The Texas Medical Center Library

DigitalCommons@TMC

The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)

The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

12-2012

FANCM AND FAAP24 MAINTAIN GENOMIC STABILITY THROUGH COOPERATIVE AND UNIQUE FUNCTIONS

Yucai Wang

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations

 Part of the Cancer Biology Commons, Molecular Biology Commons, and the Molecular Genetics Commons

Recommended Citation

Wang, Yucai, "FANCM AND FAAP24 MAINTAIN GENOMIC STABILITY THROUGH COOPERATIVE AND UNIQUE FUNCTIONS" (2012). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 309.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/309

This Dissertation (PhD) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digitalcommons@library.tmc.edu.

The TMC LIBRARY Health Sciences Resource Center

www.manaraa.com

FANCM AND FAAP24 MAINTAIN GENOMIC STABILITY THROUGH COOPERATIVE AND UNIQUE FUNCTIONS

by

Yucai Wang, M.D., M.S.

APPROVED:

_____
Lei Li, Ph.D.
Supervisory Professor

_____
Randy J. Legerski, Ph.D.

_____
Pierre D. McCrea, Ph.D.

_____
Peng Huang, M.D., Ph.D.

_____
M. James You, M.D., Ph.D.

APPROVED:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

www.manaraa.com

FANCM AND FAAP24 MAINTAIN GENOMIC STABILITY THROUGH COOPERATIVE AND UNIQUE FUNCTIONS

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Yucai Wang, M.D., M.S.

Houston, Texas

December, 2012

www.manaraa.com

DEDICATION

This dissertation is dedicated to my wife, Jia Lu,

my daughter, Julia Wang,

and

my son, Lucas Wang.

www.manaraa.com

ACKNOWLEDGEMENTS

It is my great pleasure to thank those who have made this dissertation possible.

First and foremost, I am extremely grateful to my mentor, Dr. Lei Li, for his continuous support during my PhD studies. His immense knowledge, tremendous advice and strong encouragement have guided me though over the years, and his enthusiastic attitude towards research and life has been inspiring. I am honored and greatly appreciate the opportunity to be part of his research team.

I wish to express my sincere gratitude to all my current and past committee members, Dr. Randy Legerski, Dr. Peng Huang, Dr. Pierre McCrea, Dr. James You, Dr. Mong-Hong Lee, Dr. Jean-Pierre Issa, and Dr. Khandan Keyomarsi. Their insightful advice and comments have made a great difference in my research progress.

Many warm thanks to my present and former colleagues in Dr. Li's laboratory, Dr. Xi Shen, Dr. Yingjun Jiang, Yaling Huang, Huong Do, Dr. Haoyi Chen, Dr. Xin Wang, Dr. Chang Xu, Dr. Yanyan Tian, for their helpful discussions and experimental assistances.

I sincerely thank Dr. Junjie Chen (MDACC) and Dr. Weidong Wang (NIA, NIH) for their perceptive research suggestions and valuable reagents. Special thanks to Dr. Justin Leung for rewarding collaborations, inspiring discussions and enormous help with experiments. I thank Megan Lowery and Jia Liu for help with survival assays and eChIP reagents, respectively.

I gratefully acknowledge the support from GSBS and the Department of Experimental Radiation Oncology at MDACC as well as funding provided by NIH.

www.manaraa.com

Last but not least, I am deeply indebted to my family. I express my earnest gratitude to my parents, Yangbao Wang and Mingfeng Xia, for their unconditional love. I sincerely thank my parents-in-law, Huapei Lu and Yixia Hao, for their enormous support. My daughter Julia Wang and my son Lucas Wang deserve my warmest thanks. They are the source of my strength. My most sincere thanks go to my wife Jia Lu. None of my achievements would have been possible without the dearest love and strongest support from her. In any case, she has my everlasting love.

www.manaraa.com

FANCM AND FAAP24 MAINTAIN GENOMIC STABILITY THROUGH COOPERATIVE AND UNIQUE FUNCTIONS

Publication No. _____

Yucai Wang, M.D., M.S.

Supervisory Professor: Lei Li, Ph.D.

