# Novel roles for the Fanconi Anemia pathway protein FANCD2 in the recovery of stalled replication forks

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## Dedication

This thesis is dedicated to my Papa, Mummy and Di, for having taught me to dream big and strive to achieve those dreams

**Abstract**

Fanconi Anemia (FA) is an inherited cancer predisposition syndrome that is characterized by a cellular hypersensitivity to DNA interstrand crosslinks (ICLs). To repair these DNA lesions, the 21 known FA proteins are thought to act in a linear hierarchy: Following ICL detection, an upstream FA core complex activates two central FA pathway members, FANCD2 and FANCI, via monoubiquitination. Both activated proteins then bind the ICL and recruit downstream FA proteins that repair the ICLs. Importantly, we previously found that FANCD2 has an additional independent role during the cellular replication stress response: it promotes the homologous recombination (HR) dependent restart of hydroxyurea (HU) stalled replication forks in concert with other HR DNA repair proteins such as the BLM helicase. In this work, we show that FANCD2 promotes replication fork restart in concert with downstream FA pathway proteins but independently of the upstream FA core complex and thus, independently of FANCD2 monoubiquitination. To further our understanding of how FANCD2 promotes replication fork recovery, we performed a search for S-phase specific FANCD2 interactors and we identified a novel FANCD2 interacting protein, Alpha Thalassemia Retardation X-linked factor (ATRX). ATRX is a subunit of the ATRX/DAXX histone H3 chaperone complex that plays several key roles in regulating chromatin structure and was recently identified as a replication fork recovery factor. Our new findings demonstrate that ATRX forms a constitutive complex with FANCD2 and promotes FANCD2 protein stability. Moreover, while ATRX is dispensable for DNA ICL repair, it works in concert with FANCD2 to promote HU resistance and the restart of HU-stalled replication forks. Remarkably, the

HR-dependent replication fork restart requires the histone H3 chaperone activity of both the ATRX/DAXX complex *and* FANCD2 indicating that histone exchange at stalled replication forks is a crucial step in fork restart. Altogether, our results support a novel non-linear FA pathway model where individual protein members fulfill distinct cellular roles to support genomic stability. We propose that FANCD2- and possibly other FA pathway proteins- is involved in the deposition of histone H3 variants in the vicinity of HU- stalled replication forks to mediate fork recovery.

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Table 1. Genomic instability diseases and associated cancers

## List of Abbreviations

| | |
|---|---|
| FA | Fanconi Anemia |
| DNA | Deoxy ribonucleic acid |
| ICL | Inter strand cross Links |
| ATRX | Alpha Thalassemia Retardation X-linked factor |
| ATM | Ataxia-telangiectasia mutated |
| ATR | Ataxia-telangiectasia and RAD3 related |
| DSB | DNA double stranded breaks |
| GI | Genomic instability |
| DDR | DNA damage response |
| ROS | Reactive oxygen species |
| UV | Ultra violet |
| IR | Ionizing radiation |
| MMC | Mitomycin C |
| BER | Base excision repair |
| MMR | Mismatch repair |
| NER | Nucleotide excision pathway |
| XP | Xeroderma pigmentosum |
| CS | Cockayne syndrome |
| TTD | Trichothiodystrophy |
| NHEJ | Non homologous end joining |
| HR | Homologous recombination |
| AML | acute myeloid leukemia |
| FAN1 | Fanconi associated nuclease 1 |
| TLS | Translesion synthesis |
| MRN | MRE11/RAD50/NBS1 |
| SLX4 | Synthetic lethal of unknown function protein 4 |
| BRCA1 | Breast cancer associated 1 |
| BRCA2 | Breast cancer associated 2 |
| PALB2 | Partner and localizer of BRCA2 |
| ssDNA | Single stranded DNA |
| BLM | Bloom Helicase |
| BRIP1 | BRCA1-interacting protein 1 |
| PCNA | Proliferating cell nuclear antigen |
| HU | Hydroxyurea |
| APH | Aphidicolin |
| RS-SCID | Radiosensitive severe combined immunodeficiency |
| BS | Blooms syndrome |
| WS | Werner syndrome |

| | |
|---|---|
| RTS | Rothmund Thomson syndrome |
| SS | Seckel syndrome |
| DAXX | Death domain associated protein |
| SWI2/SNF2 | Switch 2, Sucrose non- fermenting 2 |
| RPE1 | Retinal pigment epithelium-1 |
| rAAV | Recombinant adeno-associated virus |
| ALT | Alternative lengthening of telomeres |
| gRNA | Guide RNA |
| DigU | Digoxigenin-dUTPs |
| BioU | Biotin-dUTPs |
| CDK | Cyclin dependent kinase |
| iPOND | Isolation of proteins on nascent DNA |
| T-FISH | Telomere specific fluorescence in situ hybridization |
| CtIP | CtBP interacting protein |

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# CHAPTER 1

## Introduction

## 1.1 Cancer biology and genomic instability

Cells in the human body grow and divide to form new cells according to the needs of the body. As cells age or get damaged, they undergo programmed senescence or death and are replaced by newer cells. This process of cell division, aging, death and replacement is very tightly regulated and is carried out in an orderly manner by the cellular machinery. However, this orderly process can break down in some cells and allow them to gain "enabling" traits that let them override the regulatory machinery to proliferate uncontrollably. Such abnormal cells with the ability to proliferate indefinitely are termed cancer cells (Hanahan and Weinberg, 2000; Kinzler and Vogelstein, 1997; Tabassum and Polyak, 2015).

The foundation of carcinogenesis lies in the alterations of genes that control cellular growth, division and death. Hanahan and Weinberg aptly compared carcinogenesis to a process analogous to Darwinian evolution, wherein successive genetic changes confer some growth advantage, eventually leading to the conversion of a normal cell to a cancer cell (Hanahan and Weinberg, 2000). Thus, the generation of mutational changes is an early etiology for tumorigenesis. Post tumorigenesis, cancer cells have an "unstable" genome that is prone to the accumulation of many genetic mutations in a successive manner. In fact, instability of the genome is a defining characteristic of all neoplastic cells (Kastan and Bartek, 2004; Negrini et al., 2010; Shen, 2011). As further discussed by Hanahan and Weinberg in 2011, genomic instability was appended to the classic "Hallmarks of Cancer". Genomic instability is now known to be a key "enabling" characteristic that promotes the acquisition of other hallmark cancer cell

capabilities, including the ability to survive, proliferate and disseminate (Hanahan and Weinberg, 2011).

The integrity of a cell's genome is constantly threatened by various kinds of endogenous and exogenous DNA damaging sources (Helleday et al., 2014). To counteract this, cells employ an intricate network of "genomic caretaker proteins". This network encompasses several DNA damage repair (DDR) pathways that are intertwined with the "cell cycle checkpoint" machinery that can halt the cell cycle in the face of DNA damage to allow DNA repair prior to the next cell division (Kastan and Bartek, 2004; Khanna and Jackson, 2001; Kinzler and Vogelstein, 1997).

There are three cell cycle checkpoints called G1 phase, S phase and G2/M transition 'checkpoints'. G1 phase checkpoint: This checkpoint is activated in response to damaged DNA in G1 phase, and thus, prevents cells from initiating replication. Upon checkpoint activation, ATM (Ataxia-Telangiectasia Mutated) and ATR (Ataxia-Telangiectasia and RAD3 related) kinases mediate the phosphorylation of the tumor suppressor p53. Activated p53 in turn, transcriptionally silences the G1/S-transition promoting cyclin/CDK complexes, leading to G1 arrest. S phase checkpoint: This checkpoint puts a hold on DNA synthesis in response to DNA damage during S phase and ensures completion of DNA replication with a minimum of heritable mutations. The two major pathways involved at this checkpoint are ATM/ATR–Chk1/Chk2–CDC25A and ATM–NBS1–SMC1. DNA Double -stranded breaks (DSBs) in S-phase trigger the ATM mediated phosphorylation of the Chk2 kinase, whereas single stranded DNA (at stalled replication forks) triggers ATRR mediated phosphorylation of the Chk1 kinase.

The ATM/ATR–Chk1/Chk2–CDC25A pathway transmits the activation signal through a protein cascade to ultimately inactivate the S-phase-promoting Cyclin E/cdk2 complex, thus, preventing replication origin firing, and arresting replicative DNA synthesis. In response to DNA DSBs, ATM also phosphorylates NBS1 to activate the MRE11-RAD50- NBS1 (MRN) mediated DSB repair pathways. The mitotic G2/M phase checkpoint: This checkpoint prevents segregation of damaged chromosomes. Following DNA damage, ATM/ATR–Chk1/Chk2–CDC25A pathways activate a downstream protein cascade that works through p53-dependent mechanisms to cause G2 arrest that prevents cells from enetering mitosis (Houtgraaf et al., 2006; Ishikawa et al., 2006; Kastan and Bartek, 2004; Li and Zou, 2005; Shimada and Nakanishi, 2006).

Deregulation of one or more of these caretaker pathways can lead to genetic aberrations such as specific gene mutations or amplifications, deletions or rearrangements of chromosome segments, and gain or loss of entire chromosome(s) (Ferguson et al., 2015). Cancer cells downregulate these very caretaker pathways to further increase the frequency with which they acquire genetic aberrations. The “mutator phenotype” hypothesis for carcinogenesis postulates that the mutation rate in the early stages of tumor development must be significantly greater than that of normal somatic cells (Loeb and Loeb, 1999). Accumulating evidence suggests that tumorigenic cells subvert one or more caretaker pathways to fast-track their evolution by accumulating favorable genotypes that drive carcinogenesis and metastasis even more rapidly. In fact, the majority of genes within the DDR and cell cycle checkpoint pathways have been recognized as bonafide tumor suppressors (Hanahan and Weinberg, 2011; Kinzler and

Vogelstein, 1997). Accordingly, inherited mutations in caretaker genes are the underlying cause of several human genomic instability (GI) syndromes that are characterized by a significantly increased risk of developing certain types of cancer compared to the healthy population (Table 1). (Ceccaldi et al., 2016a; Ghosal and Chen, 2013; Machado and Menck, 1997; Raymond J. Monnat and Sidorova, 2014).

***Table 1. Genomic instability diseases and associated cancers***

| DDR Pathway | Gene(s) Mutated | Syndrome | Cancer predisposition |
|---|---|---|---|
| BER | MUTYH | MUTYH-associated polyposis | Colorectal cancer |
| MMR | MSH2, MSH6, MLH1, PMS2 | Lynch syndrome, Hereditary nonpolyposis colorectal cancer (HNPCC) | Colorectal cancer, carcinomas |
| NER | XPA, XPB, XPC, XPD, XPE, XPF, XPG, POLH | Xeroderma Pigmentosum | Squamous and basal cell carcinoma, melanoma |
| NHEJ | LIG4 | Ligase IV syndrome | Lymphoma |
| | ARTEMIS | Radiosensitive severe combined immunodeficiency (RS-SCID) | Lymphoma |
| HR | BLM | Bloom Syndrome (BS) | Carcinoma, lymphoma, leukemia |
| | WRN | Werner Syndrome (WS) | Sarcoma |
| | RECQL4 | Rothmund Thomson Syndrome (RTS) | Skin cancer, osteosarcoma |
| ICL repair | FANC-A, -B, -C, -E, -F, -G, L, -I, -D1, -D2, -J, -M, -N, -O, -P, -Q, -R, -S, -T, -U, -V | Fanconi Anemia (FA) | AML, squamous cell carcinoma, myelodysplasia |
| DNA damage signaling, DSB repair | ATM | Ataxia Telangiectasia (A-T) | Leukemia, lymphomas, breast cancer |
| | NBS1 | Nijmegen breakage syndrome | B cell lymphoma |
| | ATR | Seckel syndrome (SS) | AML |

Studying the clinical and cellular phenotypes observed in GI syndrome patients has provided us with significant insights into specific functions of DDR and cell cycle checkpoint genes that support genome stability and prevent carcinogenesis. However, the complex molecular mechanisms that underlie the DDR and checkpoint response network remain incompletely understood.

## 1.2 DNA damage response pathways for maintaining genomic stability

### *1.2.1 Sources and types of DNA damage*

It is estimated that each of the $10^{13}$- $10^{14}$ cells in the human body receives $10^5$ to $10^6$ DNA lesions per day (Swenberg et al., 2011). These lesions can be caused by a plethora of endogenous and exogenous sources.

Endogenous DNA damage: Some endogenous damage arises from agents that cause chemical modification of the DNA, whereas others arise from errors made by the DNA replication machinery. A few major sources that cause chemical modifications include reactive oxygen species (ROS) or reactive alkylating compounds produced in various cellular compartments ( such as mitochondria and peroxisomes) as byproducts of normal cellular metabolism (Jackson and Loeb, 2001).These reactive species can mediate oxidation, hydrolytic attack or even uncontrolled methylation of the DNA leading to base and nucleotide changes. Moreover, byproducts of alcohol or fat metabolism such as acetaldehyde can covalently cross link two bases, either on the same DNA strand or on the opposite strands resulting in a DNA intra- or inter- strand cross link respectively.

During DNA replication and DNA recombination, proofreading errors by the involved DNA polymerases can cause erroneous nucleotide insertions resulting in

mismatched DNA base pairs. Additionally, the DNA replication machinery may stall and collapse at natural impediments such as modified nucleotides giving rise to DNA DSBs. Moreover, nuclear enzymes such as Topoisomerase II (that normally function to regulate DNA supercoiling) can accidentally cleave the DNA at off-target sites to yield DNA DSBs as well. (Ceccaldi et al., 2016a; Cortez, 2015; Khanna and Jackson, 2001; Rothkamm et al., 2003).

Exogenous DNA damage: The main naturally occurring DNA damage sources include ultraviolet (UV) light and ionizing radiation (IR). UV light causes the formation of photoproducts like cyclopyrimidine dimers. Exposure to IR and X-rays causes DNA DSBs. A multitude of chemical genotoxic agents such as cisplatin, diepoxybutane, mitomycin C (MMC) and bifunctional alkylating agents such as nitrogen mustard can cause additional DNA lesions such as intra- or inter-strand crosslinks. Due to their particularly high toxicity, these crosslinking agents are frequently used as cancer chemotherapeutic drugs.(Swenberg et al., 2011).

Depending on the type of the DNA damage, cells employ different DDR pathways to remove the DNA lesion in a timely and efficient manner, thereby maintaining the integrity of the genetic information. (Figure 1.2).

**Sources of DNA damage and types of damage response pathways**

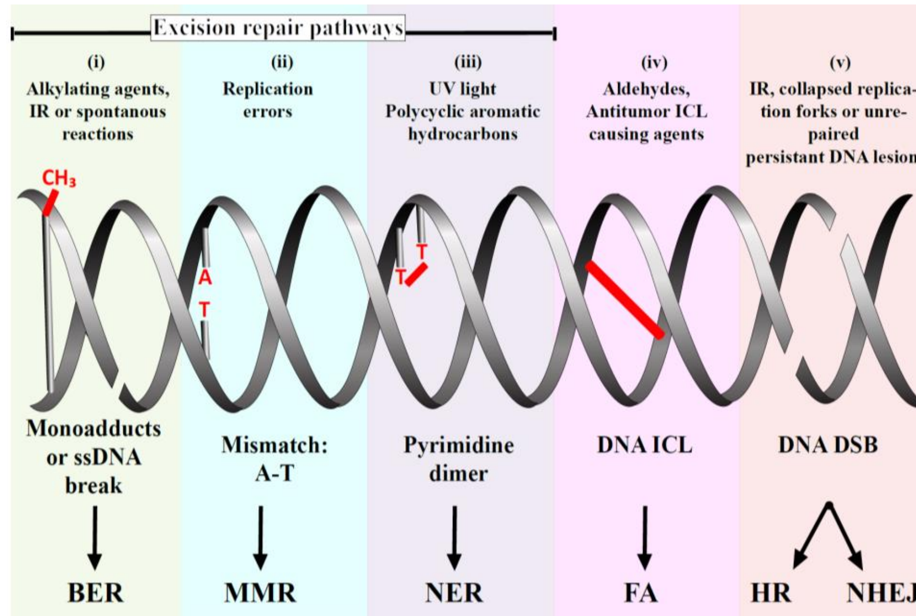


**Figure 1.1: Sources of DNA damage and associated types of damage response pathways**

Various kinds of DNA damages are resolved by a designated pathway. (i) Alkylating agents or other spontaneous reactions in the presence of reactive oxygen species can result in alkylated bases or single stranded DNA breaks. These are resolved by the BER pathway. (ii) Mis-incorporation of an incorrect base resulting in a mismatched base pair can occur during replication due to insufficient proofreading activity by the DNA polymerases. These are repaired by the MMR pathway. (iii) UV light or polycyclic hydrocarbons can cause intra-strand crosslinks or bulky adducts. These are repaired by the NER pathway. (iv) Endogenous aldehydes produced as metabolic byproducts or clinically used chemotherapeutic agents like Cisplatin or Mitomycin C can induce DNA inter-strand crosslinks. These are repaired by the Fanconi Anemia Pathway. (v) Multiple genotoxic sources including ionizing radiations, collapsed replication forks due to prolonged fork stalling, or even persistently unrepaired DNA lesions can result in DNA DSBs. The DSBs are repaired via the error prone NHEJ or the relatively error free HR pathway.

*1.2.2 DNA Repair Pathways- an overview*

***DNA Excision repair pathways:***

(**i**) The **B**ase **E**xcision **R**epair (BER) pathway.

The BER pathway repairs the majority of hydrolytic decay- mediated deamination events and bulky chemical modifications (such as methylation or oxidation) that occur at the DNA bases or the DNA sugar phosphate backbone. BER excises the damaged base carrying to create an abasic site, followed by incisions of the sugar phosphate backbone and replacement of the previously damaged nucleotide with an intact nucleotide. Biallelic mutations in BER pathway genes cause predisposition to recessively inherited adenomatous polyposis and colon cancer (Kim and Wilson, 2012; Krokan and Bjørås, 2013; Robertson et al., 2009).

(**ii**) The **M**is**M**atch **R**epair (MMR) pathway.

MMR repair is primarily used to repair base-base mismatches and insertion/deletion mis-pairings that are generated during normal DNA replication and recombination. The MMR pathway also removes mis-incorporated, chemically altered nucleotides from DNA (Li, 2008). The MMR pathway proteins identify and bind to the mismatched pair on the DNA. The mismatch-containing strand is cleaved to allow for removal of the mismatch segment by the concerted effort of an exonuclease/helicase complex, followed by resynthesis of the excised DNA tract. Mutations in the MMR pathway genes cause a genome instability disease called Lynch Syndrome (Lee et al., 2016; Li, 2008; Martín-López and Fishel, 2013).

(**iii**) The Nucleotide **E**xcision **R**epair pathway (NER).

The NER pathway primarily recognizes lesions that distort the DNA double helix structure. The primary substrates for NER are photoproducts caused by ultraviolet (UV) light (such as cyclopyrimidine dimers) and bulky adducts (Marteijn et al., 2014). The NER proteins remove a short oligonucleotide stretch from the DNA strand containing the lesion and use the undamaged single-stranded DNA as a template to re-synthesize the complementary sequence.

Mutations in three different NER pathway genes within different sub-class of the pathway, are associated with three different GI syndromes. (Xeroderma pigmentosum (mutations in global NER pathway genes), Cockayne syndrome (mutations in Cockayne syndrome NER pathway genes) and trichothiodystrophy (mutations in transcription coupled NER pathway genes). (Balajee and Bohr, 2000; Marteijn et al., 2014; Reed, 2011; Schärer, 2013).

***DNA double strand break (DSB) repair pathways:***

A DNA DSB occurs when the opposite strands of the DNA double helix are severed. DNA DSBs represent a particularly dangerous type of DNA damage that - when unrepaired or inaccurately repaired- can eventually lead to chromosomal aberrations including breaks, translocations and deletions. Human cells possess two distinct DNA DSB repair pathways- **N**on **H**omologous **E**nd **J**oining (NHEJ) and **H**omologous **R**ecombination (HR) repair (Ceccaldi et al., 2016b; Jasin and Rothstein, 2013; Rothkamm et al., 2003).

The NHEJ pathway is the primary DSB repair pathway in human cells and functions predominantly in the G1 phase of the cell cycle. The NHEJ machinery

recognizes a DSB, holds the termini together while performing limited degradation of these ends and eventually ligates even mismatched and non-cohesive broken ends of DNA back together. Since NHEJ does not rely on a homologous template for break repair it is considered to be highly error-prone (Davis and Chen, 2013; Gaymes et al., 2002; Lieber, 2010; Lieber et al., 2003).

In contrast, the HR repair pathway functions in the S-phase of the cell cycle, relies on DNA sequence homology and is therefore considered to be relatively error-free. For HR repair, both 5' ends of a DNA DSB are resected by nucleases to produce long 3' ssDNA overhangs. Subsequently, one of the 3' ends invades the intact sister chromatid, searches for the complementary sequence and uses it as the template to extend itself. The second end of the DSB is engaged, by either second end capture through DNA annealing or a second invasion event. Ultimately, the invaded strand is resolved or dissolved to individual chromatids by a group of proteins as discussed later in this chapter (Bachrati and Hickson, 2009; Jasin and Rothstein, 2013; Khanna and Jackson, 2001; Renkawitz et al., 2014; Sung and Klein, 2006).

***The DNA interstrand crosslink repair pathway:***

A DNA inter strand crosslink (ICL) is a covalent link between the Watson and Crick strands of the DNA double helix. DNA ICLs are particularly toxic to cells as they represent physical barriers to the DNA replication and DNA transcription machineries.

The repair of DNA ICLs relies heavily on the Fanconi Anemia (FA) pathway. Complete ICL removal requires concerted actions between the FA proteins and members of several other DNA repair pathways including NER and HR repair factors. In S-phase,

the FA pathway proteins incise and remove the ICL with assistance from the NER pathway members. The ensuing DSB is then repaired by the HR repair machinery. (Kee and D'Andrea, 2012; Wang, 2007). The details of DNA ICL repair are discussed in in the subsequent pages of this chapter.

***Chromatin reorganization in the context of DNA damage repair:***

In human cells, the genomic DNA is packaged with histones complexes into a highly ordered structure, the chromatin. Chromatin consists of repeating units called the nucleosomes. Each nucleosome is a nucleoprotein assembly comprised of 146 bp of DNA wrapped approximately two times around a histone octamer comprising two histone H2A-H2B dimers and a histone H3-H4 tetramer (Polo, 2015; Seeber and Gasser, 2016; Tagami et al., 2004). The role of chromatin reorganization during the cellular DNA damage response is best explained by the "Access-Repair-Restore" model described by Soria et. al., 2012. This model describes the chromatin as an integration platform orchestrating the maintenance of cellular functions while coordinating the DDR pathways. Strictly regulated chromatin disorganization events such as histone eviction and nucleosome sliding at sites of DNA damage are crucial to provide the DDR pathway members *access* to the DNA lesion. Following the *repair* of the DNA lesion, the DDR machinery dissociates from the DNA and the ordered chromatinized state is *restored*. Chromatin access and restoration are mediated by the concerted actions of the multifaceted and complex chromatin organization machinery that consists of chromatin remodelers, histone chaperones and histone modifying enzymes (Polo, 2015; Soria et al., 2012). One such example of chromatin reorganization has been well described in the

context of DSB repair. Following DNA DSB induction, the histone acetyl transferase TIP60 is recruited to the chromatin surrounding the DSB by docking onto histone H3 proteins that are trimethylated at lysine 9 (H3K9me3). Once recruited, TIP60 promotes histone H4 acetylation, which in turn creates open chromatin structures to facilitate *access* to DNA DSB repair proteins (Jacquet et al., 2016; Seeber and Gasser, 2016). Simultaneously, TIP60 acetylates and activates the ATM kinase that subsequently initiates the downstream events of DSB repair. Once the repair is initiated, the process of chromatin restoration starts concomitantly, which involves histone chaperones such as ASF1, CAF1 and possibly the ATRX/DAXX complex for histone deposition and re-chromatinization (Adam et al., 2014; Polo et al., 2006).

Thus, the coordinated actions of the chromatin reorganization machinery and the DDR machinery are important for the successful repair of DNA lesions (Hunt et al., 2013).

## 1.3 The Fanconi Anemia pathway and DNA ICL repair

### *1.3.1 Fanconi Anemia – clinical and cellular phenotypes*

Fanconi Anemia (FA) is a rare, highly heterogeneous inherited GI syndrome (1 in 100,000 people). To date, homozygous mutations in 21 different FA genes have been identified as causative of FA (Ceccaldi et al., 2016a; Walden and Deans, 2014). FA patients present with a complex spectrum of pathological manifestations (Kee and D’Andrea, 2012; Walden and Deans, 2014; Wang, 2007). Hematological defects include severe bone marrow failure, aplastic anemia, myelodysplastic syndrome, acute myeloid leukemia (AML) and pancytopenia. Other symptoms include multiple congenital

abnormalities such as skeletal defects, skin hyperpigmentation, osteopenia, endocrine and gastrointestinal abnormalities (D'Andrea and Grompe, 2003; Kee and D'Andrea, 2012). Moreover, FA adults who survive the childhood bone marrow failure via a successful bone marrow transplant show a very high incidence of squamous cell carcinomas.

FA patient cells show a delayed proliferation rate and an abnormal cell cycle distribution with a prolonged S phase (Akkari et al., 2001). Moreover, FA cells are highly sensitive to DNA interstand crosslinking agents such as MMC (Boisvert and Howlett, 2014; D'Andrea and Grompe, 2003; Garcia-Higuera et al., 2001). Upon treatment with DNA ICL inducing agents, FA cells fail to arrest during S-phase and consequently accumulate in the G2/M phase of the cell cycle, a phenomenon that is used as the diagnostic tool for FA. In addition, FA patient cells show spontaneous chromosomal aberrations including chromosome breaks, gaps and radial formations, that are further exacerbated upon DNA ICL induction.

### 1.4 The FA pathway and DNA ICL repair

DNA ICLs are predominantly recognized during S-phase, when two moving replication forks converge at the ICLs (Duxin and Walter, 2015; Knipscheer et al., 2009; Räschle et al., 2008). Per the current FA pathway model, the FA proteins act in a linear hierarchy during DNA ICL repair and can be broadly divided into three tiers: an upstream FA core complex (Tier I), a central heterodimer composed of FANCD2 and FANCI (Tier II) and several downstream FA proteins (Tier III) (Figure. 1.2) (Ceccaldi et al., 2016a; D'Andrea and Grompe, 2003; Kee and D'Andrea, 2012; Walden and Deans, 2014; Wang, 2007).

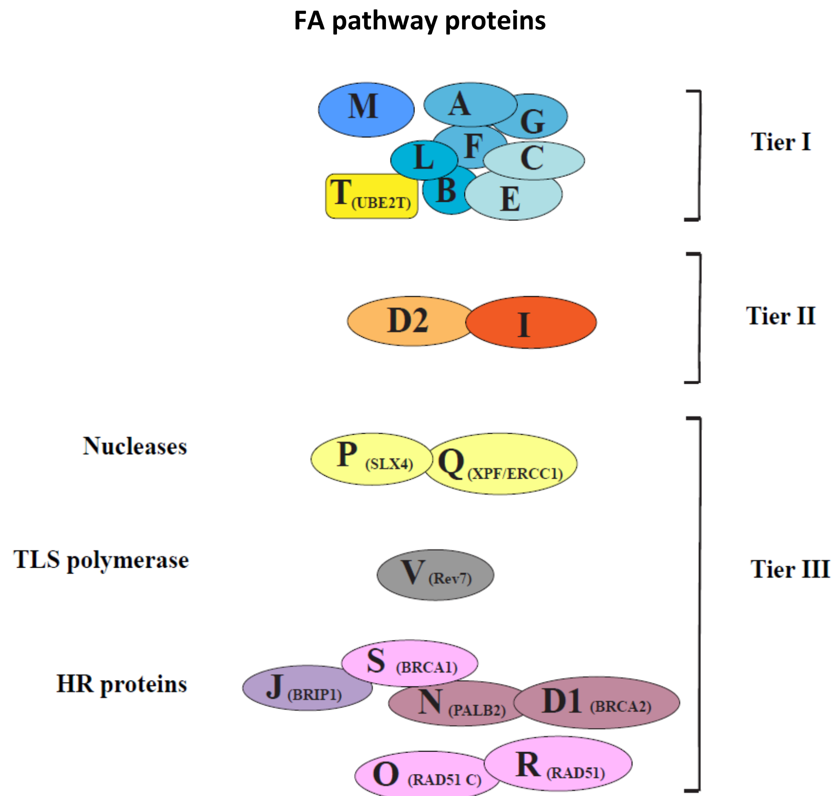


**Figure 1.2: Proteins in the FA pathway**

The FA pathway comprises 21 different proteins that can be distributed into three broad groups based on their function within the pathway. Tier I of FA pathway consists of FANCM and the FA core complex proteins that function as an E3 Ubiquitin ligase. UBE2T and FANCL are the catalytic subunits for the E2 and E3 ubiquitin ligase activity. Tier II is comprised of the FANCD2-FANCI (ID2) complex, which itself gets monoubiquitinated by Tier I proteins. Tier III of FA pathway comprises the downstream factors, that can be sub-classified as nucleases, TLS polymerase or homologous recombination repair factors. The ID2 complex facilitates the recruitment of the downstream Tier III proteins to facilitate ICL repair.

The FA pathway-mediated DNA ICL repair occurs in several steps as detailed below.

STEP I: Recognition of the DNA ICL by the upstream FA core complex (Figure 1.3 i- iii):

Upon replication fork stalling at a DNA ICL, the replicative helicase complex MCM2-7 is evicted from the stalled fork. Subsequently, the FA core complex member FANCM, in complex with the FA- associated protein 24 (FAAP24) and the histone fold proteins MHF1 and MHF2, is recruited to the ICL (Kim et al., 2008a). Once bound to the ICL, the FANCM-MHF-FAAP24 complex then assembles the complete FA core complex consisting of FANCA, -B, -C, -E, -F, -G, -L and -T (Hira et al., 2015; Hodson and Walden, 2012; Huang et al., 2014; Kim et al., 2008a; Rickman et al., 2015).

STEP II: Activation of the central FANCD2-FANCI heterodimer by monoubiquitination (Fig. 1.3 iv):

Once the FA core complex is fully assembled at the DNA ICL, it promotes the recruitment of the FANCD2-FANCI heterodimer (called the ID2 complex). The FA core complex then acts as an E3 ubiquitin ligase – with FANCL as the catalytic subunit - that monoubiquitinates both members of the ID2 complex ($ID2^{Ub}$) (Alpi et al., 2008). This step is indispensable for DNA ICL repair and is considered to be the key activation step of the FA pathway. (Garcia-Higuera et al., 2001; Garner and Smogorzewska, 2011; Gibbs-Seymour et al., 2015; Rajendra et al., 2014; Wang et al., 2004). Once activated, the chromatin-bound $ID2^{Ub}$ complex orchestrates the recruitment of downstream FA and non-FA proteins for ICL repair, as detailed below.

STEP III: Generating dual incisions at the DNA ICL site

(Fig. 1.3 v):

In order to remove the ICL lesion, incisions must be made on the same DNA strand on either side of the ICL. To this end, the $ID2^{Ub}$ complex facilitates the recruitment of FANCP/SLX4 (Synthetic lethal of unknown function protein 4) that acts as a docking platform for several DNA endonucleases including FANCQ/XPF-ERCC1 (Klein Douwel et al., 2014). FANCQ promotes the incision of the 3' side of the DNA ICL containing DNA. While it is currently not known which endonuclease is performing the incision on the other side of the DNA ICL, several endonucleases may be able to perform this role, including the SLX4-dependent endonucleases- SLX1 or MUS81-EME1, or the FAN1 nuclease (Fanconi Associated Nuclease 1) that are known to be recruited to DNA ICL-containing chromatin in an $ID2^{Ub}$-dependent manner. (Ceccaldi et al., 2016a; Kang, 2011; Kim et al., 2012b; Klein Douwel et al., 2014; Kratz et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Stoepker et al., 2011). The "unhooking" of the ICL results in a two ended DSB on the incised strand (Fig. 1.3 v) (Duxin and Walter, 2015), while the other DNA strand contains gapped dsDNA with the bulky ICL still attached (Fig. 1.3 vi).

