Unveiling the Function of Fanconi Anemia Complementation Group A Protein (FANCA)

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UNIVERSITY OF MIAMI

UNVEILING THE FUNCTION OF FANCONI ANEMIA COMPLEMENTATION GROUP A PROTEIN (FANCA)

By

Liangyue Qian

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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UNVEILING THE FUNCTION OF FANCONI ANEMIA COMPLEMENTATION GROUP A PROTEIN (FANCA)

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Fanconi anemia is a rare autosomal recessive or X-linked genetic disease characterized by progressive bone marrow failure, various developmental anomalies, and cancer predisposition. Fanconi anemia complementation group A gene is one of the 16 disease-causing genes and has been found to be mutated in more than 60% of Fanconi anemia patients. Using purified proteins, we unveil that FANCA has intrinsic affinity for nucleic acids with preference for single-strand forms and its nucleic acids binding domain is primarily located at its C-terminus. FANCA binds to RNA with an intriguingly higher affinity than its DNA counterpart. By testing the affinity between FANCA and a variety of DNA structures, we demonstrate that a 5'-flap on DNA facilitates its interaction with FANCA. We also show that FANCA stimulates the endonuclease activity of flap endonuclease 1, an important nuclease to process RNA primers during Okazaki fragment maturation, by the incision of both DNA and RNA flaps. In addition, our preliminary results indicate that deficiency of FANCA causes accumulation of late S phase cells after MMC treatment and results in insufficient activation of ATM and ATR pathways of DNA damage response.
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LIST OF ABBREVIATIONS

APE1: human ap endonuclease
ATM: Ataxia telangiectasia mutated
ATR: Ataxia telangiectasia and Rad3 related
ATRIP: ATR-interacting protein
BLM: Bloom syndrome protein
BRCA1: breast cancer type 1 susceptibility protein
BRCA2: breast cancer type 2 susceptibility protein
BrdU: Bromodeoxyuridine
BRIP1: BRCA1-interacting protein 1
BSA: bovine serum albumin
CdKs: cyclin-dependent kinases
CHK: checkpoint kinase
DSB: double strand break
dsDNA: double-stranded DNA
EDTA: ethylenediaminetetraacetic acid
EMSA: electrophoretic mobility shift assay
EXO1: Exonuclease 1
FA: Fanconi Anemia
FAAP: Fanconi Anemia associated protein
FANCA: Fanconi Anemia complementation group A protein
FBS: fetal bovine serum
FEN1: flap endonuclease 1
HES1: hairy and enhancer of split-1 protein
HR: homologous recombination
HRR: homologous recombination repair
kDa: kilodalton
ICL: interstrand crosslink
IP: immunoprecipitation
MCM: mini-chromosome maintenance
MMC: Mitomycin c
MMR: mismatch repair
MRN: Mre11-Rad50-Nbs1 protein complex
NER: nucleotide excision repair
NHEJ: non-homologous end joining
NLS: nuclear localization signal
PAGE: polyacrylamide gel electrophoresis
PALB2: partner and localizer of BRCA2
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction
PI: propidium iodide
PIKK: phosphoinositide 3-kinase related protein kinases
PKR: protein kinase regulated by RNA
Pol: polymerase
RFC: replication factor C
ROS: reactive oxygen species
RPA: replication protein A
RT: room temperature
S.D.: standard deviation
ssDNA: single-stranded DNA
SSO: single-stranded overhang
ssRNA: single-stranded RNA
SDS: sodium dodecyl sulfate
TIPIN: TIMELESS-interacting protein
TLS: translesion synthesis
TOPBP1: topoisomerase-binding protein 1
UBD: ubiquitin binding domain
UV: ultraviolet
WRN: Werner syndrome ATP-dependent helicase
WT: wild type
CHAPTER 1
INTRODUCTION

1.1 FANCONI ANEMIA

Fanconi anemia is a rare autosomal recessive or X-linked genetic disease characterized by progressive bone marrow failure, various developmental anomalies and cancer predisposition (Auerbach 2009, de Winter and Joenje 2009, Moldovan and D'Andrea 2009, Neveling, Endt et al. 2009, Rego, Kolling et al. 2009, Thompson and Hinz 2009, D'Andrea 2010). The disease was first reported as aplastic anemia since patients were described as a combination of pancytopenia with a limited number of physical anomalies and later, the discovery of somatic mosaicism extended the boundaries of the FA phenotype to cases even without any overt hematological manifestations (Neveling, Endt et al. 2009). More than two thirds of FA patients also present with a wide range of developmental abnormalities of skeleton, short stature, low birth weight and genital malformations (Tischkowitz and Hodgson 2003, Bakker, de Winter et al. 2013). Hematologic manifestations of FA individuals include a high risk of acute myeloid leukaemia in very young age (median age of 7 years) (Auerbach 2009). In addition, the incidence of nonhematologic malignancy, such as squamous cell carcinoma of the head and neck, liver tumors and gynecological cancers, in FA patients is higher and occurs earlier compared to the whole lifespan (40% by age 30, 50% by age 45 and 76% by age 60) (Alter 2003, Kutler, Singh et al. 2003, Auerbach 2009, Bakker, de Winter et al. 2013). An important cellular feature of all FA patient cells, which is also a reliable cellular marker for clinical diagnosis,
is its hypersensitivity to interstrand crosslink agents such as diepoxybutane (DEB) or MMC. Chromosome aberrations and breaks increased dramatically when FA cells encounter DNA interstrand crosslinkers (Auerbach 2009).

1.2 FANCONI ANEMICA PROTEINS

Cells that are defective in any of group I proteins are deficient in monoubiquitination of the ID complex. Downstream of the monoubiquitination of the ID complex are the group III proteins, FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4 and FANCO/XPF. These proteins are involved in the repair of double strand breaks produced during the 'unhooking' of ICLs and constitute a FA-BRCA network to guard genomic integrity (Wang 2007, de Winter and Joenje 2009, Moldovan and D'Andrea 2009, D'Andrea 2010, Vaz, Hanenberg et al. 2010, Cybulski and Howlett 2011, Bogliolo, Schuster et al. 2013). Figure 1.1 summarizes the proteins working in the FA pathways (Kottemann and Smogorzewska 2013).


**TABLE 1.1 The sixteen complementation groups of Fanconi Anemia**

<table>
<thead>
<tr>
<th>FA genes</th>
<th>Prevalence</th>
<th>Activity</th>
<th>Conservation</th>
</tr>
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<tbody>
<tr>
<td>FANCA</td>
<td>66%</td>
<td>Core complex member</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCB</td>
<td>2%</td>
<td>Core complex member</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCC</td>
<td>10%</td>
<td>Core complex member;</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCD1/BRCA2</td>
<td>2%</td>
<td>HRR mediator</td>
<td>Vertebrate, worm</td>
</tr>
<tr>
<td>FANCD2</td>
<td>2%</td>
<td>Ubiquitinated and phosphorylated following DNA damage</td>
<td>Vertebrate, worm, insect, slime</td>
</tr>
<tr>
<td>FANCE</td>
<td>2%</td>
<td>Core complex member</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCF</td>
<td>2%</td>
<td>Core complex member</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCG</td>
<td>9%</td>
<td>Core complex member</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCI</td>
<td>2%</td>
<td>Ubiquitinated and phosphorylated following DNA damage</td>
<td>Vertebrate, worm, insect, slime</td>
</tr>
<tr>
<td>FANCI/J/BRIP1</td>
<td>2%</td>
<td>HRR mediator; helicase</td>
<td>Vertebrate, invertebrate, yeast</td>
</tr>
<tr>
<td>FANCL</td>
<td>&lt;0.2%</td>
<td>Core complex member; PHD domain, ubiquitin ligase activity</td>
<td>Vertebrate, insect, slime mold</td>
</tr>
<tr>
<td>FANCM</td>
<td>&lt;0.2%</td>
<td>Helicase, core complex member</td>
<td>Vertebrate, invertebrate, yeast, archaea</td>
</tr>
<tr>
<td>FANCN/PALB2</td>
<td>2%</td>
<td>FANCD1 interactor</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>FANCO/RAD51C</td>
<td>&lt;0.2%</td>
<td>HRR mediator</td>
<td>Vertebrate, invertebrate, yeast</td>
</tr>
<tr>
<td>FANCP/SLX4</td>
<td>&lt;0.2%</td>
<td>HRR mediator; FANCOQ interactor</td>
<td>Vertebrate, invertebrate, yeast</td>
</tr>
<tr>
<td>FANCQ/XPF</td>
<td>&lt;0.2%</td>
<td>Nuclease</td>
<td>Vertebrate, invertebrate, yeast</td>
</tr>
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(Wang 2007, Moldovan and D’Andrea 2009)
FIGURE 1.1 The Fanconi anemia pathway and interstrand crosslink repair

On detection of the crosslink, the complex is activated by ATR-mediated phosphorylation (P), and one component, FANCL, ubiquitinates (Ub) the I–D2 complex. The I–D2 complex then coordinates the action of downstream repair factors. SLX4 functions as a scaffold for the three nucleaseas XPF, MUS81 and SLX1 that function at the site of DNA damage to make incisions either side of two covalently linked nucleotides. On the incised strand, TLS polymerases (TLS Pol) are recruited to bypass the unhooked crosslink. The break is then repaired through homologous recombination involving the Fanconi anaemia proteins BRCA2, BRIP1, PALB2 and RAD51C (Kottemann and Smogorzewska 2013).
1.2.1 Fanconi anemia core complex

Extensive interaction studies have shown that eight of the FA proteins form a multi-subunit nuclear core complex: FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM (Wang 2007, Moldovan and D'Andrea 2009). The FA core complex was successfully purified from Hela cells through immunoprecipitation with a FANCA-specific antibody by Weidong Wang’s group to identify three FA genes, FANCB, FANCL, and FANCM and two additional FANCA-associated proteins, namely FAAP24 and FAAP100 (Meetei, de Winter et al. 2003, Meetei, Levitus et al. 2004, Meetei, Medhurst et al. 2005, Ciccia, Ling et al. 2007, Ling, Ishiai et al. 2007, Guo, Xu et al. 2009). The 10 proteins in the FA core complex may exist in the form of subcomplexes, i.e. FANCM-FAAP24, FANCA-FANCG, FANCB-FANCL-FAAP100, and FANCC-FANCE-FANCF (Garcia-Higuera, Kuang et al. 2000, Taniguchi and D'Andrea 2002, Gordon and Buchwald 2003, Medhurst, Laghmani et al. 2006, Ciccia, Ling et al. 2007, Wang 2007)). HES1, a transcriptional repressor, is also reported to be associated with the FA core complex (Tremblay, Huang et al. 2008, Tremblay, Huard et al. 2009). In addition to its critical function of monoubiquitinating the ID complex, FA core complex is also known to be directly involved in a wide spectrum of other functions as described below.

Components of the FA core complex are phosphorylated under genotoxic stress by ATR-CHK1 kinases. Phosphorylation of FANCA on serine 1449 by ATR kinase in response to DNA damage is known to be essential for the FA pathway (Collins, Wilson et al. 2009). Although the FANCA-S1449A mutant localizes
normally to chromatin, it fails to correct a variety of FA-associated phenotypes including the FANCD2 monoubiquitination deficiency (Collins, Wilson et al. 2009). FANCE is phosphorylated at threonine 346 and serine 374 by CHK1. The non-phosphorylated mutant of FANCET346A/S374A allows normal level of FANCD2 monoubiquitination and FANCD2 foci assembly, but fails to complement the hypersensitivity of FANCE-deficient cells to the synthetic crosslinking agent, Mitomycin C (Wang, Kennedy et al. 2007). The phosphorylation of FANCE by CHK1 leads to its degradation and has been suggested to be a negative regulation mechanism of FA pathway (Moldovan and D'Andrea 2009). The putative phosphorylation of FANCM by ATR kinase increases its binding affinity for chromatin (Meetei, Medhurst et al. 2005, Kim, Kee et al. 2008, Sobeck, Stone et al. 2009). Furthermore, hyperphosphorylation of FANCM by Plk1 kinase (polo-like kinase) is involved in the cell cycle dependent recruitment of the core complex to chromatin (Kim, Kee et al. 2008, Kee, Kim et al. 2009). This phosphorylation provides an important layer of regulation that ensures the FA core complex is recruited to chromatin only during S phase, but not mitosis phase of the cell cycle. Therefore, the phosphorylation of the FA core complex components is likely to affect stability of the core complex, ubiquitin ligase activity, chromatin association, and repair functions.

FA core complex is a multi-subunit E3 ubiquitin ligase. A hallmark and convenient diagnostic marker of FA is the monoubiquitination of FANCD2 (Shimamura, Montes de Oca et al. 2002, Soulier, Leblanc et al. 2005). All 10 known subunits of the FA core complex are indispensable for the FANCD2
monoubiquitination (Garcia-Higuera, Taniguchi et al. 2001, Meetei, de Winter et al. 2003, Meetei, Levitus et al. 2004, Ciccia, Ling et al. 2007, Ling, Ishiai et al. 2007). Recently, FANCI, an interacting partner of FANCD2, was also found to be monoubiquitinated by the FA core complex (Sims, Spiteri et al. 2007, Smogorzewska, Matsuoka et al. 2007). It is now clear that FANCD2 monoubiquitination occurs via FANCL-mediated E3 ubiquitin ligase activity (Meetei, de Winter et al. 2003). FANCL modifies FANCD2 at lysine 561 by adding a single ubiquitin molecule with UBE2T acting as the E2 ubiquitin-conjugating enzyme (Machida, Machida et al. 2006). It is currently unknown how other components of the FA core complex facilitate or regulate the FANCL ubiquitin ligase in response to DNA damage. However, assembly of the FA core complex per se does not seem to trigger the FANCD2 monoubiquitination. Instead, the damage-induced recruitment of the FA core complex and the independent recruitment of UBE2T to chromatin play a critical role in regulating the FANCD2 monoubiquitination (Alpi, Langevin et al. 2007).