Fanconi anemia (FA) is a rare recessive genetic disease with an array of clinical manifestations including multiple congenital abnormalities, progressive bone marrow failure and profound cancer susceptibility. A hallmark of cells derived from FA patients is hypersensitivity to DNA interstrand crosslinking agents such as mitomycin C (MMC) and cisplatin, suggesting that FA- and FA-associated proteins play important roles in protecting cells from DNA interstrand crosslink (ICL) damage. Two genes involved in the FA pathway, FANCM and FAAP24, are of particular interest because they contain DNA interacting domains. However, there are no definitive patient mutations for these two genes, and the resulting lack of human genetic model system renders their functional studies difficult.

In this study, I established isogenic human FANCM- and FAAP24-null mutants through homologous replacement-mediated gene targeting in HCT-116 cells, and systematically investigated the functions of FANCM and FAAP24 in chromosome stability, FA pathway activation, DNA damage checkpoint signaling, and ICL repair. I found that the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double mutant was much more sensitive to DNA crosslinking agents than *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single mutants, suggesting that FANCM and FAAP24 possess

www.manaraa.com

epistatic as well as unique functions in response to ICL damage. I demonstrated that FANCM and FAAP24 coordinately support the activation of FA pathway by promoting chromatin localization of FA core complex and FANCD2 monoubiqutination. They also cooperatively function to suppress sister chromatid exchange and radial chromosome formation, likely by limiting crossovers in recombination repair. In addition, I defined novel non-overlapping functions of FANCM and FAAP24 in response to ICL damage. FAAP24 plays a major role in activating ICL-induced ATR-dependent checkpoint, which is independent of its interaction with FANCM. On the other hand, FANCM promotes recombination-independent ICL repair independently of FAAP24. Mechanistically, FANCM facilitates recruitment of nucleotide excision repair machinery and lesion bypass factors to ICL damage sites through its translocase activity. Collectively, my studies provide mechanistic insights into how genome integrity is both coordinately and independently protected by FANCM and FAAP24.

www.manaraa.com

TABLE OF CONTENTS

www.manaraa.com

www.manaraa.com

www.manaraa.com

www.manaraa.com

LIST OF ILLUSTRATIONS

www.manaraa.com

www.manaraa.com

www.manaraa.com

www.manaraa.com

LIST OF TABLES

www.manaraa.com

CHAPTER I

Background and Significance

Fanconi anemia

Fanconi anemia (FA) is a rare genetic disease first described in 1927 by a Swiss physician Guido Fanconi. The main clinical features of FA are multiple developmental abnormalities, progressive bone marrow failure, and remarkably increased risks for developing cancer (Moldovan and D'Andrea, 2009). The most frequent congenital defects among FA patients include short stature, skeletal defects and skin abnormalities, although a wide range of organ systems can be affected (D'Andrea, 2010). FA patients typically develop bone marrow failure within their first decade of life (Alter et al., 2003), which eventually leads to pancytopenia. By the age of 40, the cumulative risk of bone marrow failure is up to 90% (Kutler et al., 2003). The most common hematological and non-hematological malignancies seen in FA patients are acute myelogenous leukemia (AML) and squamous cell carcinomas of the head and neck, respectively (Kitao and Takata, 2011). Clinically, Fanconi anemia is a highly devastating disease. Even with bone marrow transplantation and advanced medical care, the median age at death was only 30 by the year 2000 (D'Andrea, 2010).

At the cellular level, Fanconi anemia is characterized by hypersensitivity to DNA interstrand crosslinking agents such as diepoxybutane (DEB), mitomycin C (MMC), and cisplatin (German et al., 1987; Poll et al., 1985). When exposed to DEB, cells from FA patients display increased chromosome breakage and accumulation in the G2 phase of cell cycle (D'Andrea, 2010). The DEB-induced chromosome-breakage assay is actually widely used for the diagnosis of Fanconi anemia using patient primary lymphocytes (Auerbach,

www.manaraa.com

1993; D'Andrea, 2010).