STEP IV: ICL lesion bypass and gap filling DNA synthesis

(Fig 1.3 vi):

The gap filling DNA synthesis across the bulky ICL cannot be carried out by either of the main replicative DNA polymerases, epsilon or delta, since the ICL adduct will block synthesis of the complementary DNA strand. To bypass such unusual DNA

lesions, cells employ a range of so-called translesion synthesis (TLS) DNA polymerases that represent low fidelity DNA polymerases with a bigger substrate binding pocket. These polymerases can insert a nucleotide across the ICL mono-adduct and thus, bypass the lesion.

The recruitment of the TLS polymerases is dependent on both, the FA core complex and FANCD2 (Fu et al., 2013; Kim et al., 2012a). The specific polymerase(s) that carry out the insertion step of adding the nucleotide complementary to the bulky ICL adduct, are still unknown. The insertion step is followed by extension of this abnormal primer template by the Rev1 and Polζ polymerase complex followed by the removal of the remaining ICL mono-adduct by one of the cellular excision repair pathways in the cell. Once restored, this intact chromatid can now serve as a template for HR mediated repair of the two ended DNA DSB on the other sister chromatid (Haynes et al., 2015). The remaining ICL mono-adduct is predicted to be removed by one of the excision repair pathways in the cell.

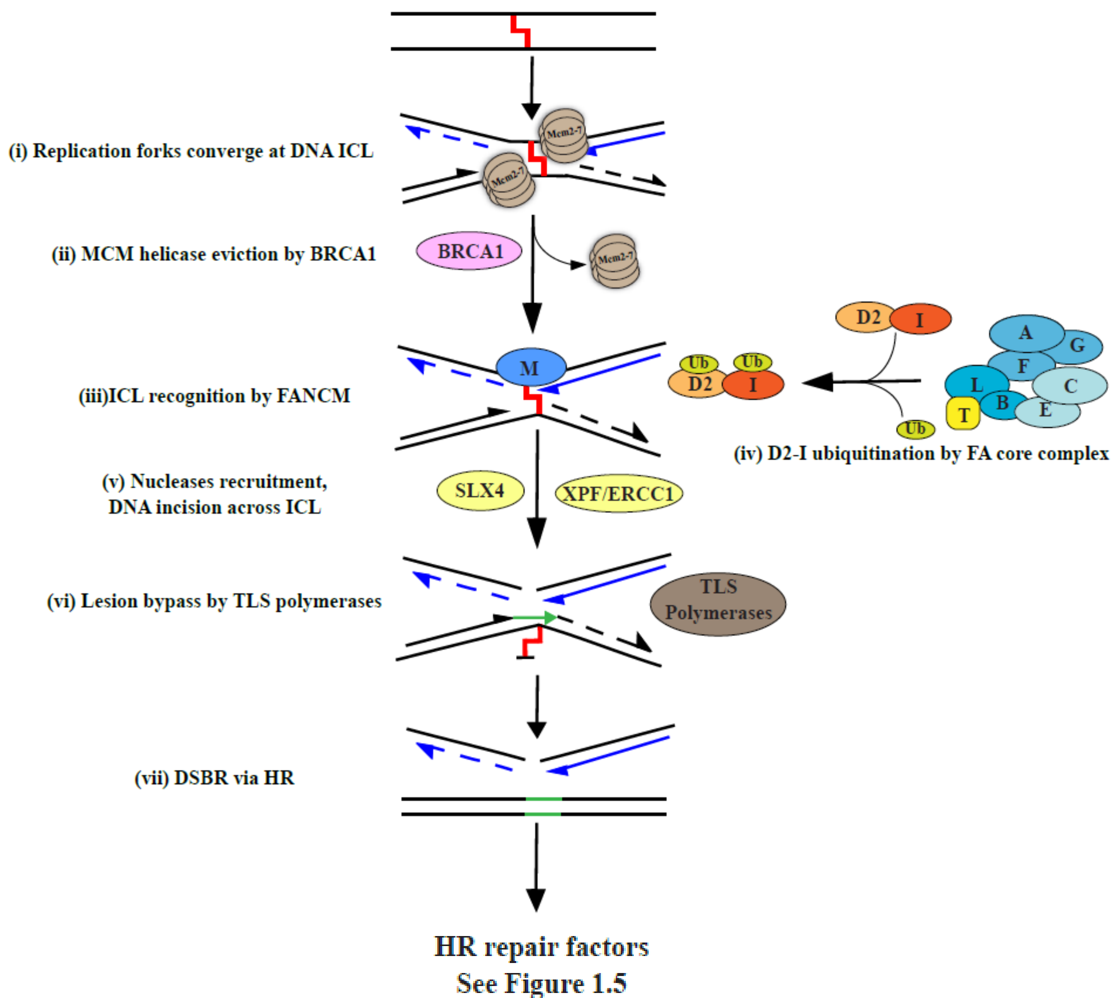


**Figure 1.3: Mechanism of ICL repair by the FA pathway-**

The 21 proteins that constitute the FA pathway work in a coherent manner to mediate ICL repair. (i) During replication, moving forks on either side of the ICL (indicated in red) converge at the ICL. (ii) The replicative helicase MCM 2-7 complex is evicted from the forks. (iii) FANCM recognizes the ICL and mediates the activation and recruitment of the FA core complex. (iv) The FA core complex mediates the chromatin recruitment and ubiquitination of FANCD2-FANCI complex. (v) Chromatin bound FANCD2 recruits the nucleases SLX4 and XPF and mediates incision across the ICL on one sister chromatid. (vi) The ICL is flipped out and TLS polymerases synthesize DNA and bypass the lesion on one chromatid. (vii) The resulting DSB in the other sister chromatid is now repaired via the HR pathway proteins

STEP V: Repair of the DNA DSB by the HR pathway

(Fig 1.3- Step (vi), Figure. 1.4):

The HR machinery that is responsible for repairing the DSB generated by the dual ICL incision step is composed of both FA and non FA pathway components. FANCD2 has been shown to participate at multiple steps within this process, as described below. BRCA1/FANCS is a key initiator of the HR machinery along with the nuclease complex MRN (MRE11/RAD50/NBS1). (Escribano-Díaz et al., 2013). BRCA1 and MRE11, along with FANCD2 recruit CtIP (CtBP-*i*nteracting protein). CtIP forms a complex with FANCD2 and MRE11 and promotes the resection of the 5' ends at the DSB to generate 3' single stranded DNA (ssDNA) overhangs (Buis et al., 2012; Escribano-Díaz et al., 2013; Yeo et al., 2014). At the resected DNA, BRCA1 and FANCD2 then recruit the FANCD1/BRCA2 (Breast Cancer Associated 2) and FANCN/PALB2 (Partner And Localizer of BRCA2) complex proteins. Subsequently, BRCA2 recruits FANCR/RAD51 and promotes the formation of RAD51-coated ssDNA nucleofilaments, with assistance from other RAD51 paralogues, including FANCO/RAD51 and XRCC2/FANCU (Buisson et al., 2010; Escribano-Díaz et al., 2013; Liu et al., 2004). The RAD51 nucleofilaments catalyze strand invasion of a homologous DNA template (the sister chromatid) and perform homology search in the form of a "D-loop" structure. The invading ssDNA is then extended by the DNA polymerases using the homologous sister chromatid DNA sequence as the template. Second end capture through DNA annealing or a second invasion event engages the second end of the DSBs, thus, resulting in a four-way junction called a Holliday Junction. The Holliday junction can then itself undergo 2

fates: 1) it can now be resolved by the nucleases- SLX1/MUS81/EME1 to result in crossover products or 2) it can be dissolved by the Bloom helicase (BLM) complex to result in a non- crossover product (Bachrati and Hickson, 2009; Kang, 2011; Sung and Klein, 2006). The BLM helicase complex is further supported by the FANCJ/BRIP1 (BRCA1-Interacting Protein 1) -5'-3' DNA helicase (Cantor and Xie, 2010; Litman et al., 2005; Suhasini and Brosh, 2012).

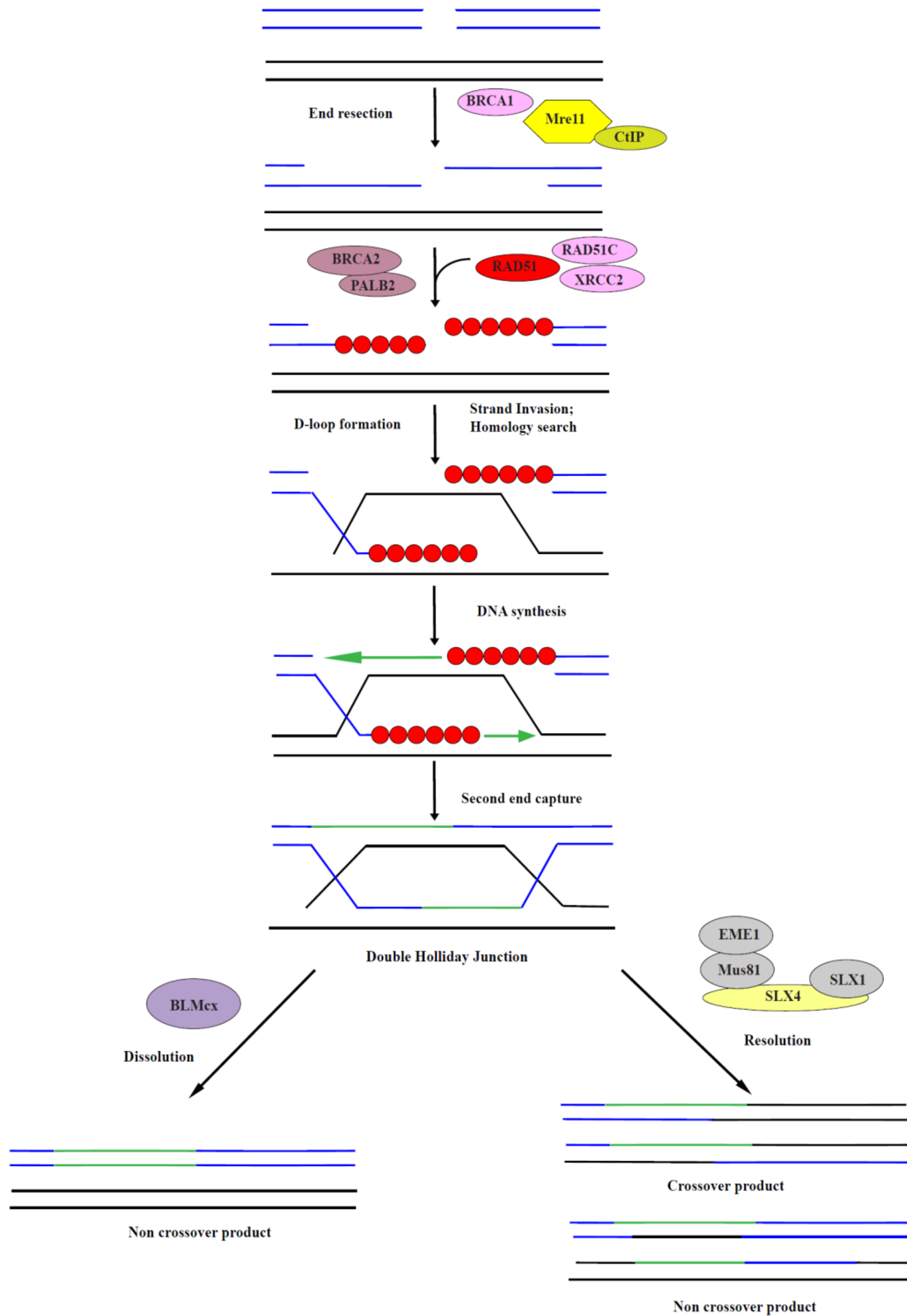


**Figure 1.4: Mechanism of HR repair pathway**

After a DSB occurs, BRCA1 and MRE11 mediate the recruitment of CtIP to the DSBs. The

Mre11-CtIP complex promotes resection of 5' ends of the DNA to generate 3' overhangs. BRCA2 and PALB2 mediate the recruitment of RAD51 to this 3' resected DNA in conjunction with the RAD51 paralogs including RAD51C and XRCC2. The RAD51 filament (filled circles) coated 3' strand invades the sister chromatid and conducts homology search. A displacement loop (D-loop) is formed during strand invasion between the invading 3' overhang strand and the homologous chromatid. Subsequently, DNA polymerase synthesizes the complimentary DNA sequence using the homologous chromatid template, converting the D-loop to a Holliday junction structure. The second end capture through DNA annealing or a second invasion event creates a double Holliday Junction (DHJ) structure. The DHJ can be dissolved by the "dissolvasome" Bloom helicase complex (BLMcx) to give non crossover product. Alternatively, the DHJ can be resolved by nucleases- (SLX4-SLX1-MUS81-EME1). Based on the orientation of the incisions, the DHJ can be resolved to give crossover or non- crossover products.

DNA ICL removal requires an additional novel FANCD2 function

Two recent studies unveiled an additional mechanism by which FANCD2 promotes DNA ICL repair. Sato et al. (2012, 2014) identified FANCD2 as a histone H3 chaperone. The authors showed that recombinant FANCD2 interacted directly with the histone H3/H4 tetramer and promoted complete nucleosome assembly *in vitro*. Furthermore, human FANCD2 mediated histone mobilization in living cells following DNA ICL induction. Strikingly, the authors showed that two FA-D2 patient-derived, histone H3 chaperone-inactive mutants, hFANCD2-R302W and hFANCD2-L231R, were unable to promote cellular resistance to DNA ICLs. These findings indicate that FANCD2's histone chaperone activity is crucial for ICL repair and hint that FANCD2 may functionally interconnect the cellular pathways for DNA repair and pathways that regulate nucleosome and chromatin structure. It is intriguing in this context that FANCD2 has been reported to interact with the chromatin modifier and histone

acetyltransferase TIP60 that is essential for the repair of DNA DSBs including those generated at DNA ICLs (Hejna et al., 2008; Jacquet et al., 2016). However, whether FANCD2 needs to interact with TIP60 or other chromatin remodelers to promote its cellular functions including DNA ICL repair, is not known.

### 1.5 Non-canonical roles of the FA pathway

The FA pathway has crucial, ICL repair-independent functions in S-phase

In recent years, we have gained insights into additional, non-canonical functions of FA pathway members during S-phase and the replication stress response. Importantly, $FANCD2^{Ub}$ formation and colocalization with other DNA repair factors such as BRCA2 and RAD51 was shown to occur during every normal S-phase of the cell cycle. (Taniguchi et al., 2002a). Moreover, Sobeck et al. previously reported that FANCD2 and FANCA accumulated on normally replicating chromatin in *Xenopus* S-phase egg extracts (Sobeck et al., 2006), suggesting that (at least some) FA pathway members function during DNA replication even in the absence of DNA ICLs. In fact, FANCD2 was shown to be required for the efficient initiation of DNA replication in primary human fibroblasts (Song et al., 2010).

Several additional functions have been found for FANCD2 and FANCI during the cellular replication stress response. FANCD2 and FANCI were shown to associate with HU-stalled replication forks, using the iPOND technology (isolation of proteins on nascent DNA) (Sirbu et al., 2013). In fact, FANCD2 was found to interact with the replicative helicase MCM2-7 complex (Lossaint et al., 2013) and the replicative polymerase clamp loader, **P**roliferating **C**ell **N**uclear **A**ntigen (PCNA) following

replication fork stalling, hinting at important roles for FANCD2 at stalled replication forks (Howlett et al., 2005).

Indeed, our laboratory and others recently employed the power of DNA fiber assay techniques to study replication events at a single molecule level to reveal novel functions for the FA pathway at replication forks stalled in the presence of the replication inhibitors hydroxyurea (HU) or aphidicolin (APH). It is now clear that FANCD2 has dual roles at these stalled replication forks. A) Fork protection: FANCD2 is required to protect nascent DNA strands at stalled replication forks from nucleolytic degradation by nucleases such as MRE11 or FAN1. FANCD2 shares these roles with the upstream FA core complex and several downstream FA proteins including FANCR/RAD51 and FANCD1/BRCA2 (Chaudhury et al., 2013, 2014, Schlacher et al., 2011, 2012; Ying et al., 2012). b) Fork restart: FANCD2 coordinates with BLM, CtIP and FAN1 to mediate restart of the stalled replication forks, while simultaneously suppressing the firing of new replication origins (Chaudhury et al., 2013, 2014, 2014; Davies et al., 2007; Yeo et al., 2014). Intriguingly, we observed that BLM, CtIP and FAN1 show a preferential interaction with the non-ubiquitinated FANCD2 isoform, hinting that – quite unexpectedly - the non-ubiquitinated FANCD2 isoform may have functions at stalled replication forks (Figure 1.5). Additionally, our studies showed that FANCD2 regulates the BLM complex recruitment to stalled forks independently of FANCI (Chaudhury et al., 2013), suggesting that FANCD2, and possibly other FA proteins, may have distinct and unique roles in the context of replication fork recovery

**Roles of FA pathway at stalled replication forks**

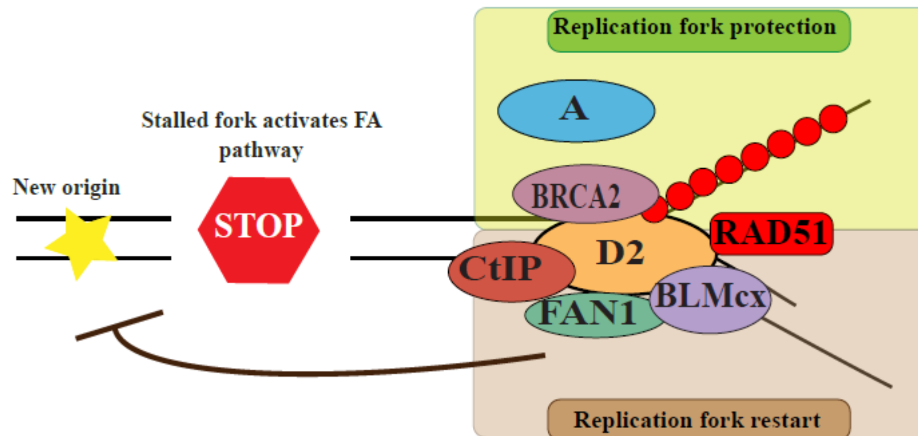


**Figure 1.5: Known roles of FA pathway proteins at stalled replication forks**
Following replication fork stalling, FANCD2 (D2) is recruited to the stalled fork. Here, FANCD2 fulfills two distinct functions: (a) it along with FANCA (A), recruits BRCA2 and stabilizes RAD51 (red circles-Rad51 nucleofilament) at the fork to prevent fork degradation and (b) it recruits the BLM complex (BLMcx), CtIP, RAD51 and FAN1 at the fork to promote fork restart and to suppress firing of new replication origins.

## 1.6 Rationale

The bone marrow failure and cancer proneness seen in FA patients has been attributed to the underlying defect in maintaining genome stability. Until recently, the FA pathway was thought to contribute to genome stability solely via its role in DNA ICL repair. However, emerging evidence from our laboratory and others strongly supports a model where FANCD2 has novel roles at HU- or APH-stalled replication forks that are independent of its functions during DNA ICL repair. Our new model raises two very important questions as described below.

**1) Does FANCD2 cooperate with other FA proteins to mediate replication fork restart and the suppression of new origin firing?**

As described above, we found that the interactions between FANCD2 and other replication fork restart proteins such as BLM, CtIP or FAN1 does not depend on FANCD2Ub formation.(Chaudhury et al., 2013, 2014; Yeo et al., 2014). This immediately raises the question of whether FANCD2's role during replication fork restart requires the upstream FA core complex whose primary function is to monoubiquitinate FANCD2. Moreover, it was previously proposed that downstream FA pathway members such as BRCA2 or RAD51 are recruited to chromatin exclusively by $FANCD2^{Ub}$ (Sato et al., 2016; Wang et al., 2004), indicating that these proteins should not be able to act in concert with the nonubiquitinated FANCD2 isoform. My work described in Chapter 2 attempted to answer these crucial questions, to dissect monoubiqitination-dependent and -independent roles of FANCD2 at HU- or APH-stalled replication forks.

**2) How does FANCD2 promote the restart of stalled replication forks?**

There are two different ways in which FANCD2 can be postulated to mediate replication fork restart:

*a) FANCD2 may serve as a docking platform for replication restart factors at stalled forks:*

FANCD2 appears to interact with and recruit several other replication restart factors including BLM, CtIP and FAN1 to stalled replication forks. It is plausible that FANCD2 may simply serve as a docking platform to recruit these factors to stalled replication forks, thereby promoting fork recovery.

*b) FANCD2 may contribute to replication fork restart via its H3 chaperone activity*

Two previous studies showed that FANCD2 possesses a histone H3 chaperone activity that is indispensable for ICL resistance. This raises the possibility that FANCD2's H3 chaperone activity is also crucial for its role in mediating replication restart and suggests that other known histone H3 chaperones may act in concert with FANCD2 to fulfill this role. My work described in Chapter 3 attempted to determine if FANCD2 functionally interacts with other histone H3 chaperone complexes to promote the restart of HU- or APH-stalled replication forks.

The overall objective of this dissertation is to discover previously unidentified roles of individual FA pathway members during the cellular replication stress response. Currently, all patients from the twenty-one different FA complementation groups undergo similar therapeutic regimens. Understanding the distinct cellular roles of individual FA proteins during S-phase is crucial towards the development of personalized treatment strategies for FA patients, which should be adjusted depending on the FA gene mutated and the type of the individual patient mutation.

# CHAPTER 2

## FANCD2, FANCJ and BRCA2 cooperate to promote replication fork recovery independently of the Fanconi Anemia core complex

(The work in this chapter was published in Raghunandan M., Chaudhury I., Kelich S.L., Hanenberg H., and Sobeck A. (2015). *Cell Cycle* 14, 342–353. PMID: 25659033)

Author contributions:

Experiments were designed by M.R. and A.S. and performed by M.R. I.C. performed some of the DNA fiber assays. S.L.K. and H.H. generated human immortalized FA patient cell lines and complemented FA patient cell lines. The data was analyzed and prepared for publication by M.R. and A.S.

**Synopsis**

Fanconi Anemia is an inherited multi-gene cancer predisposition syndrome that is characterized on the cellular level by a hypersensitivity to DNA ICLs. To repair these lesions, the FA pathway proteins are thought to act in a linear hierarchy: Following ICL detection, an upstream FA core complex monoubiquitinates the central FA pathway members FANCD2 and FANCI, followed by their recruitment to chromatin. Chromatin-bound monoubiquitinated FANCD2 and FANCI subsequently coordinate DNA repair factors including the downstream FA pathway members FANCJ and FANCD1/BRCA2 to repair the DNA ICL. Importantly, we recently showed that FANCD2 has additional independent roles: it binds chromatin and acts in concert with the BLM helicase complex to promote the restart of APH-stalled replication forks, while suppressing the firing of new replication origins.

Here, we show that FANCD2 fulfills these roles independently of the FA core complex-mediated monoubiquitination step. Following APH treatment, nonubiquitinated FANCD2 accumulates on chromatin, recruits the BLM complex, and promotes robust replication fork recovery regardless of the absence or presence of a functional FA core complex. In contrast, the downstream FA pathway members FANCJ and BRCA2 share FANCD2's role in replication fork restart and the suppression of new origin firing. Our results support a non-linear FA pathway model at stalled replication forks, where the nonubiquitinated FANCD2 isoform – in concert with FANCJ and BRCA2 – fulfills a specific function in promoting efficient replication fork recovery independently of the FA core complex.

## 2.1 Introduction

Fanconi Anemia (FA) is a recessively inherited genomic instability syndrome caused by mutation in any one of twenty one known FA genes (Kee and D'Andrea, 2012; Kupfer, 2013). Clinically, FA patients exhibit congenital abnormalities, progressive bone marrow failure and a strong predisposition to cancers such as acute myeloid leukemia and squamous cell carcinomas. Cells from FA patients are hypersensitive to DNA ICLs and show spontaneous chromosomal aberrations that are further exacerbated upon treatment with replication-inhibiting agents such as HU or APH (Kee and D'Andrea, 2010; Naim and Rosselli, 2009; Schlacher et al., 2012). To promote the repair of DNA ICLs, the twenty one FA pathway members are thought to function in a linear hierarchy that can be broadly divided into three tiers: an upstream FA core complex, a central FANCD2-FANCI protein heterodimer and several downstream FA pathway members including breast cancer-associated proteins FANCD1/BRCA2 (breast cancer associated 2), FANCN/PALB2 (partner and localizer of BRCA2) and FANCJ/BRIP1 (BRCA1-interacting protein 1) (Kee and D'Andrea, 2012; Wang, 2007). DNA ICLs are mostly repaired in S-phase when they block the progression of replication forks (Clauson et al., 2013; Knipscheer et al., 2009; Räschle et al., 2008). Detection of an ICL triggers the recruitment of the FA core complex [FANCA, -B, -C, -E, -F, -G, -L]. The FA core complex has now been identified to be composed of three modules – two ancillary modules (FANC- A, G and FANC-C, E, F) with non-redundant functions, and a catalytically active module (FANC- B, L). Once recruited, the coordinated actions of all three modules of the FA core complex – with FANCL as the catalytic subunit – enables

its function as a multi-subunit E3 ubiquitin ligase that monoubiquitinates both members of the central FANCD2-FANCI heterodimer. Monoubiquitinated FANCD2 and FANCI are then recruited to chromatin and into DNA repair foci at DNA ICLs (Garcia-Higuera et al., 2001; Meetei et al., 2003; Smogorzewska et al., 2007; Timmers et al., 2001). Subsequently, chromatin-bound FANCD2 and FANCI are thought to coordinate downstream FA nucleases like SLX4/FANCP and XPF(FANCQ)/ERCC1 with FANCJ, BRCA2 and FANCN to mediate incisions at the DNA ICL followed by HR repair of the newly generated DNA DSB (Crossan et al., 2011; Kim et al., 2008b; Klein Douwel et al., 2014; Stoepker et al., 2011; Walden and Deans, 2014; Yamamoto et al., 2011).

Recent studies from our laboratory and others revealed novel FA pathway functions at replication forks that are stalled in the presence of HU or APH. The FA core complex, FANCD2 and BRCA2 function to protect nascent DNA strands at stalled replication forks from nucleolytic degradation (Chaudhury et al., 2013; Schlacher et al., 2011, 2012; Ying et al., 2012). Importantly, FANCD2 also regulates the BLM helicase pathway to mediate restart of the stalled replication forks, while simultaneously suppressing the firing of new replication origins (Chaudhury et al., 2013; Yeo et al., 2014). Intriguingly, our previous findings also showed that FANCD2 can recruit and assemble BLM pathway members such as BLM and Topoisomerase III$\alpha$ (Top3A) on chromatin independently of FANCI, indicating a separation of function between these two central FA pathway members (Chaudhury et al., 2013). Consequently, these results raised the question of whether FANCD2 fulfills its new role in replication fork recovery

in concert with – or independently of – the upstream FA core complex and the downstream FA pathway members.

In the current study, we show that – unlike FANCD2 – the FA core complex members FANCA, FANCC, FANCG and FANCL are dispensable for the restart of APH-stalled replication forks and the suppression of new origin firing. FA core complex-deficient cells recruit nonubiquitinated FANCD2 to chromatin after APH treatment, followed by FANCD2-dependent assembly of fork restart factors and robust replication fork recovery. In contrast, cells lacking downstream FA pathway members FANCJ or BRCA2 fail to restart APH-stalled replication forks, and show a significant increase in new replication origin firing. Our results strongly support a novel, non-linear FA pathway model where nonubiquitinated FANCD2, FANCJ and BRCA2 act cooperatively, but completely independently of the FA core complex to coordinate molecular actions of the replication fork restart machinery.

## 2.2 Results

### *The FA core complex is dispensable for the restart of APH-stalled replication forks*

Using a dual labeling DNA fiber assay, we recently showed that FANCD2 promotes the restart of APH-stalled replication forks, while simultaneously suppressing new origin firing (Chaudhury et al., 2013; Yeo et al., 2014). In this assay, replication tracts are first labeled with DigUTP for 25 min, treated without or with 30 μM APH for 6 hours to cause replication fork arrest, followed by second labeling with BioUTP for 40 min (Fig. 2.1A). Reproducibly, FANCD2-deficient patient cells show an approximately

2.5-fold reduction in replication fork restart efficiency (Fig. 2.1B), accompanied by an approximately 4.5-fold increase in new origin firing events (Fig. 2.1B).

**Figure 2.1**

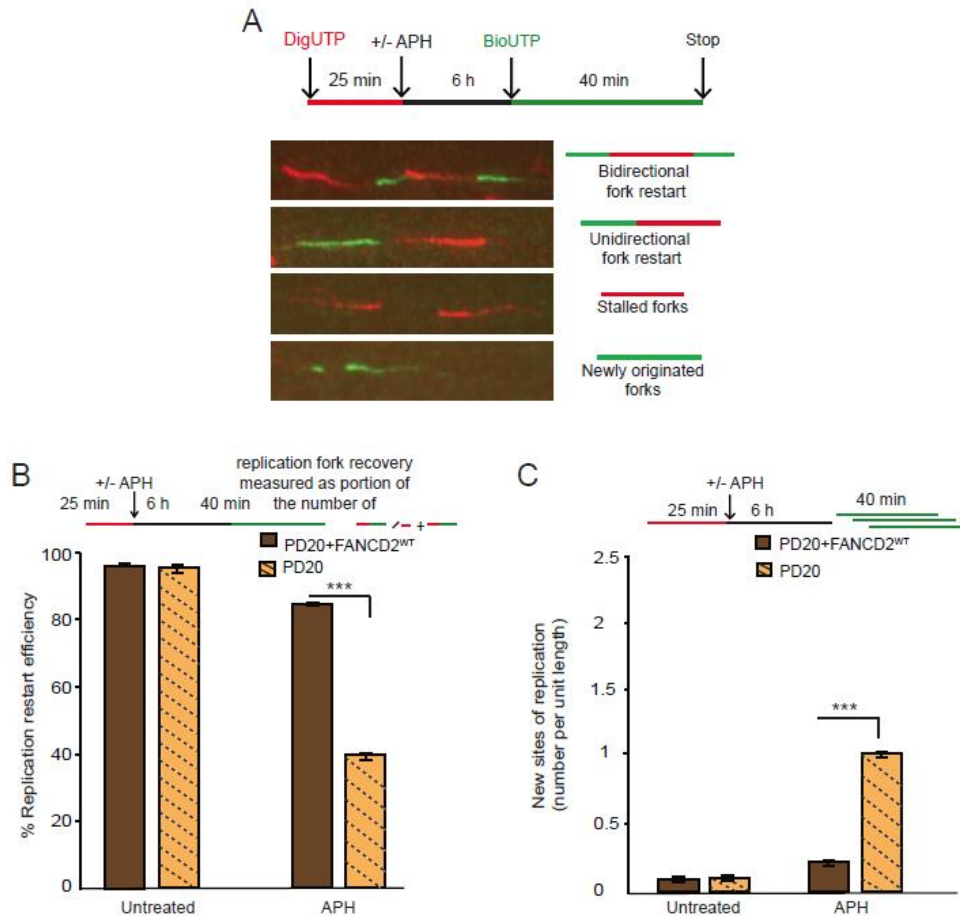


**Figure 2.1: FANCD2 is essential to mediate replication fork restart**

(A) Representative images of DNA fibers accompanied with a schematic of defining sites of replication. (B) Replication fork restart efficiencies were compared between FANCD2-proficient (PD20+FANCD2WT) and -deficient (PD20) cells. Replication restart efficiency was measured as the number of restarted replication forks after APH-mediated fork stalling (DigU-BioU tracts), compared with the total number of DigU-labeled tracts (DigU + DigU-BioU). (C) The number of new sites of replication originating during the 40 min recovery period after APH treatment was compared between PD20+FANCD2WT and PD20 cells. New origins of replication were measured as the number of green-only (BioU) tracts per unit length.