1.2.2 ID complex

FANCI is a paralog of FANCD2 and its C-terminus interacts with FANCD2 to form a complex called the ID complex (Smogorzewska, Matsuoka et al. 2007, Yuan, El Hokayem et al. 2009). It has been noted that FANCI and FANCD2 are not always found together in the ID complex. In a reconstitution analysis in insect cells, only ~5% of FANCI was found to form a complex with FANCD2 (Yuan, El Hokayem et al. 2009). Both FANCI and FANCD2 are leucine rich proteins (Yuan, El Hokayem et al. 2009) and both proteins are monoubiquitinated by the FA core
complex under genotoxic stress (Garcia-Higuera, Taniguchi et al. 2001, Meetei, Yan et al. 2004, Smogorzewska, Matsuoka et al. 2007). This modification is considered to be essential for the FA pathway to exert its effects, especially in reestablishing replication forks through homologous recombination.


The deubiquitination of FANCD2 by USP1-UAF1 is an important mechanism to keep the FA pathway in check under unstressed conditions. Down regulation of USP1 by transcriptional repression and DNA damage-dependent autocleavage shifts the ubiquitination balance toward increased monoubiquitination of FANCD2 and FANCI and therefore triggers downstream repair events (Nijman, Huang et al. 2005, Cohn, Kowal et al. 2007, Cohn and D'Andrea 2008, Cohn, Kee et al. 2009, Collins, Wilson et al. 2009, Moldovan and D'Andrea 2009).

1.2.3 Downstream partners of ID complex


FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2 are bona fide breast cancer related genes. While haplodeficiency of these three factors caused by single allelic mutation predisposes humans to breast and ovarian cancers, biallelic mutations cause Fanconi anemia (Wang 2007, Parmar, D'Andrea et al. 2009). The connection between Fanconi anemia and DSB repair factors was first shown in an elegant study by Alan D'Andrea's group (Howlett, Taniguchi et al. 2002). They established that BRCA2, a factor that facilitates formation of RAD51-
FANCO/RAD51C, FANCP/SLX4 and FANCQ/XPF are recently identified new members of Fanconi anemia complementation group proteins, but previously show involvement in the Fanconi anemia pathway. RAD51C, as a paralog of RAD51, is known to be involved in early and late stages of HR and contributes to activation of the checkpoint kinase CHK2 (Dosanjh, Collins et al. 1998, Badie, Liao et al. 2009). Vaz et al showed that biallelic mutation in RAD51C leads to FA-like disorder and the levels of FANCD2 and its monoubiquitinated form were not affected in these patient cells (Vaz, Hanenberg et al. 2010). SLX4 was first found to be required for the ICL repair as a scaffold protein that mediates interactions between endonucleases XPF-ERCC1, MUS81-EME1, and SLX1 (Andersen, Bergstralh et al. 2009, Fekairi, Scaglione et al. 2009, Munoz, Hain et al. 2009, Svendsen, Smogorzewska et al. 2009) and later two independent groups confirmed it to be a new FA genes, FANCP (Cybulski and Howlett 2011, Kim, Lach et al. 2011, Stoepker, Hain et al. 2011). And this year XPF, a structure specific nuclease usually forms as heterodimer XPF-ERCC1, complemented the phenotype of the one FA subtype cell lines (Bogliolo, Schuster et al. 2013). As an NER factor, previous study already show its distinction because its deficiency results in uniquely high sensitivity to crosslinking agents (Thompson, Rubin et al. 1980, Hoy, Thompson et al. 1985, Andersson, Sadeghi et al. 1996, De Silva, McHugh et al. 2000, Clingen, De Silva et al. 2005, Niedernhofer, Garinis et al. 2006, Ciccia, McDonald et al. 2008) and a ICL unhook candidate since purified XPF-ERCC1 protein is able to introduce damage-specific dual incisions on both 5’ and 3’ sides of the defined psoralen-

### 1.3 FANCA

FANCA is the second FA genes identified in 1996 through complementation cloning (Lo Ten Foe, Rooimans et al. 1996, de Winter and Joenje 2009). A patient mutation comparison shows that FANCA, FANCC and FANCG surprisingly make up 85% of the FA patient population and more than 60% of the patient population has mutation in FANCA (Table 1.1). Except a partial leucine zipper motif (Lo Ten Foe, Rooimans et al. 1996), computational sequence analysis of FANCA does not show other identifiable domain to indicate how it may contribute to the repair and tolerance of ICLs and pathogenesis of Fanconi Anemia (Levitus, Joenje et al. 2006, Yuan, Song et al. 2010).

Several proteins have been found interacting with FANCA. FANCA physically interacts with FANCG and the transcription factor HES1 within the FA core complex (Garcia-Higuera, Kuang et al. 1999, Christianson and Bagby 2000, Garcia-Higuera, Kuang et al. 2000, Reuter, Herterich et al. 2000, Park, Ciccone et al. 2004, Tremblay, Huang et al. 2008, Tremblay, Huard et al. 2009), which has been found to be localized to chromatin (Otsuki, Furukawa et al. 2001, Qiao,
Moss et al. 2001). A sorting nexin protein, SNX5, identified to bind FANCA and FANCA may affect its traffic with cell surface receptors (Otsuki, Kajigaya et al. 1999). A component of SWI/SNF complex, BRG1, has shown association with FANCA by yeast two hybrid analysis and immunofluorescence (Otsuki, Furukawa et al. 2001). Furthermore, direct interaction between BRCA1 and FANCA has been identified and determined to be damage independent (Folias, Matkovic et al. 2002). Recently, direct interaction between FAAP20 and FANCA was carried out to promote the functional integrity of the FA core complex (Leung, Wang et al. 2012).

Using nuclear protein extracts and complementation analysis, it has been demonstrated that FANCA is required for efficient incisions at the sites of psoralen-mediated ICLs (Kumaresan and Lambert 2000). FANCA has also been found to be involved in the spontaneous and UV-induced base substitution mutagenesis in human cells, implying its involvement in the mutagenic translesion synthesis of DNA damage (Papadopoulo, Porfirio et al. 1990, Mirchandani, McCaffrey et al. 2008). FANCA has also been shown to be required for recruiting FANCO/RAD51C and FANCD1/BRCA2 into the MMC-induced nuclear foci, indicating its role in the homologous recombination repair of ICLs (Yang, Herceg et al. 2005, San Filippo, Sung et al. 2008, Vaz, Hanenberg et al. 2010). These data imply that FANCA may play multiple roles in DNA metabolism and transactions. However, because FANCA is not evolutionarily conserved and lacks identifiable domains/motifs, it remains largely unknown how FANCA is involved in these biological processes.
FANCA has been found to exist in a multiprotein nuclear protein complex that contains the Fanconi anemia core complex and BLM complex (Meetei, Sechi et al. 2003, Wang 2007). It is localized to chromatin in a replication-dependent manner (Otsuki, Furukawa et al. 2001, Qiao, Moss et al. 2001). Xenopus egg extracts immune-depletion study shows that FANCA is directly involved in maintenance of replication forks (Sobeck, Stone et al. 2006, Wang, Stone et al. 2008, Yuan, Qian et al. 2012). Most recently, FANCA has been found to regulate MUS81-EME1 activity in a damage-dependent manner and FANCA has intrinsic affinity to nucleic acids with particularly high affinity to single-stranded RNA and DNA structures with a 5’ flap (Yuan, Qian et al. 2012, Benitez, Yuan et al. 2013). Because both FANCA and FEN1 localize to replication forks and practically share the same substrate specificity, i.e. 5’ flap structures and single-stranded RNA (Otsuki, Furukawa et al. 2001, Qiao, Moss et al. 2001, Yuan, Qian et al. 2012), we hypothesize that FANCA may affect FEN1 activity by competing or collaborating with each other on the same structures to regulate removal of RNA primers and 5’ flap structures during DNA replication and repair. In support of this hypothesis, deficiency in FEN1 causes partially similar phenotypes as FANCA, i.e. inflammation and cancers (Dufour, Corcione et al. 2003, Zheng, Dai et al. 2007, Briot, Mace-Aime et al. 2008, Du, Erden et al. 2013).

1.4 FUNCTIONS OF FEN1 IN EUKARYOTIC DNA REPLICATION AND OKAZAKI FRAGMENT MATURATION

In eukaryotic cells, replication of double-stranded DNA is a highly coordinated process that ensures accurate and efficient genome duplication.
Various structured DNA intermediates are effectively processed to avoid deleterious consequences such as genome instability and cancer. DNA replication occurs simultaneously with multiple origins on each chromosome and proceeds bi-directionally (Waga and Stillman 1998, Balakrishnan, Gloor et al. 2010, Zheng, Jia et al. 2011, Zheng and Shen 2011, Balakrishnan and Bambara 2013). Because of the anti-parallel nature of the DNA duplex, replication is semi-discontinuous (Figure 1.2). The leading strand is synthesized continuously by polymerase ε from a single initiation event at the replication origin. On the lagging strand, polymerase α/primase first synthesize an RNA primer of 8-12 nucleotides long followed by a short stretch of DNA. Then polymerase δ displaces the low fidelity Pol α via an RFC/PCNA-dependent polymerase switching mechanism and extends to synthesize a series of discrete Okazaki fragments of ~150-200 base pair in size (Pursell, Isoz et al. 2007, Nick McElhinny, Gordenin et al. 2008, Burgers 2009, Balakrishnan, Gloor et al. 2010, Zheng and Shen 2011). When the elongation complex encounter a downstream Okazaki fragments, a 5’ flap structure is generated because of strand displacement synthesis. This 5’ flap contains the RNA primer and must be removed. Thus, those Okazaki fragments are then joined into an intact lagging strand, which is called Okazaki fragment maturation (Waga and Stillman 1994, Bambara, Murante et al. 1997, Burgers 2009). Three distinct pathways during Okazaki fragment maturation and flap endonuclease 1 has been implicated to be an important nuclease during this process (Figure 1.3) (Harrington and Lieber 1994, Waga and Stillman 1994, Liu, Kao et al. 2004, Zheng and Shen 2011).
FIGURE 1.2 Enzymes and reactions in the DNA replication fork

Major proteins present in a typical replication fork include: (i) MCM proteins, which are helicases for opening up the DNA duplex to initiate a DNA replication fork; (ii) RPA, a single-stranded DNA binding protein to protect the DNA template from nuclease cleavage; (iii) primase/Pol α, which synthesizes RNA primers and a short DNA fragment to initiate Okazaki fragments; (iv) Pol δ, the DNA polymerase responsible for synthesizing the major portion of Okazaki fragments; (v) Pol ε, the DNA polymerase responsible for leading strand DNA synthesis; (vi) PCNA, which is the DNA clamp for the processivity of DNA polymerase and coordination of Okazaki fragment maturation processes; (vii) RFC, which is the clamp loader for PCNA to load onto DNA duplex; (viii) nucleases, including RNase H, DNA2 and FEN1 for removal of RNA primers and (ix) DNA Lig I, which joins processed Okazaki fragments into an intact DNA lagging strand. Black lines represent the DNA template, while pink ones are the newly synthesized DNA and light pink ones are the RNA primers (Zheng and Shen 2011).
FIGURE 1.3 Nucleases in sequential processing of RNA/DNA primers through three different pathways during Okazaki fragment maturation.

Upper panels show three different pathways involved in processing RNA primers: (i) FEN1-mediated short flap cleavage (middle); (ii) long flap degradation by sequential actions of DNA2 and FEN1 (right) and (iii) RNA primer removal by RNase H/FEN1 exonuclease (left). The bottom panel indicates the action of FEN1 or exonuclease to edit out incorporation errors of Pol α. Yellow circles represent ribonucleotides and cyan squares represent mismatched deoxyribonucleotides. Black lines correspond to DNA templates and pink lines correspond to newly synthesized DNA. Blue arrows indicate cleavage by RNase H or the exonuclease activity of FEN1 or exo-1 (Zheng and Shen 2011).

FEN1 is a structure-specific endonuclease that processes flap-structured double-stranded DNA (dsDNA) (Strathdee, Gavish et al. 1992, Dorsman, Levitus et al. 2007) and cleaves at the junction of the flap (Howlett, Taniguchi et al. 2002, Levitus, Waisfisz et al. 2005, Litman, Peng et al. 2005, Dorsman, Levitus et al. 2007). Both short flap cleavage and long flap cleavage pathway need the participation of FEN1 to cleave the flap structure generated when the replication DNA polymerase/PCNA complex encounters (Turchi, Huang et al. 1994, Waga
In the short flap cleavage pathway, Pol $\delta$ displaces the RNA primer to create a predominantly short flap of 2-10nt and later FEN1 cleaves the DNA substrate at the junction between double-stranded and single-stranded DNA (Bambara, Murante et al. 1997, Hosfield, Mol et al. 1998, Liu, Kao et al. 2004, Zheng and Shen 2011). This has been shown to be the dominant pathway in the removal of RNA primers. During long flap cleavage pathway, single-stranded RNA/DNA hybrid flaps longer than 30nt may attract RPA, which inhibits FEN1 activity but recruits Dna2 to cleave a portion of DNA in the middle of the long flap, generating a 5-7nt short flap. The remaining flap is further removed by FEN1 to produce a substrate for ligation (Bae, Choi et al. 1998, Bae and Seo 2000, Bae, Bae et al. 2001, Kim, Kim et al. 2006, Masuda-Sasa, Imamura et al. 2006). The RNA primer may also be removed by RNase H. RNase H cleaves all ribonucleotides except the last one, which is later cleaved by FEN1 through its exonuclease activity (Turchi, Huang et al. 1994, Waga and Stillman 1994, Zheng and Shen 2011). FEN1 or EXO1 further edit out incorporation error of Pol $\alpha$ and DNA ligase 1 sealed the ends to finish Okazaki fragment maturation (Shuman 2009, Balakrishnan, Gloor et al. 2010, Zheng and Shen 2011).

