DNA binding reaction of FANCD2 can be assessed in more detail. Specifically, we can reconstitute the monoubiquitination of FANCD2 *in vitro*, using purified proteins, DNA and ATP.

Historically, the first ubiquitination reactions were performed exclusively with the protein components of the reaction, mainly the E1 ubiquitin-activating enzyme (UBA1), E2 ubiquitin-conjugating enzyme (UBE2T), the E3 ubiquitin-ligase (FANCL), ubiquitin, the substrate FANCD2/FANCI complex and ATP, but the efficiency of the reaction was very low (Alpi et al., 2008; Machida et al., 2006). It was later found that the monoubiquitination of FANCD2 could be greatly stimulated in the presence of DNA, though FANCI monoubiquitination remained very low (Longerich et al., 2014; Sato et al., 2012b). Interestingly, FANCI could be monoubiquitinated *in vitro* in the absence of FANCD2 (Longerich et al., 2014; Longerich et al., 2009). In the cell, FANCL is part of a bigger protein complex, the FA core complex, which has been divided in a series of subcomplexes according to their interactions and functions:

FANCB/FANCL/FAAP100, FANCA/FANCG/FAAP20 and FANCC/FANCE/FANCF (Huang et al., 2014; Rajendra et al., 2014). The subcomplex containing FANCL was found to form a dimer through a FANCB-FANCB interaction and this was proposed as the mechanism ensuring a balanced FANCD2 and FANCI monoubiquitination (Swuec et al., 2017). The addition of FANCC/FANCE/FANCF subcomplex to FANCB/FANCL/FAAP100 could greatly enhance the monoubiquitination of both FANCD2 and FANCI, although FANCI could not reach the level of monoubiquitination of FANCD2 (van Twest et al., 2017). These data show that the *in vitro* setting can get close to recapitulating the complexity of the *in vivo* scenario and it can constitute a useful tool to study the regulation of FANCD2 function and the FA pathway.

Regarding the specific functions of FANCD2 and FANCI, some early reports described a nuclease activity for FANCD2 while others proposed a histone chaperone activity for the complex (Pace et al., 2010; Sato et al., 2012a). However, it is unclear how relevant these activities are for ICL repair *in vivo.* On the other hand, it was also found that FANCD2/FANCI have DNA binding activity and a greater affinity for branched structures, such as Holliday junctions, replication forks or D-loops that combine double and single stranded DNA (Niraj et al., 2017; Park et al., 2005; Yuan et al., 2009). Even though this is the main activity identified for the FANCD2/FANCI complex, its regulation remains unknown. It was thought that monoubiquitination regulated DNA binding (Montes de Oca et al., 2005), but the *in vitro* stimulation of monoubiquitination by DNA, together with recent evidence showing that impairing DNA binding completely abrogates monoubiquitination in cells (Liang et al., 2016), points to the reaction taking place once the FANCD2/FANCI complex is on DNA.

3.2 Effect of FANCD2 phosphorylation on its *in vitro* monoubiquitination

Our results suggest that a dynamic phosphorylation and dephosphorylation is controlling the activation of the FANCD2/FANCI complex. To gain deeper mechanistic insight into this regulatory switch, we then turned to *in vitro* experimentation. More specifically, we decided to fully reconstitute the monoubiquitination reaction *in vitro*, allowing us to dissect and uncover exactly where in the reaction phosphorylation of the FANCD2 cluster plays its role. We purified the FANCD2/FANCI, FANCD2-6A/FANCI and FANCD2-6D/FANCI complexes to homogeneity, in order to assess their activities as substrates in the reaction (Figure 3-1A). We fully reconstituted the monoubiquitination reaction *in vitro*, using purified protein components as well as plasmid dsDNA (Figure 3-1B). As expected, the FANCD2/FANCI complex was efficiently monoubiquitinated in the reaction (Figure 3-1C and appendix figure 1, lanes 1-4). Likewise, the FANCD2-6A/FANCI complex was also monoubiquitinated with similar kinetics (Figure 3-1C and appendix figure 1, lanes 5-8). In contrast, and in good agreement with the *in vivo* data, the FANCD2-6D/FANCI complex was only weakly monoubiquitinated, and with slower kinetics (Figure 3-1C and appendix figure 1, lanes 9-12).

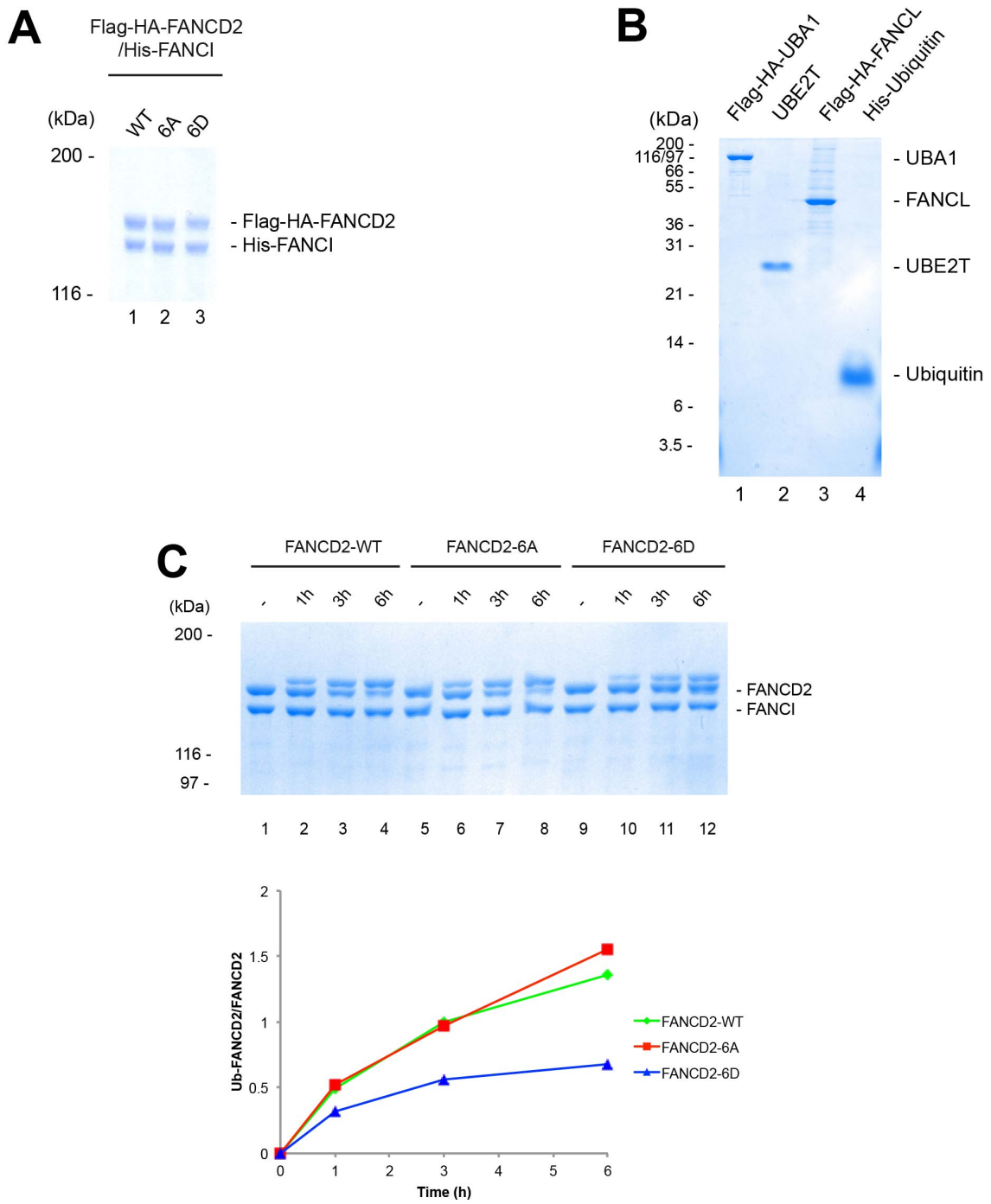


Figure 3-1. *In vitro* monoubiquitination reaction is slowed down in the phosphomimetic FANCD2 (6D). (A) Coomassie blue gel of the purification of the Flag-HA-FANCD2/His-FANCI complex co-expressed in Sf9 cells. (B) Coomassie gel of the proteins used in the *in vitro* ubiquitination assay: Flag-HA-UBA1 (E1), UBE2T (E2), Flag-HA-FANCL (E3) and His-ubiquitin. (C) Coomassie blue gel of the *in vitro* ubiquitination of the FANCD2/FANCI complex as WT, 6A or 6D forms. Quantification showing the ratio of Ub-FANCD2 to FANCD2.

These data suggest that the FANCD2/FANCI complex is kept in an inactive state via phosphorylation of the 882-898 cluster, potentially by CK2. To test this hypothesis directly, we performed an *in vitro* kinase assay of the FANCD2/FANCI complex with recombinant CK2 with radioactive γ^{32}P-ATP. In the absence of CK2, no significant signal is observed, but with CK2 FANCD2 is phosphorylated while FANCI is not (Figure 3-2A). However, FANCD2-6A and FANCD2-6D are also phosphorylated. This points to the presence of other residues on FANCD2 apart from the 882-898 cluster that can be phosphorylated (Figure 3-2A). Some of these residues might be relevant *in vivo* (some other potential CK2 targets where identified through MS (Table 2-1)), but they might also result from unspecific, artefactual phosphorylation events in the *in vitro* setting.