To investigate if these mechanisms depend on the FA core complex that is thought to act upstream of FANCD2, we considered that this complex is composed of three subcomplexes: two ancillary modules FANCA-FANCG and FANCC-FANCE-FANCF, and the actual catalytic module FANCB-FANCL (Fig. 2.2A) (Hodson and Walden, 2012; Huang et al., 2014; Medhurst et al., 2006; Rajendra et al., 2014). We chose FA patient-derived cell lines deficient for FANCA, FANCC and FANCL, each lacking one of the three FA core modules, and their isogenic counterparts corrected with the respective wild type gene (PD220 and PD220+A, PD331 and PD331+C, 913/1T and 913/1T+L). We observed robust APH-induced FANCD2 monoubiquitination in the PD220+A, PD331+C and 913/1T+L cells, confirming efficient gene correction (Fig. 2.2B). Using the same DNA fiber assay described in Fig. 2.1A, we first analyzed FANCA- and FANCC-deficient cells for fork restart after APH treatment. We found that the proportion of replication forks competent for restart was essentially unaffected in cells lacking FANCA or FANCC compared to their corrected counterparts (Fig. 2.2C, i and ii); moreover, FANCA- or FANCC-deficient cells did not show a significant increase in new origin firing (Fig. 2.2D, i and ii).

Importantly, recent studies revealed that cells lacking either one of the ancillary FA core modules still contain very low amounts of $FANCD2^{Ub}$, whereas cells deficient for the catalytic core module do not (Huang et al., 2014; Rajendra et al., 2014). Thus, we next tested if FANCL-deficient cells are capable of replication fork recovery after APH treatment. Strikingly, the FA patient-derived FANCL-deficient cells exhibited normal replication fork restart efficiencies and robust suppression of new origin firing,

comparable to the corrected cells (Fig. 2.2C, iii; Fig. 2.2D, iii). Moreover, a genetically engineered human FANCL knockout cell line (HCT116/FANCL$^{-/-}$) showed no defects in replication fork restart and no increase in new origin firing after APH treatment compared to wild type HCT116 cells (Fig. 2.3 A-C).

**Figure 2.2**

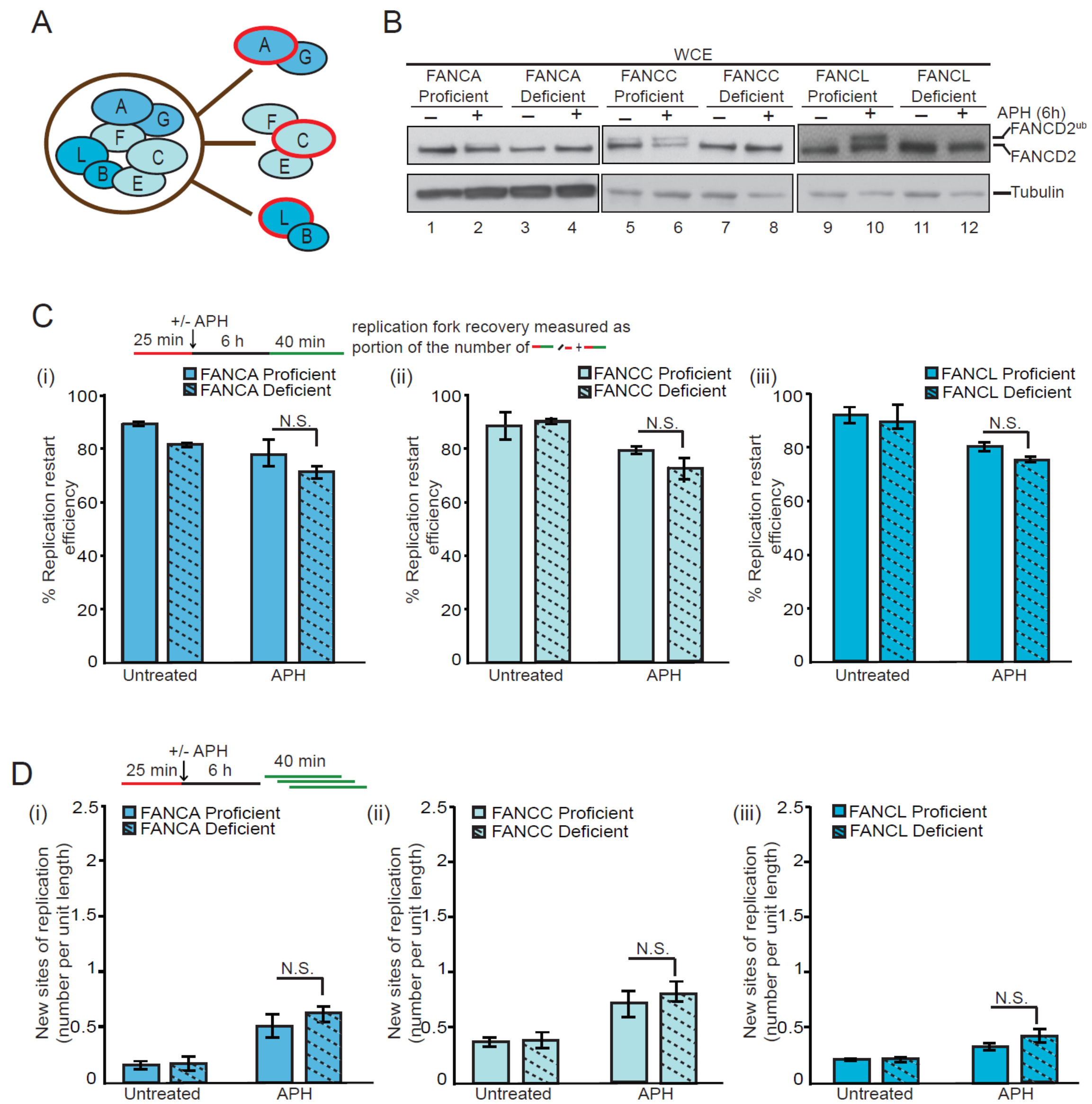

**Figure 2.2: The three FA core sub-complexes are dispensable for the restart of APH-stalled replication forks.**

(**A**) Schematic representation of the three FA core sub-complexes: *FANCA-FANCG*, *FANCC-FANCE-FANCF*, and *FANCB-FANCL*. FA patient-derived cell lines lacking FANCA, FANCC, or FANCLwere used to represent cells deficient for the respective FA core sub complex. (**B**) WCEs from untreated or APH-treated, isogenic cell pairs that were either proficient or deficient for FANCA (lanes 1-4), FANCC (lanes 5-8) or FANCL (lanes 9-12) were analyzed for the presence of FANCD2 and FANCD2$^{Ub}$. Tubulin, loading control. (**C**) Replication fork restart efficiencies after APH treatment was compared between isogenic cell pairs proficient or deficient for **(i)** FANCA, **(ii)** FANCC or **(iii)** FANCL. (**D**) The number of new replication sites originating during BioU labeling after APH treatment was compared between isogenic cell pairs proficient or deficient for **(i)** FANCA, **(ii)** FANCC or **(iii)** FANCL.

**Figure 2.3**

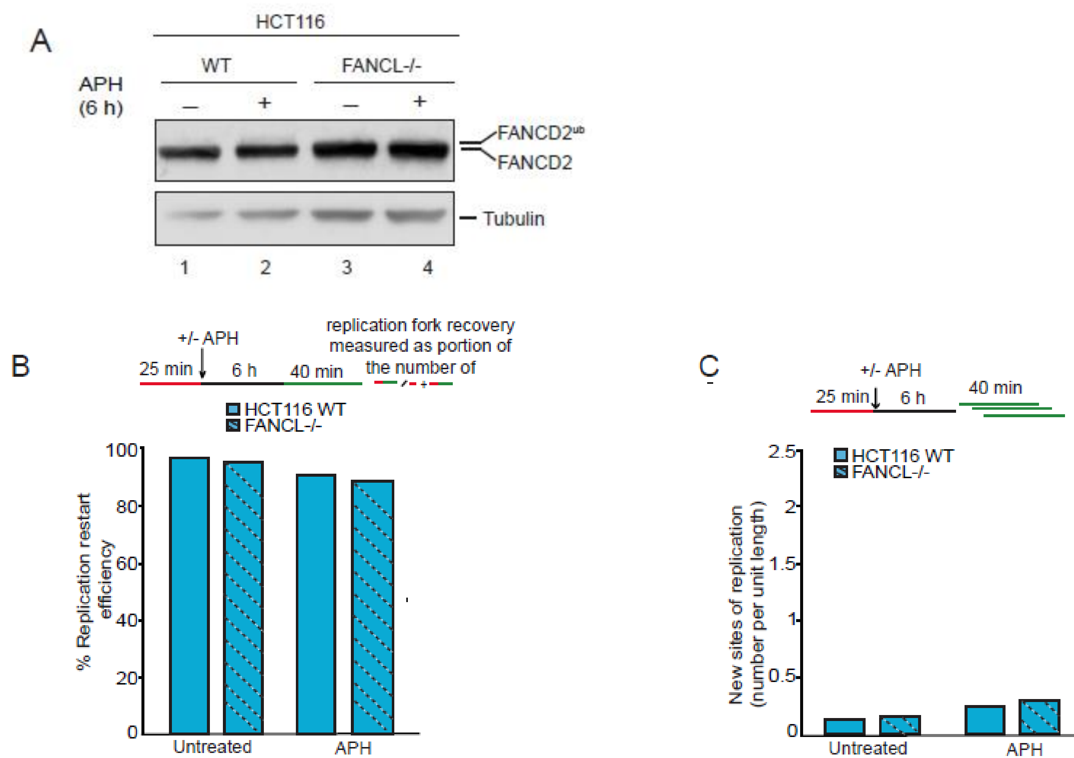

**Figure 2.3: FANCL knockout cells are competent for replication fork recovery**
(**A**) APH triggers weak FANCD2 monoubiquitination in HCT116 wild type cells. WCEs from untreated or APH-treated HCT116 WT or HCT116 FANCL-/- cells were analyzed for the presence of FANCD2 and FANCD2Ub. Tubulin, loading control. (**B, C**) FANCL is dispensable for replication fork restart and suppression of new origin firing. (**B**) Replication fork restart efficiencies after APH treatment were compared between wild type and FANCL-/- cells. (**C**) The number of new replication sites originating during Bio-U labeling after APH treatment was compared between wild type and FANCL-/- cells.

Lastly, we argued that FANCG may be required for replication fork recovery, since FANCG can act independently of other FA core complex members to regulate formation of a separate protein complex (BRCA2-FANCD2-FANCG-XRCC3) (Fig. 2.4A) (Hussain et al., 2006; Wilson et al., 2008, 2010). However, replication fork restart efficiencies and suppression of new origin firing were normal in patient-derived FANCG cells compared to wild type cells (Fig. 2.4 B-D), ruling out that FANCG or FANCG-dependent protein complexes are crucial for these mechanisms. Taken together, our results indicate that the three known FA core complex modules – and thus likely the entire FA core complex - are dispensable for the restart of APH-stalled replication forks and the simultaneous suppression of new origin firing.

## Figure 2.4

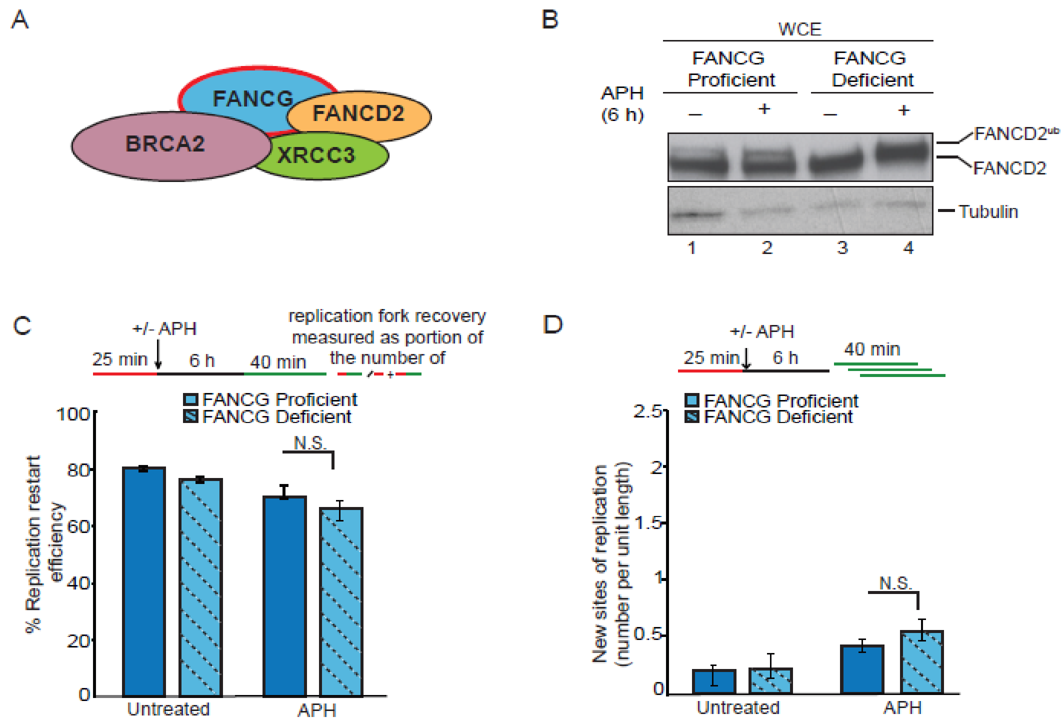


**Figure 2.4: The BRCA2-FANCD2-FANCG-XRCC3 DNA repair complex is dispensable for the recovery of APH-stalled forks.**

(**A**) Schematic representation of the BRCA2-FANCD2-FANCG-XRCC3 complex. (B) APH triggers efficient FANCD2 monoubiquitination only in FANCG proficient cells. WCEs from untreated or APH-treated wild type or FANCG-deficient cells were analyzed for presence of FANCD2 and FANCD2Ub. Tubulin, loading control. (**C, D**) FANCG is dispensable for replication fork restart and suppression of new origin firing. (**C**) Replication fork restart efficiency after APH treatment was compared between wild type (GM000637) and FANCG-deficient (PD352i) cells. (**D**) The number of new replication sites originating during the Bio-U labeling after APH treatment was compared between wild type and FANCG -deficient cells.

***The nonubiquitinated FANCD2 isoform is crucial for replication fork restart in FA core complex-deficient cells***

Our finding that the FA core complex is dispensable for the restart of APH-stalled replication forks immediately suggested that the FA core complex-dependent $FANCD2^{Ub}$ formation is dispensable for FANCD2's role during fork recovery. In that case, the nonubiquitinated FANCD2 isoform would be responsible for the successful replication fork restart observed in FA core complex-deficient cells. To test this, we treated PD331+C or PD331 cells with control siRNA or FANCD2 siRNA to generate wild type, FANCC-, FANCD2-, or FANCC/FANCD2 double-deficient cells, followed by dual-label DNA fiber analysis (Fig. 2.5A). As expected, wild type and FANCC-deficient cells exhibited normal replication fork restart efficiencies (Fig. 2.5B, wild type: 78.4%; FANCC-deficient: 74.4%) and could suppress new origin firing events (Fig. 2.5C) in response to APH.

In striking contrast, FANCD2-deficient and FANCC/FANCD2 double-deficient cells showed a significant and comparable decrease in fork restart efficiencies (Fig. 2.5BB, FANCD2-deficient: 37.8%; FANCC/FANCD2 double-deficient: 39.3%) and a significant, comparable increase in new origin firing (Fig. 2.5C, ~3.5-fold increase in FANCD2- and FANCC/FANCD2 double-deficient cells). Thus, depletion of FANCD2 renders cells deficient for replication fork restart and suppression of new origin firing, irrespective of the absence or presence of a functional FA core complex.

## Figure 2.5

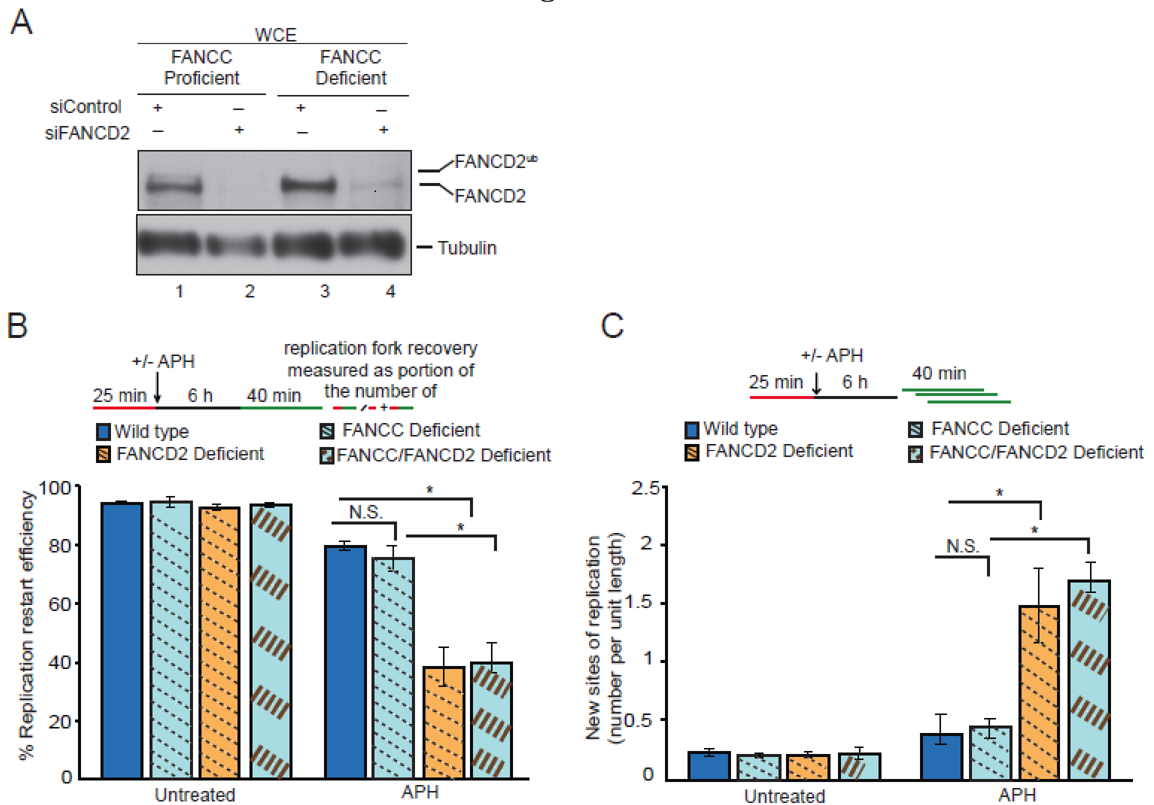


**Figure 2.5: The nonubiquitinated FANCD2 isoform promotes the recovery of APH-stalled forks in FA core complex-deficient cells**

(**A**) WCEs showing the efficiency of siRNA-mediated FANCD2 knockdown in FANCC-proficient or -deficient cells. Generated cell types: Wild type (PD331+C, siControl), FANCC-deficient (PD331, siControl), FANCD2-deficient (PD331+C, siFANCD2) and FANCC/FANCD2 double-deficient (PD331, siFANCD2). Tubulin, loading control. (**B**) Replication fork restart efficiencies after APH treatment were compared between the four cell types described in (A). (**C**) The number of new replication sites originating during BioU labeling after APH treatment was compared between the four cell types described in (A).

***Nonubiquitinated FANCD2 is recruited to chromatin in an APH-responsive manner***

Our results above indicated that cells utilize selectively the nonubiquitinated FANCD2 isoform to promote the restart of APH-stalled replication forks. Thus, one would expect the nonubiquitinated FANCD2 isoform to accumulate on chromatin in response to APH. To test this, we isolated chromatin fractions from FANCC-proficient (PD331+C) or -deficient (PD331) cells that had been untreated or treated with APH for different lengths of time (2 h, 6 h or 24 h). As expected, FANCC-proficient cells contained chromatin-bound FANCD2 even in absence of APH, and showed a continuous increase in FANCD2 chromatin binding following APH treatment (Fig. 2.6 A-B) (Sobeck et al., 2006; Taniguchi et al., 2002a). In agreement with previous studies, the chromatin fractions from FANCC-proficient cells contained predominantly the FANCD2$^{Ub}$ isoform (Montes de Oca et al., 2005); however a small amount of nonubiquitinated FANCD2 was detectable as well (Fig. 2.6A). Importantly, similar amounts of nonubiquitinated FANCD2 were chromatin-bound in FANCC-deficient cells; moreover the nonubiquitinated FANCD2 isoform accumulated further on chromatin in response to APH treatment (Fig. 2.6B). In addition, we found that Top3A, a member of the BLM complex that binds chromatin and promotes replication fork restart in a FANCD2-dependent manner, exhibited comparable chromatin binding behavior in FANCC-proficient and -deficient cells (Fig. 2.6A). These results demonstrate that nonubiquitinated FANCD2 binds chromatin in an APH-responsive manner and recruits BLM complex members independently of the FA core complex.

**Figure 2.6**

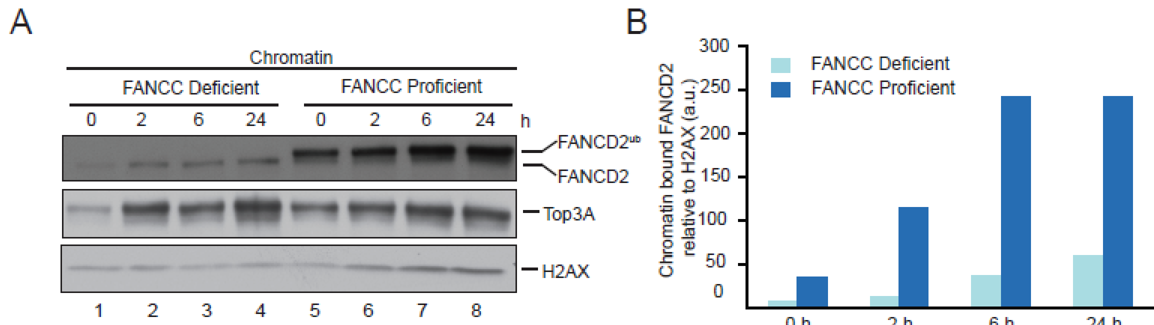


**Figure 2.6: Nonubiquitinated FANCD2 is recruited to chromatin in an APH-responsive manner**
(**A**) FANCC-deficient or -proficient cells were either untreated or treated with APH for the indicated time points. Chromatin fractions isolated from these cells were analyzed for the presence of FANCD2, FANCD2$^{Ub}$ and Top3A. H2AX, loading control. (**B**) Immunoblot signals for FANCD2 shown the in left panel were analyzed by densitometry and normalized against H2AX signals using ImageJ.

***A monoubiquitination-dead FANCD2$^{K561R}$ mutant binds chromatin and promotes replication fork restart***

To further test the role of FANCD2$^{Ub}$ formation during replication fork restart, we analyzed FANCD2-deficient patient cells (PD20) that were complemented with either (i) empty vector, (ii) wild type FANCD2 (PD20+FANCD2$^{WT}$) or (iii) a monoubiquitination-dead FANCD2 mutant (PD20+FANCD2$^{K561R}$) (Fig. 2.7A). Interestingly, we found that total protein levels of FANCD2$^{K561R}$ were significantly higher than those of FANCD2$^{WT}$ in PD20 cells (Fig. 2.7A, compare lanes 2 and 3, or 5 and 6). Concomitantly, both FANCD2$^{WT}$ and FANCD2$^{K561R}$ were present in chromatin fractions isolated from unperturbed or APH-treated cells, indicating that the FANCD2$^{K561R}$ mutant has residual chromatin binding ability (Fig. 2.7B). In agreement with this, the DNA fiber analysis revealed that the replication fork restart defect in FANCD2-deficient cells (Fig. 2.7C,

PD20: 39.3%) was significantly relieved by expressing FANCD2$^{WT}$ (Fig. 2.7C, PD20+FANCD2$^{WT}$: 84.5%) or the FANCD2$^{K561R}$ mutant (Fig. 2.7C, PD20+FANCD2$^{K561R}$: 74.8%). Simultaneously, the significant increase in new origin firing observed in PD20 cells (Fig. 2.7D; ~4.5-fold increase compared to PD20+FANCD2$^{WT}$) was partially suppressed in PD20+FANCD2$^{K561R}$ cells (Fig. 2.7D; ~2-fold increase compared to PD20+FANCD2$^{WT}$). Taken together, these results indicate that the nonubiquitinated FANCD2 isoform plays a crucial role at sites of APH-stalled replication forks to promote fork restart and suppression of new origin firing.

**Figure 2.7**

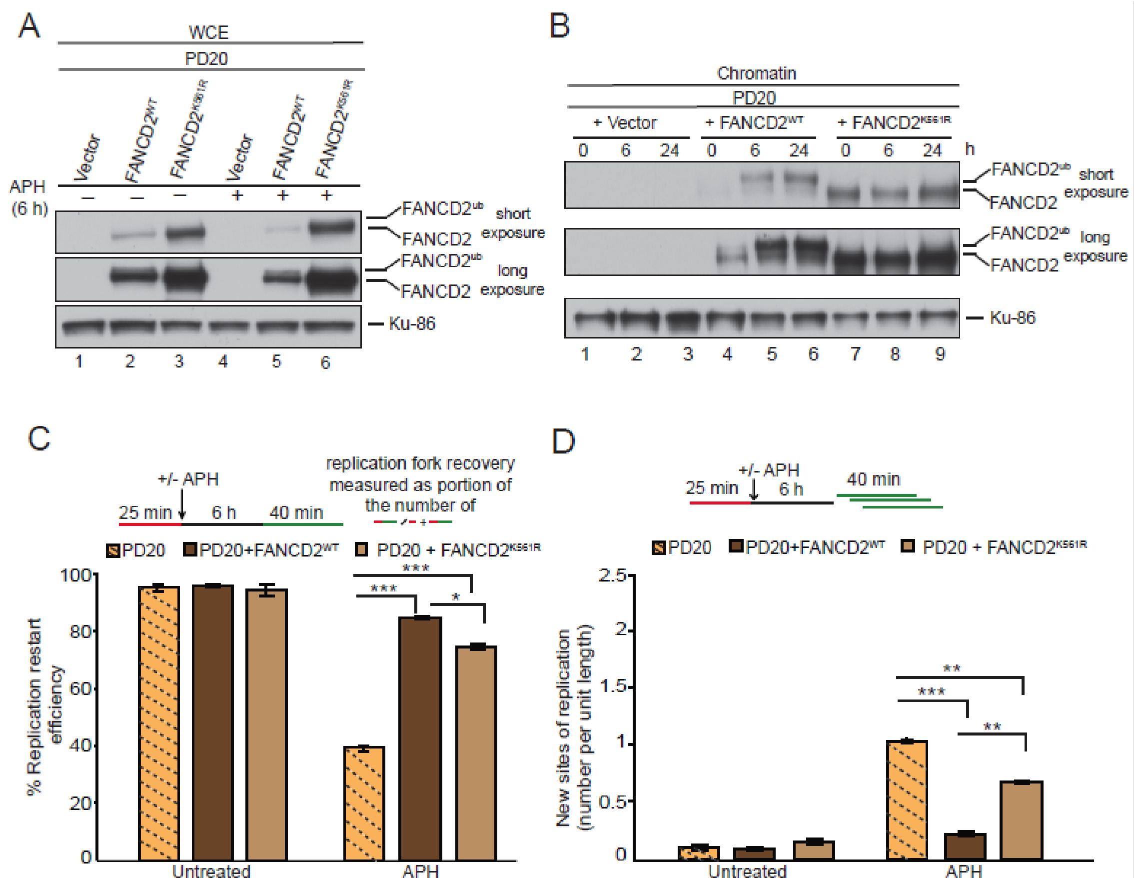

**Figure 2.7: FANCD2$^{K561R}$ binds chromatin and promotes restart of APH-stalled replication forks.**

(A) FANCD2-deficient cells (PD20) complemented with either empty vector, FANCD2$^{WT}$ or FANCD2$^{K561R}$ were either untreated or treated with APH, and analyzed for FANCD2 and FANCD2Ub. Ku-86, loading control. (B) The three cell types described in (A) were either untreated or treated with APH for 6h or 24h, and chromatin fractions from the cells were analyzed for the presence of FANCD2 and FANCD2Ub. Ku-86, loading control. (C) Replication fork restart efficiencies after APH treatment were compared between the three cell types described in (A). (D) The number of new replication sites originating during BioU labeling after APH treatment was compared between the three cell types described in (A).

***The "downstream" FA pathway members FANCJ and BRCA2 cooperate with FANCD2 to promote replication fork restart***

BRCA1 protein is a critical DNA repair factor that is responsible for the recruitment of FANCD2, as well as downstream FA pathway members FANCJ and BRCA2, to sites of DNA damage (Garcia-Higuera et al., 2001; Greenberg et al., 2006; Wang, 2007). Importantly, we recently showed that BRCA1 also functions upstream of FANCD2 to promote the restart of APH-stalled replication forks (Yeo et al., 2014). Thus, we asked if the other BRCA1-dependent DNA repair factors, FANCJ and BRCA2, are involved in FANCD2-dependent replication fork restart as well. We chose a FANCJ-deficient patient cell line (752/1T), and its corrected wild type counterpart (752/1T+J). Furthermore, we treated 752/1T+J and 752/1T cells with control siRNA or FANCD2 siRNA to generate wild type, FANCD2-, FANCJ-, or FANCD2/FANCJ double-deficient cells, followed by dual-label DNA fiber analysis. As expected, APH treatment induced FANCD2$^{Ub}$ formation normally in FANCJ- deficient cells (Fig. 2.8A) (Levitus et al.,

2005; Litman et al., 2005). Following APH treatment, the proportion of replication forks competent for restart was severely – and equally – reduced in FANCD2- and FANCJ-deficient cells (Fig. 2.8B, 40.3% and 39.5%). Moreover, this defect in fork restart was not further exacerbated in FANCD2/FANCJ double-depleted cells (Fig. 2.8B, 38.8%). In parallel, the proportion of newly originated replication tracts increased significantly and equally (Fig. 2.8C, ~5-fold) in FANCD2-, FANCJ- and FANCD2/FANCJ double-deficient cells compared with wild-type cells. Thus, FANCD2 and FANCJ cooperate to promote the recovery of APH-stalled replication forks.

**Figure 2.8**

A

WCE

FANCJ Proficient | FANCJ Deficient

si FANCD2
APH (6 h)

FANCJ
FANCD2ub
FANCD2
Tubulin

1 2 3 4 5 6 7 8

B

+/- APH
25 min 6 h 40 min
replication fork recovery measured as portion of the number of

Wild type
FANCD2 Deficient
FANCJ Deficient
FANCD2/FANCJ Deficient

% Replication restart efficiency
0 20 40 60 80 100
*** *** N.S.
Untreated APH

C

+/- APH
25 min 6 h 40 min

Wild type
FANCD2 Deficient
FANCJ Deficient
FANCD2/FANCJ Deficient

New sites of replication (number per unit length)
0 0.5 1 1.5 2 2.5
** ** N.S.
Untreated APH

.