Additionally, FEN1 has 5' to 3' exonuclease activity and gap-dependent endonuclease activity that are important during maturation of Okazaki fragments and the rescue of stalled replication forks (Turchi, Huang et al. 1994, Waga and Stillman 1994, Parrish, Yang et al. 2003, Liu, Kao et al. 2004, Zheng, Zhou et al. 2005, Dorsman, Levitus et al. 2007, Zheng and Shen 2011). FEN1 is also

More than 30 proteins have been shown to interact with FEN1 and affect its function (Liu, Kao et al. 2004, Zheng, Jia et al. 2011). For example, proliferating cell nuclear antigen stimulates FEN1 endonuclease activity through protein-protein interaction (Li, Li et al. 1995, Wu, Li et al. 1996, Tom, Henricksen et al. 2000). RecQ DNA helicases such as Werner syndrome protein WRN and Bloom syndrome protein BLM were shown to stimulate FEN1 through physical interaction (Brosh, Driscoll et al. 2002, Imamura and Campbell 2003, Liu, Qiu et al. 2006). It was also reported that MUS81-EME1 and MUS81-EME2, DNA endonucleases involved in interstrand crosslink unhooking and Holliday junction resolution, stimulate FEN1 activity (Shin, Amangyeld et al. 2012).

1.5 DNA INTERSTRAND CROSSLINK REPAIR AND DNA DAMAGE RESPONSE TO ICLS

DNA interstrand crosslinks are the most cytotoxic damage to dividing cells, because they tether the two complementary strands of the double helix through covalent bounds created by bifunctional agents such as cisplatin, nitrogen mustard, Mitomycin C and psoralen. While these agents have an important role in chemotherapy regimens for cancer, the ability of cells to repair
DNA ICLs is a critical determinant of drug sensitivity. Understanding of the cellular mechanisms that act to eliminate these toxic DNA lesions is clearly important (Dronkert and Kanaar 2001, McHugh, Spanswick et al. 2001).

The mechanisms of ICL repair have been well studied in the model organism like E. coli and S. cerevisiae. A pathway which combines nucleotide excision repair and homologous recombination repair to eliminate ICL has been characterized in E. coli, both genetically and biochemically. In S. cerevisiae, several pathways, NER, post-replication repair like translesion synthesis and HRR coordinately work together have been proposed. The main difference between the E. coli model and yeast model might be the involvement of double strand breaks triggered by ICLs. These DSBs is highly cell-cycle dependent since it happens more often in S-phase. Nonhomologous end-joining seems to be excluded in repair of the ICL-induced DSBs, which suggests that ICL repair is likely to be coupled with replication. Thus, the ICL repair in eukaryotes is more complicated than in prokaryotes (Lehoczyk, McHugh et al. 2007, McCabe, Olson et al. 2009). Studies in mammalian cells show that multiple players from established DNA repair pathways, such as FA, NER, TLS, HRR and MMR, have to work coordinately in order to remove a single interstrand crosslink lesion multiple independent pathways are involved in ICL repair (Figure 1.1) (Dronkert and Kanaar 2001, Zhang, Lu et al. 2002, Niedzwiedz, Mosedale et al. 2004, Niedernhofer, Lalai et al. 2005, Mirchandani and D'Andrea 2006, Wang 2007, Zhang, Liu et al. 2007, Bergstrahl and Sekelsky 2008, Ciccia, McDonald et al. 2009).
FIGURE 1.4 The signaling pathways of ATM, ATR and DNA-PK

The signaling pathways of ATM, ATR and DNA-PK following DNA damage. Once activated, these kinases can phosphorylate several downstream effectors to execute cellular responses that can lead either to growth arrest at the various cell cycle stages or to apoptosis. While ATM and ATR play prominent roles in mediating cell cycle checkpoints through the action of Chk1/2 and p53, DNA-PK usually activates the p53-mediated apoptosis pathway. Furthermore, a negative feedback regulation loop allows Mdm2 to down-regulate p53 (Yang, Yu et al. 2003).

DNA structure alteration by cellular metabolites and exogenous DNA-damaging agents is a common event in the life of a cell. Since these alterations
might lead to mutation, cancer and organismic death, cells developed several damage response pathways including DNA damage checkpoints, DNA repair, transcriptional response and apoptosis to deal with the damage. Defects of these pathways cause genomic instability (Zhou and Elledge 2000, Sancar, Lindsey-Boltz et al. 2004). The DNA damage response is a signal-transduction pathway that coordinates cell-cycle transitions, DNA replication, DNA repair and apoptosis. The major regulators are the phosphoinositide 3-kinase related protein kinases, including ATM and ATR (Sancar, Lindsey-Boltz et al. 2004, Yang, Xu et al. 2004, Cimprich and Cortez 2008). This pathway can be envisioned as a signal transduction cascade in which DNA lesions act as the initial signal that is detected by sensors and passed down through transducers in the presence of mediators. Eventually the effectors receive the signal and execute various cellular functions (Figure 1.4) (Yang, Yu et al. 2003, Sancar, Lindsey-Boltz et al. 2004).

The presence of any DNA damage that is bulky enough to impede the progression of replication forks is likely to be initially detected by the replication machinery. Upon stalling of the replicative DNA polymerase, the minichromosome maintenance helicase in the replication machinery continues to unwind DNA ahead of the fork, resulting in exposure of single-stranded DNA (Harper and Elledge 2007). The single-stranded DNA is quickly coated by ssDNA binding protein RPA to prevent degradation by DNA nucleases. More importantly, this RPA-coated ssDNA serves as an anchor to independently recruit ATR-ATRIP, Rad17-RFC, the 9-1-1 complex, and claspin, leading to the activation of
the ATR DNA damage response pathway (Figure 4.4A), and resulting in an intra S checkpoint (Sancar, Lindsey-Boltz et al. 2004, Byun, Pacek et al. 2005, Harper and Elledge 2007). Since ICLs present an essentially unsurmountable barrier for DNA helicases, one might expect the checkpoint activation by ICLs to be limited due to lack of ssDNA exposure. However, ICL damage actually does activate the ATR damage response pathway, resulting in an S phase checkpoint arrest (Pichierri and Rosselli 2004). The replication forks are under constant surveillance by ATR kinase that is activated during S phase to regulate the firing of replication origins and the repair of damage replication forks (Nyberg, Michelson et al. 2002, Shechter, Costanzo et al. 2004).

Extensive genetic analysis in human cells have identified proteins involved in damage sensing, signal transduction and effector steps of the DNA damage checkpoints. TOPBP1 is a crucial activator of ATR kinase activity, which is recruited to ATR by the 9-1-1 clamp (Delacroix, Wagner et al. 2007, Lee, Kumagai et al. 2007). Claspin, a mediator protein that functions to bring ATR and Chk1 together, is found at the replication fork and is crucial for the Chk1 activation (Kumagai and Dunphy 2000). Timeless and Tipin also mediates Chk1 phosphorylation by ATR, probably through interaction with RPA (Errico, Costanzo et al. 2007, Unsal-Kacmaz, Chastain et al. 2007, Stracker, Usui et al. 2009, Kemp, Akan et al. 2010). Chk1 is one of the transducers for ATR kinase. In response to replication blocks, Chk1 is phosphorylated at Serines 317 and 345 by ATR kinase (Lopez-Girona, Tanaka et al. 2001). Once phosphorylated, Chk1 is release from chromatin to phosphorylate its substrates including Cdc25, a cell-

Several groups found that DSBs or short-stranded overhangs (SSOs) can sequentially activate ATM and ATR though a MRE11 and EXO1-mediated end resection mechanism (Jazayeri, Falck et al. 2006, Myers and Cortez 2006, Shiotani and Zou 2009, Flynn and Zou 2010). Chk2 is one of the transducers for ATM kinase and phosphorylated by ATM at threonine 68 in response to ionizing radiation (Ahn, Schwarz et al. 2000, Matsuoka, Rotman et al. 2000). p53, a well-known tumor suppressor and cell cycle regulator, can be phosphorylated at Serine 15 by ATM and ATR during cellular exposure to genotoxic stress, resulting in proliferative arrest (Banin, Moyal et al. 1998, Canman, Lim et al. 1998, Tibbetts, Brumbaugh et al. 1999).

Mechanistic insight into how the FA proteins participate in an S phase checkpoint for ICL damage is emerging depending on G2/M arrest as a common phenotype of crosslinking agents treated FA cells (Neveling, Endt et al. 2009, Thompson and Hinz 2009). The inhibition of DNA synthesis in response to crosslinking is mediated by the ATR kinase (Pichierri and Rosselli 2004, Thompson and Hinz 2009). In response to DNA damage that blocks replication like the ICL, cells have developed a sophisticated ATR damage surveillance and
signaling mechanism (Figure 4.4A) to protect replication forks from collapse
(Sancar, Lindsey-Boltz et al. 2004, Cimprich and Cortez 2008, Stracker, Usui et
al. 2009). Intriguingly, this checkpoint activation requires the FA core complex
and FANCD2 (Pichierri, Averbeck et al. 2002, Pichierri and Rosselli 2004,
Pichierri and Rosselli 2004, Collis, Ciccia et al. 2008). Thus FA proteins appear
to act as replication-coupled DNA damage sensors in this scenario (Sobeck,
phosphorylate many FA proteins, such as FANCA at serine 1449 (Collins, Wilson
et al. 2009), FANCE at threonine 346 and serine 374 (Wang, Kennedy et al.
2007), FANCM (Kim, Kee et al. 2008), FANCI (Smogorzewska, Matsuoka et al.
2007) and FANCD2 at serine 222 (Taniguchi, Garcia-Higuera et al. 2002). These
phosphorylations provide an important mechanism of regulation that ensures the
FA core complex to be recruited to chromatin only during S phase, but not
mitosis phase of the cell cycle.
CHAPTER 2

FANCONI ANEMIA COMPLEMENTATION GROUP A PROTEIN HAS INTRINSIC AFFINITY FOR NUCLEIC ACIDS WITH PREFERENCE FOR SINGLE-STRANDED FORMS

2.1 SUMMARY

The Fanconi anemia complementation group A gene is one of 15 disease-causing genes and has been found to be mutated in more than 60% of Fanconi anemia patients. Using purified protein, we observed that human FANCA has intrinsic affinity for nucleic acids. FANCA binds to both single-stranded DNA and double-stranded DNAs; however, its affinity for ssDNA is significantly higher than for dsDNA in an electrophoretic mobility shift assay. FANCA also binds to RNA with an intriguingly higher affinity than its DNA counterpart. FANCA requires a certain length of nucleic acids for optimal binding. Using DNA and RNA ladders, we determined that the minimum number of nucleotides required for FANCA recognition is ~30 for both DNA and RNA. By testing the affinity between FANCA and a variety of DNA structures, we found that a 5’-flap or 5’-tail on DNA facilitates its interaction with FANCA. A patient-derived FANCA truncation mutant (Q772X) has diminished affinity for both DNA and RNA. In contrast, the complementing C-terminal fragment of Q772X, C772–1455, retains the differentiated nucleic acid-binding activity (ssRNA > ssDNA > dsDNA), indicating that the nucleic acid-binding domain of FANCA is located primarily at its C terminus. This novel property of FANCA supports its role in DNA damage repair and should be helpful in understanding how FANCA and the whole FA core complex contribute to the maintenance of genomic stability.
2.2 MATERIALS AND METHODS

2.2.1 Expression and purification of human FANCA proteins

cDNA for human FANCA was obtained by PCR amplification from a universal cDNA pool (BioChain Institute, Inc.). The full length open reading frame of FANCA was sequenced and found to exactly match NCBI Reference Sequence NM_000135. Overexpression of hexahistidine-tagged FANCA was achieved in insect High Five cells using the Bac-to-Bac expression system (Invitrogen). Truncation mutants were produced through a PCR-based method (Mao, Pan et al. 2007). Expression of FANCA and its mutants was confirmed by Western blot analysis using a Pierce ECL kit. Antibodies against FANCA were kindly provided by the Fanconi Anemia Research Fund. Monoclonal antibody THE against the His₆ tag (GenScript, Piscataway, NJ) was also used to confirm expression and subsequent purification. Upon expression of the recombinant proteins in insect cells, the cells were homogenized using a Dounce homogenizer to prepare extracts. Wild-type FANCA and various truncation mutants were purified using a HiTrap Q-Sepharose Fast Flow column; a 5ml HiTrap Blue column; a Mono S, Mono Q, and/or Superdex 200 gel filtration column (GE Healthcare); and/or a 2ml high-resolution hydroxylapatite column (Calbiochem) and by tracing FANCA protein through SDS-PAGE and Western blotting. Protein concentration was determined using the Coomassie (Bradford) protein assay reagent (Pierce). The purified proteins were stored at -80°C in aliquots. Purified replication protein A was prepared as described previously (Zhang, Yuan et al. 2005).
2.2.2 Substrate preparation

Oligonucleotides that were used to create singlestranded DNA (ssDNA; 61-mer), double-stranded DNA (dsDNA; 61bp), the 5’-tail (30-mer for the single-stranded part and 31bp for the double-stranded part), the 3’-tail (30bp for the double-stranded part and 31-mer for the single-stranded part), the splayed arm (30bp for the double-stranded part and 31-mer for the single-stranded part), the 5’-flap (with a 31-mer flap), the 3’-flap (with a 31-mer flap), the static fork (all arms are 30bp), and the static Holliday junction (all four arms are symmetrically 30bp) were adopted from Gari et al. (Gari, Decaillet et al. 2008) with the same sequences. It should be noted that there is a 1-base 5’-overhang, originally designed to label the 3’-end of the substrates, on the double-stranded area of the 5’- and 3’-tails, splayed arm, and 5’- and 3’-flaps and on one arm of the static fork and Holliday junctions. Annealing was carried out in a water bath within ~5h by slowly cooling from 85°C to 20°C. The quality of annealing was monitored by native gel electrophoresis. Proper annealing was verified by the mobility of a corresponding substrate, e.g. the static Holliday junction moves slowest because of its largest size. RNA was chemically synthesized by Integrated DNA Technologies, Inc. using the same sequence as the 61-mer ssDNA.