We, then, assessed whether CK2 can reduce the activity of the FANCD2/FANCI complex in our *in vitro* monoubiquitination assay. We phosphorylated the FANCD2/FANCI complex with recombinant CK2, and then subjected the complex to the *in vitro* monoubiquitination assay. We observed a reduction in monoubiquitination after CK2 treatment (Figure 3-2B, lanes 4-5). As expected, the FANCD2-6A/FANCI complex was less affected by the CK2 treatment (Figure 3-2B, lanes 9-10), underscoring the important role of the cluster 882-898 in this effect.

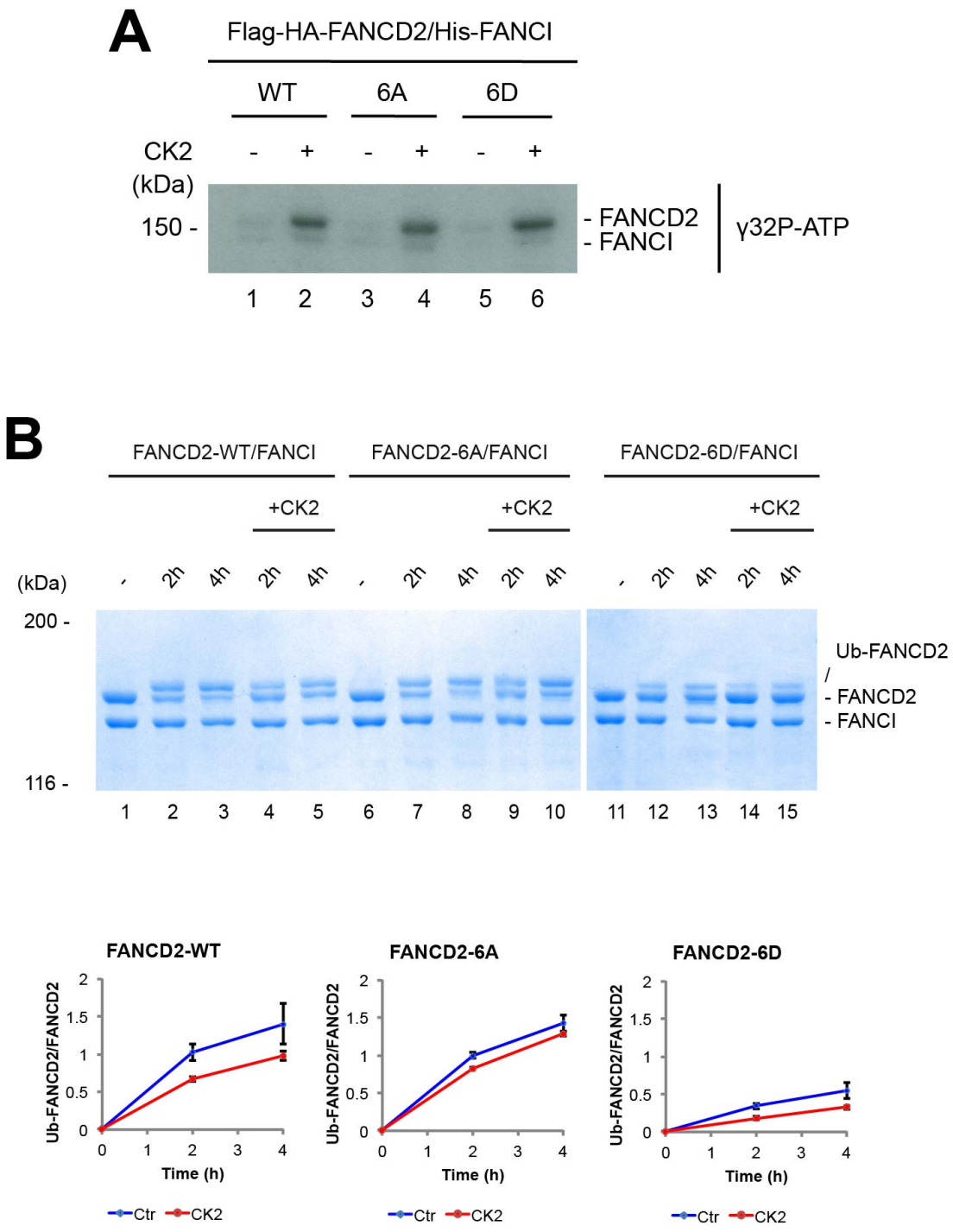


Figure 3-2. Phosphorylation of FANCD2 by CK2 inhibits its monoubiquitination *in vitro*. (A) *In vitro* kinase assay of FANCD2/FANCI (WT, 6A and 6D) by CK2. (B) Coomassie blue gel of the *in vitro* ubiquitination of the WT, 6A and 6D forms of the FANCD2/FANCI complex following a mock treatment or an *in vitro* phosphorylation by CK2. Quantification showing the ratio of Ub-FANCD2 to FANCD2 (Mean ± SD, n=2).

Taken together, these data show that the FANCD2/FANCI complex is kept in an inactive state via phosphorylation of the 882-898 cluster by CK2. If so,

activation of the complex must be achieved by a dephosphorylation event following DNA damage.

3.3 Effect of FANCD2 phosphorylation on its DNA binding activity

The inhibitory effect phosphorylation of the 882-898 cluster in FANCD2 has on monoubiquitination of the FANCD2/FANCI complex *in vitro*, could be caused by three different mechanisms. First, the integrity of the FANCD2/FANCI complex could be affected, causing dissociation. This could be causative, since monoubiquitination of FANCD2 is dependent on its heterodimerization with FANCI (Liang et al., 2016; van Twest et al., 2017). Second, the ability of the complex to interact with FANCL, the E3 ligase, could be reduced. Third, the ability of the complex to interact with DNA could be inhibited. The latter could be the cause, since interaction of the complex with DNA is required for efficient monoubiquitination (Liang et al., 2016). We decided to test which of these three options constituted the mechanism.

First, the FANCD2-6A/FANCI and FANCD2-6D/FANCI complexes were as stable as the FANCD2/FANCI complex, showing that integrity of the complexes was not the mechanism (Figure 3-1A). Second, we tested the interaction of the three complexes with recombinant FANCL. Since both FANCD2 and FANCL contain Flag-HA tags, we purified FANCI from its His tag and checked the complexes formed. We found an indistinguishable interaction of the complexes with FANCL *in vitro* (Figure 3-3A). Third, we assessed the ability of the three complexes to bind DNA. Here we applied an electrophoretic mobility shift assay, using a radiolabeled DNA structure mimicking a stalled replication fork, a Y-shaped DNA molecule containing both double stranded and single stranded DNA.

The FANCD2/FANCI complex formed a strong protein/DNA complex (Figure 3-3B, lanes 1-3). Similarly, the FANCD2-6A/FANCI complex possessed similar DNA binding properties (Figure 3-3B, lanes 4-6). In contrast, the FANCD2-6D/FANCI complex was almost defective in DNA binding (Figure 3-3B, lanes 7-9).