Fanconi anemia genes

Fanconi anemia is a highly heterogeneous disorder with at least fifteen complementation groups defined to date, each caused by mutation(s) of a distinct gene. Most of the FA genes were identified through cell fusion experiments and are organized into complementation groups. (Crossan and Patel, 2012). Newer FA genes were discovered primarily through positional cloning, functional RNAi screen, or genome sequencing (Kim et al., 2011; Levitus et al., 2005; Litman et al., 2005; Meetei et al., 2003; Meetei et al., 2005; Reid et al., 2007; Smogorzewska et al., 2007; Stoepker et al., 2011; Vaz et al., 2010; Xia et al., 2007). The fifteen FA genes defined to date are listed in Table 1. Most of the FA genes are located on autosomes, with only one exception *FANCB*, which lies on the X chromosome. Therefore, the inheritance of Fanconi anemia is in either autosomal recessive or X-linked recessive manners. The most frequently mutated genes in Fanconi anemia are *FANCA*, *FANCC*, and *FANCG*, accounting for ~66%, ~10% and ~9% of the FA patients, respectively (Moldovan and D'Andrea, 2009; Wang, 2007).

A number of genes are functionally related to and/or physically associated with FA genes. The products of such genes are therefore termed as FA-associated proteins. These include FAAP100, FAAP24, FAN1, MHF1 (FAAP16), MHF2 (FAAP10) and FAAP20 (Ali et al., 2012; Ciccia et al., 2007; Kim et al., 2012; Kratz et al., 2010; Leung et al., 2012; Ling et al., 2007; Liu et al., 2010; MacKay et al., 2010; Singh et al., 2010; Smogorzewska et al., 2010; Yan et al., 2010; Yan et al., 2012). To date, no loss of function mutations of the above genes have been found in FA patients.

www.manaraa.com

Table 1.1 Fanconi anemia genes identified to date.

FA gene	Chromosome location	Protein size (kDa)	Function in FA pathway	Protein features
FANCA	16q24.3	163	Core complex	Phosphorylated following DNA damage
FANCB	Xp22.2	95	Core complex	Contains nuclear localization sequence
FANCC	9q22.3	63	Core complex	
FANCD1/ BRCA2	13q12.3	380	Homologous recombination	Contains BRC repeats and an OB-fold DNA binding domain; regulates RAD51
FANCD2	3p26	155	ID complex	Monoubiquitylated and phosphorylated following DNA damage; exonuclease activity
FANCE	6p22-p21	60	Core complex	Phosphorylated by Chk1 following DNA damage
FANCF	11p15	42	Core complex	
FANCG	9p13	68	Core complex	Contains TPR repeats; phosphorylated following DNA damage
FANCI	15q26.1	140	ID complex	Monoubiquitylated and phosphorylated following DNA damage
FANCJ/ BRIP1	17q22.2	140	Homologous recombination	5' to 3' DNA helicase; binds BRCA1; phosphorylated following DNA damage; G-quadruplex resolution
FANCL	2p16.1	43	Core complex	PHD/ring-finger ubiquitin-ligase activity; contains WD40 repeats
FANCM	14q21.2	250	Core complex	Contains helicase and endonuclease domains; phosphorylated following DNA damage
FANCN/ PALB2	16p12.2	140	Homologous recombination	An essential partner for BRCA2 stability and nuclear localization
FANCO/ RAD51C	17q25.1	42	Homologous recombination	RAD51 paralogue
FANCP/ SLX4	16p13.3	200	Nucleolytic cleavage / Homologous recombination	Interacts with structure-specific endonuclease complexes; Holliday junction resolvase

Adapted (with permission) from Wang, 2007 and Crossan and Patel, 2012.