2.2.3 Electrophoretic mobility shift assay

DNA binding EMSA analysis was performed as described previously (Yuan, El Hokayem et al. 2009), in a 10µl reaction containing 25mM Tris-HCl (pH7.5), 100mM NaCl, 5mM EDTA, 1mM DTT, 6% glycerol, 1nM 5’-32P-labeled oligonucleotide substrates, and the indicated amounts of protein. The reactions
were incubated at 18°C for 45 min, followed by the addition of 4 µl of 50% (w/v) sucrose. The reaction mixtures were resolved by electrophoresis through a 4% nondenaturing polyacrylamide gel in 40 mM Tris acetate (pH 7.6) and 10 mM EDTA with 6% glycerol using the Owl P9DS electrophoresis system (Thermo Scientific). The setting was 100 V (~1.5 watts/gel) for 40 or 90 min as indicated. DNA substrates and shifted bands were visualized by autoradiography. Quantitation of the bands was performed using NIH ImageJ software.

2.2.4 Determination of dissociation constant of FANCA for nucleic acids

At steady state (equilibrium), $K_d$ can be determined through the following equation: $K_d = [A][B]/[AB]$, where [A], [B], and [AB] are the concentrations of FANCA, nucleic acids, and the FANCA-nucleic acid complex, respectively. Because the concentration of nucleic acids ([B]) was very low in our EMSA experiments (1 nM), the FANCA protein concentration ([A]) that shifted 50% of nucleic acids ([AB] = [B], thus [B]/[AB] = 1) was used to estimate $K_d$.

2.2.5 Determination of minimum length of nucleic acids required for FANCA binding using DNA and RNA ladders

DNA ladders (10-, 20-, 30-, 40-, 50-, 61-, 71-, and 99-mer) were created by mixing oligonucleotides with different sequences and labeling with $^{32}$P. RNA ladders (10-, 20-, 30-, 40-, 50-, 60-, 70-, 80-, 90-, and 100-mer) was created as recommended by the manufacturer (Ambion) and labeled with $^{32}$P. 10-mer, 15-mer, and 30-mer oligonucleotides were the 3'-truncated forms of a 61-mer oligonucleotide: GACGCTGCGAATTCTACCAGTCCTGCTAGGACATCTTTT
GCCCACCTGCAGGTTCACCC. Corresponding dsDNAs were prepared by annealing with the complementing oligonucleotides.

2.3 RESULTS

2.3.1 Purified human FANCA efficiently interacts with nucleic acids

To study its biochemical properties, we overexpressed wild-type human FANCA protein in insect cells using the Bac-to-Bac expression system. As shown in Figure 2.1B, we purified WT FANCA to near homogeneity. Purified WT FANCA migrated to a position corresponding to its calculated molecular mass of 164 kDa on SDS-PAGE gel, indicating that it was the full-length protein. The identity of FANCA was further confirmed by Western blotting using a FANCA-specific antibody and an anti-His$_6$ antibody (Figure 2.1B).

Because FANCA has been shown to be involved in many steps of DNA repair, we reasoned that FANCA is likely to interact directly with DNA. Indeed, EMSA analysis by incubating increasing amounts of purified FANCA with $^{32}$P-labeled ssDNA or its dsDNA counterpart showed that FANCA bound to both ssDNA and dsDNA in a concentration-dependent manner (Figure 2.2A). Intriguingly, FANCA had significantly greater affinity for ssDNA than for dsDNA. Using the paired t test, we determined the statistical significance of shifts between ssDNA and dsDNA (Figure 2.2B). The p values for 16, 32, and 64nM FANCA between ssDNA and dsDNA were 0.013, 0.031, and 0.014, respectively, which indicates a significant difference (p<0.05). Furthermore, the $K_d$ determined by protein titration showed that FANCA bound to ssDNA ~4-fold better than to dsDNA (11.1nM for ssDNA and 42.3nM for dsDNA) (Table 2.1).
FIGURE 2.1 Purification of FANCA and its mutants.

(A) schematic diagram of predicted FANCA motifs and strategy of mutagenesis. The range of numbers indicates how FANCA was truncated. NLS, predicted nuclear localization signal (amino acids 18–34 or 19–35). The leucine zipper (amino acids 1069–1090) and a phosphorylation site (serine) are also indicated. (B) SDS-PAGE of the purified proteins stained with Coomassie Brilliant Blue R-250 and Western blotting. Q772X is a C-terminal truncation mutant derived from an FA patient. C772–1455 is C-terminal residues 772–1455 of FANCA. All peptides shown were tagged with hexahistidine at their N termini. Protein markers in kilodaltons are indicated.
FIGURE 2.2 FANCA has intrinsic affinity for nucleic acids.

(A) EMSA titration of WT FANCA for nucleic acid-binding activity. Diagrams of the nucleic acid substrates are shown on top of each set of reactions. The $^{32}$P-labeled 5’-end is indicated by an asterisk. The concentrations of FANCA indicated are 0, 4, 8, 16, 32, and 64nM. The 61-mer nucleic acid substrate concentration was 1nM. The reactions were resolved in 40min nondenaturing gel electrophoresis. The protein-nucleic acid complex is indicated by an arrow. Sub, nucleic acid substrates. (B) Quantitation of three independent EMSA experiments with His6-tagged FANCA in A. Shift (%) is the ratio of the shifted band versus substrate band. Error bars represent S.D. A paired t test was performed to determine the statistical significance between ssDNA and dsDNA and between ssDNA and ssRNA. **, significant difference (p<0.05) between ssDNA and dsDNA; *, significant difference (p<0.05) between ssDNA and ssRNA.
TABLE 2.1 Dissociation constant (Kd) of FANCA for nucleic acids

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Kd  (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssRNA</td>
<td>2.8 ± 0.49</td>
</tr>
<tr>
<td>Splayed arm</td>
<td>10.2 ± 0.07</td>
</tr>
<tr>
<td>ssDNA</td>
<td>11.1 ± 1.77</td>
</tr>
<tr>
<td>5’-Flap</td>
<td>12.3 ± 0.49</td>
</tr>
<tr>
<td>5’-Tail</td>
<td>12.6 ± 0.50</td>
</tr>
<tr>
<td>3’-Tail</td>
<td>16.9 ± 4.38</td>
</tr>
<tr>
<td>3’-Flap</td>
<td>17.2 ± 1.63</td>
</tr>
<tr>
<td>Holliday junction</td>
<td>27.0 ± 6.29</td>
</tr>
<tr>
<td>Static fork</td>
<td>35.2 ± 2.33</td>
</tr>
<tr>
<td>dsDNA</td>
<td>42.3 ± 3.89</td>
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</table>

Because FA is also a developmental disease and FANCA is involved in the regulation of gene expression (Tulpule, Lensch et al., Tremblay, Huang et al. 2008, Tremblay, Huard et al. 2009), it is conceivable that FANCA could somehow be involved in RNA transactions, e.g. RNA stability, transcription, or translation, to carry out its functions. When FANCA was incubated with a single-stranded RNA (ssRNA) oligonucleotide with the same sequence as the ssDNA, we indeed observed that FANCA possessed affinity for RNA. In fact, its affinity for ssRNA determined by $K_d$ was significantly better than for ssDNA (2.8 nM for ssRNA and 11.1nM for ssDNA) (Table 2.1). The p values determined by paired t test for 4 and 8nM FANCA between ssDNA and ssRNA showed a significant difference (0.041 and 0.033 for 4 and 8nM FANCA, respectively) (Figure 2.2B).

2.3.2 FANCA requires large area of nucleic acids for optimal binding

The preferential binding of FANCA to ssDNA resembles that of RPA, a well known ssDNA-binding protein that is involved in DNA replication, damage
signaling, recombination, and repair (Wold 1997, Fanning, Klimovich et al. 2006, Oakley and Patrick 2010). EMSA analysis of purified RPA (Zhang, Yuan et al. 2005) with ssDNA and dsDNA of different lengths indicated that RPA is very specific for ssDNA and forms protein-DNA filaments with increasing amounts of RPA and increasing sizes of ssDNA (multiple shifted bands in Figure 2.3, right panel). A high concentration of RPA could shift 10-nucleotide ssDNA (10-mer ssDNA in Figure 2.3, right panel); however, it did not efficiently interact with dsDNA, even when it was a 61-mer.

**FIGURE 2.3 FANCA requires larger area of nucleic than RPA.**

EMSA assays were performed by titration of FANCA and RPA with ssDNA and dsDNA of the indicated lengths. The concentrations of both FANCA and RPA were 0, 8, 16, 32, and 64nM as indicated. The substrate concentration was 1nM. The reactions were resolved in 90min nondenaturing gel electrophoresis. The length of the DNA substrates is shown on top of each set of reactions. The protein-DNA complex is indicated by a bracket or an arrow. Sub, nucleic acid substrates.
Unlike RPA, purified FANCA did not bind to ssDNA efficiently when it was shorter than 30 nucleotides (Figure 2.3, left panel). A nitrocellulose filter binding assay using labeled 25-mer ssDNA and ssRNA also demonstrated negative results for FANCA-nucleic acid interaction. When a 30-mer ssDNA oligonucleotide was used for EMSA, we observed an unstable interaction between FANCA and the ssDNA (smear bands in Figure 2.3, left panel). However, the interaction was dramatically improved when a 61-mer oligonucleotide was used, indicating that FANCA requires a larger area of DNA compared with RPA for optimal interaction. Also apart from RPA, FANCA began to interact with dsDNA when it was a 61-mer (61-mer dsDNA in Figure 2.3, left panel).

To further define the minimum size of nucleic acids for optimal interaction, we first incubated a set of ssDNA or ssRNA ladders with purified FANCA. After EMSA, we recovered the nucleic acids in the shifted bands (Figure 2.4A&B right panel) and reran them on a denaturing sequencing gel. As shown in Figure 2.4, with increasing amounts of FANCA, more ssDNA and ssRNA species were identified in the shifted band. However, even with high concentrations of FANCA, ssDNA and ssRNA oligonucleotides shorter than 30 nucleotides were barely detectable in the shifted band, further supporting that FANCA requires ~30 nucleotides of nucleic acids for efficient interaction.

2.3.3 Multiple FANCAs can bind to DNA when it is longer

On the basis of the molecular masses and migration distances of FANCA-DNA and RPA-DNA complexes on the EMSA gel, we reasoned that only one
FANCA is bound to the 61-mer ssDNA oligonucleotide (61-mer ssDNA in Figure 2.5A, left panel). Our next question was whether longer ssDNA has the capacity to accommodate more FANCA molecules and to form a FANCA-DNA filament.

**FIGURE 2.4 FANCA requires 30nt for efficient interaction.**

EMSA were performed with RNA (A) and DNA (B) ladders (3nM). The shifted bands (arrows) were recovered and resolved on a denaturing sequencing gel (upper panels). For DNA ladders, the concentrations of FANCA were 8, 16, 32, and 64nM as indicated; for RNA ladders, the concentrations of FANCA were 2, 4, 8, 16, and 32nM as indicated. Brackets indicated DNA or RNA species shifted by FANCA. DNA and RNA inputs with the indicated sizes in nucleotides are shown on the left of each gel.
FIGURE 2.5 Multiple FANCA can bind to longer nucleic acids.

(A) EMSA using 116- and 61-mer ssDNA oligonucleotides. The 32P-labeled 5'-end is indicated by an asterisk. The concentrations of FANCA were 0, 2, 4, 8, 16, 32, and 64nM as indicated. Arrows indicate two different species of shifted bands. (B) Quantitation of EMSA experiments in A. Shift (%) is the ratio of the shifted band versus substrate band.
Using a synthetic 116-mer ssDNA oligonucleotide, which was almost double the 61-mer oligonucleotide, we found two distinct shifted bands with increasing amounts of FANCA (compare the shifted bands of the 116-mer and 61-mer oligonucleotides in Figure 2.5A), indicating two FANCA molecules on one 116-mer ssDNA molecule. In contrast, only one shifted band was observed with the 61-mer oligonucleotide which corresponding to the faster band in the 116-mer experiment. FANCA had greater overall affinity when the ssDNA was longer (Figure 2.5B).