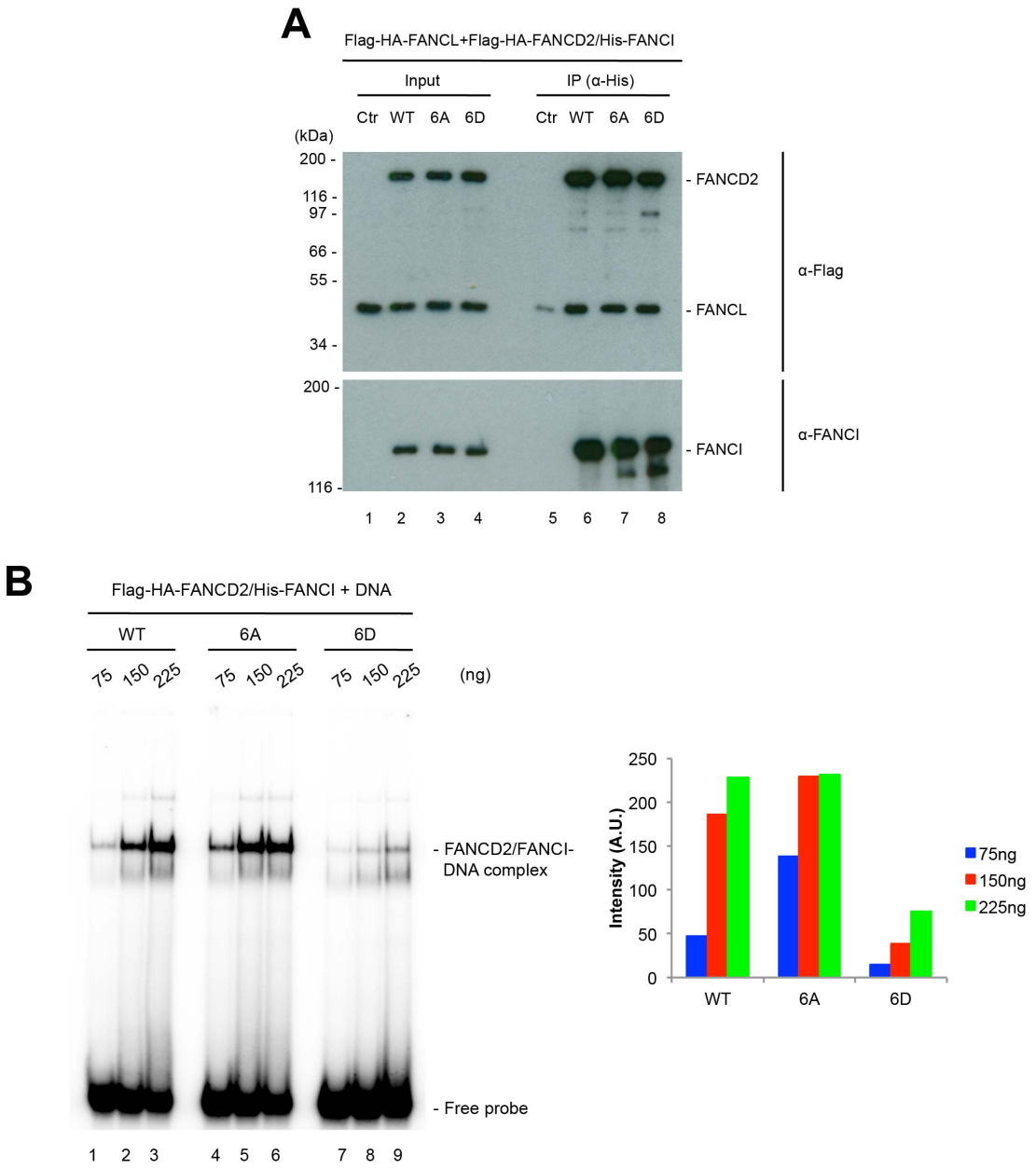


Figure 3-3. Phosphomimetic FANCD2 (6D) does not impair FANCL binding but it shows lower affinity for DNA. (A) Western blot showing the *in vitro* IP of the Flag-HA-FANCD2/His-FANCI complex (WT, 6A or 6D forms) and Flag-HA-FANCL. (B) Electrophoretic mobility shift assay (EMSA) showing the DNA binding affinity of the Flag-HA-FANCD2/His-FANCI complex (WT, 6A or 6D forms) to a Y shaped radiolabelled DNA probe.

We, then, tested whether phosphorylation of FANCD2 by CK2 could also lower the affinity for DNA of the FANCD2/FANCI complex. Phosphorylated FANCD2 showed a marked reduction in DNA binding compared to the mock treated control (Figure 3-4A, lanes 1-3 and 4-6). To assess the specific role of the

882-898 cluster in this effect we also compared CK2 phosphorylated and mock treated FANCD2-6A/FANCI. We observed a less marked defect in DNA binding of the phosphorylated FANCD2-6A/FANCI compared to the phosphorylated FANCD2-WT/FANCI (Figure 3-4A, lanes 4-6 and 10-12). However, CK2 phosphorylation of the FANCD2-6A/FANCI complex still reduced the affinity for DNA compared to the mock treatment (Figure 3-4A, lanes 7-9 and 10-12). These results point to the 882-898 cluster playing an important role in the DNA binding affinity of the complex, but it also shows that, at least *in vitro,* other sites are probably phosphorylated by CK2. Whether these other phosphosites are just an artefact of the *in vitro* setting or relevant *in vivo* remains unknown. Furthermore, this slight reduction on DNA binding of the FANCD2-6A was not linked to a similar inhibition of the *in vitro* ubiquitination (Figure 3-2B) pointing to the importance of the cluster specifically for the regulation of the DNA binding linked to the monoubiquitination reaction.

Finally, we decided to test whether this phosphorylation event could be reversed through dephosphorylation and thus, recapitulate *in vitro* the potential *in vivo* mechanism. After phosphorylating FANCD2/FANCI with CK2, we dephosphorylated the resulting product with the broad specificity lambda protein phosphatase (λPP). We could observe a clear reduction in DNA binding after CK2 treatment (Figure 3-4B, lanes 1-3 and 7-9), which could be reversed after λPP treatment (Figure 3-4B, lanes 7-9 and 10-12). Actually, the binding after λPP treatment was increased compared to the mock treated control (Figure 3-4B, lanes 1-3 and 4-6), pointing to a partial phosphorylation already present in the purified FANCD2/FANCI complex. This result shows that CK2

phosphorylation effect on DNA binding can be reversed through dephosphorylation *in vitro*, pointing to a dynamic regulatory process *in vivo*.

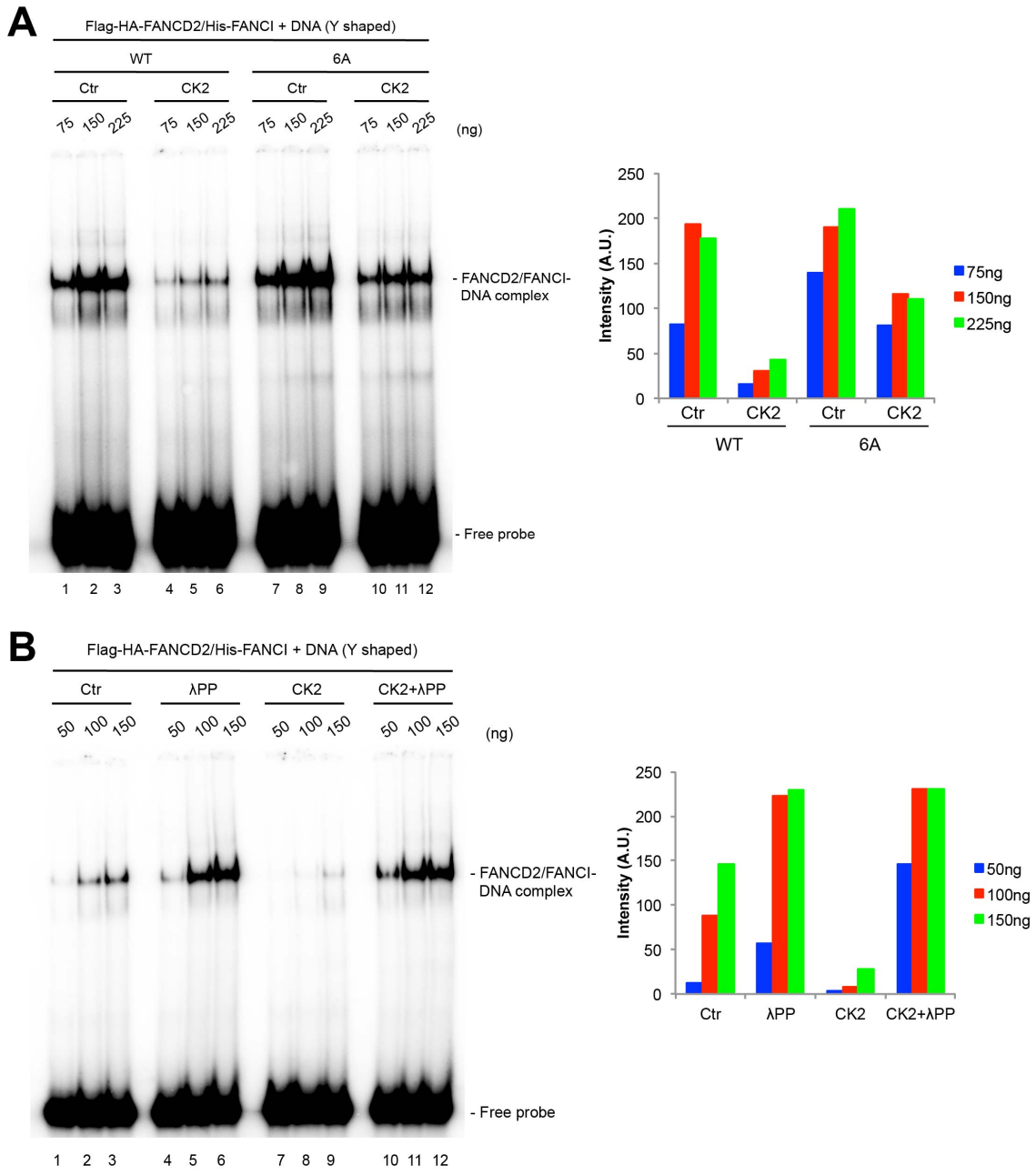


Figure 3-4. Phosphorylation of FANCD2 by CK2 impairs DNA binding and can be reverted through dephosphorylation. (A) EMSA showing the DNA binding affinity of the Flag-HA-FANCD2/His-FANCI complex (WT and 6A) after mock or CK2 treatment to a Y shaped radiolabelled DNA probe. (B) EMSA showing the DNA binding affinity of the Flag-HA-FANCD2/His-FANCI complex (WT) after mock, CK2, λPP or both treatments to a Y shaped radiolabelled DNA probe.

In conclusion, these data demonstrate that phosphorylation of the 882-898 cluster in FANCD2 by CK2 prevents binding of the FANCD2/FANCI complex to DNA, in turn preventing its activation by monoubiquitination. Furthermore, dephosphorylation of the phosphorylated FANCD2/FANCI complex can rescue the DNA binding capacity of the complex, allowing its activation by monoubiquitination.

3.4 Effect of FANCD2 phosphorylation on its *in vitro* deubiquitination

Given the effect of FANCD2 phosphorylation by CK2 on the DNA affinity of the FANCD2/FANCI complex, we asked whether phosphorylation could play a role not only in the initial recruitment of the complex to DNA but also in its dissociation from DNA later in the pathway. It is well known that deubiquitination of FANCD2/FANCI by USP1/UAF1 is necessary for ICL repair and completion of the pathway, since otherwise ubiquitinated FANCD2/FANCI accumulates on DNA (Cohn et al., 2007; Oestergaard et al., 2007). Some recent evidence showed that deubiquitination of ubiquitinated FANCD2/FANCI was stimulated in the absence of DNA (van Twest et al., 2017), thus we hypothesized that phosphorylation could enhance DNA dissociation and promote deubiquitination.