**Figure 2.8: Downstream FA pathway protein FANCJ cooperates with FANCD2 for the recovery of APH-stalled replication forks.**
(**A**) WCEs showing the efficiency of siRNA-mediated FANCD2 knockdown in FANCJ-proficient or -deficient cells. Generated cell types: Wild type (752/1T+J, siControl), FANCD2-deficient (752/1T+J, siFANCD2), FANCJ-deficient (752/1T, siControl) and FANCD2/FANCJ double- deficient (752/1T, siFANCD2). All four cell lines were untreated or treated with APH, and WCEs from these cells were analyzed for the presence of FANCJ, FANCD2 and FANCD2Ub. Tubulin, loading control. (**B**) Replication fork restart efficiencies after APH treatment were compared between wild type, FANCD2-, FANCJ- and FANCD2/FANCJ double-deficient cells (**C**) The number of new replication sites originating during BioU labeling after APH treatment was compared between Wildtype, FANCD2-, FANCJ- and FANCD2/FANCJ double-deficient cells

Next, we sought to determine whether BRCA2 promotes replication fork recovery, possibly in concert with FANCD2. To this end, we treated a wild type cell line (PD331+C) with siControl, siFANCD2, siBRCA2 or siFANCD2/BRCA2 to generate wild type, FANCD2-, BRCA2-, or FANCD2/BRCA2 double-deficient cells (Fig. 2.9A). As expected, BRCA2-deficient cells were able to support $FANCD2^{Ub}$ formation in response to APH treatment.[37] DNA fiber analysis revealed that FANCD2- and BRCA2-deficient cells exhibited strongly – and equally – reduced replication fork restart efficiencies (Fig. 2.9B, 40.4% and 39.9% that were not further reduced in the FANCD2/BRCA2 double-deficient cells (Fig. 2.9B, 42.1%). Simultaneously, we observed that FANCD2-, BRCA2- and FANCD2/BRCA2 double-deficient cells showed a significant and similar increase in newly fired replication origins (Fig. 2.9C, ~3-4 fold) compared with the wild type cells.

## Figure 2.9

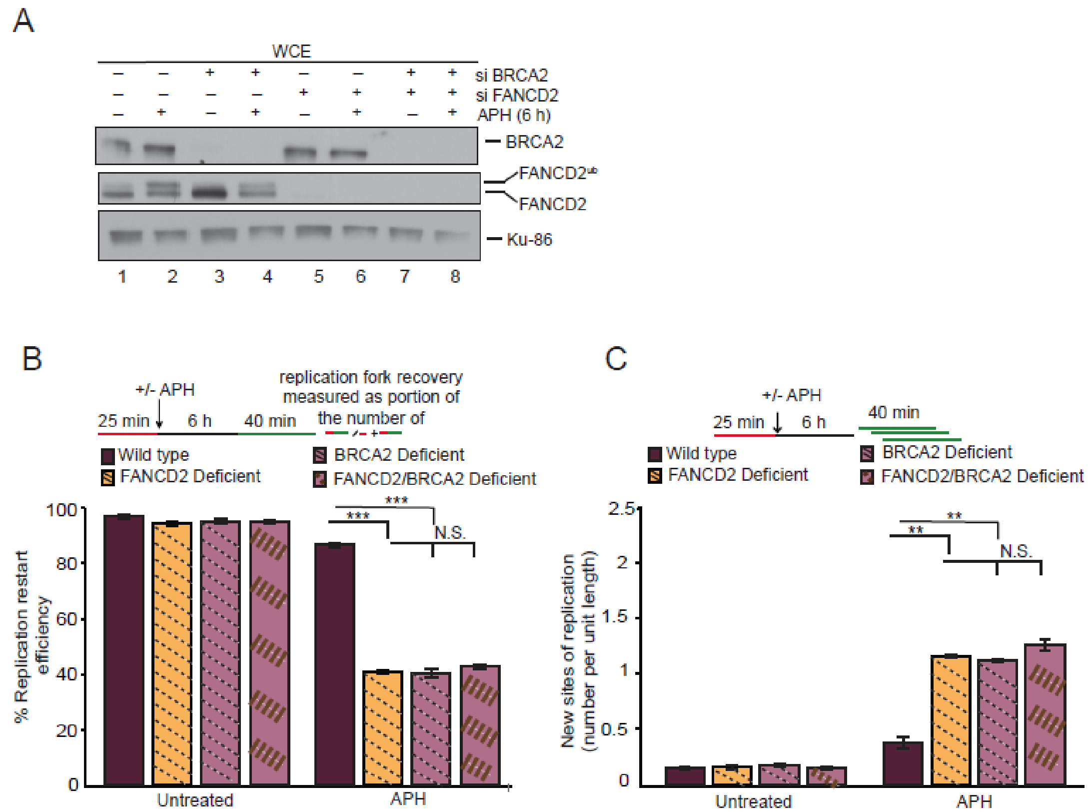


**Figure 2.9: Downstream FA pathway protein BRCA2 cooperates with FANCD2 for the recovery of APH-stalled replication forks.**

(**A**) WCEs showing the efficiency of siRNA-mediated FANCD2 and BRCA2 knockdown in Wildtype (PD331+C) cells. Generated cell types: Wild type (PD331+C, siControl), FANCD2-deficient (PD331+C, siFANCD2), BRCA2-deficient (PD331+C, siBRCA2) and FANCD2/BRCA2 double-deficient (PD331+C, siFANCD2/siBRCA2). All four cell lines were untreated or treated with APH, and WCEs from these cells were analyzed for the presence of BRCA2, FANCD2 and FANCD2Ub. Ku-86: loading control. (**B**) Replication fork restart efficiencies after APH treatment were compared between wild type, FANCD2-, BRCA2- and FANCD2/BRCA2 double- deficient cells. (**C**) The number of new replication sites originating during BioU labeling after APH treatment was compared between wild type, FANCD2-, BRCA2- and FANCD2/BRCA2 double-deficient cells.

Our finding that BRCA2 promotes replication fork restart seemingly contradicts two previous studies that used cell lines expressing BRCA2 mutants with C-terminal truncations to test the roles of BRCA2 at hydroxyurea (HU)-stalled replication forks. Both studies reported that BRCA2 was crucial to protect HU-stalled replication forks against nuclease-mediated DNA strand degradation, *but was not required* to mediate replication fork restart (Schlacher et al., 2011; Ying et al., 2012). To address this discrepancy, we first sought to exclude the possibility that our findings were due to off-target effects of the employed BRCA2 siRNA. We repeated the DNA fiber assay in cells treated with a second BRCA2 siRNA (siBRCA2$^{\#2}$) that targets a different *BRCA2* exon (Fig. 2.10A). Following APH-treatment, we observed strongly reduced replication fork restart efficiencies (Fig. 2.10B, 37.5%) accompanied by a significant increase in newly fired origins (Fig. 2.10C, 3-4-fold) in the siBRCA2$^{\#2}$-treated cells, supporting our initial results (Fig. 2.9 A-C) that BRCA2 promotes replication fork restart and the suppression of new origin firing. Simultaneously, we found that the siBRCA2$^{\#2}$-treated cells exhibited a severe shortening of nascent DNA strands at APH-stalled replication forks compared to the wild type cells (Fig. 2.10D), confirming the previous findings that BRCA2 protects stalled replication forks from nucleolytic degradation (Schlacher et al., 2011; Ying et al., 2012).

**Figure 2.10**

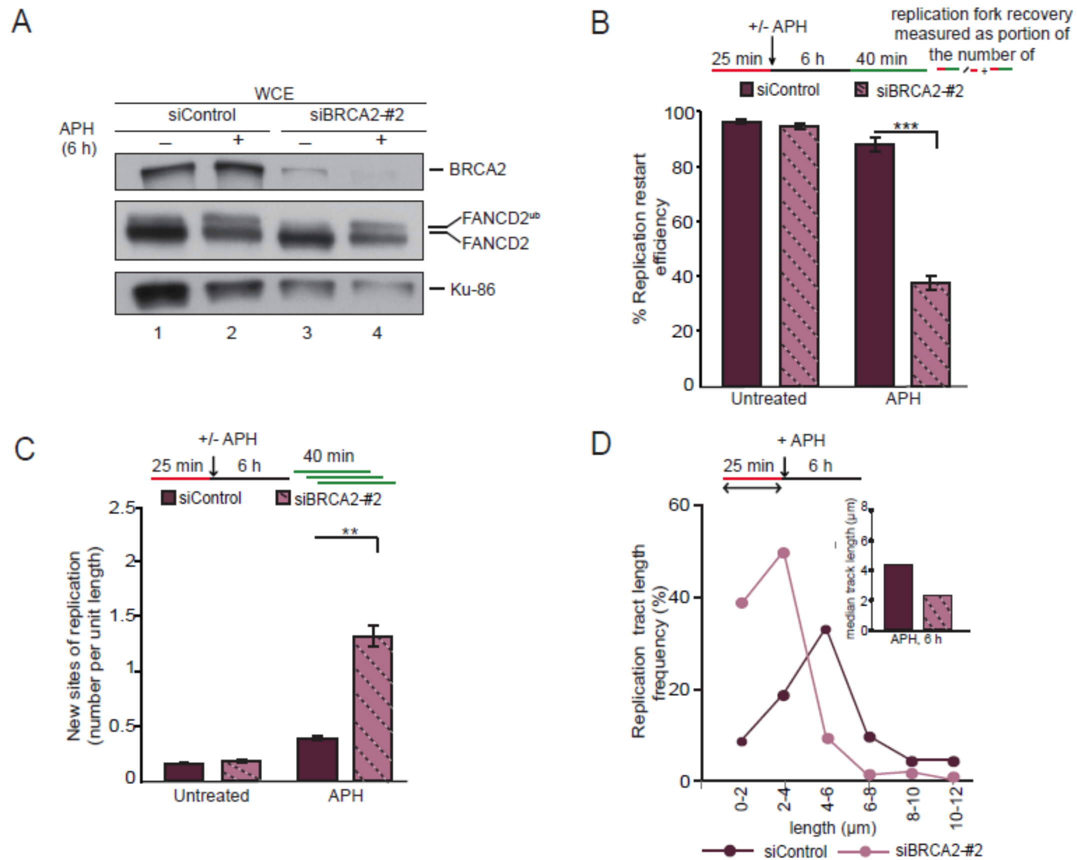


**Figure 2.10: Cellular treatment with siBRCA2$^{\#2}$ causes severe defects in replication fork recovery and in fork stability after APH treatment.**

**(A)** Western blot showing the efficiency of BRCA2 knockdown with siBRCA2$^{\#2}$. Wild type (PD331+C) cells were treated with siControl or siBRCA2$^{\#2}$, and then either untreated or treated with APH. WCEs from these cells were analyzed for the presence of BRCA2, FANCD2 and FANCD2$^{Ub}$. Ku-86, loading control. **(B, C)** siBRCA2$^{\#2}$-treatment triggers replication fork restart defects after APH treatment. **(B)** Replication fork restart efficiency after APH treatment was compared between siControl or siBRCA2$^{\#2}$ treated cells. **(C)** The number of new replication sites originating during the BioU labeling after APH treatment was compared between siControl and siBRCA2$^{\#2}$ treated cells. **(D)** siBRCA2$^{\#2}$-treatment triggers replication fork instability after APH treatment. Lengths of nascent replication fork tracts (labeled with DigU only) were measured after 6 h of APH treatment. Preformed DigU tract lengths shorten during APH-mediated replication fork stalling in siBRCA2$^{\#2}$ treated cells (median 2.3 µm) compared to siControl treated cells (median 4.8 µm).

Lastly, we wanted to test if BRCA2's role in fork restart extends to HU-stalled replication forks. We treated cells with siControl or siBRCA2 (Fig. 2.11A), followed by DNA fiber analysis. Following HU-treatment, BRCA2-deficient cells exhibited a severe reduction in replication fork restart efficiencies (Fig. 2.11B, 35.8%) and showed a significant increase in new origin firing (Fig. 2.11C, 4-fold), comparable to those phenotypes observed in APH-treated BRCA2-deficient cells (see Fig. 2.9B and C; Fig. 2.10B and C). Taken together, our results indicate that a complete absence of full length BRCA2 causes severe replication fork recovery defects. Moreover, our findings demonstrate that BRCA2 acts in concert with FANCD2 to promote the restart of APH- or HU-stalled replication forks while simultaneously suppressing the firing of new replication origins.

**Figure 2.11**

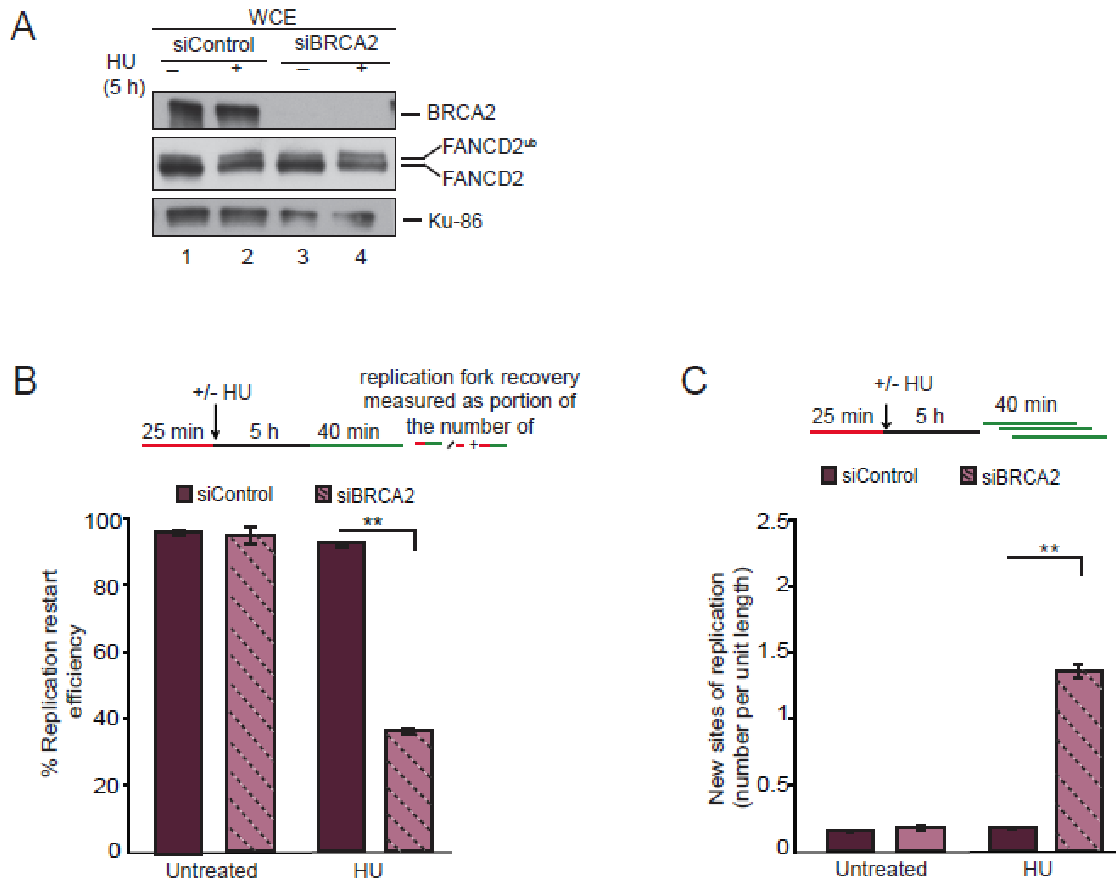


**Figure 2.11: BRCA2 is essential for the recovery of HU-stalled replication forks.**

(**A**) Western blot showing the efficiency of BRCA2 knockdown with siBRCA2. Wild type (PD331+C) cells were treated with siControl or siBRCA2, and then either untreated or treated with 4mM HU for 5h. WCEs from these cells were analyzed for the presence of BRCA2, FANCD2 and FANCD2Ub. Ku-86, loading control. (**B, C**) BRCA2 is essential for the recovery of HU-stalled replication forks. (B) Replication fork restart efficiencies after HU treatment were compared between siControl and siBRCA2 cells. (**C**) The number of new replication sites originating during BioU labeling after HU treatment was compared between siControl and siBRCA2 cells.

## 2.3 Discussion

In this study, we expand on our previous findings that FANCD2 functions in the restart of APH-stalled replication forks and the simultaneous suppression of new origin firing. Our new results show that FANCD2 shares this role with FA pathway members FANCJ and BRCA2, but not with the FA core complex proteins. Our data strongly support a non-linear FA pathway model during the recovery of APH-stalled replication forks, via mechanisms that depend selectively on the nonubiquitinated isoform of FANCD2 (Fig. 2.12).

The idea that FANCD2 does not rely on the FA core complex to recover stalled replication forks is supported by recent findings that FANCD2 binds HU-stalled replication forks, where it interacts with the MCM helicase independently of the FA core complex protein FANCA (Lossaint et al., 2013; Sirbu et al., 2013). Interestingly, it was also shown that FANCD2 and BRCA2 – but not FANCA or FANCC – promote cellular resistance to topoisomerase II inhibitors that cause DNA DSBs (Kachnic et al., 2011; Treszezamsky et al., 2007). Moreover, FANCD2 is known to promote S-phase checkpoint activation in response to ionizing radiation in a manner that depends on ATM-mediated FANCD2 phosphorylation but occurs independently of FA core complex-mediated $FANCD2^{Ub}$ formation (Taniguchi et al., 2002b). These findings indicate a functional separation between FANCD2 and the FA core complex and support the idea that $FANCD2^{Ub}$ formation is dispensable not only for replication fork restart, but for other FANCD2-dependent functions as well.

**Figure 2.12**

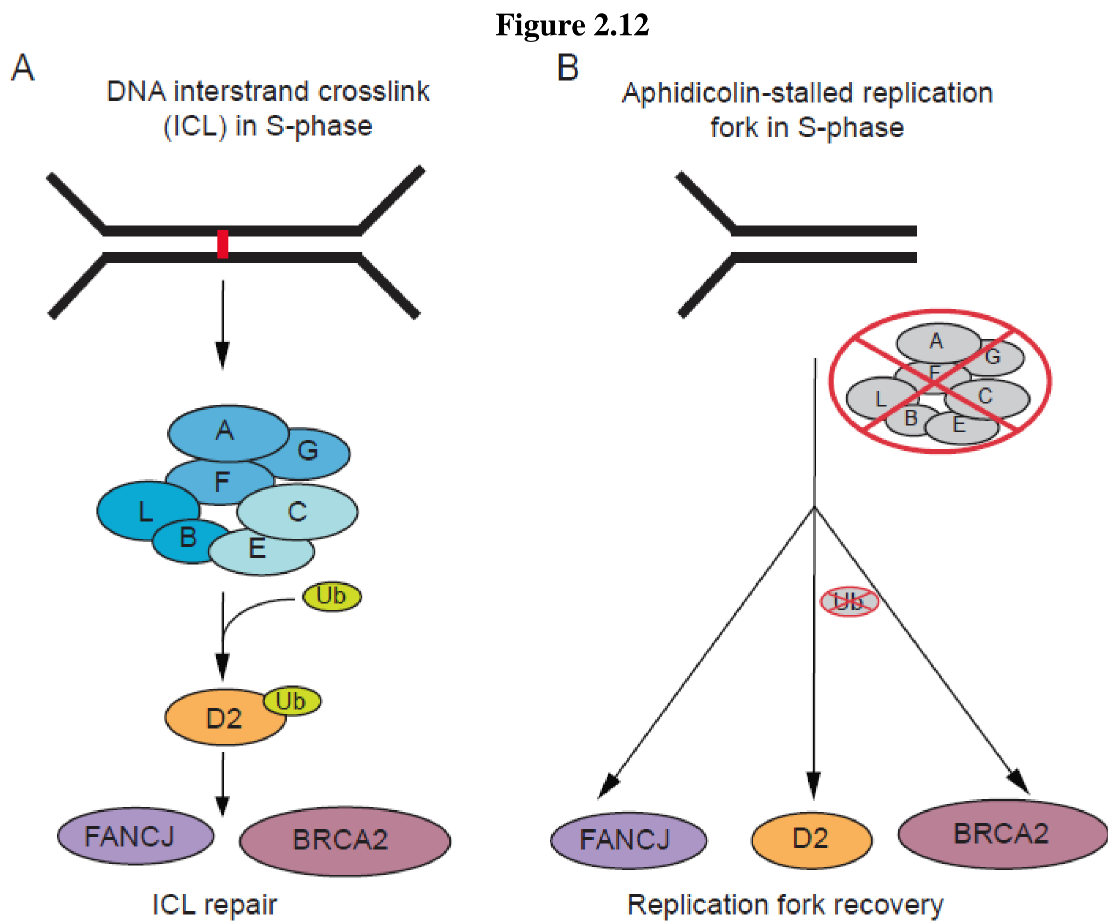


**Figure 2.12: Distinct FA pathway models during DNA ICL repair versus the restart of an APH-stalled replication fork.**

(**A**) Linear FA pathway model during DNA ICL repair. When replication forks converge at a DNA ICL (represented by a red line in the figure), the upstream FA core complex is activated and monoubiquitinates FANCD2, followed by its recruitment to the ICL on chromatin. Chromatin-bound FANCD2Ub then coordinates the actions of downstream DNA repair factors, including FANCJ and BRCA2, to facilitate ICL repair. (**B**) Non-linear FA pathway model during the recovery of APH-stalled replication forks. When a single moving replication fork is temporarily stalled by APH-treatment, nonubiquitinated FANCD2 is recruited to chromatin independently of the FA core complex. Chromatin-bound nonubiquitinated FANCD2 then functions in concert with FANCJ and BRCA2 to promote replication fork restart

Importantly, in the light of recent findings that only cells lacking the catalytic FANCB-FANCL module are truly devoid of any E3 ubiquitin ligase activity towards FANCD2, we must assume that the FANCA-, FANCC- and FANCG-deficient cells used in our study still contain trace amounts of FANCD2$^{Ub}$.[26,27] Nevertheless, our combined findings strongly support a model where replication fork restart occurs independently of FANCD2$^{Ub}$ formation: (i) FA patient cells of complementation groups A, C, G *and* L, as well as a human FANCL knockout cell line, are equally competent for replication fork restart; (ii) FANCD2$^{WT}$ *and* FANCD2$^{K561R}$ can both promote replication fork restart at similar levels and (iii) nonubiquitinated FANCD2 binds chromatin and recruits other replication fork restart proteins in an APH-inducible manner. Interestingly, a recent study also showed that human cells lacking the FANCA-FANCG *and* the FANCC-FANCE-FANCF module fail to recruit FANCB-FANCL to chromatin and do not contain any FANCD2$^{Ub}$, but still exhibit residual chromatin-bound FANCD2 (Huang et al., 2014). Together, these findings strongly indicate that nonubiquitinated FANCD2 is recruited to APH-stalled replication forks independently of the FA core complex to promote fork restart. At the same time, APH treatment *does* robustly trigger FANCD2$^{Ub}$ formation; moreover, chromatin-bound FANCD2 exists predominantly as the FANCD2$^{Ub}$ isoform, even in unperturbed cellular conditions. What then is the role of the FANCD2$^{Ub}$ formation in response to APH? Intriguingly, the Jasin laboratory showed that FANCD2 and FANCA are required to protect nascent DNA strands at HU-stalled replication forks from nucleolytic degradation (Schlacher et al., 2012). Thus, the FANCD2$^{Ub}$ isoform may specifically function in replication fork protection, whereas nonubiquitinated FANCD2

mediates fork restart. These two mechanisms appear to be functionally uncoupled, since nascent DNA strand degradation at the stalled fork does not interfere with successful restart of DNA synthesis in FA core complex-deficient cells.

It is noteworthy that while the FANCD2$^{K561R}$ mutant can support replication fork restart and suppress new origin firing after APH treatment, it is not as efficient as FANCD2$^{WT}$ (see Fig. 2.7C and D). This indicates that the FANCD2$^{K561R}$ mutant has additional defects that are not related to its inability to become monoubiquitinated. In further support of this idea, we previously found in *Xenopus* egg extracts that xFANCD2$^{K562R}$ – unlike nonubiquitinated xFANCD2$^{WT}$ – fails to stably associate with late-replicating chromatin.[21] Interestingly, several studies including ours observed that FANCD2-deficient cells complemented with the *FANCD2$^{WT}$* or *FANCD2$^{K561R}$* gene constructs contain much higher protein levels of FANCD2$^{K561R}$ than FANCD2$^{WT}$ (see Fig. 2.7A), possibly reflecting the cells' attempt to increase the amount of chromatin-bound FANCD2$^{K561R}$ (see Fig. 3B). We speculate that transient chromatin interactions of FANCD2$^{K561R}$ are sufficient to partially promote replication fork restart and suppression of new origin firing.

In contrast to the FA core complex, at least two of the "downstream" FA pathway members, FANCJ and BRCA2, now join the growing group of replication fork restart proteins including FANCD2, BRCA1, BLM, MRE11, RAD51 and CtIP (Bryant et al., 2009; Chaudhury et al., 2013; Petermann et al., 2010; Yeo et al., 2014). Intriguingly, each of these proteins has been implicated in HR repair of DNA DSBs, strongly supporting a model where the recovery of APH-stalled replication forks involves HR mechanisms.

FANCJ's role in fork recovery is not surprising, since it interacts with known fork restart factors including BRCA1, FANCD2 and BLM (Chen et al., 2014; Davies et al., 2007; Yeo et al., 2014). In fact, since FANCJ – like FANCD2 – stabilizes BLM in human cells, the replication restart defects seen in cells depleted of FANCJ, FANCD2 or both may be partly due to a lack of available BLM protein (Chaudhury et al., 2013; Suhasini et al., 2011). Unexpectedly however, another study reported only mildly delayed fork restart kinetics in FANCJ-deficient chicken DT40 cells (Schwab et al., 2013). However, this discrepancy could be due to differences in molecular mechanisms between human and chicken cells; moreover, our study analyzed fork restart after a complete DNA synthesis block, whereas the conditions used by Schwab *et al*. allowed continuous slow DNA synthesis in presence of HU, which may account for the less severe phenotype observed by these authors.

Our discovery that BRCA2 is required for the restart of APH or HU-stalled replication forks seemingly contradicts two previous reports that BRCA2 protects nascent DNA strands at stalled replication forks, but is ***not*** required for replication fork restart (Schlacher et al., 2011; Ying et al., 2012). Importantly, both studies used cells that express residual C-terminally truncated BRCA2 proteins: (i) human CAPAN-1 cells (BRCA2 truncated after aa 1981), (ii) mouse embryonic stem cells carrying the $BRCA2^{lex1/lex2}$ alleles (BRCA2 truncated after aa 3088/3139), and (iii) V-C8 hamster cells (BRCA2 truncated after aa 2494/2567) (Goggins et al., 1996; Morimatsu et al., 1998; Wiegant et al., 2006). In contrast, we observed severe fork restart defects in siBRCA2-depleted cells. This suggests that BRCA2-dependent fork recovery may not

require the BRCA2 C-terminus and predicts that separate regions on BRCA2 are responsible for its roles in fork protection versus fork restart.

Lastly, in the context of BRCA2's new role, it seems counterintuitive that FANCG, and thus the FANCG-dependent BRCA2-FANCD2-FANCG-XRCC3 complex is dispensable for fork restart, while every complex member other than FANCG is crucial for this mechanism (Chaudhury et al., 2013; Petermann et al., 2010). Additional studies will be necessary to solve this puzzle.

In summary, our study reveals a clear separation of function within the three-tiered FA pathway and demonstrates that FANCD2 and at least two of the "downstream" FA pathway proteins fulfill a crucial function at stalled replication forks that is completely independent of the FA core complex and its E3 ligase activity. The ability of FA core complex-deficient cells to promote fork restart mechanisms that rely heavily on FANCD2 and other FA proteins may contribute to the milder phenotypes typically associated with inherited mutations in FA core complex genes (Bakker et al., 2013a; Kalb et al., 2007).

## 2.4 Materials and methods

### *Cell lines and cell culture techniques*

PD220 (FANCA-deficient), PD220+A (retrovirally complemented with wild type FANCA)[57, 58] and PD352i (FANCG-deficient) patient cells (Donahue and Campbell, 2002; Jakobs et al., 1997) were obtained from the FA Cell Repository at the Oregon Health & Science University. GM00637 cells, used as control cell line for assays with PD352i cells, were purchased from the Coriell Institute, USA. PD331 (FANCC-deficient)

and PD331+C (retrovirally complemented with wild type FANCC) were a gift from Dr. Rosselli (Hirano et al., 2005; Naim and Rosselli, 2009). PD20 (FANCD2-deficient), PD20+FANCD2$^{WT}$ (complemented with wild type FANCD2) and PD20+FANCD2$^{K561R}$ (complemented with monoubiquitination-dead FANCD2 mutant) were a gift from Dr. Kupfer (Chen et al., 2014). In addition, we generated the following cell lines: 913/1T + S91IN (FANCL-deficient) and 913/1T + S91FLcoIN (retrovirally complemented with wild type FANCL); 752/1T + S91IN (FANCJ-deficient) and 752/1T + S91FJcoIN (retrovirally complemented with wild type FANCJ). The 913/1T cells carry two heterozygous *FANCL* mutations (c.1007_1009delTAT = p.I336_C337delinsS; g.35021C>G = p.K125_L126insNYELINEKEFR) (Neveling, 2012). The 752/1T cells carry a homozygous FANCJ frameshift mutation (c.308delG = pG103fsS). All cells described above were cultured in DMEM (GIBCO) with 10% FBS and 1% Penicillin-Streptomycin.

HCT116 WT cells and HCT116 FANCL-/- cells were a gift from Dr. Chen (Leung et al., 2012). These cells were cultured in Mc Coy's media (Corning) enriched with 10% FBS, 1% Pencillin-Streptomycin and 1% Glutamine. Cells were treated with Aphidicolin (Sigma; 30µM for 6 hours or Hydroxyurea (Sigma; 4mM for 5h) unless indicated otherwise.

***DNA fiber assay***

We used a DNA fiber protocol as previously described (Chaudhury et al., 2013). Moving replication forks were labeled with digoxigenin-dUTPs (DigU) for 25min and then with biotin-dUTPs (BioU) for 40min. To allow efficient incorporation of the dUTPs,

a hypotonic buffer treatment (10mM HEPES, 30mM KCl, pH 7.4) preceded each dUTP-labeling step. To visualize labeled fibers, cells were mixed with a 10-fold excess of unlabeled cells, fixed and dropped onto slides. After cell lysis, DNA fibers were released and extended by tilting the slides. Incorporated dUTPs were visualized by immunofluorescence detection using anti-digoxigenin-Rhodamine (Roche) and streptavidin-Alexa-Fluor-488 (Invitrogen). Images were captured using a Deltavision microscope (Applied Precision) and analyzed using Deltavision softWoRx 5.5 software. Replication restart efficiency was measured as the number of restarted replication forks after APH-mediated fork stalling (DigU-BioU tracts), compared with the total number of DigU-labeled tracts (DigU + DigU-BioU). New origins of replication were measured as the number of green-only (BioU) tracts per unit length. All DNA fiber results are the means of two or three independent experiments (300 DNA fibers per experiment). Error bars represent the standard error of the mean and significance was determined by student's t-test. All values for DNA fiber data analysis following recovery from APH treatment are provided in **Table S1**. Statistical significance at $P<0.01$, $P<0.001$ and $P<0.0001$ is indicated as *, ** and ***, respectively.

***siRNA experiments***

iGENOME non-targeting siRNA was used as a control for all siRNA experiments. siRNA duplexes for iGENOME non-targeting siRNA and FANCD2 (sequence- CAACAUACCUCGACUCAUUUU) were purchased from Dharmacon research (Thermo Scientific, MA, USA). Transfections were performed using DharmaFECT1 transfection reagent according to the manufacturer’s protocol. siRNA

duplex for BRCA2 was purchased from Invitrogen (siBRCA2: BRCA2HSS101095; targets exon 10; siBRCA2$^{\#2}$: BRCA2HSS101097; targets exon 18) and transfections were performed using RNAiMax lipofectamine reagent according to the manufacturer's protocol. All siRNAs were used at a final concentration of 20 nM.