2.3.4 FANCA prefers DNA structures with 5'-flap or 5'-tail

Because FA proteins are involved in maintaining the stability of replication forks (Sobeck, Stone et al. 2006, Wang, Stone et al. 2008), we reasoned that the DNA-binding activity of FANCA is likely involved in recognition of branched structures. To test this possibility, we performed EMSA with purified WT FANCA and a variety of DNA structures, including the 5'- and 3'-tails, splayed arm, 5'- and 3'-flaps, static fork, and Holliday junction (Figure 2.6) (Yuan, El Hokayem et al. 2009). Intriguingly, the affinity between FANCA and different DNA structures could be divided into three groups: Group I, the 5'-tail, 5'-flap, and splayed arm structures showed the highest close-to-ssDNA level affinity (Figure 2.6, solid red lines); Group II, the 3'-tail and 3'-flap structures had lower affinity compared with Group I (Figure 2.6, dashed green lines); and Group III, the static fork and Holliday junction possessed the lowest affinity for FANCA (Figure 2.6, solid blue lines). The apparent $K_d$ constants of FANCA for all structures also support this grouping (Table 2.1).
FIGURE 2.6 FANCA prefers DNA structures with 5'-flap or 5'-tail.

EMSA assays were performed by titration of FANCA with different DNA structures. The concentrations of FANCA were 8, 16, 32, and 64nM. The substrate concentration was 1nM. The reactions were resolved in 40min nondenaturing gel electrophoresis, quantitated, and expressed as shift (%), which is the ratio of the shifted band versus substrate band. Diagrams of the DNA structures are shown below. Three groups of DNA structures are color-coded. Red asterisks indicate $^{32}$P labeling. RNA, ssDNA, and dsDNA were used as references for comparison. Error bars represent S.D.

Whereas the common feature of Group I structures is that they contain either a 5'-flap or a 5'-tail (31-mer), Group II DNAs have a 3'-flap or a 3'-tail (31-mer as well), and Group III structures barely have any free ends (only a 1-base 5'-overhang designed for 3' labeling). On the basis of these observations, we conclude that FANCA prefers DNA structures with a 5'-flap or a 5'-tail.
2.3.5 Nucleic acid-binding domain of FANCA is located at its C terminus

FANCA does not have any identifiable domains for its interaction with nucleic acids. To define the nucleic acid binding domain of FANCA, we first analyzed the primary structure of FANCA using the PSIPRED protein structure prediction server (University College London) and FANCA is predicted to contain extensive α-helices with a few short β-strands. On the basis of this prediction, we first chose a truncation mutant of FANCA (Q772X) derived from FA patients. Q772X is located roughly in the middle of the 1455-amino acid protein and in the middle of a coiled structure. EMSA of the purified truncation mutant (Figure 2.1A and 2.1B) indicated that the N-terminal moiety of FANCA had diminished nucleic acid binding activity (Figure 2.7, first panel). However, the complementing fragment of Q772X, i.e. C-terminal amino acids 772–1455 of FANCA (Figure 2.1A and 2.1B), bound to nucleic acids more efficiently and retained the differentiated binding activity in the order of RNA>ssDNA>dsDNA (Figure 2.7, second panel).

To further define the nucleic acid-binding domain, we created two additional C-terminal truncation mutants (Figure 2.1A and 2.1B). EMSA tests indicated that the DNA binding activity of both mutants was severely compromised (Figure 2.7, third and fourth panels), although the leucine zipper containing C772–1197 fragment retained partially affinity for ssDNA. The nucleic acid binding results are summarized in Figure 2.1A. Overall, these data demonstrate that the nucleic acid-binding domain is located primarily at the C terminus of FANCA.
FIGURE 2.7 Nucleic acid-binding domain of FANCA.

EMSA assays were performed with titration of the indicated FANCA truncation mutants. The concentrations of the mutants were 0, 8, 16, 32, and 64nM as indicated. The 61-mer substrate concentration was 1nM. The reactions were resolved in 40min nondenaturing gel electrophoresis. Diagrams of the nucleic acid substrates are shown on top of each set of reactions. The $^{32}$P-labeled 5'-end is indicated by an asterisk. The protein-nucleic complex is indicated by brackets or an arrow. Sub, nucleic acid substrates.
2.4 DISCUSSION

An intriguing observation of this study is the preferential binding activity of FANCA for ssDNA over dsDNA. It has been known that recruitment of the FA core complex to chromatin relies strictly on replication (Sobeck, Stone et al. 2006, Wang, Stone et al. 2008). Therefore, it is likely that FANCA recognizes the exposed ssDNA in the stalled replication forks, which look like a 5’-flap structure or a splayed arm, and contributes to assembly of the FA core complex on the stalled forks. It would be interesting to further investigate whether and how the DNA-binding activity of FANCA facilitates repair of crosslinks through damage recognition, translesion synthesis (Mirchandani, McCaffrey et al. 2008), and homologous recombination events (Yang, Herceg et al. 2005).

Thus far, FANCM-FAAP24 is the only identified DNA-binding component in the FA core complex (Meetei, Medhurst et al. 2005, Ciccia, Ling et al. 2007). FANCM can remodel stalled replication forks through fork reversal and branch migration, thus stabilizing the stalled replication forks and providing temporal and spatial access for the damage to be repaired (Gari, Decaillet et al. 2008, Gari, Decaillet et al. 2008). FANCM appears to be responsible for recruitment of the FA core complex to chromatin (Garcia-Higuera, Kuang et al. 2000, Meetei, Medhurst et al. 2005, Medhurst, Laghmani el et al. 2006, Ciccia, Ling et al. 2007, Ling, Ishiai et al. 2007, Ciccia, McDonald et al. 2008, Kim, Kee et al. 2008). The monoubiquitinated FANCI-FANCD2 complex may also be recruited to chromatin through a FANCM-dependent mechanism (Garcia-Higuera, Taniguchi et al. 2001, Montes de Oca, Andreassen et al. 2005, Mosedale, Niedzwiedz et al.
2005, Smogorzewska, Matsuoka et al. 2007). However, unlike other factors in the core complex, FANCM is not required for the formation of the eight-subunit (but not the 10-subunit) core complex (Kim, Kee et al. 2008), and FANCM<sup>−/−</sup> cells are only partially deficient in damage-induced FANCD2 monoubiquitination (Rosado, Niedzwiedz et al. 2009, Singh, Bakker et al. 2009). Fancm<sup>−/−</sup> knock-out mice further support that FANCM may have a stimulatory but not essential role in monoubiquitinating FANCD2 (Bakker, van de Vrugt et al. 2009). Additionally, a direct interacting partner for FANCMFAAP24 in the FA core complex has not been identified thus far, although FANCM-FAAP24 was originally identified through protein association in a FANCA-specific immunoprecipitation assay (Meetei, Sechi et al. 2003, Meetei, Medhurst et al. 2005, Thompson and Hinz 2009). FANCM<sup>−/−</sup> cells are sensitive to camptothecin, a topoisomerase inhibitor. Susceptibility to camptothecin is a unique feature identified only for FANCD1/BRCA2 and FANCN/PALB2 but not for components of the FA core complex (Singh, Bakker et al. 2009). These observations suggest that FANCM may act downstream of FANCD2, and therefore, the upstream FA core complex may be recruited to DNA through other mechanisms, such as the DNA-binding activity of FANCA. Based on our observations, FANCA is capable of recruiting the FA core complex to stalled replication forks through its ssDNA-binding activity and its preferential recognition of 5’-flap and splayed arm structures.

Another interesting insight to emerge from these studies is that FANCA has a higher affinity for ssRNA than for ssDNA. There is currently limited information to explain how the RNA binding activity of FANCA could be linked to
its functions. However, we think this activity may be physiologically relevant to RNA-related processes, such as transcription, translation, and RNA stability. First, the FA core complex has been reported to be involved in regulating gene expression through transcriptional (Tremblay, Huard et al. 2009) and post-transcriptional (Li and Youssoufian 1997) mechanisms. Second, besides the nucleus, FANCA does localize to the cytoplasm (Kruyt, Waisfisz et al. 1997, Naf, Kupfer et al. 1998, Du, Li et al. 2010), which supports a possible function in RNA metabolism. Third, FANCA has been shown to functionally interact with PKR, a critical factor in translational control as well as regulation of cell proliferation and apoptosis (Zhang, Li et al. 2004). We speculate that, through its RNA-binding activity, FANCA may actively participate in these important biological processes. Further investigation into this issue should help us understand the unusually disproportional contribution of FANCA to FA.

The nucleic acid-binding domain of FANCA is located at its C terminus, where an imperfect leucine zipper and an ATR phosphorylation site are found (Figure 2.1A) (Collins, Wilson et al. 2009). It would be interesting to test whether the partial leucine zipper and the phosphorylation site have any effect on nucleic acid binding. It is very intriguing that, by analyzing the FANCA variants (1380 public entries as of March 23, 2011) available in the Fanconi Anemia Mutation Database, we found that ~90% of the reported disease-causing point mutations of FANCA are located at the C terminus (from amino acids 772 to 1455), where the nucleic acid-binding domain is identified, further supporting the idea that FANCA is likely to exert its functions through its affinity for nucleic acids.
CHAPTER 3

HUMAN FANCONI ANEMIA COMPLEMENTATION GROUP A PROTEIN
STIMULATES THE 5’ FLAP ENDONUCLEASE ACTIVITY OF FEN1

3.1 SUMMARY

In eukaryotic cells, Flap endonuclease 1 is a major structure-specific
endonuclease that processes 5’ flapped structures during maturation of lagging
strand DNA synthesis, long patch base excision repair, and rescue of stalled
replication forks. Here we report that Fanconi anemia complementation group A
protein, a protein that recognizes 5’ flap structures and is involved in DNA repair
and maintenance of replication forks, constantly stimulates FEN1-mediated
incision of both DNA and RNA flaps. Kinetic analyses indicate that FANCA
stimulates FEN1 by increasing the turnover rate of FEN1 and altering its
substrate affinity. More importantly, six pathogenic FANCA mutants are
significantly less efficient than the wild-type at stimulating FEN1 endonuclease
activity, implicating that regulation of FEN1 by FANCA contributes to the
maintenance of genomic stability.

3.2 MATERIALS AND METHODS

3.2.1 Expression and purification of proteins

cDNAs for human FANCA and FEN1 were obtained by PCR amplification
from a universal cDNA pool (BioChain Institute, Inc.). The full-length open
reading frames were confirmed by sequencing and found to exactly match NCBI
Reference Sequence NM_000135 and NM_004111 respectively. Overexpression
and purification of hexahistidine-tagged FANCA was achieved in insect High Five
cells using the Bac-to-Bac expression system (Invitrogen) as previous described
Truncation mutants of FANCA were produced through a PCR-based method (Mao, Pan et al. 2007). Point mutations were produced through a site-directed Mutagenesis Kit (Agilent). Expression of FANCA and its mutants was confirmed by Western blot analysis using FANCA Antibody (Santa Cruz Biotech.). Monoclonal antibody against the His6 tag (GenScript, Piscataway, NJ) was also used to confirm expression and subsequent purification. Protein concentration was determined using the Coomassie protein assay reagent (Pierce). The purified proteins were stored at -80 °C in aliquots. Purified FEN1 was prepared as described previously (Zheng, Li et al. 2002, Zheng, Zhou et al. 2005) and confirmed by western blot using a FEN1 antibody (Epitomics).

3.2.2 Preparation of substrates

Oligonucleotides that were used to create the 15-nt 5’ flap substrates were adopted from a design by Fisher et al with the same sequences (Figure S3.1) (Fisher, Bessho et al. 2008). RNA/DNA hybrid oligos were chemically synthesized by Integrated DNA Technologies, Inc. with the flap as RNA. All DNA oligos were purified by 10% denaturing PAGE gel. The 5’ ends in the flap structures were labeled by $^{32}$P (Figure S3.1). Annealing was carried out in a water bath within 5 h by slowly cooling from 70 °C to 20 °C.

3.2.3 Endonuclease assay

The endonuclease assay was performed as previously described (Harrington and Lieber 1994). 2nM of 5’ $^{32}$P-labeled 5’ flap substrates were incubated with purified proteins as indicated amount in a 10µl reaction with the
buffer containing 30mM HEPES PH 7.5, 1 mM dithiothreitol, 3mM MgCl₂, 5% glycerol, 100 ng/mL bovine serum albumin and 100mM KCl at 37°C for 15min. The reaction was stopped by adding 10µl 2x sequencing dye (10mM EDTA, 0.2% SDS, 0.03% Xylene cyanol and Bromophenol blue). Reaction products were separated on a 10% or 15% denaturing polyacrylamide gel. The incision products were visualized by autoradiography and quantified by using NIH ImageJ software. The incision rate was calculated by dividing the intensity of product band by the total substrate band of each reaction.

3.2.4 Determination of kinetic parameters

To measure kinetic parameters, kinetic analyses were repeated three times using increasing amounts (described under the figure) 15nt both DNA and RNA 5’ flap substrates. Kinetic parameters were obtained based on the Michaelis-Menten equation: \( v = \frac{V_{\text{max}}[S]}{K_m+[S]} \), where \( v \) is the reaction rate and \([S]\) is the concentration of substrates. \( K_m \) and \( V_{\text{max}} \) were gained by plotting \( v \) against \([S]\) using Origin software through nonlinear curve fit.