In order to test this, we co-expressed and purified recombinant Flag-HA-USP1/Strep-UAF1 from insect Sf9 cells (Figure 3-5A). USP1/UAF1 were able to efficiently deubiquitinate FANCD2 after monoubiquitination *in vitro* (Figure 3-5B, lanes 2-5). We tested whether degrading DNA after ubiquitination, but before deubiquitination, would promote the deubiquitination reaction by treatment with benzonase. However, we saw the opposite effect with a slower

deubiquitination reaction (Figure 3-5B, lanes 6-9). This could be due to a reduced processivity or accessibility of the FANCD2/FANCI complex to the USP1/UAF1 complex after DNA degradation, since this step would fragment the DNA with the ubiquitinated FANCD2/FANCI complexes bound. We finally tested the effect of CK2 treatment after ubiquitination, but we saw no effect on deubiquitination (Figure 3-5B, lanes 10-13). Since benzonase treatment did not stimulate deubiquitination, it is possible that DNA dissociation would not stimulate it either but that will require further testing. Nevertheless, we cannot discard a role of phosphorylation of FANCD2 by CK2 on DNA dissociation. In fact, CK2 phosphorylation following FANCD2/FANCI ubiquitination fails to reduce the DNA binding of the complex (Figure 3-5C, lanes 3 and 4), while deubiquitinating the complex after the phosphorylation step seems to promote further DNA dissociation compared to a non phosphorylated control (Figure 3-5C, lanes 5 and 6). However, this is a preliminary experiment that must be taken with caution since ubiquitination of FANCD2/FANCI did not seem to stimulate DNA binding as would be expected (Figure 3-5C, lanes 1 and 2).

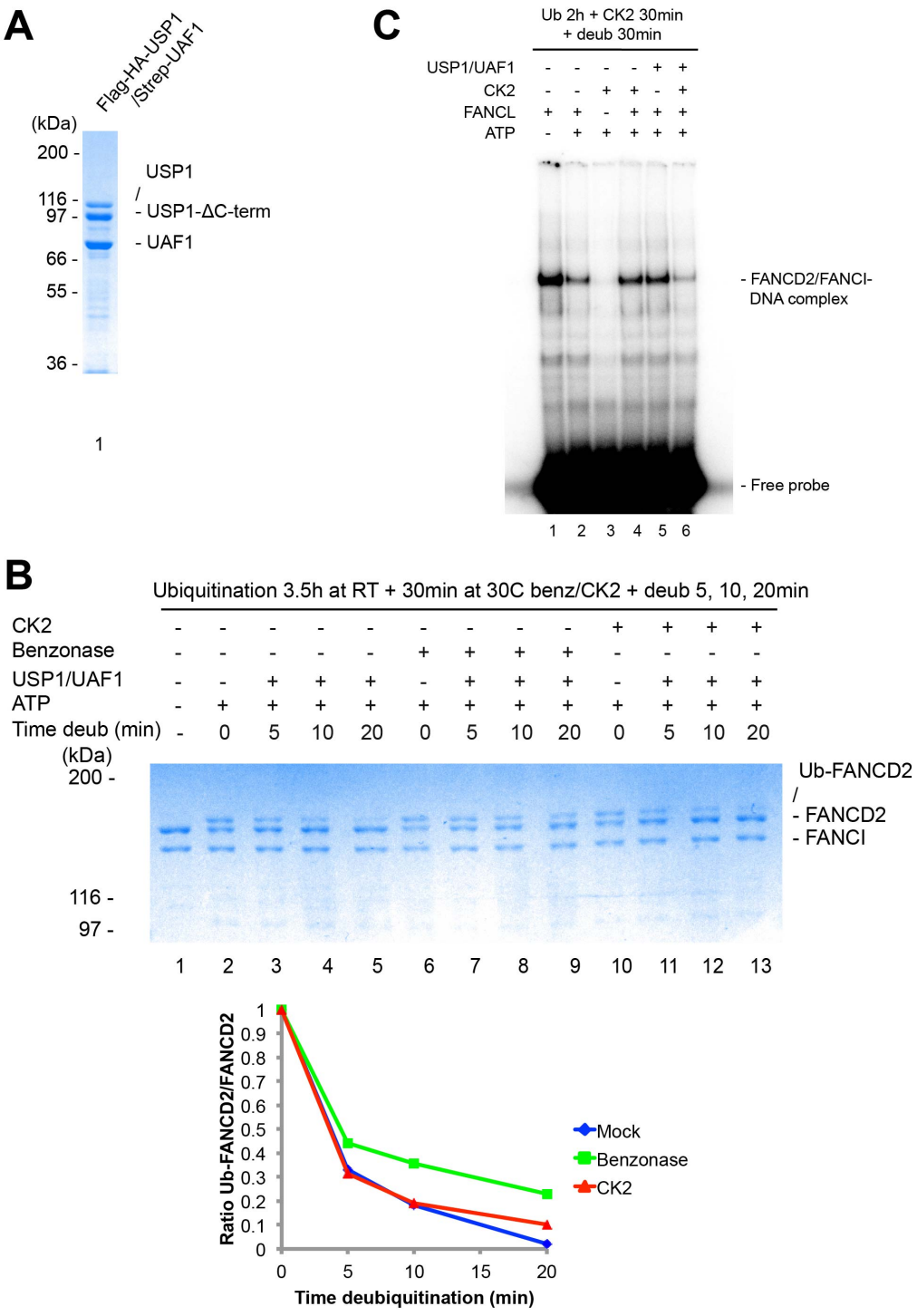


Figure 3-5. CK2 phosphorylation does not promote deubiquitination *in vitro*. (A) Coomassie gel of the purification of Flag-HA-USP1/Strep-UAF1 coexpressed in insect Sf9 cells. USP1 can be cleaved to form a C terminal deleted USP1 that is also active. (B) Coomassie gel of the deubiquitination *in vitro* reaction of the FANCD2/FANCI complex following *in vitro* ubiquitination and either mock, CK2 or benzonase treatments. Quantification showing the ratio of Ub-FANCD2 to FANCD2 normalized to time 0 of deubiquitination. (C) EMSA showing the DNA binding affinity of the Flag-HA-FANCD2/His-FANCI complex after mock, ubiquitination, CK2 or deubiquitination treatment.

3.5 Discussion

We could reconstitute FANCD2 monoubiquitination *in vitro* with purified proteins and plasmid dsDNA. Furthermore, the inhibitory effect of the phosphorylation of the 882-898 cluster on FANCD2 *in vivo* could also be observed *in vitro*, allowing a deeper mechanistic study. We observed that the phosphomimetic form of FANCD2 showed a less efficient and slower monoubiquitination. However, the interaction with either FANCI or the E3 ligase FANCL was intact in all of the phospho-mutant forms of FANCD2. That left the interaction with DNA as the main candidate to explain the phenotypes observed.

We could confirm that the DNA binding activity of FANCD2/FANCI was greatly inhibited both in the phosphomimetic form of FANCD2 and in the CK2 phosphorylated FANCD2. This impairment of DNA binding could also explain the lack of recruitment to ICLs and reduced monoubiquitination previously observed in the cells. Moreover, we could reverse the inhibitory effect of CK2 phosphorylation by dephosphorylation, suggesting that this modification constitutes a regulatory mechanism and potentially reconstituting the process that takes place in the cell prior to FANCD2 recruitment and monoubiquitination.

Interestingly, a similar mechanism was described for the protein HP1α (heterochromatin protein 1α), which together with other HP proteins play important roles not only in heterochromatin assembly, but also in transcription, DNA repair, cell cycle regulation, etc. It was found that CK2 phosphorylation of the N-terminus of HP1α interfered with its DNA binding, but paradoxically this same phosphorylation event promoted HP1α binding to H3K9me3 modified nucleosomes and actually increased recruitment to chromatin (Nishibuchi et al.,

2014). A very similar mechanism was found to regulate the binding of CBX2, a member of the polycomb repressive complex 1 (PRC1), to H3K27me3, which leads to the repression of the gene *p21* (Kawaguchi et al., 2017). In the case of FANCD2, however, phosphorylation by CK2 led to both reduced DNA binding *in vitro* and abrogated chromatin recruitment in the cells. There is growing evidence that histone modifications may also play a role in regulating FANCD2 loading onto chromatin. Recently, it was found that H3K27me2/3 tends to exclude FANCD2 from chromatin and thereby promotes NHEJ. On the other hand, lack of H3K27me2/3 or mutation to methionine (H3K27M), which is found in tumours, led to increased FANCD2 loading on chromatin, 53BP1 exclusion and increased genomic instability (Zhang et al., 2018). FANCD2 has some histone chaperone activity and can promote nucleosome assembly *in vitro* (Sato et al., 2012a). This activity could play a role in DNA repair, mobilising histones after DNA damage. It could also be a mechanism for FANCD2 recruitment onto chromatin. Although FANCD2 can bind H3K4me, and promote replication fork stability, lack of this methylation event only mildly affects its recruitment to chromatin after DNA damage (Higgs et al., 2018). DNA binding activity of FANCD2/FANCI is critical for ICL repair, probably recruitment of FANCD2/FANCI to chromatin is initially mediated through a protein-protein interaction or through binding to a modified histone and then DNA binding ensures the accumulation of FANCD2/FANCI on DNA around the lesion, where it is locked through further modifications such as monoubiquitination.