***Preparation of whole cell extract (WCEs) and chromatin fractions from human cells***

To prepare whole cell extracts, cells were washed in PBS, resuspended in lysis buffer (10mM Tris, pH 7.4, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 1mM EDTA, 1mM DTT, complete protease inhibitor cocktail) and incubated on ice for 10 min. Cell extracts were centrifuged for 5 min at 10000g, and the supernatant was used for further analysis. Chromatin fractions were prepared as described (Ge et al., 2007). Briefly, $3x10^6$ cells were washed in PBS, pelleted and resuspended in 1 ml cytoskeleton buffer. After 10 min incubation on ice, the suspension was centrifuged at 5000g for 3 min. The supernatant was discarded and the resultant pellet containing chromatin-bound sample was washed 3 times with CSK buffer. The chromatin pellet was resuspended in 150 µl CSK buffer and mixed with equal volume of 2X LDS Nupage LDS sample buffer. Samples were sonicated briefly prior to PAGE analysis.

***Immunoblotting***

Protein samples were separated on denaturing gradient gels and transferred to Immobilon P membranes (Millipore). After blocking in 5% milk, membranes were incubated overnight with the primary antibodies. Subsequent incubation with Horseradish peroxidase-conjugated rabbit secondary antibodies (Jackson Labs) or mouse secondary

antibodies (Bio-Rad) was carried out for 1 hour. Protein bands were visualized using an ECL Plus system (Millipore).

### *Antibodies*

Commercial antibodies were used against human FANCD2 (Santa Cruz, sc-20022), Tubulin (Abcam, ab7291), H2AX (Bethyl, A300-083A), BRCA2 (Calbiochem, OP95), Top3A (Proteintech 14525-1-A), Ku86 (Santa-Cruz, sc-5280). Antibodies were used at the following dilutions in WB: FANCD2 (1:1000), BRCA2 (1:500), H2AX (1:10000), Tubulin (1:10000), Top3A (1:1000), Ku-86 (1:500).

# CHAPTER 3

## A functional interaction between the Fanconi anemia protein FANCD2 and the chromatin remodeling factor ATRX promotes replication fork recovery.

(Maya Raghunandan, Adam J. Harvey, Jung Eun Yeo, Kai Saito, Stacie Ittershagen, Maureen E. Hoatlin, Eric A. Hendrickson and Alexandra Sobeck)

Author contributions:

Experiments were conceived by M.R. and A.S. and majority of experiments were performed by M.R. A.J.H. generated the ATRX- null and FANCD2/ATRX -null cell lines. J.Y. generated the mutant FANCD2 complemented cells lines and performed some of the DNA fiber assays. K.S. performed some of the nuclear foci formation and protein stability experiments. S.I. and M.E.H. designed and performed the mass spectrometry experiments in *Xenopus* egg extracts. The data were analyzed and prepared for publication by M.R. and A.S.

## Synopsis

Fanconi Anemia (FA) is an inherited cancer predisposition syndrome characterized by cellular hypersensitivity to DNA interstrand crosslinks (ICLs). The 21 known FA proteins act in a linear pathway to repair the ICLs: Following ICL detection, an upstream FA core complex monoubiquitinates and activates two central FA pathway members, FANCD2 and FANCI, that subsequently orchestrate the recruitment of downstream FA proteins to repair the ICL. The molecular mechanisms that allow FANCD2 to promote ICL repair are not completely understood, however it was shown that FANCD2 possesses a histone H3 chaperone activity that is crucial for cellular ICL resistance. Beyond FANCD2's role in ICL repair, recent findings from our laboratory and others demonstrated that FANCD2 functions at hydroxyurea (HU) or aphidicolin (APH) stalled DNA replication forks to promote fork restart via homologous recombination mechanisms. In this study, we identified a novel FANCD2 interactor named ATRX (Alpha Thalassemia Retardation X-linked factor). ATRX is known to function in a constitutive complex with DAXX (Death domain associated protein) to act as a histone H3.3 chaperone that regulates chromatin structure. Importantly, ATRX – like FANCD2 – is known to promote replication fork recovery, hinting at overlapping roles of ATRX and FANCD2 during the replication stress response. Our new findings demonstrate that ATRX forms a constitutive complex with FANCD2 and promotes FANCD2 protein stability. Whereas ATRX is dispensable for the cellular DNA ICL resistance, it works in concert with FANCD2 to promote cellular resistance to HU. Moreover, the ATRX/DAXX complex cooperates with FANCD2 and the key HR factor MRE11 to

promote the restart of HU-stalled replication forks, suggesting that ATRX/DAXX-mediated histone H3.3 deposition is required for HR mechanisms at stalled replication forks. Supporting this idea, ATRX and FANCD2 also cooperate to recruit the HR factor CtIP into DNA repair foci following fork stalling. Strikingly, FANCD2's own histone H3 chaperone activity is indispensable for replication fork restart as well, indicating that histone H3 exchange at stalled replication forks is a crucial step in HR-mediated fork restart.

## 3.1 Introduction

Fanconi Anemia (FA) is a recessively inherited genomic instability syndrome caused by mutations in any one of twenty one known FA genes (Ceccaldi et al., 2016a; Duxin and Walter, 2015; Kee and D'Andrea, 2012; Kupfer, 2013). FA patients exhibit congenital abnormalities, progressive bone marrow failure and a strong predisposition to cancers such as acute myeloid leukemia and squamous cell carcinomas. Cells from FA patients are hypersensitive to DNA interstrand crosslinks (DNA ICLs) and show spontaneous chromosomal aberrations that are further exacerbated upon treatment with replication-inhibiting agents such as hydroxyurea (HU) or aphidicolin (APH) (Kee and D'Andrea, 2010; Naim and Rosselli, 2009; Schlacher et al., 2012). To promote the repair of DNA ICLs, the FA pathway members are thought to function in a linear hierarchy that can be broadly divided into three tiers: an upstream FA core complex (at least 8 proteins), a central FANCD2-FANCI protein heterodimer (named the ID2 complex) and several downstream FA pathway members including the Breast Cancer Associated proteins, BRCA1/FANCS and BRCA2FANCD1 (Ceccaldi et al., 2016a; Duxin and Walter, 2015; Kee and D'Andrea, 2012; Wang, 2007). DNA ICLs are mostly repaired in S-phase when they block the progression of replication forks (Clauson et al., 2013; Knipscheer et al., 2009; Räschle et al., 2008). Detection of an ICL triggers the recruitment of the FA core complex, followed by the ID2 complex. The FA core complex then acts as an E3 ubiquitin ligase that monoubiquitinates both subunits of the ID2 complex ($ID2^{Ub}$). (Garcia-Higuera et al., 2001; Liang et al., 1998; Meetei et al., 2003; Smogorzewska et al., 2007; Timmers et al., 2001; van Twest et al., 2017). Subsequently, $ID2^{Ub}$ coordinate the

recruitment of downstream FA proteins to promote incisions at the DNA ICL, followed by HR-mediated repair of the resulting DNA double strand break (DSB) (Crossan et al., 2011; Kim et al., 2008b; Klein Douwel et al., 2014; Stoepker et al., 2011; Walden and Deans, 2014; Yamamoto et al., 2011).

Intriguingly, a recent study showed that the recombinant FANCD2 protein can act as a histone H3 chaperone that forms a complex with the H3/H4 histone tetramer and promotes complete nucleosome assembly *in vitro*. The authors also showed that cells expressing FA-D2 patient-derived FANCD2 missense mutants (FANCD2-R302W and FANCD2-L231R) lacking H3 chaperone activity, were as ICL sensitive as FANCD2-depleted cells (Sato et al., 2012, 2014). Thus, FANCD2's histone H3 chaperone activity is crucial for cellular DNA ICL resistance and may be required for other cellular functions of FANCD2 as well.

Recent studies from our laboratory and others revealed a novel dual role for FANCD2 at HU- or APH- stalled DNA replication forks: first, FANCD2 protects nascent DNA strands at stalled replication forks from nucleolytic degradation (Chaudhury et al., 2013; Schlacher et al., 2011, 2012; Ying et al., 2012). Second, FANCD2 acts in concert with several HR factors to promote the restart of the stalled replication forks, which simultaneously suppresses the firing of new replication origins (Chaudhury et al., 2014; Raghunandan et al., 2015a; Yeo et al., 2014).

To gain a better understanding of how FANCD2 may function during the S-phase replication stress response, we performed mass spectrometry analysis of FANCD2 interacting proteins and identified a novel FANCD2-associated protein named <u>A</u>lpha

Thalassemia Retardation X-linked factor (ATRX). ATRX belongs to the Switch 2, Sucrose Non- Fermenting 2 (SWI2/SNF2) family of helicases/ATPases that are known to have chromatin modifying roles. Hereditary mutations in ATRX induce a complex neurological syndrome (ATRX syndrome) characterized by skeletal deformities, distinctive craniofacial features, genital anomalies, severe developmental delays, intellectual disability and alpha-thalassemia (Gibbons et al., 1995, 2003; Stevenson, 1993). ATRX forms a constitutive complex with DAXX (Death domain associated protein), which acts as a specific chaperone for the histone H3 variant, H3.3. The ATRX/DAXX complex is known to deposit H3.3 at repetitive DNA sequence regions such as the telomeric, pericentric and ribosomal DNA sequence repeats to promote heterochromatin formation (Drané et al., 2010; Elsässer et al., 2012; Goldberg et al., 2010; Lewis et al., 2010; Wong et al., 2010). Recently, ATRX and DAXX were identified, quite unexpectedly, to function during the HU-induced replication stress response, similar to FANCD2: Following replication fork stalling, ATRX and DAXX protect the stalled forks from nucleolytic degradation; moreover, ATRX was shown to promote the restart of these stalled forks (Clynes et al., 2014; Huh et al., 2016; Leung et al., 2013).

Here, we asked if FANCD2 and the ATRX/DAXX complex act in concert during the replication stress response in S-phase. To this end, we utilized a combination of rAAV and CRISPR/Cas9 gene targeting technologies to generate isogenic FANCD2-, ATRX-and FANCD2/ATRX double knockout cell lines in an HCT116 cell background. A functional characterization of our cell lines revealed that ATRX forms a constitutive

complex with FANCD2 and stabilizes FANCD2 protein levels. Importantly, ATRX is not required for the FANCD2-dependent cellular DNA ICL resistance. In contrast, ATRX and FANCD2 cooperate with the homologous recombination (HR) factor MRE11 to promote replication fork restart. ATRX and FANCD2 also cooperate to recruit the HR factor CtIP to chromatin following replication fork stalling, indicating that ATRX has a new role in promoting FANCD2- dependent HR mechanisms. Lastly, we find that both the histone H3.3 depositor DAXX, as well as the histone H3 chaperone function of FANCD2, are required for replication fork restart. Our findings implicate that a functional protein complex containing ATRX/DAXX, FANCD2 and MRE11 utilizes histone H3 variant chaperone activities to promote the HR-driven restart of stalled replication forks.

## 3.2 Results

### *FANCD2 interacts with ATRX to form a constitutive protein complex in the presence or absence of DNA damage*

To better understand S-phase specific functions of FANCD2 we set out to identify novel, S-phase specific interactors of FANCD2. To this end, we performed pulldown studies from S-phase *Xenopus laevis* S-phase egg extracts that are stockpiled with DNA replication and repair proteins and allow for the detection of even less abundant protein complexes. We used a *Xenopus laevis* FANCD2 (xFANCD2) specific antibody (Sobeck et al., 2006) to pull down xFANCD2 from high-speed S-phase egg extracts (Sobeck et al., 2006, 2007), followed by mass spectrometric identification of the xFANCD2-associated proteins (a detailed description of the procedure and the analysis of the identified

FANCD2 protein interactors will be published elsewhere). One of the proteins identified by our mass spectrometry screen was ATRX. Interestingly, the human ATRX and FANCD2 proteins share a known interactor, the MRN (MRE11-NBS1-RAD50) complex (Clynes et al., 2014; Leung et al., 2013; Roques and Masson, 2010; Roques et al., 2009). In fact, it was previously shown that MRE11 interacts with FANCD2 in human cells to protect FANCD2 from proteosomal degradation (Roques et al., 2009). To identify if ATRX and FANCD2 have a comparable relationship, we immunoprecipitated (IP-ed) FANCD2 from human wild type cells (PD20+3xFLAG-FANCD2; an FA-D2 patient-derived cell line complemented with triple FLAG-tagged wild type FANCD2). As shown in Fig. 3.1A, an anti-FLAG antibody) co-immunoprecipitated (co-IPed) ATRX with FANCD2. In the reciprocal experiment, we successfully co-IPed FANCD2 with an anti-ATRX antibody from the same wild type cells (Fig. 3.1B). Of note, we performed this experiment in the presence of the DNase Benzonase and the DNA intercalator ethidium bromide, indicating that the ATRX-FANCD2 interaction is not mediated by DNA. To test whether the ATRX-FANCD2 interaction was improved following DNA damage, we IP-ed ATRX from wild type cells that were either untreated or treated with HU. We observed that DNA damage did not significantly alter the degree of interaction between FANCD2 and ATRX (Fig. 3.1B, lanes 4 and 5). As previously described, we were also able to co-IP the MRN complex member, NBS1, with both ATRX and FANCD2 (Fig. 3.1C). These findings indicate that ATRX, FANCD2 and MRN participate in a constitutive protein complex that forms independently of the presence or absence of DNA damage.

**Figure 3.1**

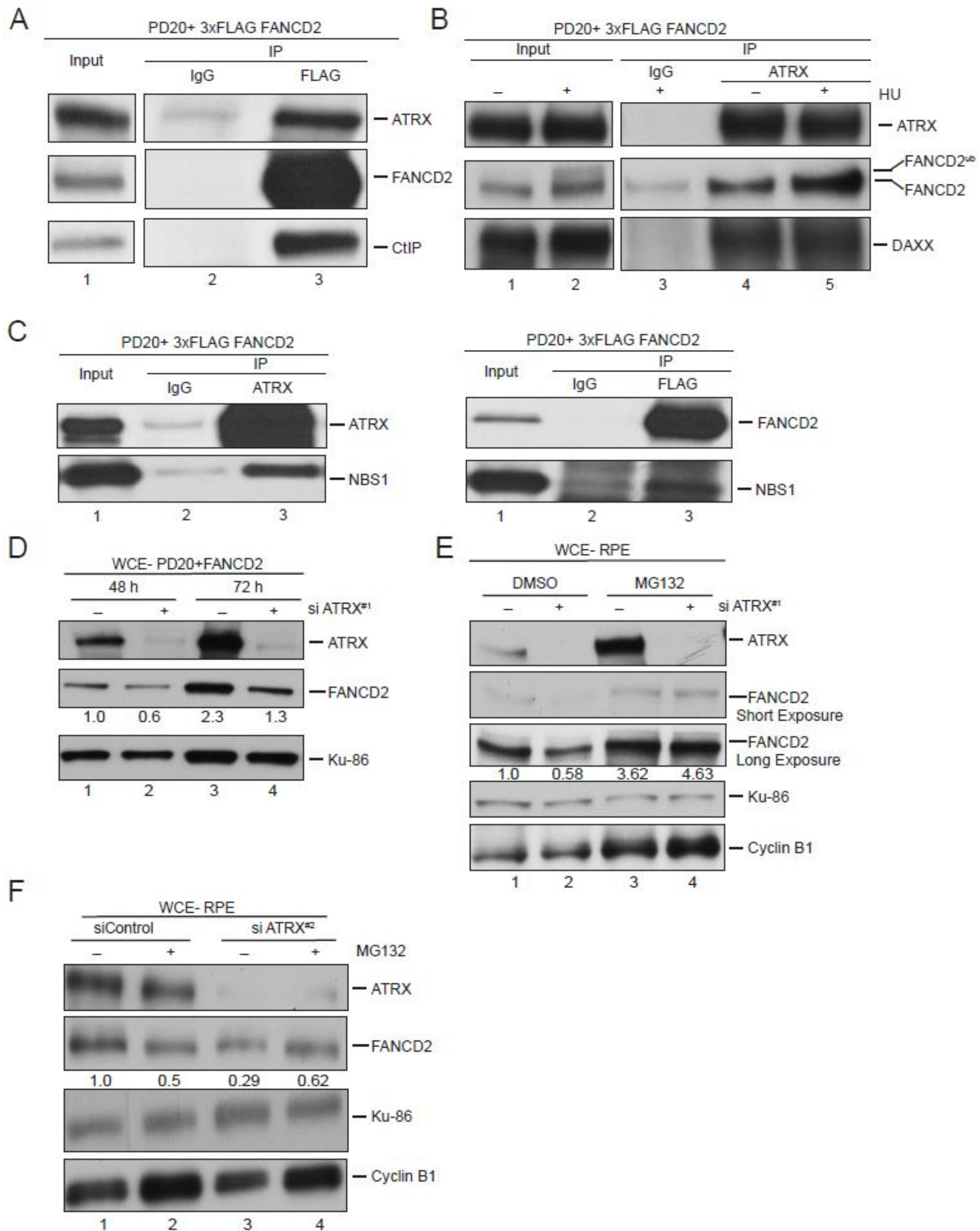

**Figure 3.1: FANCD2 interacts with ATRX.**

All IP experiments were performed from nuclear extracts (NE) prepared from PD20 + 3x FLAG-FANCD2 cells, in the presence of Benzonase and EtBr. (**A**) ATRX co-IPs with FANCD2. NEs were subjected to IP with mouse IgG (lane 2; neg. control) or the anti-FLAG antibody (lane 3). NE and IP samples were analyzed for the presence of FANCD2 and ATRX by western blot. (**B**) FANCD2 co-IPs with ATRX. NE were either treated without or with 2mM HU for 24h (lanes 1 and 2) and subjected to IP with rabbit IgG (lane 3; neg. control) or the anti-ATRX antibody (lanes 4 and 5). (**C**) NBS1 co-IPs with ATRX and FANCD2. NEs were subjected to IP with: left panel- rabbit IgG (lane 2; neg. control) or the anti-ATRX antibody (lane 3); right panel- mouse IgG (lane 2; neg. control) or the anti-FLAG antibody (lanes 3).

ATRX promotes FANCD2 protein stability. (**D**) Whole cell extract (WCEs) were prepared from human PD20+D2 cells that had been treated with control siRNA (siControl- lanes 1 and 3) or ATRX siRNA ($siATRX^{\#1}$- lanes 2 and 4) for the indicated time points and analyzed for ATRX and FANCD2. Ku-86 loading control. (**E**) WCEs were prepared from human hTERT-RPE cells that had been treated with control siRNA (siControl- lanes 1 and 3) or ATRX siRNA ($siATRX^{\#1}$- lanes 2 and 4) for 72 hours. At 67 hours, these cells were treated with either DMSO (lanes 1 and 2) or MG132 (10uM for 5 hours- lanes 3 and 4). WCEs was analyzed for ATRX and FANCD2. Ku-86 loading control, Cyclin B- positive control for MG132 activity. (**F**) Same as (E), but using a second siRNA against ATRX- $siATRX^{\#2}$. At 67 hours, these cells were treated with either DMSO (lanes 1 and 3) or MG132 (10uM for 5 hours- lanes 2 and 4). WCEs were analyzed for ATRX and FANCD2. Ku-86: loading control, Cyclin B: positive control for MG132 activity.

For D-F, immunoblot signals for FANCD2 were analyzed by densitometry and normalized against Ku-86 signals using ImageJ. The normalized values are provided under the corresponding lanes below the FANCD2 blot in each figure

To test if ATRX – like MRE11 – stabilizes FANCD2 protein levels, we treated wild type cells (PD20+ FANCD2) with either control siRNA or an siRNA against ATRX ($siATRX^{\#1}$) for 48 hr or 72 hr. An analysis of ATRX and FANCD2 protein levels revealed that FANCD2 protein levels were significantly reduced in ATRX-depleted cells, compared to the control cells. (Fig 3.1D). We confirmed these findings in a second cell

line, RPE1 (Retinal Pigment Epithelium-1). Similar to the siRNA-mediated ATRX knockdown in PD20+ FANCD2 cells (Fig. 3.1 D), ATRX knockdown with two different siRNAs ($ATRX^{\#1}$ and $ATRX^{\#2}$) caused a reduction of FANCD2 protein levels in RPE1 cells (Fig 3.1E, compare lanes 1 and 2; Fig. 3.1F, compare lanes 1 and 3). Importantly, treatment with the proteasome inhibitor, MG132, restored the FANCD2 protein levels in ATRX-depleted RPE1 cells (Fig 3.1E, compare lanes 3 and 4; Fig. 3.1F, compare lanes 2 and 4). Thus, ATRX interacts with and stabilizes FANCD2 protein molecules, protecting them from proteasomal degradation.

***Generation of human isogenic $FANCD2^{-/-}$, $ATRX^{-/o}$ and $FANCD2/ATRX^{-/-}$ cell lines***

To determine the functional interactions between FANCD2 and ATRX, we set out to generate isogenic FANCD2, ATRX and FANCD2/ATRX knockout (KO) cells using HCT116 as the parental cell line. To this end, we used a combination of CRISPR/Cas9 and rAAV (recombinant adeno-associated virus)-mediated gene targeting techniques. *To create the FANCD2 KO ($D2^{-/-}$) cell line* (Thompson & Yeo et. al, 2017, submitted) we targeted the *FANCD2* exon 12. For the first round of targeting, conditional rAAV vectors that contained LoxP sites flanking both a *NEO* gene and the targeted exon were designed. An additional straight knockout vector to replace exon 12 with a *NEO* gene flanked by LoxP sites was also designed for *FANCD2*. The $D2^{-/-}$ cell line was generated using two rounds of rAAV gene targeting, first with the conditional vector and second with the knockout vector. Cre recombinase was subsequently used to remove the NEO gene and the conditional allele (Fig. 3.2A). *To generate the ATRX KO cell line*, a guide RNA was designed to target the Cas9 endonuclease to cleave within an endogenous restriction

enzyme (SmlI) recognition site in *ATRX* exon 9 as described previously. ATRX, being an X-linked gene, is mono-allelic in the male HCT116 cells line (Napier et al., 2015) (Fig. 3.2B). *To generate the FANCD2/ATRX KO (D2AX$^{-/-}$)* cell line, the same CRISPR/Cas9 gene targeting system was used to knock out *ATRX* in the *D2$^{-/-}$* cells. For the generation of the *ATRX$^{-/o}$* and *D2AX$^{-/-}$* cell lines, we screened the genomic DNA of the targeted clones for the ones with complete resistance to digestion with SmlI within *ATRX* exon 9, indicating disruption of ATRX (Fig. 3.2B and C). Sequence analysis of the targeted region was used to confirm that the *D2AX$^{-/-}$* clone had CRISPR/Cas9-induced frameshift mutations in ATRX. Western blot analysis of whole cell extracts (WCEs) prepared from WT, *D2$^{-/-}$, ATRX$^{-/o}$* and *D2AX$^{-/-}$* cells confirmed that the genetically null cells lacked protein expression of FANCD2, ATRX, or both. (Fig. 3.2D).

In agreement with our ATRX knockdown experiments described above, we found that the ATRX$^{-/o}$ cells contained reduced FANCD2 protein expression levels compared to the WT cells independently of the presence or absence of HU-induced DNA damage (Fig. 3.2D, compare lanes 1 with 3 and 5 with 7). In contrast, ATRX protein levels remained unchanged in *D2$^{-/-}$* cells compared to WT cells, demonstrating that ATRX does not rely on FANCD2 for its own protein stability. Despite the reduced overall FANCD2 protein levels in ATRX$^{-/o}$ cells, FANCD2$^{ub}$ formation following HU treatment was robustly and equally induced in WT and ATRX$^{-/o}$ cells, indicating that FANCD2 monoubiquitination occurs independently of ATRX (Fig. 3.2D, compare lanes 5 and 7).

**Figure 3.2**

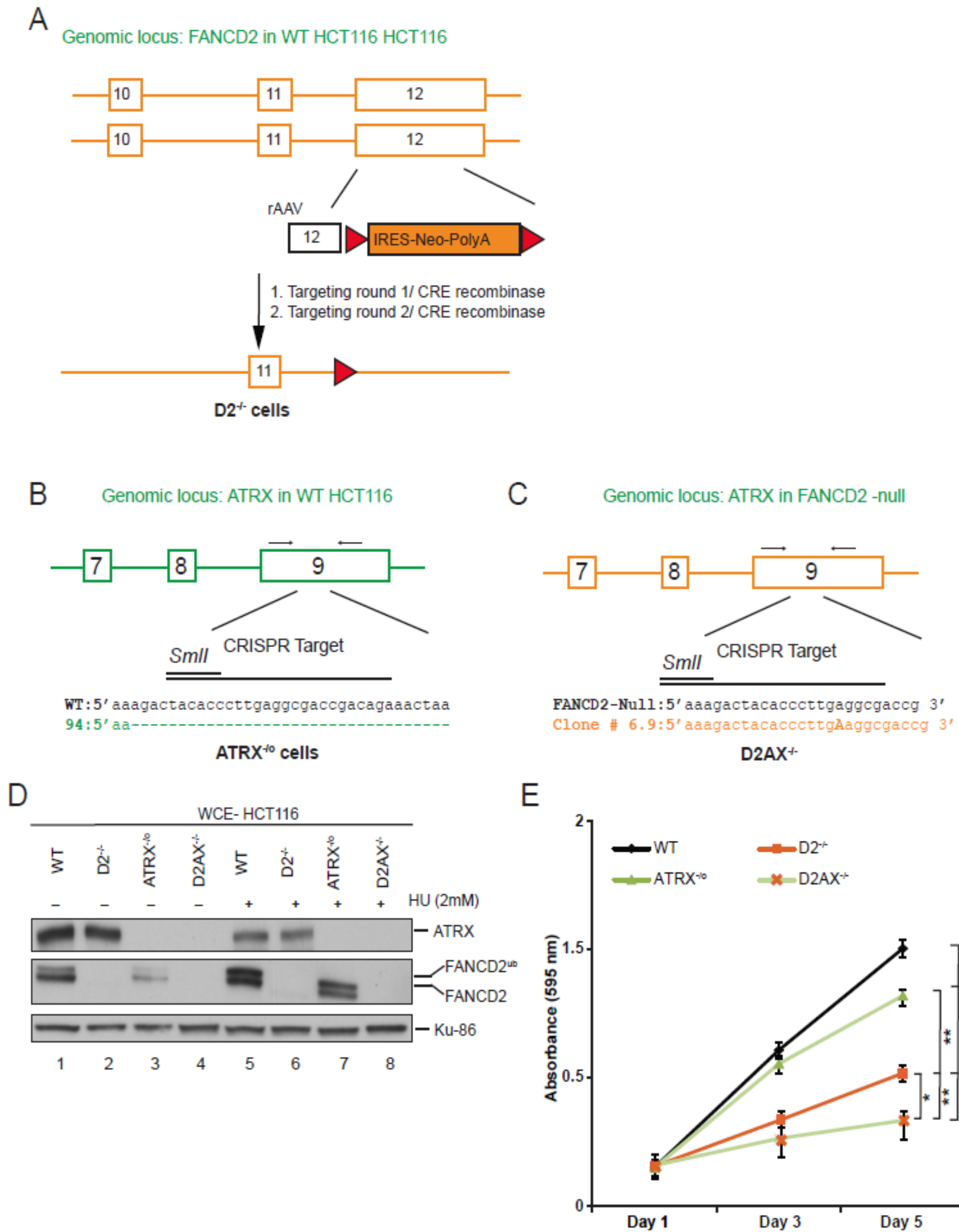


**Figure 3.2: Generation of somatic genetic knock out cell lines for FANCD2, ATRX and FANCD2/ATRX.**

(**A**) $D2^{-/-}$ cell line generation: rAAV (recombinant adeno-associated virus)-mediated gene targeting was used to knock out FANCD2 from HCT116 cells. The first round of targeting with

the conditional vector replaced FANCD2 exon 12 with a conditional, floxed allele along with a Neo selection cassette, also flanked by LoxP sites. G418 resistant clones were screened by PCR to confirm targeting, and *Cre* recombinase was used to remove the Neo selection cassette. The FANCD2 second round of targeting was performed in the $D2^{flox/+}$ cells with the knock-out rAAV vector to replace exon 12 with a Neo selection cassette. *Cre* recombinase was used to remove both the Neo selection cassette and the conditional allele(s) resulting in viable $D2^{-/-}$ clones. (**B**) $ATRX^{-/o}$ cell line generation. Following single cell cloning of HCT116 cells co-transfected with CRISPR/Cas9 correctly targeted clones were identified by restriction enzyme analysis of a PCR product that encompassed the CRISPR target (Figure 1A). HCT116 clones with a disrupted ATRX gene were resistant to digestion with the SmlI restriction enzyme due to removal of the SmlI recognition site, which was verified by Sanger sequencing. Clone #94 was used in subsequent experiments. (**C**) $D2AX^{-/-}$cell line generation. The CRISPR described in (B) was transfected in the $D2^{-/-}$ cells generated from (A). $D2^{-/-}$ clones with a disrupted ATRX gene were resistant to digestion with the SmlI restriction enzyme due to removal of the SmlI recognition site, which was verified by Sanger sequencing. Clone #6.9 was used in subsequent experiments. (**D**) WCEs were prepared from HCT116- WT, $D2^{-/-}$, $ATRX^{-/o}$ or $D2AX^{-/-}$ cells. All four cell lines were untreated (lanes 1-4) or treated with 2mM HU for 24 h (lanes 5-8) and analyzed for the presence of FANCD2 and ATRX by western blot. Tubulin: loading control. (**E**) FANCD2-, ATRX- or FANCD2/ATRX- deficiency results in cellular proliferation defects. Proliferation rates for WT, $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells were compared over five days using MTS assay. The values are shown as absorbance readings at 595 nm, after treatment with the MTS reagent.

***FANCD2 and ATRX can contribute separately to cell proliferation***

Previous studies suggested that FANCD2 supports normal cell proliferation (Thomposon and Yeo et. al., submitted, Houghtaling et al., 2003). Similarly, ATRX was previously shown to contribute to cellular proliferation as well (Bérubé et al., 2005; Huh et al., 2012; Watson et al., 2013). To test if FANCD2 and ATRX cooperate to promote cell proliferation, we analyzed the cellular growth of WT, $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells using an MTS assay. The three knockout cell lines exhibited significantly reduced

cellular growth rates compared to the WT cells, indicating that FANCD2 and ATRX both contribute to cell proliferation. Strikingly, the cellular growth rates of the three knockout cell lines were also significantly different from one another, with decreasing viabilities in the following order: $ATRX^{-/o}$ > $D2^{-/-}$ > $D2AX^{-/-}$ cells (Fig. 3.2E). These results indicate that FANCD2 and ATRX have partially non-overlapping roles to promote cellular proliferation in otherwise unperturbed conditions.

***FANCD2 functions independently of ATRX to mediate ICL repair***

A hallmark of FA is the cellular hypersensitivity to DNA ICL-inducing agents such as MMC (Garcia-Higuera et al., 2001; Kee and D'Andrea, 2010). To test if ATRX participates in the FANCD2-mediated cellular resistance to DNA ICL agents, we performed a colony formation assay. WT, $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells were plated and either left untreated or treated with increasing concentrations of MMC. Colony formation ability of each cell line was determined after 12-14 days. The $D2^{-/-}$ and $D2AX^{-/-}$ cells exhibited a severe hypersensitivity to MMC even at the lowest MMC concentration (5nM) compared to WT cells (Fig. 3.3A, $D2^{-/-}$ cells: 15% survival; $D2AX^{-/-}$ cells: 17% survival; WT cells: 82% survival). In stark contrast, the $ATRX^{-/o}$ cells did not exhibit any MMC sensitivity compared to the WT cells (Fig. 3.3A, $ATRX^{-/o}$ cells, 81% survival). These results indicate that ATRX is dispensable for the FANCD2-mediated cellular DNA ICL resistance.