3.2.5 Co-IP assay

FANCA-null (RA3087) and the FLAG-FANCA-complemented cells were generously provided by Agata Smogorzewska at the Rockefeller University (Kim, Spitz et al. 2013). Cells were grown in DMEM (sigma) with 10% FBS and harvested at 80% confluence by trypsinization. Cells were washed once by PBS and dissolved in Lysis Buffer (50 mM Tris-HCl, pH 7.6, 500 mM NaCl, 0.5 % NP-40, 2 mM EDTA, 2 mM DTT, 1x proteinase inhibitor, and 1mM Sodium orthovanadate) for sonication by using a Qsonica sonicator. Lysates were
centrifuged and pre-cleaned with 10 µl activated Staph.aureus cells. 600 µg pre-cleaned extracts were incubated in buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM DTT, 50 µg/mL BSA) overnight at 4 ºC with the following antibodies or IgG: mouse monoclonal Anti-FLAG M2 antibody (Sigma Aldrich), rabbit polyclonal anti-FEN1 antibody (Bethyl), mouse IgG1 antibody and rabbit IgG antibody (Santa Cruz). Next, they were incubated with protein G magnetic beads (Millipore) for 1h at 4 ºC, followed by 3 washes with ice cold Wash Buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1x proteinase inhibitor, and 1 mM Sodium orthovanadate). 10µl of Lysis Buffer was used to elute the protein complexes from the beads and the protein complexes were resolved by 10% SDS-PAGE and transfer to nitrocellulose membranes (Bio-Rad). Blots were incubated with the following primary antibodies: goat polyclonal anti-FANCA (C-20) antibody (Santa Cruz), rabbit monoclonal anti-FEN1 antibody (for mouse FLAG co-IP, Epitomics), and mouse monoclonal anti-FEN1 (4E7) antibody (for rabbit FEN1 co-IP, Abcam), followed by incubation with HRP conjugated secondary antibodies and visualization using a Thermo Supersignal detection kit.

3.3 RESULTS

3.3.1 FEN1 incises 5' RNA flap differently from the DNA counterpart

In order to test whether FANCA interacts with FEN1, we overexpressed and purified full-length human FEN1 protein (Figure S3.1A). Considering the length of the in vivo substrate of FEN1, we designed 15-nt 5' DNA and RNA flaps (Figure S3.1B).
**FIGURE S3.1 Purification of FEN1 and FANCA and diagrams of substrates**

(A) SDS-PAGE analysis of purified FANCA and FEN1 proteins. Proteins were subjected to a 10% gel and the gel was stained with Coomassie Brilliant Blue R-250. Protein markers in kilodaltons were indicated. (B) Diagrams and sequence of the 15-nt DNA and RNA flaps.

Initial incubation of the purified protein with the flap structures showed that FEN1 incised both the DNA and RNA flap structures (Figure 3.1A). Intriguingly, FEN1 incised the DNA flap differently from the RNA counterpart. FEN1 had two major incision sites on the 15-nt DNA flap with one right at the junction site (Figure 3.1A, DNA panel, arrow 2) and the other at -1 base inside the junction site (Figure 3.1A, DNA panel, arrow 1). However, FEN1 cut the RNA flap only at the -1 position inside the junction (Figure 3.1A, RNA panel). Furthermore, FEN1 incised the RNA flap significantly more efficiently than the DNA flap (Figure 3.1A, compare lanes 1-7 with 8-14; Figure 3.1B).
FIGURE 3.1 FEN1 incises 5’ RNA flap differently from the DNA counterpart.

(A) FEN1 endonuclease assays were performed with increasing amounts of FEN1 (0.25, 0.5, 1, 2, 4, 8 nM) with both the 15-nt DNA (black) and RNA (green) 5’ flaps (2 nM). Diagrams of the 5’ flap substrates were shown on top of each set of reactions. Reaction products (indicated by an arrow) were resolved in 10% denaturing polyacrylamide gel.

(B) Quantitation of FEN1 endonuclease assays in A by incision rate. Error bars represent standard deviations of three independent experiments. *, p<0.05 when compared the RNA and DNA flap reactions within the same FEN1 concentration.

3.3.2 FANCA stimulates the 5’ flap endonuclease activity of FEN1

Since FANCA and FEN1 share the same substrate specificity, i.e. 5’ flap structures and single-stranded RNA (Yuan, Qian et al. 2012), we hypothesize that FANCA physically and functionally interacts with FEN1. Physical interaction between FANCA and FEN1 has never been reported previously. In order to examine whether FANCA interacts with FEN1 in cells, we prepared whole-cell extracts of the FANCA-null and FLAG-FANCA-complemented cells (FANCA +/-)
and +/+ respectively in Figure 3.2). Next, we performed a co-immunoprecipitation assay of the extracts using a mouse anti-FLAG antibody and detected FANCA and FEN1 using a goat anti-FANCA and a rabbit anti-FEN1 antibodies respectively (Figure 3.2, top panel). As shown in Figure 3.2, When FANCA was pulled down by the anti-FLAG antibody, FEN1 followed, indicating that FANCA interacts with FEN1 in cells. To confirm the physical interaction, we performed the co-immunoprecipitation assay using a FEN1 antibody (Figure 3.2, bottom panel). Again, FANCA was steadily detected in the pull-down lysate. These results indicated that FANCA and FEN1 interact with each other in human cells.

Reciprocal co-immunoprecipitation was performed in FANCA-null (-/-) and FLAG-FANCA-complemented (+/+ ) cells. FLAG-FANCA was pulled down by a mouse FLAG antibody and FEN1 was pulled down by a rabbit FEN1 antibody (Bethyl). Detection of the FANCA and FEN1 was carried out by antibodies with different origins as described in 3.3 Materials and methods.
FIGURE 3.3 FANCA stimulates FEN1 activity

Both the DNA and RNA flap substrates were used at 2 nM. Red asterisks represent the oligos end labeled by $^{32}$P. The concentration of FEN1 was 0.2 nM for DNA flap and 0.1 nM for RNA flap. The concentration of FANCA indicated were 2.5, 5, 10 nM. FANCA+/FEN1- lanes: 10 nM of FANCA. Reaction products were resolved in 10% denaturing polyacrylamide gel. Arrows point to the incision sites. The incision rate is quantified and shown on the bottom. **, p<0.01; *, p<0.05 when compared to the reaction with FEN1 alone.

To test whether FANCA functionally affects the catalytic activity of FEN1, we purified human wild-type FANCA to near homogeneity (Figure S3.1A). Next, we titrated the purified FANCA in a flap endonuclease assay using suboptimal amounts of FEN1 (Figure 3.3, 0.2 nM for DNA flap and 0.1 nM for RNA flap
respectively). To rule out the possibility of the stabilizing effect of proteins on FEN1, we diluted purified FEN1 and FANCA proteins in a buffer with 1 µg/µl BSA. Incision of the 15-nt DNA and RNA flaps by FEN1 alone is ~11% and ~4% of the total substrate respectively.

However, addition of increasing amount of FANCA greatly enhanced the flap endonuclease activity of FEN1 by up to 8-fold for RNA flap and 4.5-fold for DNA flap within the titration range (Figure 3.3). These data establish that FANCA physically interacts with FEN1 and functionally stimulates the flap endonuclease activity of FEN1 in a concentration-dependent manner.

3.3.3 FANCA increases the enzyme efficiency of FEN1

To determine how FANCA affects the flap endonuclease activity of FEN1, we performed a steady-state analysis of FEN1 by titration of the DNA and RNA flap substrates in the presence or absence of FANCA (Figure S3.2). The obtained incision rate (v) and the substrate concentration [S] were fit into the Michaelis-Menten equation $v = \frac{V_{\text{max}}[S]}{+[S]}$ in a nonlinear manner (Figure 3.4) to determine $V_{\text{max}}$ and $K_m$ (as described in “3.3 Material and methods”) and obtain other kinetic parameter such as $k_{\text{cat}}$ (Table 3.1). As shown in Table 3.1, FANCA resulted in ~4-5 fold increase in FEN1 enzyme turnover rate on the DNA flap ($k_{\text{cat}}$ 0.125 vs. 0.026). However, FANCA reduced the DNA flap substrate affinity of FEN1 ($K_m$ 13.5 vs. 33.9). Overall, FANCA increased the enzyme efficiency of FEN1 by 2-fold ($k_{\text{cat}}/K_m$ 1.9 pM⁻¹s⁻¹ vs. 3.7 pM⁻¹s⁻¹). A similar result was obtained with the RNA flap substrate (Figure 3.4, Table 3.1), i.e., FANCA increased the turnover as well as Km of FEN1 with a 1.7-fold overall increase in
enzyme efficiency. This result is distinct from that of PCNA which decreased the Km of FEN1 (Tom, Henricksen et al. 2000), as well as that of WRN or RFC, which increased the Vmax but did not alter substrate binding (Brosh, Driscoll et al. 2002, Cho, Kim et al. 2009).

**FIGURE S3.2 Kinetic analyses of FANCA stimulation of FEN1 activity**

Kinetic analyses of FEN1 endonuclease activity were performed with or without WT FANCA protein for both $^{32}$P-labeled 15-nt length DNA and RNA 5' flap substrates with increasing amount of non-labeled “cold” substrates to a final concentration of 0, 2, 6, 20, 60, 200 nM. The concentration of $^{32}$P-labeled “hot” substrates was 1 nM. The concentration of FEN1 was 0.2 nM for DNA flap and 0.1 nM for RNA flap. The concentration of FANCA was 10 nM. Reaction products were resolved in 15% denaturing polyacrylamide gel. Arrows point to the incision products. The incision product band is quantified, converted to the final product concentration and shown on the bottom.
FIGURE 3.4. FANCA stimulates the 5’ flap endonuclease activity of FEN1.

Steady-state analysis was perform by increasing the flap substrate concentration (0, 2, 6, 20, 60, 200 nM) and fixing amounts of FEN1 (0.2 nM for DNA flap, 0.1 nM for RNA flap) and/or FANCA (10 nM) in three independent FEN1 endonuclease assays (Figure S3.2). Error bars represent stand errors. A paired t-test was performed to determine the statistical significance of FEN1 endonuclease activity between with and without FANCA. **, p<0.01; *, p<0.05.
### TABLE 3.1 Kinetic parameters of FEN1 incision with or without FANCA

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</table>

#### 3.3.4 Both N- and C-terminals of FANCA are required for the stimulation of FEN1 activity

We previously showed that FANCA has a nucleic acid binding domain at the C terminal and this domain confers the preferential binding of FANCA to ssRNA, ssDNA, and 5' flaps (Yuan, Qian et al. 2012). To test whether the nucleic acid binding domain of FANCA affects the stimulation of FEN1 by FANCA, we used two truncation mutants of FANCA, Q772X and C772-1455 for FEN1 assay. Q772X is a Fanconi anemia disease-causing C-terminal truncation mutant. C772-1455 is the complementing C-terminal fragment of Q772X (Figure S3.3). Using 10 nM of protein that is sufficient for the WT protein to exert its stimulation, we found that both mutants showed drastic reduction in stimulating FEN1 endonuclease activity (Figure 3.5A and 3.5B, last two lanes in each panel). These results indicate that both the N-terminal and the nucleic acids binding C-terminal of FANCA are indispensable for FEN1 stimulation and that the DNA binding domain by itself is insufficient to regulate FEN1 activity.

#### 3.3.5 Pathogenic FANCA mutants are significantly less efficient in stimulating FEN1 activity

To evaluate whether stimulation of FEN1 by FANCA is physiologically relevant to Fanconi anemia, we created 5 more FANCA point mutations and
purified them to near homogeneity (Figure S3.3). D598N, R951W, R1055W, R1117G, and F1263Δ are selected from FANCA mutations that cause Fanconi anemia (Adachi, Oda et al. 2002, Levran, Diotti et al. 2005). D598N, R951W, R1055W, and R1117G are pathogenic missense point mutations. F1263Δ is a one residual deletion mutant representing one of the most prevalent pathogenic point mutations. Using 10 nM of protein, we found that all of the FANCA disease-causing mutants have defects in stimulating the endonuclease activity of FEN1 (Figure 3.5A and 3.5B). These results clearly demonstrate that mutations in FANCA significantly affect its ability to stimulate FEN1 and the interaction between FANCA and FEN1 is relevant to the etiology of Fanconi anemia.

**FIGURE S3.3 SDS-PAGE analysis of purified FANCA mutant proteins.**

Proteins were subjected to 10% gel electrophoresis and the gel was stained with Coomassie Brilliant Blue R-250. D598N, R951W, R1055W, R1117G, F1263Δ, and Q772X are pathogenic FANCA mutants. C772–1455 is C-terminal residues 772–1455 of FANCA. All peptides shown were tagged with hexahistidine at their N termini. Protein markers in kilodaltons were indicated on the left.
FIGURE 3.5 Pathogenic FANCA proteins are inefficient in stimulation.

(A) FEN1 endonuclease assays were performed with WT and mutant FANCA protein for both 15nt length DNA and RNA 5’ flap substrates. The concentration of FEN1 was 0.2 nM for DNA flap and 0.1 nM for RNA flap. The concentration of FANCA was 10 nM. Reaction products were resolved in 15% denaturing polyacrylamide gel. Arrows point to the incision products. The incision rate is quantified and shown on the bottom. (B) Quantitation of three independent FEN1 endonuclease assays in B by incision rate. Error bars represent standard errors. A paired t-test was performed to determine the statistical significance of FEN1 endonuclease activity between WT and mutant FANCA. *, p<0.05.

3.4 DISCUSSION

In this study, we aimed to unveil the function of the preferential 5’ flap and RNA binding activity of FANCA (Yuan, Qian et al. 2012) and found a novel physical and functional interaction between FEN1 and FANCA. We demonstrated that FEN1 incises a RNA flap more efficiently than its DNA counterpart and FANCA further stimulates this incision catalyzed by FEN1. More importantly, all six pathogenic FANCA mutant proteins we tested were defective in this
interaction to different degrees, indicating that the interaction between FEN1 and FANCA physiologically contributes to the pathogenesis of Fanconi anemia.