Whether CK2 mediated phosphorylation of FANCD2 could be responsible for regulating DNA dissociation during ICL repair is still unknown. Since FANCD2 deubiquitination is essential for a complete ICL repair (Oestergaard et al., 2007),

it is possible that DNA dissociation following deubiquitination of FANCD2 is needed to allow other factors to be recruited and culminate ICL repair. It is possible that phosphorylation signals for deubiquitination to happen or promotes DNA dissociation once deubiquitination occurs. We could not observe an enhancement of FANCD2 deubiquitination following CK2 phosphorylation in *vitro*, but it is still possible that phosphorylation plays a role in DNA dissociation and ensuring that rebinding does not occur.

Chapter 4. Discussion.

We have shown that phosphorylation of the 882-898 cluster on FANCD2 greatly lowers the affinity of the FANCD2/FANCI complex for DNA, thereby keeping the complex away from DNA in the absence of damage. We propose that CK2 is responsible for this phosphorylation event, which could happen constitutively on all free FANCD2/FANCI. It is, therefore, necessary that the regulated event upon DNA damage is the dephosphorylation of FANCD2/FANCI by a still unidentified phosphatase that creates a facultative active form of the FANCD2/FANCI complex with high affinity for DNA. This form of the complex can then be recruited onto DNA where further modifications can happen, mainly its monoubiquitination by FANCL that leads to its stable locking on DNA (Figure 4-1). In this way the binding of FANCD2/FANCI to DNA could be regulated prior to its monoubiquitination and act as a safeguard against spurious activation of DNA repair.

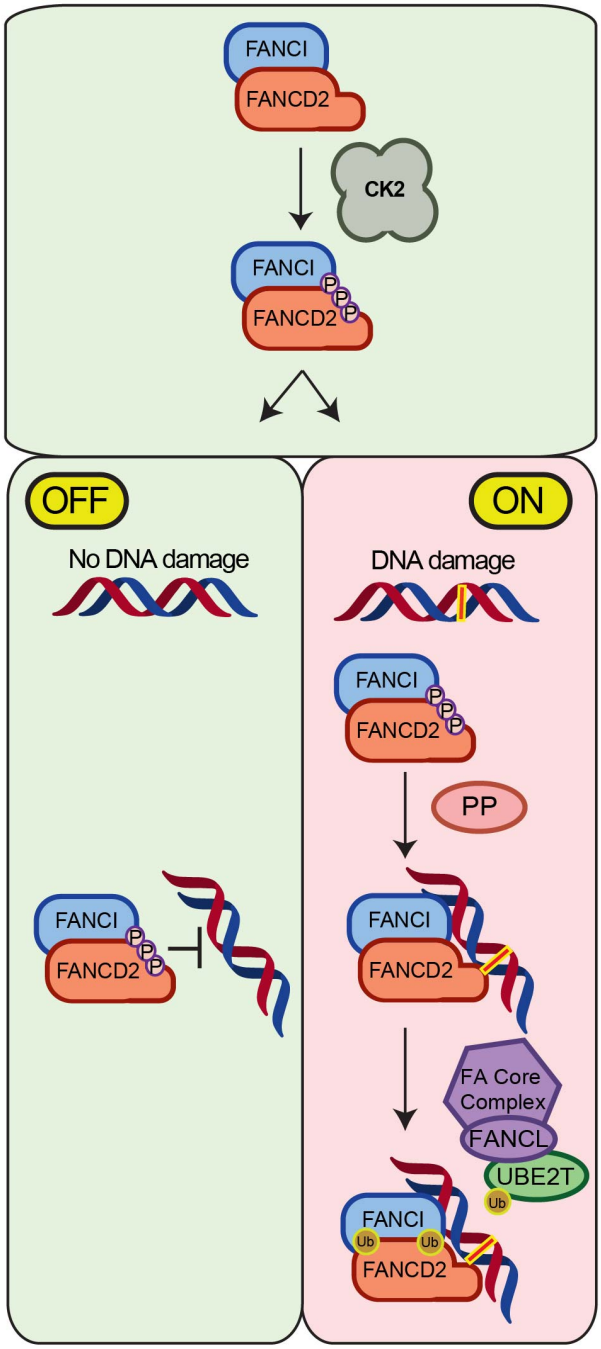


Figure 4-1. Model of the regulation of the FA pathway by CK2 phosphorylation of FANCD2. CK2 phosphorylates FANCD2 constitutively on the 882-898 cluster and prevents DNA binding of the FANCD2/FANCI complex in the absence of DNA damage. However, upon the detection of DNA damage, FANCD2 gets dephosphorylated, allowing the FANCD2/FANCI complex to bind DNA where their monoubiquitination by the core complex containing the E3 ligase FANCL can take place. This event locks the FANCD2/FANCI complex on DNA, completing its activation and allowing for the downstream events leading to the repair to happen.

4.1 Role of dephosphorylation in DNA damage repair

There is mounting evidence that dephosphorylation is not only needed for the shutdown of the DDR once the repair is complete but also to provide for an efficient repair to happen. The master regulators of the DDR are kinases such as ATR and ATM, which are linked to the activation of repair factors in the initial stages of the DNA repair (Blackford and Jackson, 2017). A key event in the early response to DSBs is the phosphorylation of the histone variant H2AX, which is thought to mediate the accumulation of repair factors around the damaged region that lead to the formation of foci (Fernandez-Capetillo et al., 2004; Rogakou et al., 1998). The phosphatase responsible for reversing this event was later proposed to be a PP2A complex (Chowdhury et al., 2005), although later evidence showed that PP4 could also dephosphorylate γH2AX and that this was required for completion of repair (Chowdhury et al., 2008). More importantly, PP4 was necessary for the repair of DNA replication-associated damage, pointing to other repair factors being dephosphorylated at the sites of stalled replication forks and subsequent damage (Chowdhury et al., 2008).

Proteomics studies have shown that following DNA damage more than 750 phosphosites in close to 400 proteins were modified and almost half of them were actually dephosphorylation events (Bennetzen et al., 2010; Bensimon et al., 2010). In fact, several phosphatases have already being identified as promoting the DDR (Zheng et al., 2015). PP4C, for example, dephosphorylates RPA2, which is necessary for a timely binding to ssDNA, resuming DNA synthesis after damage and performing HR (Lee et al., 2010). PP4 also dephosphorylates 53BP1 at T1609 and S1618, sites located on its UDR (ubiquitination-dependent recruitment) domain. This allows 53BP1 recruitment onto DSBs by promoting its

interaction to ubiquitinated H2A (Figure 4-2) (Lee et al., 2014). PP4C has also been shown to counteract ATM phosphorylation of 53BP1, which in turn regulates DSB repair pathway choice. Mainly, phosphorylation of 53BP1 by ATM at, at least, 7 N-terminal sites leads to binding to RIF1, which inhibits strand resection. Upon dephosphorylation by PP4C, a process stimulated by BRCA1, RIF1 is released and 53BP1 can reposition and promote resection and HR (Figure 4-2) (Isono et al., 2017). In yeast, the phosphatase CDC14 dephosphorylates the nuclease YEN1 counteracting the kinase CDK. During S phase phosphorylation of YEN1 by CDK promotes its nuclear exclusion and avoids untimely nucleolytic activity on DNA. In anaphase, dephosphorylation by CDC14 promotes YEN1 nuclear localization and increases its DNA binding affinity, which helps resolve Holliday junctions in mitosis (Figure 4-2) (Blanco et al., 2014).