**Figure 3.3**

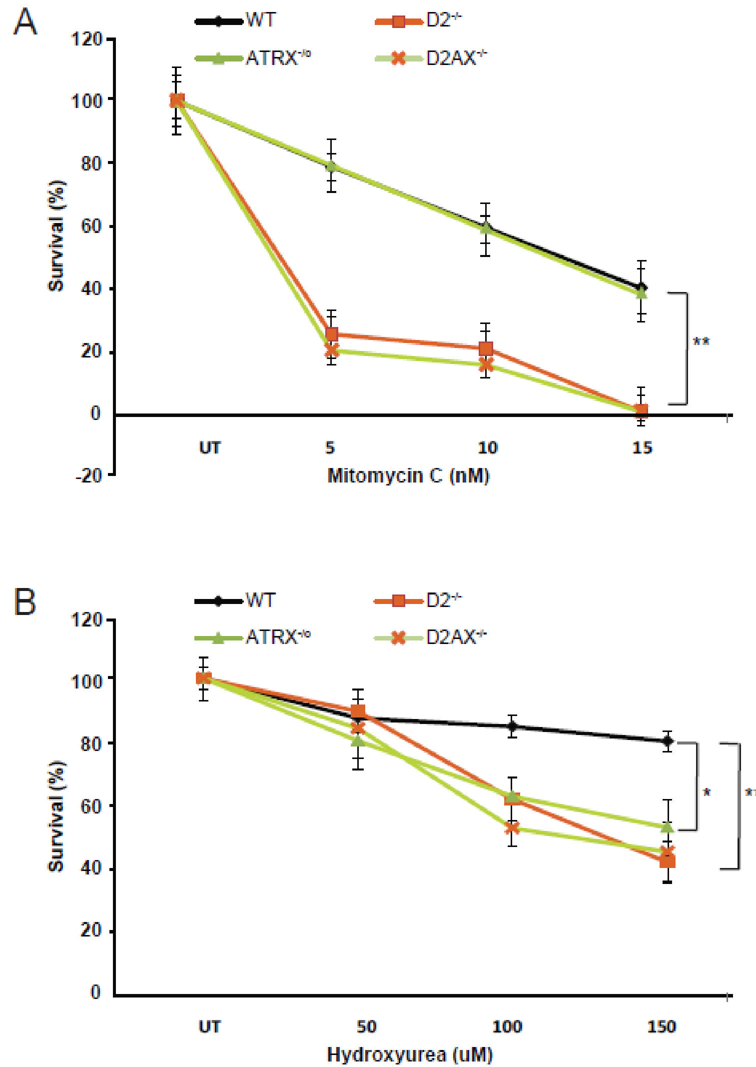


**Figure 3.3: FANCD2 functions independently of ATRX for ICL resistance, but works in concert with ATRX for HU resistance.**

(**A**) FANCD2 promotes ICL resistance independently of ATRX. WT, D2$^{-/-}$, ATRX$^{-/o}$, and D2AX$^{-/-}$ cells were treated with 5, 10 or 15 nM MMC, and assayed for colony forming ability after 10 days. (**B**) FANCD2 acts in concert with ATRX to promote HU resistance. WT, D2$^{-/-}$, ATRX$^{-/o}$, and D2AX$^{-/-}$ cells were treated with 50, 10 or 150 μM HU, and assayed for colony forming ability after 10 days.

For both (A) & (B), only colonies containing more than 50 cells were considered. The percentage of colony forming cells was normalized to the plating efficiency of the respective cell line in untreated conditions as 100% survival.

***FANCD2 and ATRX act in concert to promote cellular resistance to HU***

Recent findings from Chen et. al., 2016 shows that FANCD2 promotes cellular resistance to HU-mediated replication stress (Chen et al., 2016). Similarly, ATRX was previously reported to promote cellular HU resistance (Clynes et al., 2014; Leung et al., 2013). To test if ATRX and FANCD2 act in concert to perform this function, WT, $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells were plated and left either untreated or treated with increasing concentrations of HU. Colony formation of each cell line was determined after 12-14 days. Strikingly, we observed that the $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells were all considerably and equally sensitive to HU compared to the WT cells (Fig. 3.3B, 150uM HU: WT cells, 80% survival; $D2^{-/-}$ cells, 41.7% survival; $ATRX^{-/o}$ cells, 52.6 % survival; $D2AX^{-/-}$ cells, 45.4% survival). Thus, FANCD2 and ATRX act within the same pathway to mediate cellular HU resistance.

***ATRX cooperates with FANCD2 to promote replication fork restart.***

Previous findings from our laboratory showed that FANCD2 functions to mediate the restart of APH-stalled replication forks (Chaudhury et al., 2013; Raghunandan et al., 2015a; Yeo et al., 2014). Similarly, studies from other laboratories showed that ATRX promotes the restart of HU-stalled replication forks (Clynes et al., 2014; Leung et al., 2013). Thus, we asked if FANCD2 and ATRX act in concert to fulfill this role. We monitored the replication events in WT, $D2^{-/-}$, $ATRX^{-/-}$ and $AXD2^{-/-}$ cells using a dual-labeling DNA fiber assay, as described before (Chaudhury et al., 2013; Davies et al., 2007; Raghunandan et al., 2015a). Replication tracts were first labeled with DigU (red label) for 25 min, then either kept untreated or treated with HU for 5h to cause replication

fork arrest, followed by second labeling with EdU (green label) for 40 min. We found that compared to the WT cells, the proportion of replication forks competent for restart was reduced significantly and equally in $D2^{-/-}$ and $ATRX^{-/-}$ cells (Fig.3.4A, WT cells: 84.4%; $D2^{-/-}$ cells: 37.6%; $ATRX^{-/-}$ cells: 41.6%). In parallel, the proportion of newly originated replication tracts increased significantly and equally (Fig. 3.4B, ~2-fold) in $D2^{-/-}$ and $ATRX^{-/-}$ cells compared to the WT cells. Somewhat unexpectedly, the $D2AX^{-/-}$ cells – compared to the $D2^{-/-}$ cells - showed a milder defect in replication fork restart (Fig. 3.4A, $AXD2^{-/-}$: 48.2%), accompanied by a moderate, but significant increase in the proportion of newly originated replication tracts (Fig. 3.4B, 1.5 fold). To confirm these results in a different genetic background, we repeated our experiments in FA-D2-deficient patient fibroblasts (PD20) that were complemented with either empty vector or wild type FANCD2 (PD20+$FANCD2^{WT}$). To generate WT, FANCD2-deficient, ATRX-deficient and FANCD2/ATRX-double deficient cells, we treated the PD20+$FANCD2^{WT}$ or PD20 cells with either control siRNA or ATRX siRNA, followed by the dual-label DNA fiber analysis. In agreement with our findings in the knockout cell lines, we found that compared to the WT cells, the proportion of replication forks competent for restart was reduced significantly and equally in FANCD2-deficient and ATRX-deficient (Fig.3.4C, WT: 82.4%; FANCD2-deficient: 40%; ATRX-deficient: 40.67%), accompanied with a similar increase in the number of newly fired replication origins that fire (Fig. 3.4, ~3 fold). Similar to $D2AX^{-/-}$ cells, FANCD2/ATRX-double deficient cells compared to the FANCD2-deficient cells - showed a milder defect in replication fork restart (Fig. 3.4A, FANCD2/ATRX-double deficient: 56.6%), accompanied by a

moderate increase in the proportion of newly originated replication tracts (Fig. 3.4B, 2.5 fold).

**Figure 3.4**

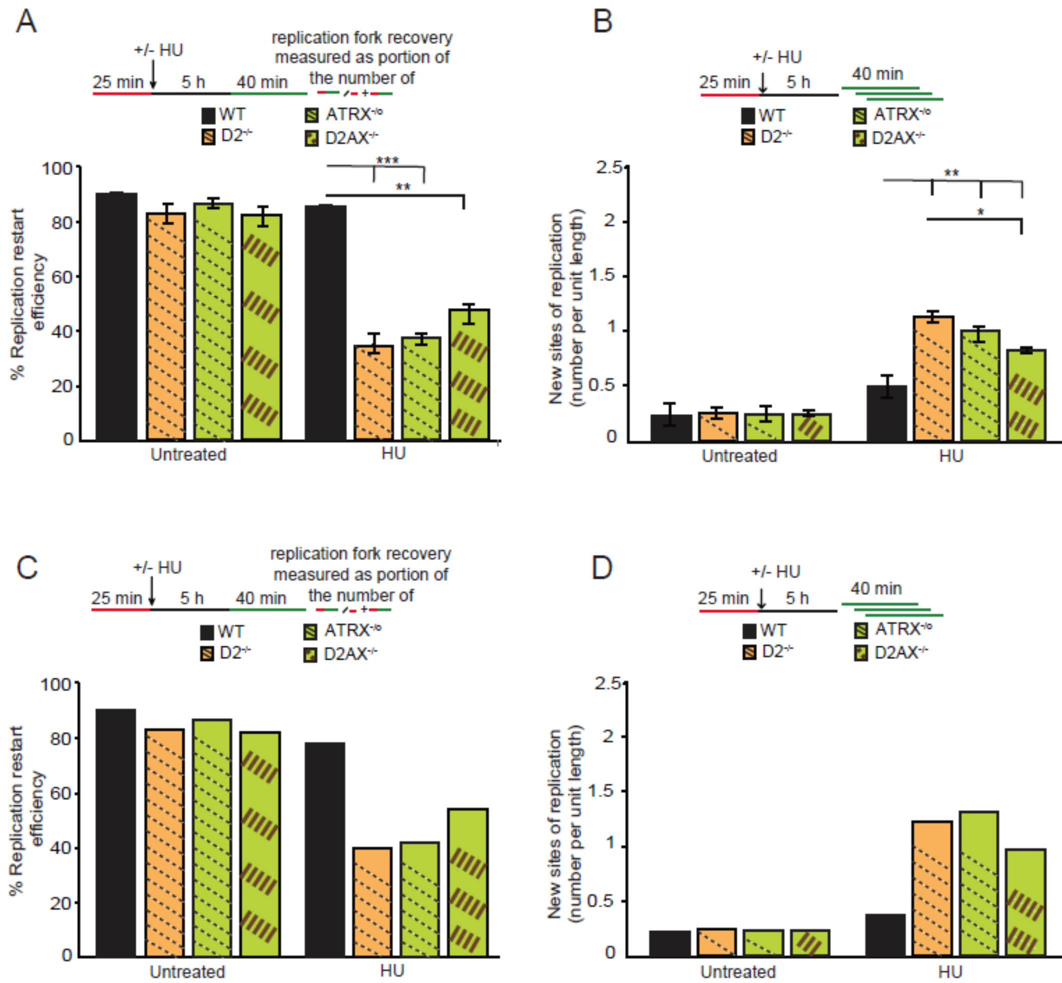


**Figure 3.4: FANCD2 cooperates with ATRX to mediate replication fork restart**

(**A-B**) FANCD2- and ATRX- null cells show replication restart defects after HU treatment. (**A**) Replication fork restart efficiencies were compared between WT, D2$^{-/-}$, ATRX$^{-/o}$, and D2AX$^{-/-}$ cells. (**B**) The number of new sites of replication originating during the 40 min recovery period after HU treatment was compared between WT, D2$^{-/-}$, ATRX$^{-/o}$, and D2AX$^{-/-}$ cells. (**C-D**) Generated cell types: Wild type (PD20+FANCD2, siControl), FANCD2-deficient (PD20, siControl), ATRX-deficient (PD20+FANCD2, siATRX) and FANCD2/ATRX double-deficient (PD20, siATRX). FANCD2- and ATRX- deficient

cells show replication restart defects after HU treatment. (**C**)Replication fork restart efficiencies after HU treatment were compared between the four cell types described. (**D**) The number of new replication sites originating during EdU labeling after HU treatment was compared between the four cell types described in (C)

*NOTE (for all DNA fiber assays):* *Replication tracts are first labeled with DigU label for 25 min, treated without or with 4 mM HU for 5 hours to cause replication fork arrest, followed by a second labeling with EdU label for 40 min. Replication restart efficiency was measured as the number of restarted replication forks after APH-mediated fork stalling (DigU-EdU tracts), compared with the total number of DigU-labeled tracts (DigU + DigU-EdU). New origins of replication were measured as the number of EdU-only tracts per unit length.*

These results indicate that FANCD2 and ATRX cooperate to promote the restart of HU-stalled replication forks and suppressing firing of new origins. Moreover, they indicate that cells doubly deficient for FANCD2 and ATRX may activate an alternative path towards replication fork restart.

***The ATRX-interactor DAXX cooperates with FANCD2 to promote replication fork restart.***

ATRX forms a constitutive complex with DAXX to act as a histone H3.3 chaperone (Drané et al., 2010; Lewis et al., 2010). We asked if DAXX – like ATRX – promotes the FANCD2-dependent restart of HU-stalled replication forks. To generate wild type, FANCD2-, DAXX-, or FANCD2/DAXX double-deficient cells, we treated PD20+ $FANCD2^{WT}$ or PD20 cells with control siRNA or DAXX siRNA (Figure 3.5A). DNA fiber analysis after HU mediated replication fork stalling revealed that compared to the WT cells, the proportion of replication forks competent for restart was reduced significantly and equally in FANCD2-deficient and DAXX-deficient cells (Fig 3.5B,

WT: 82.1%; FANCD2-deficient: 44.8%; DAXX-deficient: 38.1%), accompanied with a similar increase in the number of newly fired replication origins (Fig.3.4, ~3 fold). Similar to D2AX$^{-/-}$ cells, FANCD2/ATRX-double deficient cells compared to the FANCD2-deficient cells - showed a milder defect in replication fork restart (Fig. 3.4A, FANCD2/ATRX-double deficient: 56.6%), accompanied by a moderate, but significant increase in the proportion of newly originated replication tracts (Fig. 3.5B and C, new origin firing, 1.5 fold). These results indicate that both subunits of the ATRX/DAXX complex cooperate with FANCD2 to mediate the restart of HU-stalled replication forks while suppressing the firing of new origins. This in turn suggests that the histone H3.3 deposition function of the ATRX/DAXX complex may be crucial for replication fork restart. Moreover, these results support a model where a cellular deficiency in both FANCD2 and the ATRX/DAXX complex triggers a backup path towards replication fork restart.

**Figure 3.5**

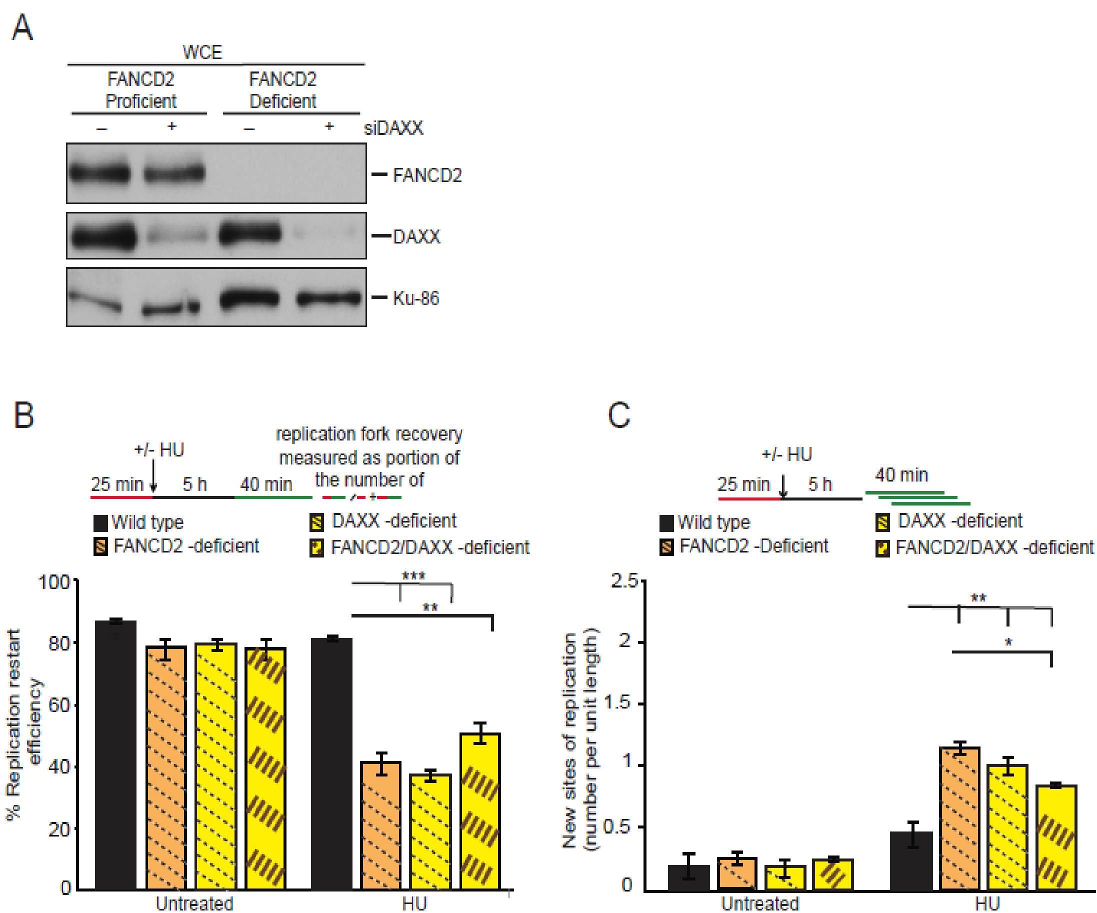


**Figure 3.5: FANCD2 cooperates with H3.3 chaperone DAXX to mediate replication fork restart**

(**A**) WCEs showing the efficiency of siRNA-mediated DAXX knockdown in FANCD2-proficient or -deficient cells. Generated cell types: Wild type (PD20+FANCD2, siControl), FANCD2-deficient (PD20, siControl), DAXX-deficient (PD20+FANCD2, siDAXX) and FANCD2/DAXX double-deficient (PD20, siDAXX). Ku-86, loading control. (**B-C**) FANCD2- and DAXX- deficient cells shows replication restart defects after HU treatment. (**B**) Replication fork restart efficiencies after HU treatment were compared between the four cell types described in (A). (**C**) The number of new replication sites originating during EdU labeling after HU treatment was compared between the four cell types described in (A).

***Histone chaperone activity of the non-ubiquitinated FANCD2 isoform is required to mediate replication fork restart.***

Previous studies showed that FANCD2 has histone H3 chaperone activity, albeit it remains unclear, which of the histone H3 variants is actually deposited by FANCD2. These studies also showed that FANCD2's histone chaperone activity is required to promote cellular resistance to DNA ICLs. Inspired by our findings above suggesting that replication fork restart requires the ATRX/DAXX histone H3.3 chaperone activity (Fig 3.5), we asked if FANCD2's own histone chaperone activity is also required for mediating the restart of HU-stalled replication forks. We took advantage of a recently described patient-derived FANCD2 missense mutant, $FANCD2^{L231R}$, that lacks histone H3 deposition activity (Sato et al., 2012, 2014). We reconstituted the $D2^{-/-}$ cells with $FANCD2^{WT}$ or $FANCD2^{L231R}$ (Fig 3.6A). Importantly, since $FANCD2^{L231R}$ is known to exhibit reduced chromatin binding activity (Sato et al., 2014), we first compared FANCD2 chromatin binding of cells that expressed normal, WT-like levels of $FANCD2^{L231R}$ ($D2^{-/-}$ + $FANCD2^{L231R_NOR}$) or cells that overexpressed $FANCD2^{L231R}$ ($D2^{-/-}$ + $FANCD2^{L231R_HI}$) to that of cells reconstituted with the WT FANCD2 ($D2^{-/-}$ + $FANCD2^{WT}$). Compared to the $D2^{-/-}$+ $FANCD2^{WT}$ cells, $D2^{-/-}$ + $FANCD2^{L231R_NOR}$ showed a significantly reduced chromatin binding. However, the overexpression of the mutant protein in the $D2^{-/-}$ + $FANCD2^{L231R_HI}$ cells was able to compensate for the weaker chromatin affinity of the $FANCD2^{L231R}$, resulting in equal chromatin recruitment compared to the $FANCD2^{WT}$ protein.

To test whether FANCD2's histone chaperone activity promotes replication fork restart, we performed the DNA fiber assay using WT cells and $D2^{-/-}$ cells complimented with either empty vector, $FANCD2^{WT}$ or $FANCD2^{L231R_HI}$. In agreement with our previous findings, the $D2^{-/-}$ cells show a significant reduction in replication fork restart efficiency after HU treatment (Fig. 3.6C, $D2^{-/-}$: 49.3%). This defect was relieved in $D2^{-/-}$ cells expressing $FANCD2^{WT}$ (Fig. 3.6C, $D2^{-/-}$ + $FANCD2^{WT}$: 78.8%), but not in cells expressing the $FANCD2^{L231R_HI}$ mutant (Fig. 3.6C, $D2^{-/-}$ + $FANCD2^{L231R}$: 54.4%). Simultaneously, the significant increase in new origin firing observed in $D2^{-/-}$ cells (Fig. 3.6D; ~2-fold increase compared to $D2^{-/-}$ + $FANCD2^{WT}$) was suppressed in the $D2^{-/-}$ + $FANCD2^{WT}$ cells, but not in the $D2^{-/-}$ + $FANCD2^{L231R_HI}$ cells (Fig. 3.6D; ~2-fold increase compared to $D2^{-/-}$ + $FANCD2^{WT}$).

**Figure 3.6**

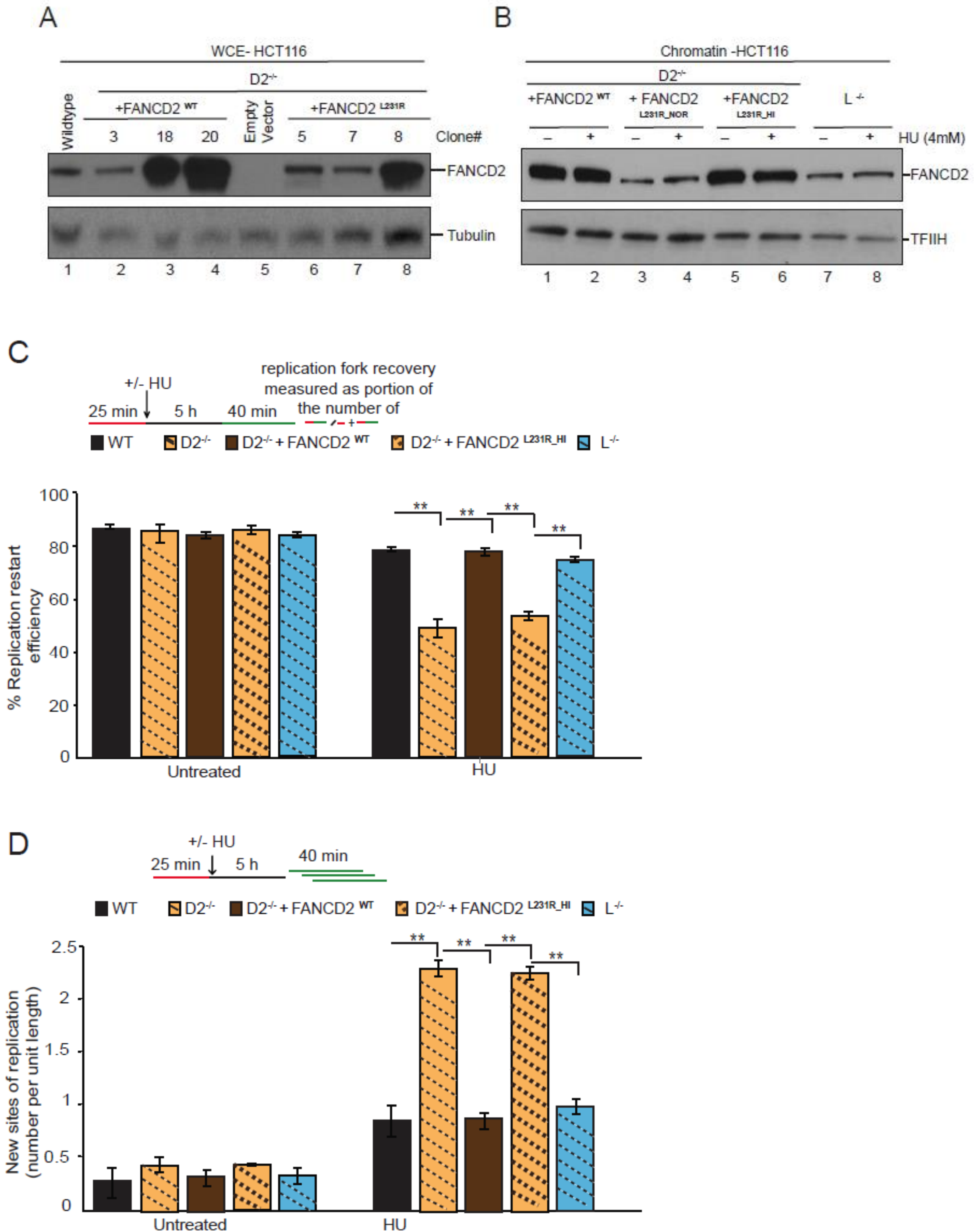


**Figure 3.6: FANCD2's histone H3 chaperone activity is required to mediate replication fork restart**

(**A**) WCEs extracts examining FANCD2 levels in the following HCT116 derived cell

lines: WT or $D2^{-/-}$ cells complemented with either $FANCD2^{WT}$ (clone # 3, 18 20; lanes 2-4) or the histone chaperone dead mutant $FANCD2^{L231R}$ (clone # 5, 7, 8; lanes 6-8). Tubulin-loading control. (**B**) $D2^{-/-}$ cells complimented with either $FANCD2^{WT}$ (lanes 1-2) or $FANCD2^{L231R}$ mutant ($FANCD2^{L231R_NOR}$, lanes 3-4; $FANCD2^{L231R_HI}$, lanes 5-6); and $L^{-/-}$ cells (lanes 7-8) were either untreated or treated with HU, and chromatin fractions from the cells were analyzed for the presence of FANCD2. TFIIH: loading control. (**C** and **D**) $FANCD2^{L231R_HI}$ shows replication restart defects after HU treatment. (**C**)Replication fork restart efficiencies after HU treatment were compared between WT, $L^{-/-}$, or $D2^{-/-}$ cells complemented with empty vector, $FAND2^{WT}$ or $FANCD2^{L231R_HI}$. (**D**) The number of new replication sites originating during EdU labeling after HU treatment was compared between the four cell types described in (C).

It was previously shown that the $FANCD2^{L231R}$ mutant protein cannot get monoubiquitinated (Sato et al., 2014), raising the possibility that it is this modification defect that blocks the $FANCD2^{L231R}$ mutant from promoting fork recovery. However we previously presented evidence that monoubiquitination of FANCD2 is not required for replication fork restart (Raghunandan et al., 2015). In agreement with these findings, we observed that HCT116 cells deficient in the FA core complex E3 ubiquitin ligase subunit, FANCL ($L^{-/-}$ cells) were completely proficient for replication fork restart (Fig3.6B, $L^{-/-}$: 78.6%), and the suppression of new origin firing (Figure 3.6C, $L^{-/-}$: 0.8-fold) in the same experiment. Notably, the $L^{-/-}$ cells exhibited significantly less chromatin-bound WT FANCD2 than the $D2^{-/-}$+ $FANCD2^{L231R_HI}$ cells (Fig. 3.6B, compare lanes 2 and 3, or 5 and 6). Thus, although the nonubiquitinated $FANCD2^{L231R_HI}$ has a higher chromatin binding capability than the nonubiquitinated WT FANCD2 in $L^{-/-}$ cells, it fails to promote replication fork restart. We conclude that the histone H3 chaperone activity of

FANCD2, and not FANCD2 monoubiquitination, is required to mediate the restart of stalled replication forks.

***FANCD2 and ATRX cooperate with MRE11 to promote replication fork restart.***

Both FANCD2 and ATRX have been independently shown to interact with the MRE11 nuclease (Clynes et al., 2014; Leung et al., 2013; Roques et al., 2009). Moreover, it is known that MRE11 is one of many HR factors that are required to promote replication fork restart (Bryant et al., 2009; Raghunandan et al., 2015b; Somyajit et al., 2015; Yeo et al., 2014). To test if ATRX and FANCD2 work in concert with MRE11 to promote HR-mediated fork recovery, we made use of a small molecule inhibitor of the MRE11 exonuclease activity, mirin, which completely blocks MRE11's ability to promote fork restart (Chaudhury et al., 2014). WT, $D2^{-/-}$, $ATRX^{-/o}$ and $AXD2^{-/-}$ cells were either untreated or pre-incubated with 50µM mirin for one hour. The cells were subsequently treated with 4 mM HU for 5 hours and subjected to DNA fiber analysis. As expected, mirin-treated WT cells were unable to restart HU-stalled replication forks, compared to the untreated WT cells (Chaudhury et al., 2014).

**Figure 3.7**

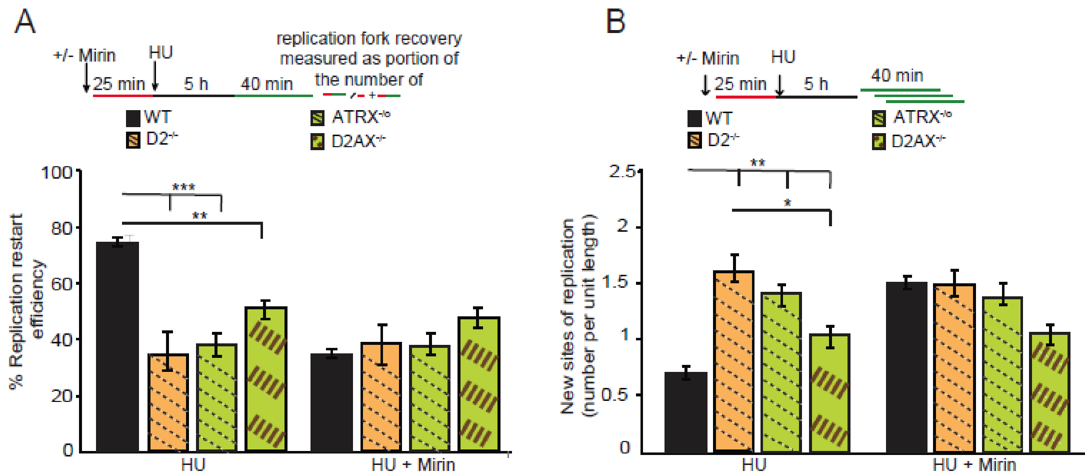


**Figure 3.7: FANCD2 and ATRX cooperate with MRE11 to promote replication fork restart** (**A**) Replication fork restart efficiencies were compared between WT, D2$^{-/-}$, ATRX$^{-/o}$, and D2AX$^{-/-}$ cells after HU treatment. Cells were either untreated or pre-incubated with 50µM mirin for one hour. For mirin treated samples all subsequent steps for the DNA fiber assay were carried out in the presence of mirin. (**B**) The number of new sites of replication originating during the 40 min recovery period after HU treatment was compared between WT, D2$^{-/-}$, ATRX$^{-/o}$, and D2AX$^{-/-}$ cells.