Intriguingly, before we study how FANCA may affect FEN1 activity, we observed that FEN1 alone, different from a previous study reporting that FEN1 does not cleave the 5' RNA flap structure (Harrington and Lieber 1994), incises the 5' RNA flap more efficiently than its DNA counterpart (Figure 3.1). This is obviously beneficial to its function in RNA primer removal during maturation of Okazaki fragment. Additionally, the incision pattern for the RNA flap is different from the DNA flap (Figure 3.1). It showed that FEN1 only cleaves the RNA flap substrate at the -1 position inside the junction (Figure 3.1A, RNA panel) which is actually a DNA base pair. However, there were two cleavage sites on the DNA flap substrate: one at the junction site and the other at -1 base inside the junction site (Figure 3.1A, DNA panel). This is different from a previous study showed that mammalian FEN1 cuts DNA flaps only at the -1 position inside the junction site (Harrington and Lieber 1994). Our data supports that the nature of the 5' flap affects the incision sites and efficiency of FEN1 initially proposed by Bambara’s group (Murante, Rumbaugh et al. 1996).

Besides the physical interaction we showed in Figure 3.2 and the shared substrate specificity of FEN1 and FANCA for 5' flap and RNA, our confocal microscopy result showed that FANCA perfectly colocalizes with replication forks in unstressed human cells (Qian et al, unpublished data), indicating that FANCA is associated with the normal replication machinery where FEN1 is known to be found for regular maintenance of replication forks. Additionally, it is estimated that more than 10^6 replication-stalling DNA lesions per cell per day form in humans. Because the number of Okazaki fragments per cell cycle is about 20-50 X 10^6 in humans, one replication fork stalling event may associate with about 20-50 Okazaki fragments theoretically (Lodish, Berk et al. 2004, Zheng and Shen 2011, Shin, Amangyeld et al. 2012). This estimate suggests that FEN1 is likely to encounter and interact with FANCA that is recruited to maintain stability of replication forks (Otsuki, Furukawa et al. 2001, Qiao, Moss et al. 2001, Yuan, Qian et al. 2012). Our in vitro result with the 15-nt 5' RNA and DNA flaps demonstrated that FANCA is likely to be involved in the removal of RNA/DNA primers by facilitating FEN1 action during maturation of Okazaki fragments.

FEN1 participates in the long-patch base excision repair of non-bulky DNA lesions (oxidation, methylation, base loss, etc) by interacting with Pol β, APE1, Lig 1, PCNA and Neil1 for efficient removal of the damaged bases and 5' flaps (Kim, Biade et al. 1998, Gary, Kim et al. 1999, Prasad, Dianov et al. 2000, Dianova, Bohr et al. 2001). FANCA was also shown to be involved in base
excision repair through stabilizing the glycosylase Neil1 (Mace-Aime, Couve et al. 2010). Our result indicated that FANCA additionally interacts with FEN1 to facilitate removal of 5’ DNA flap catalyzed by FEN1. Our findings suggest that the Fanconi anemia pathway may directly regulate the excision repair of DNA lesions caused by oxidative stress explaining the oxidative stress sensitive phenotype of Fanconi anemia cells (Yuan, Song et al. 2010).

Because of the preferential binding of FANCA to 5’ flap structures (Yuan, Qian et al. 2012), we hypothesized that FANCA may facilitate loading FEN1 to the 5’ flap substrate and therefore increase the substrate affinity of FEN1. However, FANCA increased both the turnover rate and Km of the endonuclease activity of FEN1 (Table 3.1). Based on these results, we speculate that FANCA may regulate the endonuclease activity of FEN1 through two possible mechanisms: (i) Direct protein-protein interaction between FANCA and FEN1 that changes FEN1 conformation and increases its endonuclease turnover and (ii) competition for the 5’ flap substrate between FANCA and FEN1 that reduces substrate affinity of FEN1. It is conceivable that reduced substrate affinity of FEN1 in the presence of FANCA may also cause faster release of the incision product and therefore help FEN1 to turnover. Overall, these possible mechanisms result in about two-fold increase in FEN1 enzyme efficiency. This possible mechanism is supported by the data that both non-nucleic-acids-binding N- and nucleic-acids-binding C-terminals of FANCA are required for FEN1 stimulation (Figure 3.5). It remains to be determined how FANCA exactly interacts with FEN1, but it is distinct from WRN, RFC, and PCNA in interacting
with FEN1. Both WRN and RFC increased the turnover of FEN1 without affecting the substrate affinity, on the other hand, PCNA increased the substrate affinity of FEN1 without changing the turnover rate (Tom, Henricksen et al. 2000, Brosh, Driscoll et al. 2002, Cho, Kim et al. 2009).

The next interesting question remaining to be answered is whether FANCA affects other activities of FEN1. Like FANCA, the gap-dependent endonuclease as well as the exonuclease of FEN1 is important for rescue of stalled replication forks (Sobeck, Stone et al. 2006, Wang, Stone et al. 2008, Zheng, Jia et al. 2011). It has been shown that defects in the gap-dependent endonuclease and exonuclease of FEN1 cause chronic inflammation and cancers (Zheng, Dai et al. 2007). Coincidently, deficiency in FANCA also results in inflammation and cancers (Dufour, Corcione et al. 2003, Briot, Mace-Aime et al. 2008, Du, Erden et al. 2013). Based on the physical and functional interactions between FANCA and FEN1, it is conceivable that FANCA may regulate the gap-dependent endonuclease as well as exonuclease activities of FEN1 and therefore contribute to suppression of inflammatory responses and maintenance of genomic stability.
CHAPTER 4

FANCA IS INVOLVED IN THE DAMAGE SENSING AND SIGNALING IN THE ATR PATHWAY OF DNA DAMAGE RESPONSE

4.1 SUMMARY

DNA damage response is a signal transduction pathway that coordinates cell cycle transitions, DNA replication, DNA repair and apoptosis. Mechanistic insight into how the FA proteins participate in an S phase checkpoint for ICL damage is emerging depending on G2/M arrest as a common phenotype of crosslinking agents treated FA cells. Here, we observed that deficiency of FANCA causes accumulation of late S phase cells after MMC treatment and those late S phase arrest cells are not replicating. In addition, FANCA deficiency results in insufficient activation of ATM and ATR pathways of DNA damage response. Thus, FANCA protein may play a critical role in the ICL-induced ATM and ATR-mediated damage signaling and cell cycle checkpoint pathways.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture and antibodies

GM02977A and GM01309C cells were obtained from Coriell Institute. Both human fibroblast cells were maintained in EMEM (Sigma) plus 20% FBS (Sigma) at 37°C with 5% CO₂. Phosphorylation antibodies that Chk1 Ser345, Chk2 T68 and p53 Ser15 are all from Cell Signaling; β-actin is from Abcam.

4.2.2 Cell cycle arrest assay

Human fibroblast cells were set at 50% confluence one day before and add 1µM MMC. Cells were then harvested at indicated time point, trypsinized and transferred to 15ml centrifuge tube. Pellet the cells by centrifugation 2000rpm,
5min and discard the supernatant. 1ml RT PBS was added to resuspend cells. Resuspended cells were then transferred to 4ml 100% -20°C cold ethanol and put in -20°C for 15min. Pellet the cells and tap the tube to loosen the pellet. 5ml RT PBS were added and incubate RT for 15min. Cells were pelleted and incubated in PI stating buffer (10mM Tris, pH 7.4, 15mM NaCl, 0.1mM CaCl₂, 0.05mM MgCl₂, 0.01% Nonidet P-40, 20µg/ml PI and 50µg/ml RNase A) at RT for 30min with light protection. Then transfer no more than 300µl solution to 5ml flow cytometry tube and use BD LSRII analyzer for FACS analysis. At least 5000 cells were sorted to get the represent pictures in Figure 4.1.

4.2.3 Checkpoint signaling assay

Human fibroblast cells were set at 50% confluence on day before and 1µM MMC. Cells were then harvested at indicated time point, trypsinized and transfer to 1.5ml centrifuge tube. Cells were washed by PBS once and frozen in -80°C. When all the time point cells were collect, cells were thawed on ice and dissolved in 50µl Lysis buffer (50 mM Tris-HCl, pH 7.6, 500 mM NaCl, 0.5% NP-40, 2 mM EDTA, 2 mM DTT, 1x proteinase inhibitor, 1mM sodium orthovanadate, 1mM sodium pyrophosphate, 1mM sodium fluoride and 1mM β-glycerophosphate) for sonication by using a Qsonica sonicator. Measure the protein concentration in the cell lysate and 40µg total protein was loaded onto the SDS-PAGE gel for western blot analysis.

4.2.4 BrdU and PI duel labeling assay

Human fibroblast cells were set at 50% confluence one day before and add 1µM MMC. 10µM BrdU were added 2h before harvesting. Cells were then
harvested at indicated time point, trypsinized and transferred to 15ml centrifuge tube. Cells were pelleted, fixed in 80% ethanol and incubation in -20°C for 15min. Pellet the cells and wash with 1ml wash buffer (PBS with 0.5% BSA), aspirate the supernatant and loosen the pellet. Pellet were then resuspend in 1ml 2M HCL and incubate for 20min at RT. Wash the pellet once and resuspend the pellet in 0.5ml 0.1M sodium borate, pH 8.5 for 2min at RT. Wash the pellet once and resuspended the pellet in 50µl dilution buffer (PBS with 0.5% Tween-20 and 0.5% BSA) with anti-BrdU FITC antibody. Incubate for 20min at RT and wash once with wash buffer. Pellet was then resuspended in PI solution (10µg/ml in PBS with 50µg/ml RNase A) for 30min at RT. Then transfer no more than 300µl solution to 5ml flow cytometry tube and use BD LSRII analyzer for FACS analysis. At least 5000 cells were sorted to get the represent pictures in Figure 4.2.

4.3 RESULTS

4.3.1 FANCA deficient cells show prolonged S phase arrest after MMC Treatment

We first tested response of FANCA proficient (WT) and deficient cells to DNA crosslinking agents by investigating cell cycle variation. GM02977A (WT) is normal human fibroblast cell line and GM01309C (fanca-/-) is patient cell lines derived from FA complementation group A, so here we would like to use it as FANCA deficient cell line. As show in Figure 4.1, we observe that WT cells accumulate significant amount cells in S phase at 16h and subsequently shift to and arrest at the G2/M phase. FANCA deficient cells show accumulation of S
phase (early and late) cells at the 16h point as well, however, a significant amount of these cells arrest themselves at the late S phase after 16h when compared with the wild type cells (Figure 4.1A, 32h and 64h). This observation is more obvious in Figure 4.1B where we isolated and quantitated cells at each of the three individual phases. These results indicate that FANCA deficiency is likely to cause defective replication, preventing DNA synthesis from being accomplished when under genotoxic stress.

GM02977A (normal human fibroblast cell line) and GM01309C (FANCA deficient fibroblast cell line) cells were harvested at indicated time point after treating with 1μM MMC, and then fixed with 80% ethanol and final stained with Propidium iodide (20ug/ml). (A) Histograms show cell cycle variation. (B) Quantitation of G1, S and G2 phase of cells in panel A.
4.3.2 Late S phase arrest FANCA deficient fibroblast cells are not replicating.

Recent studies suggest an involvement of the FA pathway in the activation of dormant origins when replication forks stall. In response to replication stress, FANCC-/-, FANCD2-/- and FANCM deficient cells show increased frequency of new origin firing (Phelps, Gingras et al. 2007, Wang, Stone et al. 2008, Schwab, Blackford et al. 2010). Thus, the firing of dormant origins is likely an alternative mechanism to overcome the stalling of replication fork. Based on that, we thought that the accumulation of late S phase cells in FANCA deficient fibroblast cell line might be reasoned to unfinished and damaged replication forks.

To understand the reason of the arrest of late S phase cells in FANCA deficient fibroblast cell line, we use propidium iodide (PI) and Bromodeoxyuridine (BrdU) duel labeling method to monitor the cell cycle variation more clearly and accurately. In Figure 4.2, FANCA deficient fibroblast cells arrest at late S phase after continuous MMC treatment as we previously observed. Those arrested cells stay at the S phase after 24h MMC treatment and they are BrdU negative cells. BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication (Yuan, Qian et al. 2012). As we added BrdU 2h before harvesting, BrdU negative stand for no incorporation of BrdU. Thus, replication is not happening in those cells. As those S phase arrested cells in FANCA deficient fibroblast cells are not replicating, their arrest are not caused by new origin firing.
FIGURE 4.2 Late S phase arrest FANCA deficient cells are not replicating.

Histograms of PI and BrdU dual labeling between FANCA WT and deficient human fibroblast cells. GM02977A (normal human fibroblast cell line) and GM01309C (FANCA deficient fibroblast cell line) cells were harvested at indicated time point after treating with 1uM MMC, BrdU were added 2h before harvesting, and then fixed with 80% ethanol and final stained with PI and BrdU antibody. Following flow cytometry analysis were done on BD LSRII analyzer.

4.3.3 FANCA deficiency results in insufficient activation of the ATM and ATR pathways of DNA damage response.

Accumulation of late S phase cells as a result of MMC treatment may also be a checkpoint response to MMC-induced ICL damage that blocks DNA replication. Because both ATR and ATM have previously been shown to play critical roles in initiating DNA damage checkpoints and maintaining the stability of replication forks (Trenz, Smith et al. 2006, Cimprich and Cortez 2008), we examined the changes of phosphorylation status in two ATR downstream targets
(Chk1 and p53) and one ATM downstream target (Chk2) in response to MMC treatment (Figure 4.3).

![Figure 4.3 Insufficient activation of ATM and ATR in fanca^- cells.](image)

GM02977A (normal human fibroblast cell line) and GM01309C (FANCA deficient fibroblast cell line) cells were harvested at indicated time point after treating with 1uM MMC. 40ug cell lysate were loading to SDS-PAGE and follow by western blot analysis by different checkpoint markers.