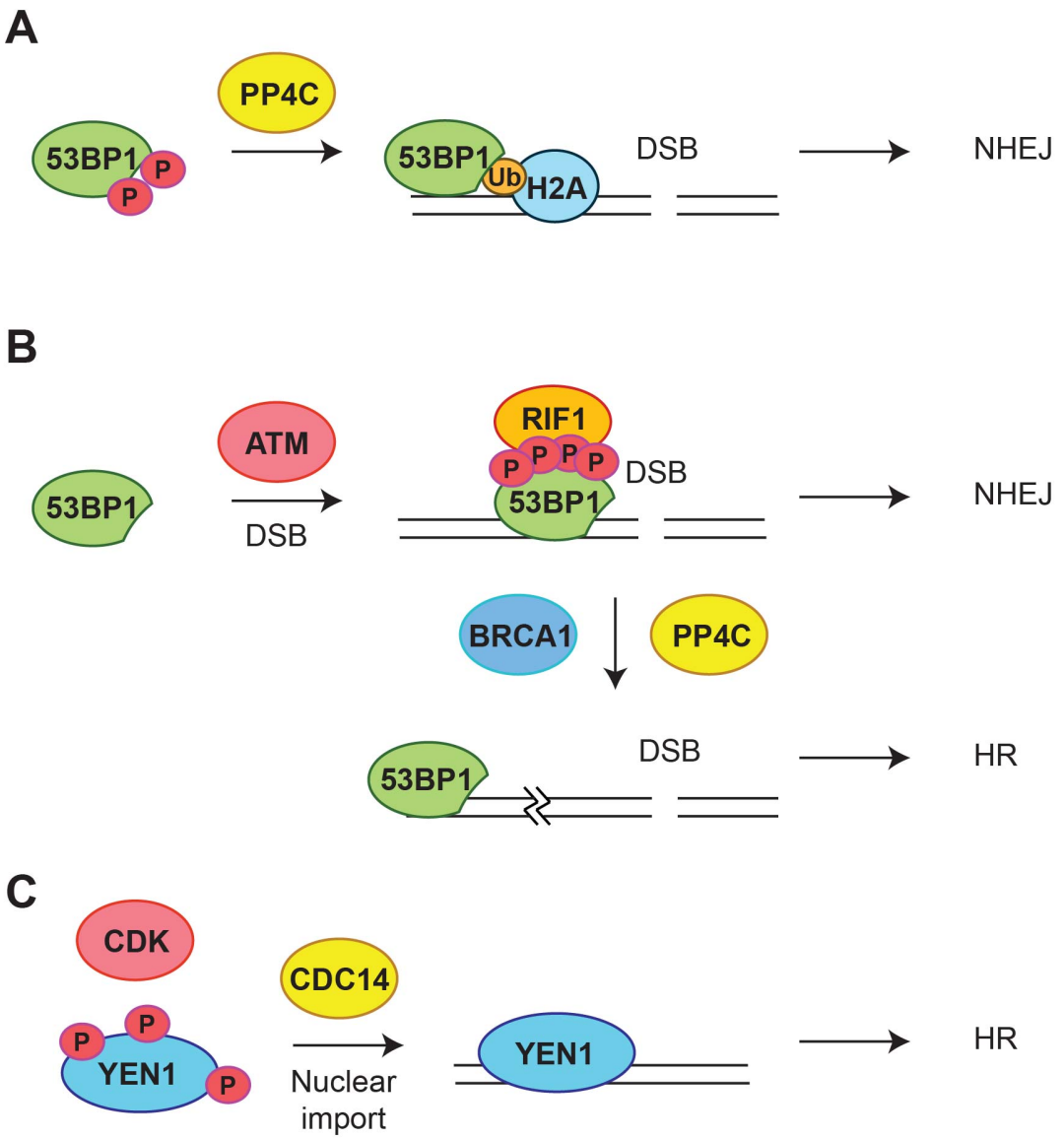


Figure 4-2. Models of DNA damage repair regulation by dephosphorylation. (A) 53BP1 is dephosphorylated by PP4C at T1609 and T1619 of its UDR domain. This in turn promotes 53BP1 binding to ubiquitinated histone H2A on chromatin and its function in NHEJ. (B) 53BP1 phosphorylation by ATM at 7 N-terminal sites (between residues 300 and 900) upon DSB generation leads to its binding to RIF1, immobilization on the DSB and promoting of NHEJ. Dephosphorylation of 53BP1 by PP4C, promoted by BRCA1, leads to RIF1 liberation and 53BP1 repositioning away from the DSB, allowing HR to take place. (C) In yeast, CDC14 dephosphorylates the nuclease YEN1 leading to its mobilization into the nucleus and increasing its DNA binding affinity in anaphase where it resolves Holliday junctions during HR.

A role in the FA pathway has been proposed for the phosphatase PTEN since lack of PTEN led to sensitivity to crosslinking agents in an epistatic

pathway with FANCD2. It was found that lack of PTEN led to increased phosphorylation of FANCM by PLK1, which in turn led to its SUMOylation and degradation (Vuono et al., 2016).

4.2 CK2 and the DNA damage response

The inactivation of the FANCD2/FANCI complex by constitutive phosphorylation by CK2 is a new regulatory step in the FA pathway, but CK2 has already been linked to other DNA repair pathways. Surprisingly, there are instances of CK2 both promoting and inhibiting the DDR, depending on which substrates are phosphorylated (summarised in Table 4-1).

The phosphorylation of XRCC1 by CK2 was the first instance of CK2 playing a role in DNA repair. This event was implicated in single strand break (SSB) repair by promoting the interaction of XRCC1 with PNK through the fork-head associated (FHA) domain on PNK (Loizou et al., 2004). Similarly, in DSB repair, CK2 phosphorylates XRCC4, promoting XRCC4 interaction with PNK and APLF (aprataxin and PNK-like factor) also through their FHA domains (Koch et al., 2004; Macrae et al., 2008). CK2 phosphorylation of XRCC1 was also found to promote the stability of the XRCC1-LigIII complex implicated in the ligation step of both SSB repair and BER (Parsons et al., 2010). Interestingly, it was later shown that XRCC1 phosphorylation could promote chromatin dissociation, allowing XRCC1 to form protein repair complexes needed for the incision in BER (Strom et al., 2011). It was recently found that XRCC1 phosphorylation by CK2 was necessary for interaction with aprataxin (APTX), that like PNK also contains a FHA domain, and it is implicated in SSB repair (Horton et al., 2018). XRCC1 can also interact with CtIP and MRE11, leading to DSB repair through MMEJ. Again,

phosphorylation of XRCC1 by CK2 was shown to promote these interactions and MMEJ (Dutta et al., 2017). It seems that XRCC1 would normally be chromatin-associated and immobile, but upon DNA damage CK2 phosphorylates XRCC1, decreasing its DNA binding affinity and liberating it from chromatin to form repair complexes for SSB repair, BER or even MMEJ.

Phosphorylation of H2AX is one of the first events known to happen following the generation of a DSB (Rogakou et al., 1998). CK2 was found to mediate an even earlier event, the phosphorylation of HP1β (heterochromatin protein 1β) (Ayoub et al., 2008). Phosphorylation of HP1β on T53 by CK2 led to its release from H3K9me, which in turn promotes chromatin relaxation and downstream events such as γH2AX phosphorylation (Ayoub et al., 2008). For such a quick regulatory event to happen, CK2 must be recruited very early to DSBs, and some evidence for this was later found (Olsen et al., 2012), though the exact mechanism or the mediators remain unknown.

Also during DSB repair, CK2 has been shown to constitutively phosphorylate MDC1 promoting the recruitment of the MRN (MRE11-RAD50-NBS1) complex to the sites of damage through its interaction with NBS1 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008). CK2 is also required for the accumulation of 53BP1 at sites of damage (Guerra et al., 2014). The mechanism was through the phosphorylation of LSD1 (lysine-specific demethylase) by CK2, reversible by the phosphatase WIP1. Phosphorylated LSD1 is then recruited through enhanced interaction with RNF168, leading to H3K4me2 demethylation and H2AX ubiquitination, enhancing 53BP1 recruitment and ubiquitination (Peng et al., 2015). CK2 was also found to be part of the sequential phosphorylation of RAD51, following phosphorylation by PLK1,

which leads to the recruitment of RAD51 to DSBs through its interaction with NBS1 in a BRCA2-independent manner (Yata et al., 2012). A similar mechanism has been recently found for MRE11 but with a very different outcome. Phosphorylation by PLK1 first, led then to CK2 phosphorylation of MRE11 rendering it unable to bind DNA and perform repair, which could potentially shutdown the repair (Li et al., 2017).

Recently, a new role for CK2 in NER has been described. XPC has been shown to be ubiquitinated in response to damage. It has also been reported to be phosphorylated by CK2 on S94 after damage induction (UVB), which promotes its ubiquitination, its recruitment and the recruitment of downstream effectors (Shah et al., 2018).

Table 4-1. CK2 targets implicated in DNA damage repair and the role of their phosphorylation.

Repair factor	Pathway	Role of CK2 phosphorylation
XRCC1	SSB/BER /MMEJ	Decrease DNA affinity and promote formation of repair complexes
XRCC4	DSB	Promote interaction with PNK and APLF
HP1β	DSB	Release from nucleosomes and increase chromatin relaxation, access of repair factors
MDC1	DSB	Promote recruitment of MRN complex
LSD1	DSB	Promote accumulation of 53BP1 at sites of damage
Rad51	DSB	Promote its recruitment to DSBs independently of BRCA2
MRE11	DSB	Decrease DNA binding affinity and shutdown repair
XPC	NER	Promote ubiquitination and recruitment to damage

A common feature arising from the role of CK2 in DDR is that usually the regulation of that event relies on some other component, whether it is another kinase, a phosphatase or spatial and compartmental separation. In the case of

FANCD2/FANCI the most likely answer is that dephosphorylation is the key, since the phosphomimetic mutants are still localized to the nucleus and the phosphorylation events already described by ATR on FANCD2 and FANCI are probably taking place already on DNA (like the monoubiquitination, which they usually promote) since ATR has been shown to be recruited to sites of damage (Cuadrado et al., 2006; Jazayeri et al., 2006).

4.3 FANCD2 roles outside of ICL repair

Although we have focused on ICL repair in this study, there is mounting evidence for roles of FANCD2 and the FANCD2/FANCI complex in numerous other processes (Federico et al., 2018; Palovcak et al., 2017). In many of these, DNA binding is still key for the function of FANCD2, so CK2 phosphorylation of FANCD2 could also play an important role regulating DNA binding in these instances.

A link between the FA pathway and DNA replication was one of the first novel functions to be identified. Components of the FA core complex, as well as FANCD2, are recruited to chromatin during S phase in the absence of induced DNA damage (Mi and Kupfer, 2005; Wang et al., 2008). These FA proteins are needed for replication fork restart after chemically induced collapse (Wang et al., 2008). FANCD2 was shown to help stabilise and restart replication forks in combination with other repair factors (such as RAD51 and BRCA1/2 (Schlacher et al., 2012)) and, interestingly, also with nucleases; mechanisms reminiscent of FANCD2 proposed functions in ICL repair. FANCD2 could associate with stalled replication forks, in an ATR-dependent manner, through association with the MCM2-MCM7 replicative helicase (Lossaint et al., 2013). FANCD2, then, mediates

recruitment of CtIP, MRE11 and FAN1 to stalled replication forks and regulates their nucleolytic activity, promoting fork restart but limiting fork degradation (Chaudhury et al., 2014; Lachaud et al., 2016; Yeo et al., 2014). FANCD2 was also shown to promote recruitment of BLM to stalled replication forks and to coordinate fork restart and suppression of new origin firing together with FANCJ and BRCA2, but independently of FANCI and the FA core complex (Chaudhury et al., 2013; Raghunandan et al., 2015). This is in contrast to the described function of FANCD2 in ICL repair where its binding to FANCI as well as its monoubiquitination by the FA core complex are essential. Nonetheless, there is also mechanistic evidence for a role for the FANCD2/FANCI complex at stalled forks. It was recently reported that FANCD2/FANCI stabilise RAD51-DNA filaments at stalled forks and this protects them from degradation by FAN1 (Sato et al., 2016).