www.manaraa.com

Fanconi anemia pathway

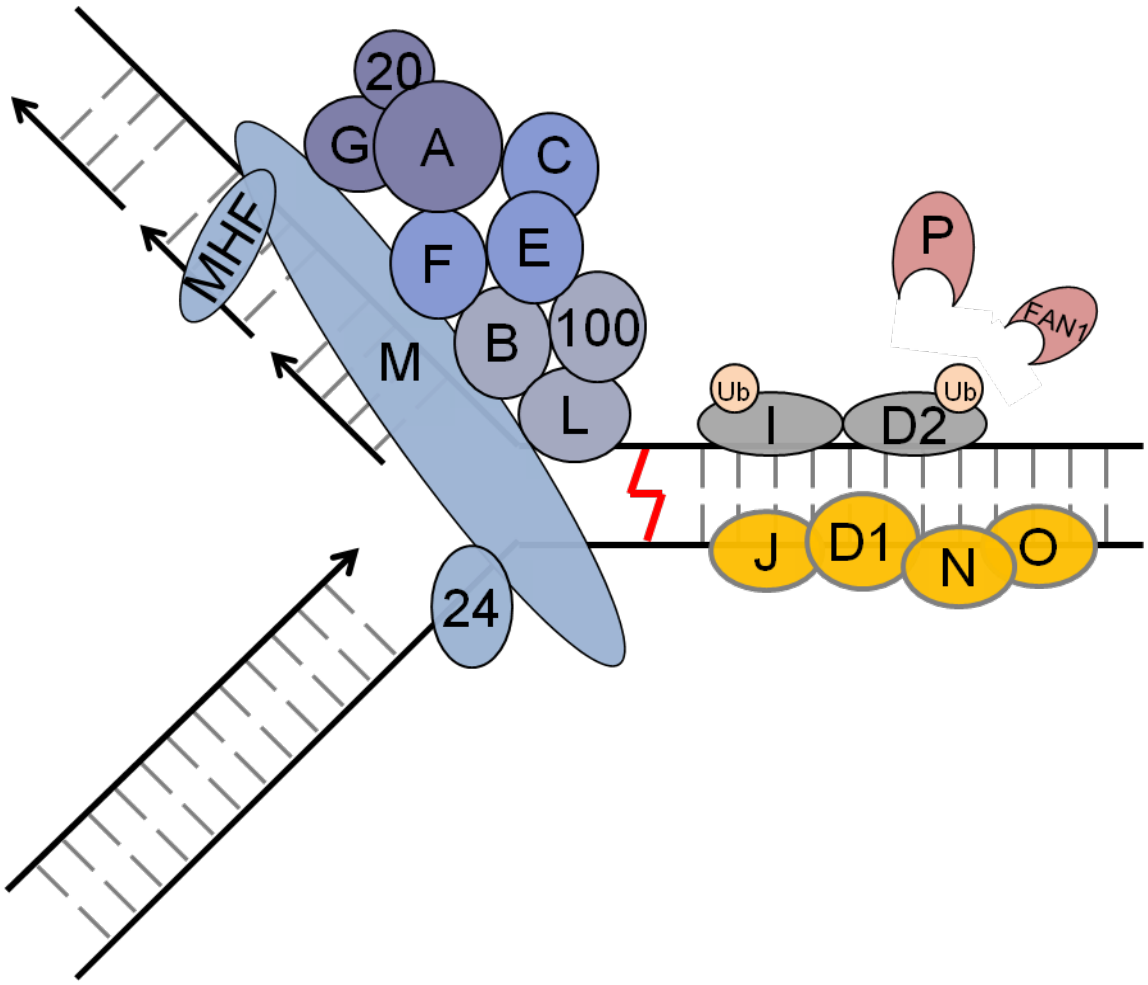
The fact that cells from FA patients are hypersensitive to DNA cross-linking agents suggests that genes mutated in Fanconi anemia play important roles in DNA damage response and repair. Indeed, accumulating evidences suggest that FA and FA-associated proteins constitute a FA pathway which is important in protecting cells from DNA interstrand crosslink (ICL) lesions and maintaining genomic stability (Fig 1.1).

Eight of the FA proteins FANCA, B, C, E, F, G, L, and M, together with five FA-associated proteins (FAAP100, FAAP24, MHF1, MHF2 and FAAP20), form a FA core complex (Ali et al., 2012; Ciccia et al., 2007; Kim et al., 2012; Leung et al., 2012; Ling et al., 2007; Singh et al., 2010; Yan et al., 2010; Yan et al., 2012). In response to DNA damage, the FA core complex monoubiquitinates a heterodimeric ID complex composed of FANCD2 and FANCI (Meetei et al., 2004; Smogorzewska et al., 2007; Wang, 2007). Central to the FA pathway, this monoubiquitination reaction triggers the recruitment of the FANCD2-associated nuclease FAN1 and the FANCP gene product SLX4, a scaffolding protein involved in assembly of structure-specific nucleases (Crossan et al., 2011; Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Stoepker et al., 2011), followed by ICL processing by multiple nucleases. Four other FA proteins BRCA2/FANCD1, BRIP1/FANCJ, PALB2/FANCN, and Rad51C/FANCO, also known as breast cancer susceptibility proteins (Howlett et al., 2002; Levitus et al., 2005; Reid et al., 2007; Vaz et al., 2010; Wang, 2007; Xia et al., 2007), are mainly associated with downstream homologous recombination (HR)-mediated repair of DNA double strand breaks formed as a result of ICL processing (Shen et al., 2009).

www.manaraa.com

Fig. 1.1 The Fanconi anemia pathway.

www.manaraa.com



www.manaraa.com

FANCM and FAAP24

How the FA pathway acts to protect cells from DNA interstrand crosslink (ICL) lesions and to maintain genomic stability remains largely unclear. Further understanding of this pathway has been restricted, partially because most of the FA proteins are only conserved in vertebrates and lack well known functional domains (Wang, 2007).