Under the interstrand crosslink stress triggered by MMC, Chk1 is indeed phosphorylated at Serine 345 in WT cells (Figure 4.3, WT). Additionally, this phosphorylation shows a peak at ~24 hours after MMC treatment. Intriguingly, this phosphorylation diminished in FANCA deficient cells, so Chk1 Serine 345 phosphorylation seems to be dependent upon FANCA (Figure 4.3, fanca^-^-). This observation resembles the deficiency of Chk1 phosphorylation in FANCM (another member of the FA core complex) deficient cells reported recently (Schwab, Blackford et al. 2010).

In the same Western blot membrane, we found that Chk2 was surprisingly phosphorylated earlier than Chk1 in WT cells (Figure 4.3, WT, 6h). However, Chk2 activation was significantly delayed in FANCA^-^- cells (Figure 4.3, FANCA-
This piece of preliminary result indicates that ATM activation is likely ahead of ATR activation in response to interstrand crosslinking agents and FANCA is required for the sequential activation of ATM and ATR.

Very interestingly, p53 phosphorylation seems to depend on the presence of FANCA. Without FANCA, p53 phosphorylation at Serine 15 is greatly reduced (Figure 4.3, p53 S15 panel). The p53 phosphorylation at serine 15 seems to be catalyzed by Chk1 because in WT cells, at 24h, Chk1 Serine phosphorylation peaks while p53 Serine 15 phosphorylation just begin to show up (Figure 4.3, 24h).

4.4 DISCUSSION AND FUTURE DIRECTION

In this study, we would like to elucidate how FANCA involved in ATM and ATR-mediated damage signaling and cell cycle checkpoint pathways. Our results suggest that ATM-mediated Chk2 phosphorylation and ATR-mediated phosphorylation of both Chk1 and p53 are greatly compromised in the absence of FANCA protein. It supports the idea that FANCA works as a sensor or mediator after the damage response trigger by ICL. Signal will thus transfer to activate ATM and ATR-mediated cell cycle arrest (S phase arrest in my results) in order to allow sufficient time for repairf DNA damage. We hypothesize that FANCA acts upstream of the ATM and ATR damage response pathways to maintain replication forks and regulate cell cycles (Figure 4.4B). This hypothesis is in line with a recent observation in Xenopus eggs show that Fanconi anemia pathway act upstream of RPA-ATR-Chk1 to generate signals for ICL damage.
Thus, we proposed an ICL-induced ATM and ATR damage signaling activation model shown in Figure 4.4B.

**FIGURE 4.4 Two different forms of ATR-mediated DNA damage.**

(A) Canonical ATR activation: stalling of replication forks by a single-stranded DNA adduct causes uncoupling of DNA unwinding and synthesis, exposure of large ssDNA areas, and ATR activation. (B) Our Hypothesis: Stalling of replication forks by interstrand crosslinks results in incision at the junction and production of DSBs or SSOs. DSBs or SSOs activate ATM kinase. Further resection of DSBs or SSOs by MRN or EXO1 produces longer ssDNA that promotes activation of ATR and inactivation of ATM. Red bar: single-stranded DNA adduct; Red zigzag: ICL damage.

One interesting finding is the late S phase cell arrest after continuous MMC treatment in FANCA deficient cell line, which is different from the G2/M arrest as a common phenotype of FA cells (Akkari, Bateman et al. 2001, Pang and Andreassen 2009). Several reports support the idea that the G2/M arrest of FA cells is likely to be result of failed damage checkpoint signaling during S
phase (Sala-Trepat, Rouillard et al. 2000, Akkari, Bateman et al. 2001, Thompson and Hinz 2009). Failure of cell cycle checkpoint signals at S phase will put cells to go through without repairing which finally turned out to be increased sister chromosome exchanges during mitosis (Auerbach 2009). FANCA, as a early step Fanconi anemia pathway protein, may have multiple function and act upstream of the checkpoint signaling pathways. This is in line with the finding that Fanconi anemia pathways acts upstream of RPA-ATR-Chk1 to generate the ICL signal (Ben-Yehoyada, Wang et al. 2009). Further study need to perform to understand how the S phase checkpoint signals was transferred through FANCA under ICL stress. Some G1/S checkpoint markers, e.g. p21 and p16 expression, and Rb phosphorylation (Pestell, Albanese et al. 1999, Sherr 2001, Lowe and Sherr 2003, Wang, Schulte et al. 2006, Abbas and Dutta 2009) may also need to be tested to know whether the late S phase arrest is resulted from that.

The BrdU and PI duel labeling assay in our study implicated that those MMC-induced last S phase arrest cells is not replicating. So it will be interesting to know whether those cells (with unfinished and stalled replication forks) will result in senescence or apoptosis and therefore depletion of affected cells, such as hematopoietic stem cells. Hematopoiesis is impaired in FANCA knockdown human embryonic stem cells from the earliest stages of development, suggesting that deficiencies in embryonic hematopoiesis may underlie the progression to bone marrow failure in FA (Tulpule, Lensch et al. 2010). Our observation may further help understand how FANCA functions in hematopoietic stem cell
regulation and generation of acute myeloid leukemia and myelodysplastic syndrome (Lensch, Rathbun et al. 1999, Soulier 2011).

More downstream targets of ATM and ATR, such as BRCA1, γH2Ax, 53BP1, MCM2 and Cdc2 (Brown and Baltimore 2003, Furuta, Takemura et al. 2003, Okada and Ouchi 2003, Ward, Minn et al. 2003, Yang, Yu et al. 2003, Cimprich and Cortez 2008, Trenz, Errico et al. 2008) need to be tested in both FANCA proficient and deficient cells to confirm whether ATM and ATR activation is compromised because of FANCA deficiency. Compromised ATM and ATR activation in FANCA deficient cells under MMC treatment may accumulate DSBs (by products during incision of ICL) in cells and lead to chromosome instability.

Our data shows that FANCA is required for sequential activation of ATM and ATR kinases (Figure 4.3). Subsequent activation of ATR after ATM requires resection of DNA ends by MRN and EXO1 (Shiotani and Zou 2009, Flynn and Zou 2010). Further study of knockdown MRE11 (nuclease component of MRN) or EXO1 with crosslinking agents may need to confirm this sequential activation of ATM and ATR. KU55933 (ATM but not ATR inhibitor (Hickson, Zhao et al. 2004)), caffeine (PIKKs inhibitor (Sarkaria, Busby et al. 1999, Cortez 2003)) and CGK733 (ATM and ATR exclusive PIKK inhibitor (Crescenzi, Palumbo et al. 2008, Cruet-Hennequart, Glynn et al. 2008, Goldstein, Roos et al. 2008, Alao and Sunnerhagen 2009, Bhattacharya, Ray et al. 2009)) might also be useful to help define how FANCA functions in the MMC-induced ATM and ATR damage response pathway.
CHAPTER 5
DISCUSSION AND SIGNIFICANCE

In 1927, Swiss pediatrician Guido Fanconi first described a family with physical abnormalities and aplastic anemia in which three male children between the ages of 5 and 7 had pancytopenia and birth defects. Based on his observations, chief criteria for the diagnosis of Fanconi anemia included pancytopenia, hyperpigmentation, skeletal malformations, small stature, urogenital abnormalities and familial occurrence (Auerbach 2009, de Winter and Joenje 2009). Clinical research and therapy were then focus on the regenation of bone marrow of hematopoietic stem cells, such as bone marrow transplantation (Muller and Williams 2009). Until many years later, Traute Schroeder observed spontaneous chromosomal breakage during routine cytogenetic analysis of FA patients and this abnormalities in FA cells were drastically enhanced by the DNA crosslinking agent MMC by Masao Sasaki’s discovery. These observation not only extend diagnostic criteria to the widely used chromosomal breakage test but also connect FA genes to a cellular pathway that is activated when DNA replication becomes blocked through crosslinking agents and that regulates mechanisms leading to recovery from this harmful situation (Sasaki 1975, Auerbach and Wolman 1976, de Winter and Joenje 2009). Thus, study of Fanconi anemia switch to understand how FA proteins functions in the DNA interstrand crosslink repair pathways. However, FANCA, a gene contribute more than 60% of the FA patient population (Table 1.1), does not show to have a clear function in the DNA ICL repair pathways in previous study. Our discovery of
several biomedical functions of FANCA may help understand etiology of Fanconi anemia and partial elucidate the phenotype in clinical observation.

One intriguing discovery of my thesis work is that FANCA has a significantly higher affinity for ssRNA than for ssDNA. Even though Fanconi anemia is a developmental disease, limited study has shown direct involvement of RNA metabolism with Fanconi anemia. This unique function of FANCA may be a bridge for further study of how FA proteins link to transcription, translation and RNA stability. HES1 was previously shown to interact with FA core complex and FA core complex regulate the gene expression of HES1 on transcriptional level (Tremblay, Huang et al. 2008, Tremblay, Huard et al. 2009). Therefore, we speculate that FA core complex may directly localize to the HES1 promoter region through FANCA and FANCA may further recruit other components of FA core complex to regulate HES1 expression. HES1 plays an essential role in the development of many organs by promoting the maintenance of stem/progenitor cells in an undifferentiated state, by regulating both cell fate decisions and by control the timing of several developmental events (Kageyama, Ohtsuka et al. 2007, Tremblay, Huard et al. 2009). It will be interesting to investigate how FANCA and HES1 work together to operate gene networks during vertebrate embryogenesis. Another important connection to the RNA binding activity of FANCA may be the functionally control of PKR activity. PKR is a critical factor in translational control as well as regulation of cell proliferation, survival and apoptosis (Zhang, Li et al. 2004). The possible senescence or apoptosis phenomena carried out in FANCA deficient cells (Figure 4.2) may also result
from inappropriate activation of PKR. In this way, the RNA binding activity of FANCA might be crucial to cell differentiation and proliferation, especially the hematologic stem cells regeneration.

The major biochemical function of FANCA shown in my thesis work was that FANCA activates FEN1 endonuclease that helps remove RNA primer during Okazaki fragment maturation in DNA replication. And the nucleic acid binding activity of FANCA might not critical for this stimulation as both N-terminal and C-terminal (nucleic acid binding domain) are required. It is surprising that even a point mutation of FANCA proteins will disrupt the whole function of FANCA to regulate FEN1 endonuclease activity. As all our point mutation sequence change derived from FA patients, the stimulation of FANCA to FEN1 seems to be important for the generation of Fanconi anemia. One possible explanation might be that FEN1 is a principal nuclease for DNA replication during Okazaki fragment maturation to process stalled replication fork (Zheng, Zhou et al. 2005, Zheng, Jia et al. 2011). This is consistent with the fact that FANCA localize to the chromatin during replication and maintain the stability of replication forks (Sobeck, Stone et al. 2007, Wang, Stone et al. 2008). Our cell cycle arrest assay for FANCA deficiency cell lines also implicate a portion of nonreplicating cells arresting in the S phase after continuous MMC treatment. Thus, this arrest may also result from improper regulation of FEN1 because of FANCA deficiency under genotoxic damage situation. In addition, the gap-dependent endonuclease as well as the exonuclease of FEN1 is important for rescue of stalled replication forks (Zheng, Zhou et al. 2005). It is important to further investigate whether
FANCA also regulate the gap-dependent endonuclease as well as exonuclease activities of FEN1. Failure to rescue stalled replication fork by FEN1 may either cause cell death (apoptosis) or genomic instability (chromosome breakage) during mitosis in FANCA deficient cells. Therefore, the stimulation of FANCA to FEN1 activity might implicate an important clue to understand and elucidate the generation of Fanconi anemia.

Cancer predisposition was found in heterozygous FEN1 depletion mice because of haploinsufficiency of FEN1 (Kucherlapati, Yang et al. 2002, Zheng, Jia et al. 2011). Although we do not know whether FANCA contribute to the regulation of the amount of FEN1 in cells, FA patients also diagnose for early cancer predisposition. The stimulation mechanism of FANCA to endonuclease activity of FEN1 seems to regulation the turnover rate of FEN1 for efficient incision of 5’ DNA or RNA flap. Cells deficient of FANCA may accumulate malignant cell division which has slow turnover rate of FEN1 and therefore cause insufficient amount of FEN1 for other function. Further study of the stimulation mechanism of FANCA to FEN1 activity may be helpful to understand why FA patients are likely to have cancers.

FANCA have previously shown to be directly influenced by redox state and cells from Fanca double knockout mice are hypersensitive to oxidants (Pagano, Degan et al. 2005, Rani, Li et al. 2008). Oxidative base damage is a type of genomic DNA lesion that commonly results from the action of reactive oxygen species and usually removed by base excision repair pathways (Liu and Wilson 2012). The participation of FEN1 in long-patch base excision repair of
oxidation lesion FEN1 is carried out in maintaining trinucleotide repeat stability though interacting with Pol β (Kim, Biade et al. 1998, Prasad, Dianov et al. 2000, Liu and Wilson 2012). There, failure to remove of 5’ DNA flap catalyzed by FEN1 in FANCA deficient cells impede the repair of oxidative base damage. This also explains the oxidative stress sensitive phenotype of Fanconi anemia cells and further irregular hematopoietic cells generation turned out to be bone marrow failure (Pagano, Degan et al. 2005).

In conclusion, my thesis study provides a new angle to study the function of FANCA, which has few identifiable domains for primary sequence and conserved only in vertebrates. The biochemical functions of FANCA beyond to the DNA ICL pathway might be important to understand the evolvement of the disease itself. The innovative knowledge will either shift or challenge the current paradigms of the Fanconi anemia field. Our discovery might provide evidence for understanding the phenotype observed in FANCA patient population, which is also determine the major diagnose criteria during clinical investigation.
REFERENCES