Some genomic regions are particularly subject to replication stress, which can lead to breaks and genomic instability. These regions are called common fragile sites (CFS) and constitute hotspots for genomic deletions, translocations, etc. FANCD2 associates with these regions and it was recently shown to facilitate replication through CFS by inhibiting dormant origin firing and DNA:RNA hybrid formation that follow replication stalling at CFS (Howlett et al., 2005; Madireddy et al., 2016). This association of FANCD2 with CFS is especially marked in introns of large transcribed genes and it is conserved in other organisms where novel CFS have been identified through their FANCD2 interaction (Okamoto et al., 2018; Pentzold et al., 2018). The formation of DNA:RNA hybrids or R-loops during transcription is a normal process but it can cause damage when replication forks encounter them. The FA pathway has been linked also to the

resolution of these replication-transcription conflicts. FANCM has been shown to promote R-loop resolution through its translocase activity and lack of FANCD2 promotes the accumulation of R-loops, pointing to a FANCD2-dependent R-loop resolution also in place (Garcia-Rubio et al., 2015; Schwab et al., 2015).

When replication stalling is not resolved, it can lead to under-replicated regions linking two sister chromatids during mitosis. These linkages form ultra-fine bridges (UFB) of DNA that can be detected through the presence of proteins such as BLM, RPA or PICH (PLK1-interacting checkpoint helicase). Interestingly, FANCD2 forms foci at the bases of this type of UFB, but the role of FANCD2 there is still unknown even though it helps in the identification of UFBs. The presence of these FANCD2 foci is independent of FANCJ, BRCA2 or ATR, but depends on monoubiquitination and the FA core complex, in contrast to the described situation at stalled replication forks (Chan et al., 2009; Chan et al., 2018).

Some FA proteins, including FANCD2, have also been shown to play a role in the alternative lengthening of telomeres (ALT), a telomere maintenance process independent of telomerase that relies on HR present in some tumour cells. FANCD2 colocalizes with ALT telomeres in a FANCL and ATR dependent manner (Fan et al., 2009; Spardy et al., 2008). The exact role of FANCD2 in ALT is still unclear, but it was recently proposed to help resolve replication stalling at ALT telomeres and regulate the loading of BLM, that leads to the resections and chromosome exchanges in ALT (Root et al., 2016).

Since DNA binding is vital in all of these processes, whether FANCD2 phosphorylated by CK2 can still perform these functions or a similar activation by dephosphorylation is needed would be interesting to know and would show whether this event is a master regulator of the activity of FANCD2.

4.4 Future approaches and conclusion

Another question is what is the phosphorylation status of the population of FANCD2/FANCI in the cell before and after damage. A quantitative approach through MS/MS has proven difficult for a 6-residue cluster since many combinations of phosphorylated peptides can co-exist at any one point, making their detection especially complicated. Probably only a fraction of the free FANCD2/FANCI is dephosphorylated to allow for its recruitment on DNA and repair to happen. The precise mechanism behind the DNA binding affinity changes is also open to further study. The phosphorylated cluster could directly repel the DNA through electrostatic force or interfere with DNA binding regions nearby (residues 857-876) (Niraj et al., 2017), or further away. In fact, some positively charged regions have already been identified on FANCD2 Tower domain that mediate DNA binding (Liang et al., 2016). An alternative mechanism could be envisaged, where the phosphorylated cluster lies on a mobile loop able to bind to the Tower domain and block the DNA binding, like a DNA-mimetic loop. Or even a bigger conformational change could take place where the whole Tower domain bends and closes the complex to bind the phosphorylated cluster, thus creating an open versus close conformation.

The presence of a new layer of regulation on FANCD2 activation through CK2 phosphorylation opens the question on what negative effects could arise from the lack of this regulation. According to the results presented here, the expression of the non-phosphorylable FANCD2 mutant did not severely affect the survival to crosslinking agents. However, it is possible that under perturbed conditions the lack of phosphorylation does not lead to decreased survival because the repair can still proceed, unlike with the phosphomimetic mutant,

which keeps the protein in its off state, shutting down the repair pathway. Lack of phosphorylation on FANCD2 could pose a problem in unperturbed conditions for some of the processes previously described, such as DNA replication or CFS stability. The presence of a constitutive dephosphorylated form of FANCD2 should lead to an enhanced presence on chromatin, spurious activation and potential generation of damage.

Chapter 5. Materials and Methods.

Cell lines, antibodies and plasmids

HeLa cells were grown in DMEM (D5796, Sigma) supplemented with 2.5-10% FBS. Antibodies used were as follows: anti-FANCD2, 1:200 (sc-20022, Santa Cruz Biotechnology); anti-α-Tubulin, 1:2000 (5829, Millipore); anti-FANCI, 1:500 (G4270, Merck-Millipore); anti-Lamin B, 1:1000 dilution (sc-6216, Santa Cruz Biotechnology); anti-HA, 1:1000 (mouse monoclonal antibody clone 12CA5); anti-UHRF1, 1:1000 dilution (sc-373750, Santa Cruz Biotechnology); anti-Flag, 1:1000 (M5, F4042, Sigma-Aldrich). EGFP-fused FANCD2 and mCherry-fused UHRF1 cDNA were expressed using a derivative of the pOZ-N plasmid (Sato et al., 2012b). Transfections of plasmid DNA were carried out using FuGENE6 (Promega) according to the manufacturer's instructions.

CRISPR-Cas9 gene editing

HeLa FANCD2-/- cells were generated using plasmid pX459 (Addgene #48139) as described in (Ran et al., 2013). The targeting sequence used in the sgRNA was: 5´G TTT GTC TTG TGA GCG TCT GC 3´. Primers:

5´CACCGTTTGTCTTGTGAGCGTCTGC3´ and 5´AAACGCAGACGCTCACAAGACAAAC3´ were annealed and introduced into the pX459 plasmid through its BbsI site. HeLa cells were transfected with 2 μg of pX459 plasmid and selected after 24 h with 4 μg/ml puromycin. After 24 h cells were plated at low densities and clones were picked after 2 weeks. Clones were tested with Western blot. HeLa Flag-HA-FANCD2 knock-in cells were generated using plasmid pX459. The targeting sequence used in the sgRNA was:

5´CATGCCTCACACATTGCTA3´. Primers: 5´CACCCATGCCTCACACATTGCTAC 3´ and 5´AAACGTAGCAATGTGTGAGGCATG 3´ were annealed and introduced into the pX459 plasmid through its BbsI site. A donor plasmid was generated using pBS-SK (+) as the backbone containing 1000bp homology arms upstream (cloned with primers: 5'CACTTTGGGAGTCCGAGG 3' and 5'TTTGACCAATGTCTTGTGC 3') and downstream (cloned with primers: 5'ATGGTTTCCAAAAGAAGACTG 3' and 5'AATCACCACTAGAGAACTTATTTATG 3') of exon 1 of *FANCD2* gene. HeLa cells were co-transfected with 2 μg of pX459 plasmid and 2 μg of donor plasmid and selected after 24 h with 4 μg/ml puromycin. After 24 h cells were plated at low densities and clones were picked after 2 weeks. Clones were tested with Western blot.

Preparation of radiolabelled DNA substrates

DNA molecules used for EMSA were prepared as described (Liang et al., 2016). In brief, the DNA oligos were annealed in a buffer containing 10 mM Tris-HCl pH 7.5, 100 mM NaCl and 1 mM EDTA. ICL14 was created by annealing the following DNA oligonucleotides: ICL14 (+): 5'CATTGTGAATTCGCCTCTCTGTCTAGCCGAAGCTCGAAACGATCTTGTGC-3' ; ICL14 (-): 5'GTCCATCAAAGTTCGACTGTGCGGCTAGACAGAGAGGCGAATTCACAAG3'.

Mass spectrometric analysis

Initial phosphorylation events on FANCD2 were identified as follows. Flag-HA-FANCD2 purified from HeLa cells was reduced with DTT, cysteine residues were derivatized with iodoacetamide, and the proteins were separated by SDS-PAGE. Proteins from silver stained gel bands were in-gel digested with

trypsin (Chen et al., 2014). The generated peptide mixtures were subjected to LC-MS/MS using a hybrid linear ion trap/FT-ICR mass spectrometer (LTQ FT, Thermo Electron) essentially as described previously (Longerich et al., 2014). MS/MS spectra were assigned by searching them with the SEQUEST algorithm (Daee et al., 2012) against the human International Protein Index sequence database.