As expected based on our findings above (Fig 3.4A), the D2$^{-/-}$, ATRX$^{-/o}$ and D2AX$^{-/-}$ cells showed a significant reduction in their replication restart efficiency after HU treatment (Fig 3.7A WT: 76.5%; D2$^{-/-}$: 36.7%; ATRX$^{-/o}$: 37.6%, D2AX$^{-/-}$: 51.9%). Strikingly, this fork restart defect was (a) not exacerbated upon mirin treatment, and (b) comparable to that observed in mirin-treated WT cells (Fig 3.7B WT: 36.25%; D2$^{-/-}$: 40.8%; ATRX$^{-/o}$: 37.9%, D2AX$^{-/-}$: 48.1%). In parallel, mirin caused an upregulation of new origin firing by >2 fold in the HU-treated WT cells, but did not further increase new origin firing in the HU-treated D2$^{-/-}$, ATRX$^{-/o}$ and D2AX$^{-/-}$ cells. These results suggest

that the protein complex composed of FANCD2, ATRX/DAXX and MRE11 acts as a functional entity to mediate the HR-dependent restart of stalled replication forks.

#### ***FANCD2 and ATRX cooperate to recruit the HR factor CtIP to stalled replication forks***

We previously showed that FANCD2 recruits CtIP to chromatin and into HU-induced DNA repair foci at stalled replication forks (Buis et al., 2012; Yeo et al., 2014). To test if ATRX cooperates with FANCD2 to promote CtIP chromatin recruitment, we analyzed CtIP foci formation in untreated or HU-treated WT, $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells. As expected, the WT cells showed an increase in HU-triggered CtIP foci formation (Fig 3.8A, cells with >5 CtIP foci, untreated: 10%, HU-treated: 56.3%). In contrast, the $D2^{-/-}$ cells showed severely reduced CtIP foci formation in HU-treated conditions (Fig.3.8A (HU) WT cells: 56.3%, $D2^{-/-}$cells: 3.5%). Importantly, the $ATRX^{-/o}$ cells showed a slightly less severe yet highly significant defect in CtIP foci formation (Fig. 3.10A, (HU) $ATRX^{-/o}$: 15.2%). Moreover, the $D2AX^{-/-}$ cells closely mirrored the CtIP foci formation defect seen in the $D2^{-/-}$ cells (Fig. 3.8A, $D2AX^{-/-}$cells: 4.1%), suggesting that FANCD2 and ATRX act in concert to recruit CtIP to sites of stalled replication forks. We attempted to confirm our finding in an independently generated ATRX null cell line (${}^{rAAV}ATRX^{-/o}$) that was generated using rAAV-mediated gene targeting as previously described (Napier et al., 2015). The ${}^{rAAV}ATRX^{-/o}$ cells showed a severe defect in HU-triggered CtIP foci formation, similar to the $ATRX^{-/-}$ cells described above. (Fig. 3.8B, (HU) WT: 50%, ${}^{rAAV}ATRX^{-/o}$: 20%). These finding suggest that both ATRX and FANCD2 are required to recruit CtIP to sites of stalled replication forks, likely to promote HR-mediated restart events.

**Figure 3.8**

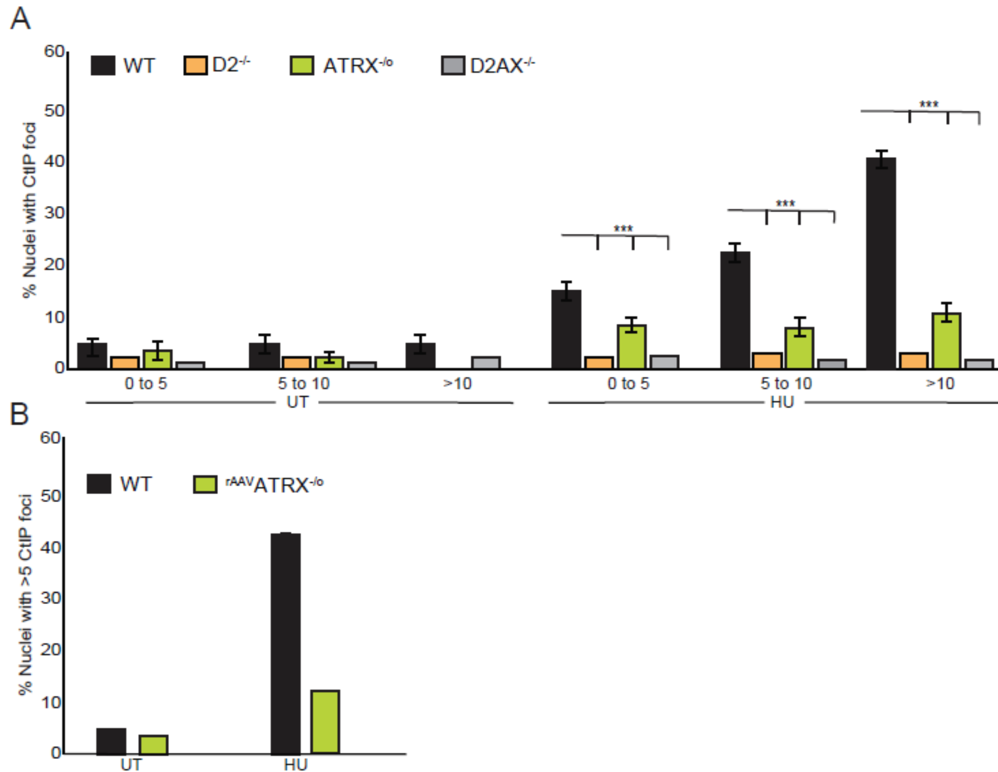


**Figure 3.8: FANCD2 and ATRX cooperate to recruit CtIP to sites of prolonged HU damage to promote HR**

(**A**) ATRX and FANCD2 cooperate for CtIP foci formation WT, $D2^{-/-}$, $ATRX^{-/o}$, and $D2AX^{-/-}$ cells were either untreated or treated with 2mM HU for 20 hours. Nuclear CtIP foci formation was analyzed by fluorescence microscopy. CtIP foci formation was quantified for distribution into % of nuclei with 0-5, 5-10 or >10 foci per nucleus. (**B**) CtIP depends on ATRX for foci formation. WT and $^{rAAV}ATRX^{-/o}$ cells were untreated or treated with 2mM HU for 20 hours. Nuclei with > 5 foci were considered positive for CtIP foci formation.

The finding that ATRX cooperated with FANCD2 to promote HU-induced CtIP foci formation, combined with our earlier observation that ATRX stabilizes FANCD2 protein levels, led us to speculate that ATRX may in fact regulate FANCD2's own recruitment

into HU-induced chromatin foci. To test this, we compared FANCD2 foci formation in untreated and HU-treated WT and $ATRX^{-/o}$ cells. As expected (Castella et al., 2015; Garcia-Higuera et al., 2001; Yeo et al., 2014), FANCD2 re-localization into DNA repair foci increased significantly post HU treatment in WT cells (Fig 3.8C, cells with >5 FANCD2 foci UT: 10%, HU: 47%). Interestingly, the $ATRX^{-/o}$ cells showed a mild reduction in the HU-triggered FANCD2 foci formation (Fig 3.8C, cells with >5 FANCD2 foci, WT: 47%, $ATRX^{-/o}$: 33%), suggesting that re-localization of FANCD2 to HU-stalled replication forks is partially dependent on ATRX. Next, we asked if FANCD2 also reciprocally regulates ATRX foci formation following cellular HU treatment. To this end, we monitored ATRX foci formation in untreated or HU-treated WT and $D2^{-/-}$ cells. Similar to the findings by Leung et. al., 2013, the percentage of WT cells containing ATRX foci did not increase significantly after HU treatment (UT: 55%, HU: 62%). Intriguingly however, we observed that following HU treatment, the D2-/- cells exhibited significantly increased ATRX foci numbers compared to the WT cells (Fig 3.8D, (HU) % cells with >10 ATRX foci: WT, 3.09%; $D2^{-/-}$, 19.9%). We further confirmed these findings in a second set of FA patient-derived FANCD2-deficient cells (PD20) and their complemented counterpart ($PD20+FANCD2^{WT}$). We monitored ATRX foci formation in untreated or HU-treated FANCD2-proficient and -deficient cells. Similar to our findings in the HCT116 $D2^{-/-}$ cells versus WT cells (Fig 3.8D), we found that the PD20 cells exhibited a strongly increased HU-induced ATRX foci formation compared to the PD20+D2 cells (Fig 3.8E, (HU) % cells with >10 ATRX foci: $PD20+FANCD2^{WT}$: 1%, PD20: 11%). Together, these findings indicate that FANCD2 cooperates with – and

partly depends on – ATRX to promote DNA repair at stalled replication forks. Furthermore, our data suggest that ATRX accumulates at sites of HU-stalled replication forks in FANCD2-deficient cells, possibly in an attempt to compensate for the absence of FANCD2 during the cellular HU response.

**Figure 3.9**

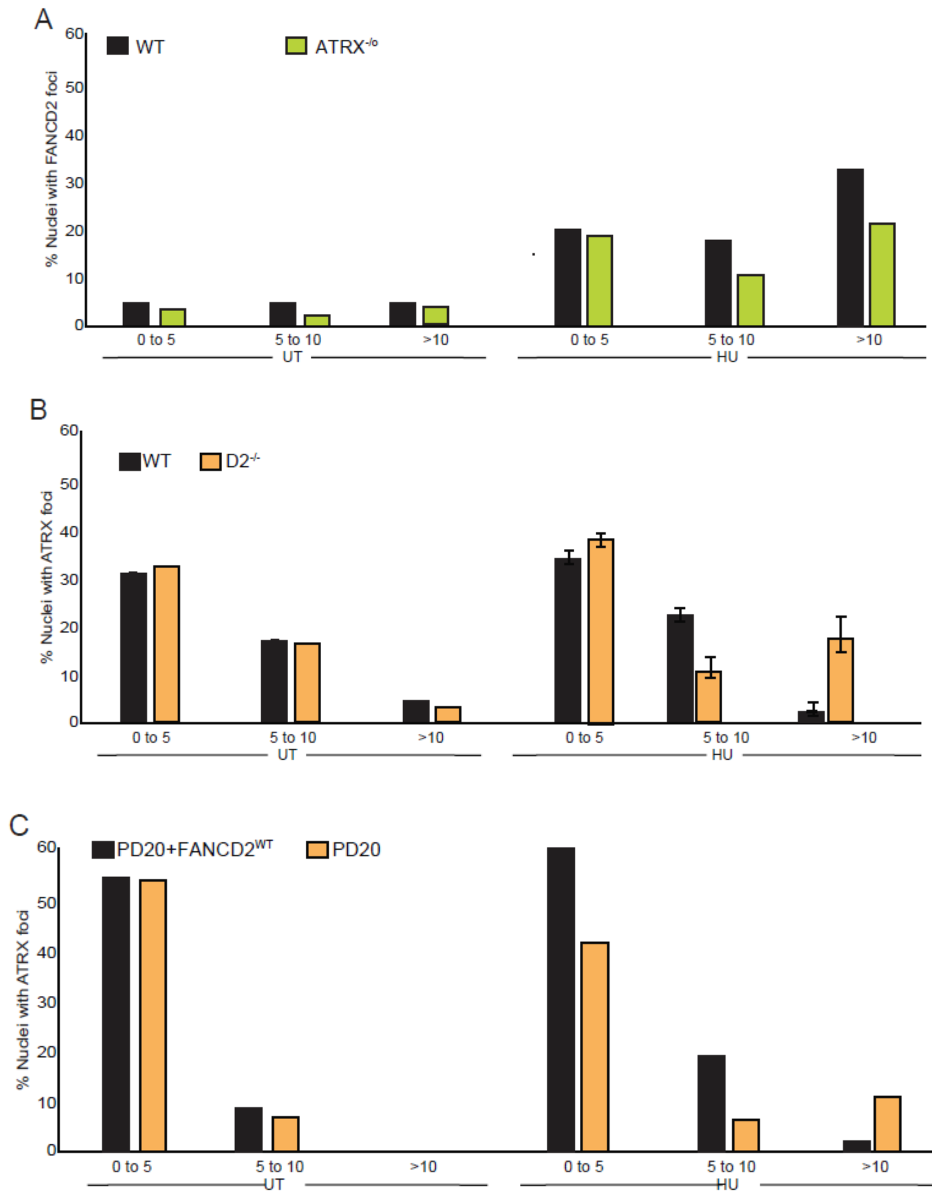

**Figure 3.9: FANCD2 and ATRX show altered foci formation in $ATRX^{-/-}$ and $D2^{-/-}$ cells respectively**

(**A**) FANCD2 is partially dependent on ATRX for foci formation. WT and $ATRX^{-/o}$ cells were untreated or treated with 2mM HU for 20 hours. Nuclear FANCD2 foci formation was analyzed by fluorescence microscopy. (**B and C**) ATRX accumulates in DNA repair foci in the absence of FANCD2. (**B**) WT and $D2^{-/-}$ cells were untreated or treated with 2mM HU for 20 hours. Nuclear ATRX foci formation was analyzed by fluorescence microscopy. (**C**) Same as (B), in PD20+FANCD2WT and PD20 cells. Nuclei positive for FANCD2 and ATRX foci formation were quantified for distribution into % of nuclei with 0-5, 5-10 or >10 foci per nucleus.

## Discussion

In this study, we identify a novel physical and functional interaction between FANCD2 and the ATRX/DAXX histone H3.3 chaperone complex, using newly generated isogenic knockout cell lines. Our results indicate that ATRX/DAXX and FANCD2 act in concert to promote the restart of HU-stalled DNA replication forks. Moreover, they suggest that this role of ATRX/DAXX and FANCD2 involves a histone chaperone activity that is crucial for the recruitment of HR factors to promote fork restart. To our knowledge, this is the first study that utilizes human isogenic D2-, ATRX- and D2AX double-null cell lines to analyze functional cooperation between the FA and ATRX pathways. The successful generation of viable $D2^{-/-}$, $ATRX^{-/o}$ and $D2AX^{-/-}$ cells demonstrates that neither of the two genes are essential in human somatic cells. However, this observation does not rule out the possibility that these genes may have important roles during normal development; in fact, developmental functions seem likely considering the development abnormalities observed in both FA and ATRX patients. Though not essential, both FANCD2 and ATRX contribute to cellular proliferation and

FANCD2 appears to be more crucial for this feature than ATRX. Importantly, the fact that D2AX$^{-/-}$ cells proliferate significantly more slowly than either of the singly null cells demonstrates that FANCD2 and ATRX have some partially non-overlapping cellular roles. In support of this idea, patients suffering from ATRX or FA syndrome exhibit several distinct, non-overlapping phenotypes. For example, ATRX patients present with mental retardation and thalassemia, whereas FA patients are clinically characterized by bone marrow failure and aplastic anemia. This is further supported by our observation that ATRX is completely dispensable for the FANCD2-dependent cellular DNA ICL resistance, in agreement with previous studies (Clynes et al., 2014; Leung et al., 2013).

That said, a literature search revealed that some intriguing phenotypical similarities have been described for the ATRX and FA syndromes. Both syndromes are associated with congenital skeletal abnormalities including brachydactyly, genital anomalies, short stature, epicanthal folds, microcephaly and an increased cancer predisposition. (Ceccaldi et al., 2016a; Kalb et al., 2007; Solomon et al., 2013; Stevenson, 1993).

On the cellular level, deficiencies in ATRX or FANCD2 expression have been associated with spontaneously elevated levels of chromosome instability that manifest as an accumulation of DNA DSBs, increased micronuclei formation and elevated telomere fusions (Baumann et al., 2010; Chan et al., 2009; Huh et al., 2012, 2016; Joksic et al., 2012; Lovejoy et al., 2012; Naim and Rosselli, 2009; Reliene et al., 2010a, 2010b; Ritchie et al., 2008; Watson et al., 2013; Wong et al., 2010), hinting that the two proteins cooperate to protect genomic stability, thus preventing carcinogenesis.

In strong support of this idea, our findings indicate that FANCD2 and ATRX can participate in a common protein complex that likely also contains DAXX and the MRE11 nuclease. It should be pointed out that the interaction between ATRX and FANCD2 appears to be relatively weak; however, this is typical for all FANCD2 protein interactions that have been identified so far. For example, the interactions between FANCD2 and BLM, CtIP, FAN1 or even FANCD2's heterodimerization partner, FANCI, have all been shown to be weak and likely rather transient (Chaudhury et al., 2013, 2014; Hussain et al., 2004; Murina et al., 2014; Naim and Rosselli, 2009; Pichierri et al., 2004; Smogorzewska et al., 2007; Unno et al., 2014; Yeo et al., 2014). In fact, protein complex size fractionation experiments from our laboratory (unpublished data) and others (Zhi et al., 2010) indicate that FANCD2 can participate in quite a few different protein complexes that may additionally only form transiently during S-phase. Somewhat puzzlingly, this model is not quite supported by our finding that ATRX prevents FANCD2 from proteosomal degradation, which rather suggests that the majority of FANCD2 molecules should exist in a robust and constitutive complex with ATRX. However, a previous study from the Masson laboratory demonstrated that MRE11- albeit not being a strong FANCD2 interactor either – is absolutely crucial to promote FANCD2 protein stability (Roques et al., 2009). Interestingly MRE11 can interact with both FANCD2 and ATRX (Clynes et al., 2014; Leung et al., 2013; Roques et al.,2009), hinting that ATRX may only indirectly protect FANCD2 from proteolytic degradation by stabilizing the FANCD2-MRE11 interaction.

In agreement with the idea of a physical and functional protein complex composed of ATRX/DAXX, MRE11 and FANCD2, our findings demonstrate that all four proteins act in one pathway to mediate the HR-dependent restart of HU-stalled replication forks, which simultaneously suppresses the firing of new replication origins. Several additional HR factors are known to promote replication fork restart and recent findings from our laboratory showed that several of these factors including CtIP are recruited in a FANCD2- and MRE11-dependent manner (Buis et al., 2012; Murina et al., 2014; Unno et al., 2014; Yeo et al., 2014). Our finding that ATRX works in concert with FANCD2 to promote CtIP re-localization to nuclear DNA repair foci, in response to cellular HU treatment, strengthens a model where an ATRX/DAXX/MRE11/FANCD2 protein super-complex assembles in a stepwise manner at stalled replication forks to recruit additional HR factors to ultimately promote fork recovery (Fig. 3.10). It is interesting in this regard that foci formation for FANCD2 foci appears to be minimally affected in absence of ATRX, but for CtIP, depends heavily on ATRX and FANCD2. This suggests to us that ATRX and FANCD2 are recruited to sites of stalled replication forks largely independently of one another, but then act as a complex to recruit CtIP.

On the other hand, our finding that HU-induced ATRX foci formation increases in $D2^{-/-}$ cells compared to WT cells suggests that ATRX is positioned upstream of FANCD2 and ATRX accumulates on chromatin in $D2^{-/-}$ cells in an attempt to partially compensate for the absence of FANCD2. A similar relationship has been reported for FANCD2 and its downstream target, BRCA2 (Hussain et al., 2004; Wang et al., 2004): BRCA2-deficient cells exhibit a large increase in FANCD2 foci formation during DNA

ICL repair, presumably because cells are trying to compensate for the absence of BRCA2. (Kais et al., 2016). Future studies will reveal if ATRX is also required for the recruitment of other, FANCD2-dependent HR proteins such as BLM, FAN1 or RAD51 (Chaudhury et al., 2013, 2014; MacKay et al., 2010; Sato et al., 2016). We predict that cells deficient in these factors may exhibit an increased HU-triggered ATRX foci formation as well.

The fact that ATRX/DAXX, MRE11 and FANCD2 cooperate to promote HR-mediated replication fork restart and the recruitment of HR factors following fork stalling, strongly suggests that the ATRX/DAXX complex has a hitherto unrecognized role in promoting cellular HR mechanisms. This is a highly unexpected finding since the current literature suggests that ATRX does in fact act as an inhibitor of HR. This prediction is based on findings in ATRX-deficient cancer cells that utilize a different, supposedly HR-mediated mechanism of telomere maintenance named ALT (Alternative Lengthening of Telomeres) (Dilley and Greenberg, 2015). Based on the correlative relationship between ATRX deficiency and hyper-recombinogenic telomeres in ALT cells, ATRX has been predicted to be an active suppressor of HR, at least at the telomeres. Future studies should reveal whether ATRX fulfills separate functions at non telomeric stalled or even collapsed DNA replication forks and at telomeric DNA sequences, possibly by associating with different protein complexes at intra-chromosomal regions versus the telomeric regions.

Additional support for a role for ATRX in genome-wide HR repair stems from its role as an H3.3 chaperone in complex with DAXX. A recent study demonstrated that

H3.3 deposition at sites of DNA DSBs is crucial for NHEJ and HR-mediated DSB repair, indicating that H3.3 variant deposition is generally involved in HR mechanisms throughout the genome (Luijsterburg et al., 2016). It is quite intriguing in this context that FANCD2 was previously shown to have histone H3 chaperone activity itself, although it remains unknown whether FANCD2 has a binding preference for a particular H3 variant such as H3.3 (Sato et al., 2012). Nevertheless, based on its cooperative roles with the ATRX/DAXX complex during fork recovery, it seems highly plausible that FANCD2 is an H3.3-specific chaperone itself. On the other hand, it is possible that FANCD2 has a different histone H3 variant specificity that is required – alongside with ATRX/DAXX-dependent H3.3 deposition – to enable appropriate nucleosome restructuring steps at sites of stalled replication forks.

Remarkably, neither of these two models can account for the perhaps most unusual finding of our study, i.e. that the restart of stalled replication forks, while equally defective in $D2^{-/-}$ and $ATRX^{-/o}$ cells, became actually more efficient in the $D2AX^{-/-}$ cells. While this phenomenon requires future investigations, we would like to point out that we did recently observe a somewhat similar phenotype in cells doubly null for FANCD2 and FANCI, compared to $D2^{-/-}$ cells. We found that depletion of FANCI significantly improved the replication forks restart efficiencies in HU-treated $D2^{-/-}$ cells (Thompson and Yeo et. al., 2017, submitted). One possible explanation for this “improved” phenotype is that FANCD2 and ATRX both still try to promote the commonly used, major HR-mediated fork restart mechanisms, whereas in the absence both proteins, a compensatory/back-up pathway is engaged to promote fork recovery. An obvious

candidate for this backup would be the NHEJ DNA repair machinery that is upregulated in HR-deficient cells (Ceccaldi et al., 2015; Tutt et al., 2001). In fact, NHEJ factors such as DNA-PKcs and Ku have been implicated in the replication stress response in recent years (Miyoshi et al., 2009; Ying et al., 2016). Additional studies are underway to determine which backup pathway is activated to promote replication fork recovery in cells lacing both ATRX and FANCD2.

In summary, we propose a model (Fig. 3.10) wherein the ATRX/DAXX complex form a super-complex with MRE11 and FANCD2 at sites of stalled replication forks to support a histone H3.3 deposition-dependent, HR-mediated mechanism of replication fork restart.

**Figure 3.10**

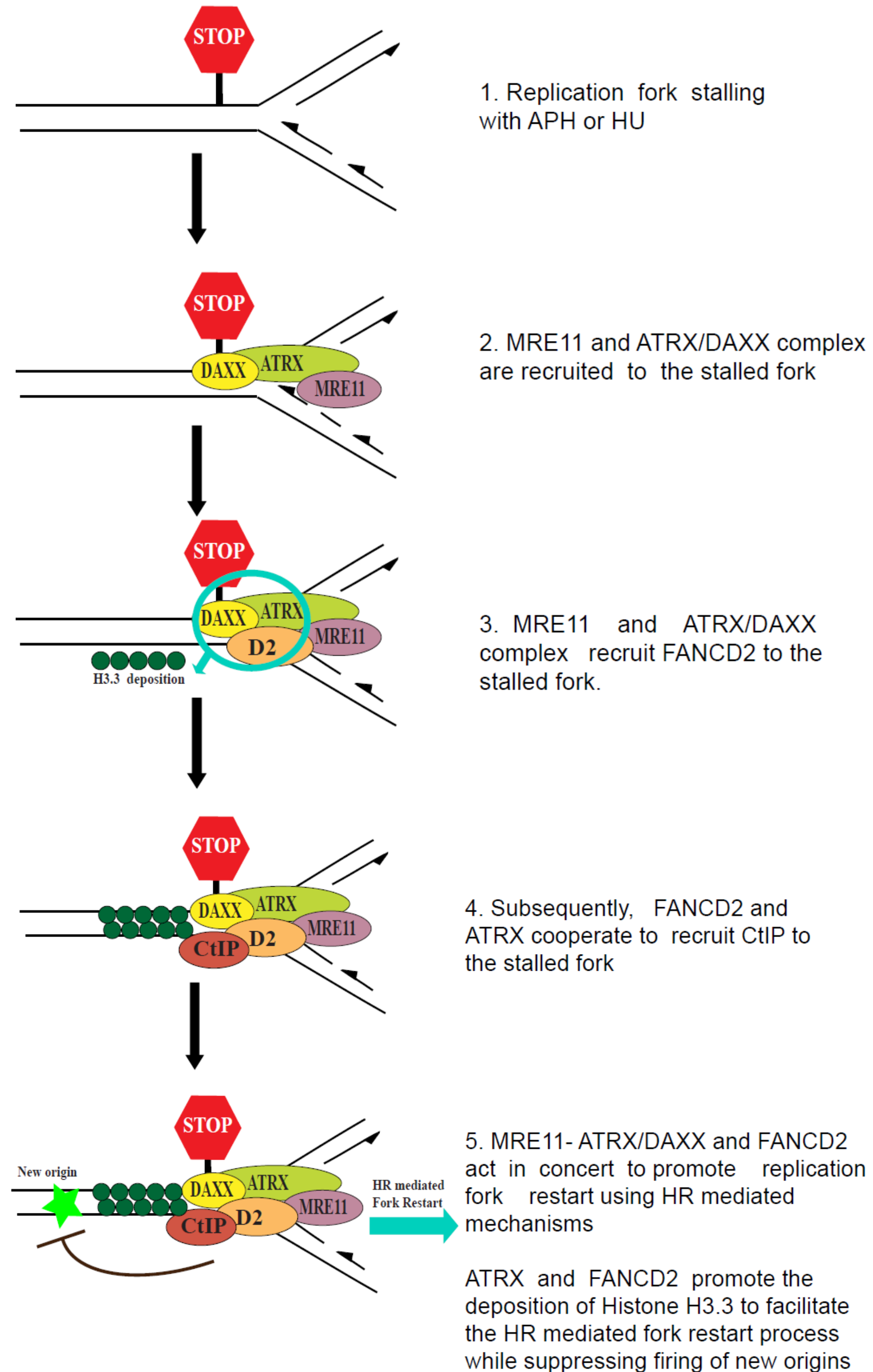


**Figure 3.10: FANCD2 and ATRX promote H3.3 deposition at stalled replication forks and promote HR mediated fork restart**

(Text in figure)

## 3.4 Materials and Methods

***Cell lines and cell culture techniques***

FANCD2 deficient (PD20) and complemented cell lines (PD20+FANCD2) were obtained from the FA Cell Repository at the Oregon Health & Science University. PD20 cells complemented with 3xFLAG FANCD2 were a kind gift from Dr. Tosiyashu Taniguchi at the Fred Hutchinson Research Centre. All cells described above were cultured in DMEM (GIBCO) with 10% FBS and 1% Penicillin-Streptomycin. All HCT116 derived cell lines were cultured in Mc Coy's media (Corning) enriched with 10% FBS, 1% Pencillin-Streptomycin and 1% Glutamine.

***Gene targeting for generating FANCD2-, ATRX- and FANCD2/ATRX- knockouts in HCT116 cells.***

*rAAV gene targeting mediated generation of FANCD2 - null cells*

FANCD2 null HCT116 cells were generated using rAAV (recombinant adeno-associated virus)-mediated gene targeting (Kohli et al., 2004). The detailed method for this cell line will be published elsewhere (Thompson and Yeo et. al.). Briefly, conditional and knock-out rAAV vectors targeting FANCD2 exon 12 were constructed using Golden Gate cloning technology and designed as previously described (Kohli et al., 2004; Oh et al., 2014). The first round of targeting with the conditional vector replaced FANCD2 exon 12 with conditional, floxed allele along with a floxed Neo selection cassette. G418 resistant clones were screened by PCR to confirm targeting, and Cre recombinase was used to remove the Neo selection cassette. The FANCD2 second round of targeting was performed in the FANCD2flox/+ cells with the knock-out rAAV vector to replace exon

12 with a Neo selection cassette. G418 resistant clones were screened by PCR for correct targeting. Cre recombinase was used to remove both the Neo selection cassette and the conditional allele(s) and resulted in viable FANCD2-/- clones.

*CRISPR/Cas9 generation of ATRX- and FANCD2/ATRX- null cells*

ATRX-null cells were generated as previously described (Napier et al., 2015). The same ATRX guide RNA (gRNA) containing CRISPR/Cas9 plasmid (targeting ATRX exon 9) was transfected into the FANCD2- null HCT116 cells using Lipofectamine 3000 (Life Technologies). Two days after transfection the cells were sub-cloned, and individual sub-clones were screened for targeting by amplification of exon 9 and subsequent digestion with SmlI (New England BioLabs, Inc.). Sub-clones that were resistant to digestion with SmlI were TOPO TA cloned (Life Technologies). Sanger sequencing of the TOPO TA clones confirmed targeted insertion of a single nucleotide, that would induce a frameshift in FANCD2, generating a FANCD2/ATRX- null cell line. Western blot analysis further confirmed that these clones were null for FANCD2 and ATRX expression. The information for ATRX gRNA and the primers used for amplification of targeted exon 9 can be found in Napier et. al., 2015.

The generation of FANCD2 -null cells complemented with FANCD2 is described in Thompson and Yeo et. al. Briefly, a human codon optimized FANCD2 cDNA was cloned into Piggyback CAG promoter expression vector using Gateway cloning technology (Invitrogen). G418 clones were screened for FANCD2 expression by Western blot. FANCD2-L231R mutant was generated using site directed mutagenesis kit from Stratagene.

***Immunoprecipitation Assay:***

Nuclear extracts from PD20+3xFLAG FANCD2 cells were generated using a protocol generously shared by Dr. Andrew Deans at St. Vincent's institute of Medical research, Australia. Freshly isolated cells were washed in PBS and nuclei were isolated using CSK buffer (10 mM PIPES pH6.8, 100 mM NaCl, 300 mM sucrose, 3mM MgCl2, 0.1% Triton-X100 and complete protease inhibitor). The isolated nuclei were lysed in Nuclear lysis buffer (10 mM HEPES pH 7.9, 100 mM NaCl, 1.5mM MgCl2, 25% glycerol, 1mM DTT and complete protease inhibitor) using a Dounce homogenizer. The nuclear lysates were treated with 50U/ml Benzonase for 1 hour at 4 degrees, with rotation. The lysates were spun at maximum speed in table top centrifuge at 4 degrees for 30 min, twice. The supernatant obtained is the NE used for downstream IP. NE was precleared with rabbit or mouse IgG and subjected to immunoprecipitation with anti-ATRX or anti-FLAG or IgG antibody in presence of EtBr (10 μg/ml) at 4°C overnight. Next day, 100ul of Sepharose 4B beads (50% slurry) was added to the NE and rotated for 30 min at 4°C. The beads were pelleted from solution, washed in nuclear lysis buffer, boiled in 1× NuPAGE buffer (Invitrogen) and analyzed by SDS–PAGE and western blotting.

***Cell proliferation assay***

Wild type, FANCD2-, ATRX- or FANCD2/ATRX-null cells were plated in 96 well plates based on their plating efficiencies. Wild type cells and ATRX-null cells were plated at 200 cells/well, FANCD2-null cells were plated at 500 cells/well, FANCD2/ATRX-null cells were plated at 1000 cells/well in 96 well plates. Cell growth

was analyzed using MTS assay (Manufacturer's instructions, Promega) performed in triplicate on Days 1, 3 and 5 after seeding.