Among the FA core complex components, FANCM is of particular interest. It was initially identified as a novel component (FAAP250) of the FA core complex during tandem affinity purification of FANCA. The gene encoding FAAP250 was later found to be mutated in one Fanconi anemia patient that needed to be classified into a new complementation group (M), and therefore the gene was named as *FANCM* (Meetei et al., 2005). Structurally, human FANCM resembles archaeal DNA repair protein Hef and eukaryote endonuclease XPF. It contains a DEAH helicase domain at its N-terminus as well as an ERCC4-like endonuclease domain at the C-terminus (Meetei et al., 2005). Translocase activity, instead of helicase activity, was found to be associated with the helicase domain. The endonuclease domain is degenerated and thus no nucleolytic activity was found in FANCM (Meetei et al., 2005).

Given that the flap-specific endonuclease family typically forms heterodimers such as XPF/ERCC1 and Mus81/Eme1, a bioinfomatics study was conducted to search for FANCM binding partner. A novel protein with ERCC4-like endonuclease domain was identified as a potential new XPF family member. It was then found to specifically interact with FANCM and to be an intrinsic component of the FA core complex, and was later named as FAAP24 (Ciccia et al., 2007).

FANCM and FAAP24 promote FA pathway activation

www.manaraa.com

Being an integral component of the FA core complex, FANCM was initially thought to be required for the stability of the core complex and monoubiquitylation of FANCD2. This was supported by the fact that in the potential FANCM patient-derived cell line (EUFA867), levels of FANCA and FANCG were reduced, and FANCD2 monoubiquitylation was completely absent (Meetei et al., 2005). However, siRNA mediated depletion of FANCM did not lead to significant decrease of FANCA and FANCG levels or disruption of FANCA/FANCG/FANCL association in HeLa cells, arguing against the necessity of FANCM in maintaining FA core complex integrity (Kim et al., 2008). This discrepancy was not resolved until identification of biallelic *FANCA* mutations in EUFA867 cells (Singh et al., 2009), suggesting that FANCA and FANCG defects in EUFA867 cells are due to *FANCA* mutations, and FANCM may not be required for maintaining the integrity of the core complex. Importantly, re-expression of FANCA in EUFA867 lymphoblasts only partially restored FANCD2 monoubiquitylation, suggesting that FANCM promotes FANCD2 monoubiquitylation although it is not indispensable for this process (Singh et al., 2009). This finding was consistent with subsequent genetic studies in mouse models as well as chicken DT40 cells (Bakker et al., 2009; Rosado et al., 2009).

The fact that FANCM is not essential for FA core complex integrity or FANCD2 monoubiquitylation distinguishes it from earlier identified FA core complex proteins. It is likely that FANCA, B, C, E, F, G, L, FAAP100 and FAAP20 first form a sub-complex, while FANCM/FAAP24 heterodimer interacts with and targets this subcomplex to chromatin where they form the larger active core complex (Thompson and Hinz, 2009). This appears to be an attractive model, since FANCM and FAAP24 possess DNA-binding domains and activities. FANCM has a DEAH-helicase domain at the N-terminus, and both FANCM and