Protein purification

UBA1 and FANCL proteins purified from Sf9 cells were expressed using the pFastBac1 vector (Life Technologies) with an engineered N-terminal Flag-HA tag. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 0.1 M KCl, 10% glycerol, 0.1% Tween-20, 2 mM β-mercaptoethanol and 0.2 mM PMSF). Lysates were clarified by centrifugation, and the supernatants were incubated with M2 anti-Flag agarose resin for 2 h. The resin was washed extensively, and the protein was eluted in the same buffer containing 0.5 mg/ml Flag peptide, however excluding Tween-20. For FANCD2/FANCI complex and USP1/UAF1 complex, Sf9 cell pellets were re-suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 0.1 M KCl, 10% glycerol and 0.2 mM PMSF), and sonicated. Lysates were clarified by centrifugation (17,000 g), and the supernatants were incubated with M2 anti-FLAG agarose resin (A2220, Sigma) for 2 h. The resin was washed extensively, and the protein was eluted in the same buffer containing 0.5 mg/ml Flag peptide. UBE2T was expressed in *E. coli* BL21 cells (Invitrogen). Cells were cultured in Lysogeny broth (LB) supplemented with antibiotics. Once OD_{600} had reached 0.6, protein expression was induced by the addition of 0.5 mM isopropyl-1-thio-b-d-galactopyranoside (IPTG). Cells were cultured overnight at

16°C and harvested the following day by centrifugation. Harvested cells were lysed by sonication of 4 X 10s bursts on ice, in buffer containing 0.5 M NaCl, 0.1 M Tris (pH 8), 0.02 M imidazole and 0.25 mM tris(carboxyethyl)phosphine (TCEP). Cell debris was removed by centrifugation at 32,000 g. Supernatants were added to equilibrated Ni-NTA agarose (QIAGEN) and incubated on a roller for 1 h at 4°C. 6xHis-Smt3 tags were removed overnight at 4°C by Ulp1 protease at a w/w ratio of 1:15, Ulp1: protein.

In vitro protein binding assay

Recombinant proteins were expressed and purified from Sf9 insect cells as indicated above. 4 μg of Flag-HA-FANCD2/His-FANCI and 1 μg Flag-HA-FANCL were mixed in the reaction buffer containing 100 μg/ml BSA (NEB), 20 mM Tris-HCl pH 7.5, 100 mM KCl, 5% glycerol, 2mM β-mercaptoethanol and 0.2 mM PMSF. The mixture was first incubated at 30°C for 1 h for protein complex formation. Ni^{2} -NTA (30310, QIAGEN) beads were added subsequently, and the mixture was incubated at 4°C with gentle mixing for 30 minutes. The mixture was then transferred to Micro Bio-Spin Chromatography Columns (Bio-Rad), and washed with the reaction buffer supplemented with 0.1% Tween-20. The proteins were eluted in SDS-BME buffer at 37°C and run on an SDS-PAGE gel.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described (Zheng et al., 2011). In brief, the binding reaction contained indicated amounts of FANCD2/FANCI and 1 nM radiolabeled DNA in 10 μl containing 2 5mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 6% glycerol, 1 mM DTT (dithiothreitol). Binding reaction was left at room temperature for 1 h, and 0.025% BPB (bromophenol blue) was added. A

4% polyacrylamide (30:1) 0.4xTBE gel was pre-run at 150 mV for 1 h at 4°C. The samples were then loaded into the gel and run at 10 mA (100-200 mV) for 3-4 h at 4°C. The gel was then dried and exposed to a photo-stimulable phosphor imager plate.

In vitro phosphorylation and dephosphorylation assays

Phosphorylation reaction containing FANCD2/FANCI and CK2 (P6010, NEB) were incubated at 30°C for 30 min in 50 mM Tris pH 7.5, 10 mM $MgCl_2$ and 2 mM DTT. Dephosphorylation reaction containing FANCD2/FANCI and λPP (P0753, NEB) were incubated at 30°C for 30 min in 50 mM HEPES pH 7.5, 100 mM NaCl and 2 mM DTT.

In vitro ubiquitination and deubiquitination assays

Reaction contained 17 nM UBA1, 0.64 mM UBE2T, 0.372 mM FANCL or FLAG-HA-FANCL, 4.2 mM His-Ub, 0.25 mM FANCD2–FANCI complex or derivatives thereof, 4 mM pBlueScript SKII (+) when indicated, in the following reaction buffer: 50 mM Tris (pH 7.5), 100 mM KCl_2, 2 mM $MgCl_2$, 0.5 mM DTT and 2 mM ATP. Reactions were incubated at room temperature for the indicated time. If deubiquitination reaction followed, the reactions were stopped with 4 mM EDTA and 40 nM Flag-HA-USP1/Strep-UAF1 was added. 6x SDS loading buffer containing BME was used to terminate reactions. Samples were loaded onto an SDS-PAGE gel and subjected to Coomassie blue staining or immunoblotting. The described *in vitro* ubiquitination assay is a derivative of a previously published method (Hodson et al., 2014).

Clonogenic survival assay

Cells (200–1,000) were plated in 6-well plates and, after 24 h, treated with different dosages of MMC (mitomycin C). Colony formation was scored after 10-14 days using 1% (w/v) crystal violet in methanol.

Preparation of whole cell lysate and cell fractionation

Cells were scraped off the dishes, and centrifuged at 1,000 rpm for 5 minutes. Cell pellets were resuspended and incubated in equal volume of Benzonase buffer (2 mM $MgCl_2$, 20 mM Tris pH 8.0, 10% glycerol, 1% Triton X-100 and 12.5 units/ml benzonase (E1014, Sigma)) on ice for 10 minutes. The cells were then lysed by the addition of an equal volume of 2% SDS to reach a final concentration of 1%. Samples were heated at 70°C for 2 minutes. The protein concentration was determined by Bradford assay (Bio-Rad Life Science). For cell fractionation, cell pellets were permeabilized with CSK buffer containing 200 mM NaCl, 10 mM PIPES, 300 mM Sucrose, 1 mM $MgCl_2$, 1 mM EDTA and 0.5% Triton X-100 on ice for 10 min. CSK fraction (supernatant) and nuclear pellet were separated by centrifugation at 900 g at 4°C for 10 min. Nuclear pellet was processed the same way as whole-cell lysate described above.

Live-cell imaging

EGFP-fused FANCD2 and mCherry-fused UHRF1 cDNA were inserted into the pOZ vector as described above. Live cell imaging were carried out with an OLYMPUS IX81 microscope connected to PerkinElmer UltraView Vox spinning disk system equipped with a Plan-Apochromat 60x/1.4 oil objective using Volocity software 6.3 for image capturing. EGFP and mCherry were excited with

488 nm and 561 nm laser lines, respectively. Throughout the experiment, these cells were maintained at 5% CO_2, and 37°C using a live cell environmental chamber (Tokai hit). Confocal image series were typically recorded with a frame size of 512x512 pixels and a pixel size of 139 nm. For localized DNA damage induction, cells were seeded in glass bottom dish (MatTek) and sensitized by incubation in DMEM supplemented with 10% FBS and 20 µg/ml 4,5′,8-trimethylpsoralen (TMP) for 30 min at 37°C. Microirradiation was performed using the FRAP preview mode of the Volocity software by scanning (each irradiation time was 100 ms) a preselected area (50x3 pixels) within the nucleus 20-75 times with a 405 nm laser set to 100% laser power. The mCherry and EGFP intensities at microirradiated sites were quantified using ImageJ with Fiji, and normalized by their intensities before microirradiation.

Chapter 6. References

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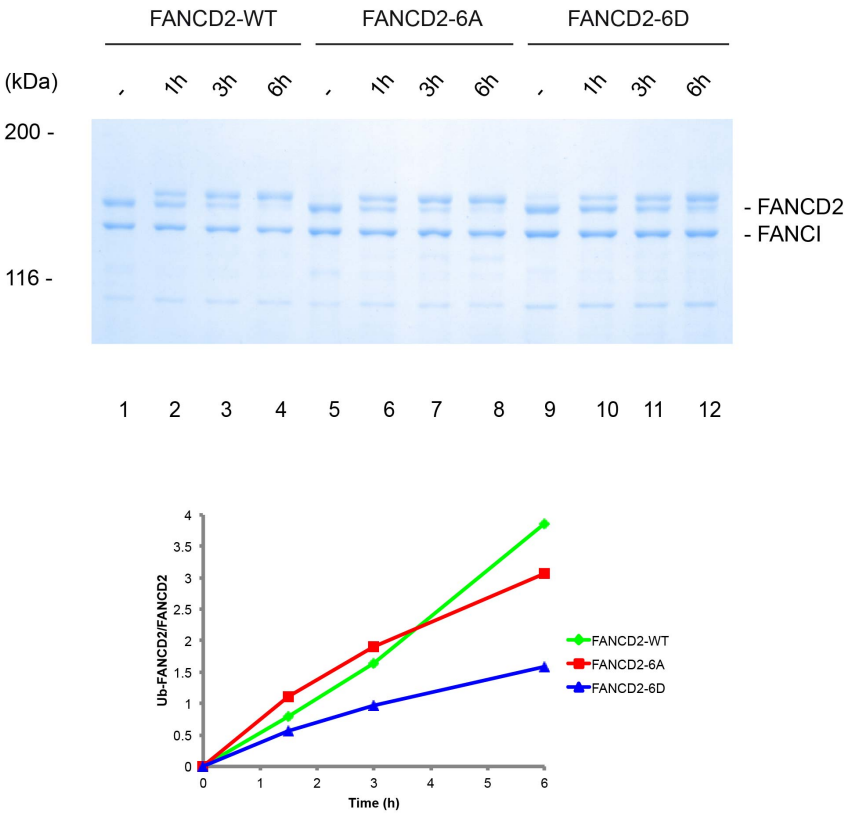
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Appendix



Appendix figure 1. *In vitro* monoubiquitination reaction is slowed down in the phosphomimetic FANCD2 (6D). Coomassie blue gel of the *in vitro* ubiquitination of the FANCD2/FANCI complex as WT, 6A or 6D forms. Quantification showing the ratio of Ub-FANCD2 to FANCD2.