***MMC and HU sensitivity***

Wild type, FANCD2-, ATRX- or FANCD2/ATRX-null cells were plated in 6 well plates according to plating efficiency. Wild type and ATRX- null cells were plated at 350 cells/well, FANCD2-null cells were plated at 1000 cells/well, and FANCD2/ATRX- null cells were plated at 1200 cells/well in 6-well plates. The next day, media removed and media containing 0, 5, 10, and 15 nM MMC OR 0, 50, 100 and 150 uM HU was added in triplicate to each cell line. Cells were allowed to grow for 10-12. The resulting colonies were then washed in PBS, fixed in 10% Acetic Acid/10% methanol, and stained with crystal violet. Colonies reaching a minimum size of 50 cells were counted and normalized to the average colony number in untreated wells.

***Antibodies***

Commercial antibodies were used against human FANCD2 (Santa Cruz, sc-20022, for WB) (abcam, ab1287- for IFA), ATRX (SC, 15408 for IP and IFA) (Genetex, GTX101310- for WB), Ku86 (Santa-Cruz, sc-5280), FLAG (Sigma M2 monoclonal), DAXX (sc-7152), Tubulin(XX), Cyclin B(sc-245). Antibodies against CtIP have been described previously (Yu X., Baer R. J. Biol. Chem.2000).

***Immunoblotting***

Protein samples were separated on gradient gels and transferred to Immobilon P membranes (Millipore). After blocking in 5% milk, membranes were incubated with primary antibodies overnight. Horseradish peroxidase-conjugated rabbit secondary antibody (Jackson Labs) or mouse secondary antibody (Biorad) were used at dilutions of 1:10000 and 1:3000, respectively. Protein bands were visualized using an ECL Plus system (Amersham).

***Preparation of whole cell extracts (WCEs) from human cells***

For WCEs preparation, cells were washed in PBS, re-suspended in lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 1mM EDTA, 1mM DTT, 0.5mg/ml pefabloc protease inhibitor) and incubated on ice for 20 min. Cell extracts were centrifuged for 5 min at 10,000 rpm, and the supernatant was used for further analysis.

***DNA fiber assay***

We used a previously described DNA fiber protocol (Sugimura K, et al 2007). Moving replication forks were labeled with digoxigenin-dUTPs (DigU) for 25 min and then with EdU-dUTPs (BioU) for 40 min. To allow efficient incorporation of the DigUs, a hypotonic buffer treatment (10 mM HEPES, 30 mM KCl, pH 7.4) preceded the DigU - labeling step. To visualize labeled fibers, cells were mixed with a 10-fold excess of unlabeled cells, fixed and dropped onto slides. After cell lysis, DNA fibers were released and extended by tilting the slides. EdU labelled DNA was conjugated to Biotin using

Click reaction with Biotin Azide. Incorporated dUTPs were visualized by immunofluorescence detection using anti-digoxigenin-Rhodamine (Roche) and streptavidin-Alexa-Fluor-488 (Invitrogen). Images were captured using a Deltavision microscope (Applied Precision) and analyzed using Deltavision softWoRx 5.5 software. All shown DNA fiber results are means of two or three independent experiments (300 DNA fibers/experiment). Error bars represent the standard error of the mean and significance was determined by t-test and Mann-Whitney test. Statistical significance at $P<0.01$, $P<0.010$, and $P<0.0001$ are indicated as *, **, ***, respectively.

***siRNA experiments***

siRNA duplexes were purchased from Dharmacon research (Thermo Scientific, MA, USA). The sequence of ATRX#1 and ATRX#2 siRNA are 5’ GAGGAAACCUUCAAUUGUAUU 3' and 5' GCAGAGAAAUUCCUAAAGAUU 3' respectively. The DAXX siRNA was obtained from Invitrogen (HSS102654). siGENOME non-targeting siRNA from Dharmacon was used as a control. Transfections were performed using DharmaFECT1 transfection reagent according to the manufacturer's protocol.

***Immunofluorescence***

Indirect immunofluorescence was carried out essentially as described (Yeo et al., 2014). Primary antibodies used: FANCD2 (Abcam, ab2187, 1:4000), CtIP (mouse monoclonal, 1:400). Secondary antibodies used: alexa Fluor 594-conjugated goat anti-rabbit (1:1000) and Alexa Fluor 488-conjugated goat anti-mouse (1:1000; Molecular Probes). For statistical analysis of nuclear foci formation, images were taken using a

Leica DM LB2 microscope with a Hamamatsu Orca-ER camera. Quantification of foci was carried out using ImageJ.

# CHAPTER 4

## Discussion and Future Directions

## 4.1 Discussion

Since the identification of FA as an inherited genomic instability disease, the molecular machinery of the FA pathway has been studied intensively in the context of DNA ICL repair. Accumulating evidence strongly supports a model where the FA proteins act in a linear hierarchy during ICL repair. In this model, monoubiquitination of the central FANCD2/FANCI heterodimer by the FA core complex is a key step that is indispensable for the subsequent recruitment of downstream FA pathway members, such as BRCA2, FANCJ and other HR factors to promote nucleolytic ICL removal and repair of the ensuing DNA DSB (Ceccaldi et al., 2016a; Walden and Deans, 2014; Wang, 2007). While this model is widely accepted in the FA research field, it does not account for the fact that FA patients do phenotypically differ from one another. Perhaps most strikingly, patients with mutations in proteins that act downstream of the FA core complex exhibit much more severe phenotypes, such as more frequent congenital anomalies and early manifestations of the hematological and neoplastic defects (Bakker et al., 2013b; Kalb et al., 2007; Litman et al., 2005). This in turn suggests that the FA pathway members from the central or downstream tiers must have additional cellular functions beyond DNA ICL repair.

Indeed, there has been a surge in the identification of alternative roles of the FA pathway members (Ceccaldi et al., 2016a). Several additional roles were assigned to FANCD2, such as 1) a transcriptional regulatory role to promote gene expression of the TAp63 tumor suppressor; 2) a role in resolving DNA–RNA hybrids arising from collisions of the transcription and replication machineries (García-Rubio et al., 2015); 3)

a role in the so-called alternative NHEJ pathway (a DSB repair pathway that utilizes microhomology-mediated end joining (Kais et al., 2016)) and 4) a role in promoting the initiation of normal DNA replication in S-phase (Chaudhury et al., 2013; Song et al., 2010). Additionally, the downstream members of the FA pathway such as FANCJ and BRCA2 have been shown to play an important role in promoting DNA DSB repair during S-phase, using the HR pathway (Costanzo, 2011; Levitus et al., 2005; Zhang et al., 2009)

Previous work from our laboratory provided additional insights into S-phase specific roles of FANCD2 and FANCI. Using the *Xenopus* egg extract system, we showed that FANCD2 and FANCI dissociate during the replication stress response in S-phase. Moreover, we showed that chromatin recruitment and monoubiquitination of FANCD2 and FANCI occurs in a consecutive, not simultaneous, manner (Sareen et al., 2012). Subsequently, we demonstrated that FANCD2- but not FANCI - has important functions at replication forks that are stalled in the presence of the replication inhibitors APH or HU. We showed that FANCD2 assembles the BLM helicase complex at stalled replication forks independently of FANCI (Chaudhury et al., 2013). Moreover, we demonstrated that FANCD2 promotes replication fork restart and suppresses the firing of new replication origins in concert with the BLM helicase complex, CtIP and FAN1 (Chaudhury et al., 2013, 2014; Yeo et al., 2014). This role for FANCD2 at replication forks is likely to be more fundamental than ICL repair for the cellular survival and the prevention of carcinogenic genome alterations. Spontaneous stalling of the replication machinery can occur during every S-phase at numerous natural impediments such as

seconday DNA structures, late replicating heterochromatic zones, or DNA-bound protein complexes such as the transcription machinery (Branzei and Foiani, 2010).

The work described in this thesis builds on these newly identified roles of FANCD2 at stalled replication forks and attempts to answer two major outstanding questions: 1) Does FANCD2 promote the restart of stalled replication forks independently of the other FA pathway members? 2) What is the underlying molecular mechanism that allows FANCD2 to support the restart of these stalled forks?

Through the studies described in Chapter 2, I sought to answer whether other FA pathway members cooperate with FANCD2 to support the replication fork restart process. In complete agreement with the idea that the central and downstream FA pathway members do have roles that are not shared by the FA core complex, I demonstrated that FANCD2 is recruited to APH-stalled replication forks to promote fork restart independently of the FA core complex. Since FANCD2 relies strictly on the FA core complex for its own monoubiquitination, my findings also demonstrated – for the first time – that the nonubiquitinated FANCD2 isoform has a crucial role during the S-phase of the cell cycle. Moreover, this nonubiquitinated FANCD2 isoform acts in concert with at least two downstream FA proteins, BRCA2 and FANCJ, to promote fork restart, reemphasizing that FANCD2 and its downstream targets participate in crucial cellular pathways that act independently of the FA core complex. Collectively, my results support a novel, non-linear FA pathway model during the cellular replication stress response.

In further support of my findings, a follow up study showed that FANCD2 maintains the stability of common fragile sites (genomic regions that are prone to break

under replication stress) independently of the FA core complex proteins and of the FANCD2 monoubiquitination event, but in concert with BRCA2, (Madireddy et al., 2016). The importance of the nonubiquitinated FANCD2 isoform during the replication stress response is also supported by a recent finding that the nonubiquitinated FANCD2 protein is crucial to support cellular resistance to HU-mediated replication stress (Chen et al., 2016).

It should be pointed out that two other studies seemingly contradict my model that FANCD2 and BRCA2 cooperate to promote fork restart (Kais et al., 2016; Michl et al., 2016). In contrast to my observations, these studies found that FANCD2 and BRCA2 can contribute independently to replication fork recovery. Some of these discrepancies could be due to the difference in the cell lines used in these studies; for example one study analyzed FANCD2 and BRCA2 protein behavior in a lung carcinoma (H2199) cell line instead of using human FA patient-derived cells. Moreover, both studies utilized a different strategy to analyze replication fork recovery: the authors quantified replication fork progression following replication fork stalling, thus analyzing fork speed after restart. In contrast, my study directly quantified the actual number of fork restart events, raising the possibility that fork restart and fork speed following restart are differently regulated by FANCD2 and BRCA2.

Following the identification of nonubiquitinated FANCD2 as a key factor to mediate the restart of APH- or HU-stalled replication forks, I attempted to investigate the actual mechanism of FANCD2-mediated fork restart in Chapter 3. I determined that FANCD2 interacts and cooperates with the histone H3.3 chaperone complex

ATRX/DAXX, to mediate the restart of HU-stalled replication forks. Intriguingly, my findings support a model where histone chaperone activity of both the ATRX/DAXX complex and FANCD2 are required to promote fork recovery, suggesting that the deposition of one (or more) histone variants in the vicinity of stalled replication forks is a crucial step towards fork recovery. Moreover, my findings support a model where ATRX/DAXX, FANCD2, and several other HR factors cooperate as part of a larger protein complex to ultimately mediate HR-dependent mechanisms of replication fork restart and repair.

While my findings in these chapters identify novel roles for the central and downstream tiers of the FA pathway at stalled replication forks, it raises several new questions regarding the molecular basis of these newfound roles. In the remainder of this chapter, I address outstanding questions resulting from this work that should provide interesting avenues for future research.

## 4.2 Future Directions

### *4.2.1 How do the remaining downstream FA pathway members fit into my proposed FANCD2/FANCJ/BRCA2-dependent replication fork restart model?*

Accumulating evidence indicates that the restart of APH or HU stalled replication forks relies on HR mechanisms that may involve D-loop formation and Holliday junction dissolution (Petermann and Helleday, 2010). Importantly, seven of the ten currently known downstream FA pathway members (Tier III, Fig. 1.3) are known to be crucial for HR. Studies from other laboratories as well as from our own have already implicated six of them, namely BRCA1, BRCA2, FANCJ, RAD51, RAD51C and XRCC2, in the restart

of HU- or APH-stalled replication forks (Petermann et al., 2010; Raghunandan et al., 2015a; Somyajit et al., 2015; Yeo et al., 2014). The seventh downstream HR factor, FANCN, is a known interactor of BRCA1 and BRCA2 (Zhang et al., 2009), and thus highly likely to be required for fork recovery as well. In fact, we have unpublished preliminary results indicating that FANCN acts in concert with the other downstream FA HR proteins to promote the recovery of replication stalled forks.

What about the three other downstream FA pathway proteins, namely FANCP/SLX4, its binding partner and endonuclease FANCQ/XPF-ERCC1 and the TLS DNA polymerase ζ subunit, FANCV/Rev7? During HR-dependent fork restart, I envision that the FA pathway endonuclease could be involved in the resection of the dsDNA strand at a reversed fork ("Chicken Foot") to generate single stranded 3' overhangs that facilitate RAD51 mediated strand invasion (D-loop) and subsequent fork regeneration and restart. I hypothesize that SLX4 and FANCQ/XPF/ERCC1 may behave similar to the FAN1 endonuclease that – albeit not an FA protein itself – promotes replication fork restart in a FANCD2-dependent manner (Chaudhury et al., 2014). It seems less likely that fork recovery after APH- or HU-mediated fork stalling should require a translesion DNA polymerase such as DNA pol ζ, however additional studies are needed to answer this question.

Perhaps the most interesting question in the context of FA pathway-mediated fork recovery is whether the FANCD2 heterodimerization partner, FANCI, is required for replication fork restart. In the canonical FA pathway model, the ID2 heterodimer acts as a functional entity that must be monoubiquitinated to promote ICL repair. However, data

from our laboratory indicated that FANCD2 and FANCI actually dissociate in response to replication stress, and bind chromatin separately, in a step-wise manner (Sareen et al., 2012). Moreover, we previously showed that FANCD2 assembles the BLM helicase fork restart complex independently of FANCI and therefore independently of the FANCI-dependent FANCD2 monoubiquitination (Chaudhury et al., 2013). These findings suggest that FANCI may in fact be dispensable for fork restart. To address this question, we recently generated, in addition to the already available FANCD2$^{-/-}$ cells, FANCI$^{-/-}$ and ID2 double knockout (DKO) cells in the isogenic HCT116 cell background. We are currently using our standard DNA fiber assay to dissect the contributions of FANCD2 and FANCI to HR-mediated replication fork recovery.

#### *4.2.2 How does FANCD2 monoubiquitination regulate its functions at a stalled replication fork?*

Based on my findings and findings from other laboratories, it is now clear that at both the FA core complex and FANCD2 are required to protect the nascent DNA strands at stalled replication forks from nucleolytic degradation. In contrast, FANCD2 – but not the FA core complex - is required to promote fork restart (Chaudhury et al., 2013; Raghunandan et al., 2015; Schlacher et al., 2012). This immediately suggests a strict separation of function between the monoubiquitinated and the nonubiquitinated FANCD2 isoforms: monoubiqitinated FANCD2 acts to protect nascent DNA at the stalled fork, whereas nonubiquitinated FANCD2 functions to promote fork restart. Interestingly, two recent studies showed that following DNA ICL induction in human cells, the non-ubiquitinated FANCD2 protein is recruited to the chromatin prior to being

monoubiquitinated by the FA core complex. These new findings hint that the nonubiquitinated and monoubiquitinated FANCD2 isoforms may fulfill distinct roles even during DNA ICL repair (Liang et al., 2016; van Twest et al., 2017).

How can one envision this separation of function at a molecular level? Firstly, nonubiquitinated, but not monoubiquitinated, FANCD2 may serve as a docking platform to recruit other replication restart proteins. This idea is strongly supported by our previous findings that fork restart factors such as BLM or CtIP interact preferentially with the nonubiquitinated FANCD2 isoform even after $FANCD2^{Ub}$ induction with APH or HU (Chaudhury et al., 2013; Yeo et al., 2014). Moreover, the FANCD2-FAN1 protein interaction that occurs in an APH- or HU- stimulated manner and is crucial for replication fork recovery, occurs independently of the FA core complex and thus independently of $FANCD2^{Ub}$ formation. Somewhat provocatively, these findings actually suggest that $FANCD2^{Ub}$ formation may actively interfere with FANCD2's ability to serve as a platform for the replication fork restart machinery.

To test this idea, we plan to perform *in vitro* interaction studies using recombinant FANCD2, BLM, CtIP and FAN1 proteins. Since the human recombinant FANCD2 protein is quite unstable *in vitro* (our observation and personal communication with Dr. Andrew Deans, St Vincent's Institute of Medical Research, Australia), we plan to perform these experiments with the *Xenopus* homologs, xFANCD2, xBLM, xCtIP and xFAN1. Specific antibodies are at hand for all four *Xenopus* proteins (Chaudhury et al., 2013; Klein Douwel et al., 2014; Peterson et al., 2013). To achieve xFANCD2 monoubiquitination *in vitro*, we will incubate recombinant xFANCD2 with the

appropriate recombinant E2 and E3 ubiquitin ligases, UBE2T and FANCL, respectively (Alpi et al., 2008), followed by interaction studies with one of the three predicted interaction partners. Additionally, we are planning to use eight already generated truncation mutants of xFANCD2 to map the interaction surfaces that FANCD2 utilizes to make physical contact with BLM, CtIP or FAN1. Currently, no protein interaction sites (other than those with FANCI) have been mapped onto the FANCD2 protein. Ultimately, selective interaction-deficient human FANCD2 mutants expressed in our $D2^{-/-}$ cells would provide us with a powerful model to dissect the importance of individual FANCD2 protein interactions during the replication stress response.

If our model holds true, the monoubiqitinated FANCD2 isoform should not interact with fork restart factors, but should somehow protect the nascent DNA strands from nucleolytic attack by nucleases such as MRE11 or DNA2, as previously reported (Schlacher et al., 2012; Thangavel et al., 2015; Ying et al., 2012). How can FANCD2 perform this protective role? $FANCD^{2Ub}$ appears to have a higher affinity to chromatin than the nonubiquitinated FANCD2, hinting that $FANCD2^{Ub}$ may bind more tightly to DNA at stalled replication forks, thus, protecting the nascent DNA ends. Intriguingly, a recent DNA ICL repair study in *Xenopus* egg extracts found that approximately 50 $FANCD2^{Ub}$ molecules bound to each ICL (Douwel et al., 2014), indicating that $FANCD2^{Ub}$ tends to bind to a target region on DNA and then spread to the surrounding DNA. If similar actions occurred at stalled replication forks, one could envision that $FANCD2^{Ub}$ "filaments" spread out behind the stalled replication fork to stabilize the

nascent DNA, while nonubiquitinated FANCD2 molecule(s) engages in replication fork restart.

#### ***4.2.3 What is the underlying molecular mechanism of FANCD2 and ATRX/DAXX-dependent, histone H3 variant-mediated replication fork restart?***

To answer this question, I propose the following step-wise strategy:

a) Does FANCD2 share the same histone H3.3 variant specificity as the ATRX/DAXX complex?

FANCD2 has been previously identified to possess histone H3 chaperone activity using *in vitro* nucleosome assembly assays. However, the authors did not clarify which of the common histone H3 variants (3.1, 3.2 or 3.3) were actually analyzed in their *in vitro* or *in vivo* analyses (Sato et al., 2012, 2014). In chapter 3, I showed that replication fork restart relies on a functional interaction between FANCD2 and the ATRX/DAXX complex that is known to selectively deposit the H.3.3 variant onto chromatin (Drané et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). This suggests that FANCD2's own histone chaperone activity may be targeted (directly or indirectly via ATRX/DAXX) towards the H3.3 variant. To investigate this, I plan to use several approaches: (**1**) Test if FANCD2 participates specifically in nuclear histone H3.3, but not histone H3.1 or H3.2 protein complexes *in vivo*. To this end, I am currently generating cell lines that stably express a combination of 3xFLAG-FANCD2 with EGFP-H3.1, EGFP-H3.2 or EGFP-H3.3 (the EGFP-histone plasmids are a kind gift from Dr. Luis M. Schang, University of Alberta (Conn et al., 2013)). Following stable expression, we will isolate H3.1, H3.2 or H3.3-containing protein complexes using tandem-affinity purification from soluble,

chromatin free nuclear extracts using protocols described previously for the identification of DAXX-H3.3 protein complex (Lewis et al., 2010). (**2**) Test if FANCD2 interacts selectively with histone H3.3 *in vitro*. We will perform *in vitro* immunoprecipitation assays using recombinant xFANCD2 and recombinant histone H3.1, H3.2 or H3.3 (the H3.1, H3.2 and H3.3 amino acid sequences are identical between the human and *Xenopus* homologs) purified from SF9 insect cells, as previously described (Chaudhury et al., 2013; Sareen et al., 2012). In this context, we also need to consider that FANCD2 may only interact with a post translationally modified form of histone H3.3. All histone variants can be targeted by a multitude of posttranslational modifications (PTMs) *in vivo* (Hunt et al., 2013; Szenker et al., 2011). Should FANCD2 only bind to post translationally modified H3.3, we will not pick up on this interaction in an assay that uses non-modified histone H3 variants. As an alternative approach, we plan to screen for binding of recombinant xFANCD2 against a commercially available histone peptide array that is composed of H3 variant peptides that carry distinct PTMs including acetylation, mono-, di- or tri-methylation, phosphorylation etc. (Moore et al., 2013).

Additionally, there is no direct evidence of any histone H3 variant deposition specifically at or behind the stalled forks. To test for histone deposition at or in the vicinity of stalled replication forks, we will perform the iPOND assay (isolate proteins on nascent DNA (Sirbu et al., 2012)). The iPOND enables the purification of proteins bound to the nascent synthesized DNA and obtain a high-resolution spatiotemporal analysis of proteins at replication forks or on chromatin following DNA replication fork stalling.

b) Is histone H3.3 deposition required to promote fork restart?

My results described in Chapter 3 demonstrate that the ATRX/DAXX complex promotes replication fork restart, indirectly suggesting that it is the ATRX/DAXX-mediated H3.3 deposition at stalled forks that is crucial for fork restart. However, it is possible that ATRX and DAXX promote restart independently of their function as histone H3.3 chaperone. To test this, we recently received a *H3.3B* knock-out MEF cell line (a generous gift from Dr. Paul Knoepfler, University of California, Davis (Bush et al., 2013)). This cell line exhibits very low H3.3 protein levels that stem from residual expression from the *H3.3A* gene locus. We will use the DNA fiber assay in WT and *H3.3B* knock-out MEFs to analyze replication fork restart efficiencies following HU treatment. Simultaneously, we will perform the same DNA fiber assays in human cells treated with control siRNA or H3.3 siRNA. Our idea that H3.3 may be required to promote the restart of HU-stalled replication forks, is supported by previous studies that report a crucial role for H3.3 to promote the progression of the replication machinery in the presence of UV light-induced DNA damage in vertebrate cells (Adam et al., 2013; Frey et al., 2014).

#### *4.2.4 Does FANCD2 share ATRX's role in telomere maintenance?*

ATRX has been closely associated with the maintenance of genomic integrity at telomeric DNA (Koschmann et al., 2016; Wang et al., 2016; Wong et al., 2010). Telomeres are the specialized nucleoprotein structures (DNA coated with "shelterin" proteins such as TRF1) that protect the ends of the chromosomes from being recognized as a DNA DSB (Doksani and de Lange, 2014; Sfeir et al., 2009). Since the telomeric

DNA consists of G-rich repeat sequences (TTAGGG), it has a high propensity to form secondary DNA structures called G4 quadruplexes. G4 structures are natural impediments for the DNA replication machinery (Bochman et al., 2012). ATRX binds G4 quadruplexes *in vitro* and associates with the G-rich telomeric and subtelomeric repetitive DNA *in vivo* (Law et al., 2010). The ATRX/DAXX complex promotes incorporation of the histone variant H3.3 at telomeric repeats, which was shown to counteract replication fork stalling events. It has been proposed that ATRX/DAXX mediated H3.3 deposition and H3.3K9me3 formation at telomeric regions likely increased telomere chromatinization, which in turn should limit the formation of G4 structures (Clynes et al., 2015; Goldberg et al., 2010; Lewis et al., 2010; Lovejoy et al., 2012). Since FANCD2 and ATRX have shared roles at APH- or HU-stalled replication forks, it seems plausible to hypothesize that FANCD2 may shares ATRX's role in promoting H3.3-dependent chromatinization at the repetitive G4-prone DNA sequences to support telomere maintenance. In agreement with possible protective roles of FANCD2 at telomeres, previous reports showed that cells from FA-D2 patients and patients of other FA complementation groups (such as BRCA2) exhibit shorter telomeres with an average length in the lower quarter of the "normal" range as well as telomere loss/breakage in peripheral leukocytes (Adelfalk et al., 2001; Alter et al., 2015; Joksic et al., 2012).

In ATRX null cells, a reduction in H3.3 density at the telomeres suggests decreased chromatinization (Clynes et al., 2015). If FANCD2's contribution to telomere integrity maintenance is via its role as a histone chaperone, we would expect to see

reduced H3.3 densities at the telomeric region in FANCD2-deficient cells as well. This can be tested by performing a chromatin immunoprecipitation (ChIP) assay for H3.3 in our WT and $D2^{-/-}$ cells, to probe for enrichment of telomeric DNA using slot blotting techniques (Clynes et al., 2015). Additionally, a reduction in H3.3 density at telomeres is typically accompanied with an increase in G4 quadraplex formation observed in ATRX deficient cells (Wang et al., 2016). Using commercially available G4 antibodies in a co-staining experiment using a Telomere specific- Fluorescence in situ hybridization (T-FISH) analysis, we can examine 1) if more G4 structures accumulate in the $D2^{-/-}$ cells compared to WT cells and 2) if the accumulating G4 structures in $D2^{-/-}$ cells occur predominantly at telomere structures.

The idea that FANCD2 may cooperate with ATRX/DAXX to support DNA replication through G4 structure-prone regions at telomeres is additionally supported by recent findings that the BLM helicase, a known functional interactor of FANCD2 during global replication fork restart (Chaudhury et al., 2013; Davies et al., 2007) is crucial in facilitating telomere DNA replication (Drosopoulos et al., 2015). To examine if BLM, FANCD2 and ATRX/DAXX function in concert to promote telomere replication, we plan use the telomere SMARD (Single molecule analysis of replicated DNA) assay (Sfeir et al., 2009). Telomere-SMARD is a modified DNA fiber assay which can further specifically enrich for telomeric DNA. This telomeric DNA enrichment step enables analyzing replication events exclusively at telomeric DNA sequences. Should I observe that the BLM helicase, FANCD2 and ATRX/DAXX are required to support timely replication of telomeric DNA, it would be quite interesting to test if other known

FANCD2 interactors, such as MRE11, CtIP, FAN1 and additional downstream FA pathway proteins share this new role of FANCD2 as well.

### *4.2.5 Does FANCD2's predicted role in telomere maintenance in normal cells change in ALT cancers?*

ATRX's role in maintaining telomeric integrity becomes particularly evident upon the examination of cancer cells lacking ATRX. Approximately 5-15% of cancers utilize an alternative, HR based telomere maintenance mechanism named ALT (Alternative Lengthening of Telomeres). ALT cells lack any detectable telomerase activity and show a characteristic heterogeneous pattern of telomere length (ranging from 1 kb to 20 kb). A previous study that used uniquely tagged telomeres in an ALT cell line showed that the tag is dispersed to the telomeric ends of different chromosomes, strongly suggesting that ALT cells use HR mechanisms to maintain their telomeres. However, the molecular details of ALT are yet to be elucidated (Pickett and Reddel, 2015; Zhong et al., 2007).

Strikingly, 90% of all ALT cancers lack the expression of ATRX, DAXX or H3.3 (Pickett and Reddel, 2015). In these ALT cancer cells, FANCD2 and other HR proteins such as BLM and MRE11 are known to localize excessively to the telomeres, visible as telomere associated foci (Clynes et al., 2015; Fan et al., 2009; Root et al., 2016). Within these foci, FANCD2 and MRE11 are predicted to promote ALT mechanisms and support telomere length heterogeneity (Fan et al., 2009; Zhong et al., 2007). This peculiar observation suggests that ATRX/DAXX may in fact prevent an inappropriate over-accumulation of HR factors at the telomeres in normal cells. In fact, it was recently

shown that re-expression of ATRX in an ALT cancer cell line, U2OS, annihilated MRE11 telomere foci formation. (Clynes et al., 2015). Should ATRX re-expression also block telomere formation of FANCD2 and other HR factors in ALT cells, one would predict that ATRX negatively regulates the recruitment of FANCD2 and other HR proteins to chromatin, thus repressing HR. In complete contrast, my findings in Chapter 3 suggest that ATRX and FANCD2 cooperate to recruit HR factors, such as CtIP, to stalled replication forks and to support HR mechanisms towards fork restart. Additionally, the possibility that FANCD2 and ATRX/DAXX may both support DNA replication through G4 structure-prone regions at telomeres via their histone H3 chaperone activities (Section 4.4) provides further support that FANCD2and ATRX/DAXX complex work as a team at normal telomeres.

One potentially unifying model for these seemingly opposing findings would predict that ATRX and FANCD2 cooperate at stalled replication forks – including those that occur within the telomeric regions – to promote HR-dependent restart mechanisms. At the same time, ATRX may very strictly regulate how many FANCD2 molecules are permitted to access the telomeric DNA, perhaps via controlling additional FANCD2 protein interactions, to prevent over-accumulation of FANCD2 and other HR factors.

To test if ATRX does in fact prevent overloading of FANCD2 molecules at telomeres, we plan to perform a T-FISH analysis of our non-ALT $ATRX^{-/o}$ cells and their complemented counterpart, $ATRX^{-/o+ATRX}$.

Simulataneously, we plan to test if the mode of interaction between ATRX and FANCD2 occurs differently at the telomeres compared to the rest of the genome. To this end, we will employ a recently developed DR-GFP assay using Cas9 nucleases to create site specific DSBs (Vriend et al., 2014). We will use our WT, $ATRX^{-/o}$, $D2^{-/-}$ and $D2AX^{-/-}$ cells and engineer into each cell line the DR-GFP cassette either within a telomeric/subtelomeric DNA sequence or at a non-telomeric genomic DNA sequence. Subsequently, Cas9 transfection in the presence of a sceGFP guide RNA will induce a DNA DSB within the *GFP* gene present in the distinct genomic contexts (Vriend et al., 2014). The percentage of HR-proficient, GFP-positive cells can then be determined by flow cytometric analysis. Using this assay, we will determine the contributions of ATRX and FANCD2 to HR mediated-DNA DSB repair at the non-telomeric regions versus the telomeric ones. If ATRX does indeed prevent FANCD2-mediated HR events at telomeres, we expect to observe an increase in HR-mediated telomeric GFP repair in $ATRX^{-/o}$ cells compared to the WT, $D2^{-/-}$ and $D2AX^{-/-}$ cells.

In conclusion, the studies presented in this dissertation have greatly enhanced our understanding of the molecular FA pathway. This work demonstrates novel and non-canonical roles for the FA pathway proteins at stalled replication forks. Moreover, it sets a strong precedent for examining the role of individual FA proteins as they may have additional, not yet recognized functions independently of their canonical DNA ICL repair roles. In my studies, I show convincing evidence for a functional relationship of FANCD2 with the histone chaperone, ATRX/DAXX. These findings argue for putative roles of at least one FA protein in regulating chromatin dynamics to promote error-free

and timely DNA replication. Importantly, this novel view of the FA pathway has immense therapeutic potential for the development of novel future treatment strategies for FA patients and FA-type cancers in the normal population. Histone methylation for example is a therapeutically actionable realm, with several chemicals that activate or inhibit histone modifiers currently undergoing phase II clinical trials. Beyond the realm of FA mutated cancers, additional studies that further our understanding of ALT cancers and the role of FANCD2 in this cancer subtype, will enable the development of alternative cancer therapies, and ultimately strengthen our fight against cancer.

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