www.manaraa.com

FAAP24 have ERCC4-like endonuclease domains at their C-termini (Ciccia et al., 2007). FANCM was shown to have translocase activity *in vitro*, making it a plausible candidate damage sensor that moves along DNA to detect replication fork blockage or other types of abnormal structures (Meetei et al., 2005). *In vitro* assays demonstrated that FAAP24 could bind to ssDNA, splayed-arm, and 3'-flap DNA substrates, and while $FANCM_{1727-2048}$ alone exhibits very little DNA binding activity, $FANCM_{1727-2048}$/FAAP24 complex binds to a variety of substrates with a preference to splayed-arm DNA, suggesting that FAAP24 may play an important role in facilitating damage recognition and binding by the FANCM/FAAP24 heterodimer (Ciccia et al., 2007). It is possible that following detection of DNA damage such as replication fork blockage by ICL, this heterodimer would target FA core complex to chromatin. Indeed, *in vivo* studies demonstrated that FANCM constitutively associates with chromatin and is tethered to the latter by FAAP24 as an anchor, and the FANCM/FAAP24 heterodimer is required for chromatin association of the FA core complex during S phase and in response to DNA damage (Kim et al., 2008). It is likely that FANCM and FAAP24 do not significantly affect the E3 ligase activity of the core complex, but rather its availability for substrate binding.

Another two FANCM-associated proteins, MHF1 and MHF2, have been identified via their co-purification with FANCM (Singh et al., 2010; Yan et al., 2010). The MHF proteins also possess DNA binding activity, and they can promote the DNA interacting activities of FANCM (Yan et al., 2010). Importantly, they have been shown to act together with FANCM to activate the FA pathway (Singh et al., 2010; Yan et al., 2010). Therefore, it appears that FANCM, FAAP24 and the MHF proteins coordinately activate the FA pathway by recruiting the FA core complex upon DNA damage recognition.

www.manaraa.com

FANCM and FAAP24 in DNA damage checkpoint activation

FANCM and FAAP24 are implicated in the activation of ATR-mediated checkpoint by several groups. It was shown that FANCM and FAAP24 interact with the ATR-ATRIP-HCLK2 complex, and depletion of FANCM or FAAP24 leads to defective ATR/Chk1 signaling, manifesting as increased spontaneous S phase DNA damage, diminished foci formation of phosphorylated Chk1 and other substrates, and failure to prevent mitotic entry in response to replication stress (Collis et al., 2008; Luke-Glaser et al., 2009; Schwab et al., 2010). Mechanistically, FANCM has been shown to retain TopBP1 on chromatin in response to replication stress, which is important for ATR activation (Schwab et al., 2010). It was also suggested that FANCM and Chk1 mutually stabilize each other, thus sustaining necessary checkpoint signaling in response to DNA damage (Luke-Glaser et al., 2009). In addition, the function of FANCM in ATR/Chk1 signaling requires its translocase activity, suggesting that the putative replication fork remodeling activity of FANCM may be important for efficient checkpoint activation (Collis et al., 2008).

A recent study suggests that the potential function of FANCM and FAAP24 in ATR-mediated checkpoint activation appears to be specifically linked to ICL damage response (Huang et al., 2010). It was shown that cells lacking FANCM or FAAP24 are deficient in RPA foci assembly in response to ICL damage but not to replication stress induced by hydroxyurea. This leads to an ICL damage specific ATR-mediated checkpoint defect. Furthermore, the DNA binding activity of FAAP24 but not the translocase activity of FANCM is important for their function in ICL-induced activation of ATR-mediated checkpoint (Huang et al., 2010). Given the apparent discrepancies in data obtained from different studies, further studies are warranted to elucidate the role of FANCM and FAAP24

www.manaraa.com

in DNA damage checkpoint activation.

FANCM remodels stalled replication fork

In FANCM deficient cells, expression of a K117R translocase mutant of FANCM could restore FANCD2 monoubiquitylation but could not fully correct MMC sensitivity (Xue et al., 2008). This suggests that the translocase activity allows FANCM to function in parallel to or downstream of FANCD2 monoubiquitylation to promote or directly mediate ICL repair.

FANCM was observed to promote branch migration of Holliday junctions and replication forks *in vitro* (Gari et al., 2008b), suggesting that FANCM either functions to facilitate Holliday junction branch migration during homologous recombination or to regress stalled replication forks to facilitate ICL repair. Given that FANCM was dispensable for the repair of I-SceI induced double strand breaks (DSBs) by homologous recombination in DT40 cells (Mosedale et al., 2005), it is more likely that FANCM remodels the structure of stalled replication forks in response to replication stress. Indeed, *in vitro* studies demonstrated that FANCM has the ability to convert a replication-fork structure to a four-way junction, causing a replication fork regression (Gari et al., 2008a). In addition, *in vivo* studies demonstrated that in the absence of FANCM, the progression of replication forks are unstable in both mammalian cells and chicken DT40 cells, suggesting that FANCM is critical for the control of replication fork movement (Luke-Glaser et al., 2009; Schwab et al., 2010). Importantly, the translocase activity of FANCM is required for recovery of stalled replication forks (Blackford et al., 2012; Luke-Glaser et al., 2009; Schwab et al., 2010), indicative of a role of FANCM in remodeling replication forks in response to replication stress. Consistently, cells lacking FANCM are hypersensitive to agents that inhibit replication fork progression such as camptothecin, hydroxyurea and aphidicolin (Schwab et al., 2010; Singh et al., 2009).

www.manaraa.com

FANCM limits crossovers and suppresses sister chromatid exchange

Yeast orthologs of FANCM, namely Fml1 in *S. Pombe* and Mph1 in *S. cerevisiae*, limit crossovers during homologous recombination by dissociating Rad51-induced D-loops, thereby suppressing sister chromatid exchanges (SCEs) (Prakash et al., 2009; Sun et al., 2008). Chicken DT40 cell depleted of FANCM and *Fancm*$^{-/-}$ MEFs both displayed increased basal SCE levels as well (Bakker et al., 2009; Mosedale et al., 2005; Rosado et al., 2009). Consistently, human cells deficient for FANCM also showed increased SCE formation both at basal level and after MMC treatment (Deans and West, 2009).

How FANCM functions to suppress SCE is not fully understood. A recent study demonstrated that FANCM connects the FA core complex and the Bloom's complex through direct interaction with FANCF and RMI1, respectively, and the coordinated recruitment of both complexes are important for SCE suppression (Deans and West, 2009). However, while the BLM helicase is well known for its function in SCE suppression, whether FA core complex is required for suppressing SCE remains controversial (Bakker et al., 2009; Rosado et al., 2009). Therefore, alternative mechanisms of FANCM's function in SCE suppression need to be explored.

In the absence of FANCM, insufficient replication fork regression and stabilization in response to replication stress will result in increased DSB formation due to fork collapse, the repair of which may result in SCEs (Bakker et al., 2009). In addition, like its yeast orthologs, recombinant FANCM protein is capable of dissociating D-loop structures *in vitro* (Gari et al., 2008a). Furthermore, FANCM orthologs in fission yeast and plants have been shown to be important for limiting crossovers during meiosis (Crismani et al., 2012; Knoll et al., 2012; Lorenz et al., 2012). Collectively, it is therefore possible that human FANCM plays an

www.manaraa.com

important role in limiting crossover and suppressing SCE formation via direct enzymatic activities.

Posttranslational regulation of FANCM

Given the multiple roles FANCM plays in DNA damage response and repair, it would be important to understand the regulation mechanisms of FANCM function. FANCM is a phosphoprotein that becomes hyperphosphorylated in response to DNA damage, and this phosphorylation is independent of the FA core complex (Meetei et al., 2005). In addition, FANCM phosphorylation is dynamically regulated throughout cell cycle, with moderate and extensive phosphorylation during S phase and mitosis respectively while dephosphorylated after mitotic exit. This dynamic change negatively correlates with FANCD2 monoubiquitylation level and FA core complex-chromatin association, suggesting that FANCM phorsphorylation regulates the FA pathway (Kim et al., 2008). Further studies demonstrated that the Plk1 kinase is responsible for FANCM phorsphorylation during mitosis, and FANCM phorsphorylation is followed by its degradation mediated by the β-TRCP component of the SCF E3 ligase, which may be important for releasing the FA core complex and inactivation of DNA repair machinery (Kee et al., 2009). In xenopus, FANCM phosphorylation is shown to be under control of ATR and ATM, and more interestingly, FANCD2, but whether this is also true for human proteins needs to be addressed.

Genetic models for FANCM

An avian cellular model for FANCM was constructed in DT40 lymphoblastoid platform. DT40 cells lacking FANCM display sensitivity to ICL damage, camptothecin and UV light (Rosado et al., 2009). Interestingly, UV sensitivity of human cells deficient for FANCM has also been reported, suggesting that FANCM has functions related to the repair

www.manaraa.com

of UV lesions (Kelsall et al., 2012). A mutation in the translocase domain of avian FANCM does not affect FA pathway activation but impairs SCE suppression (Rosado et al., 2009), suggesting the enzymatic activity of FANCM is indeed important for suppressing SCE.

A mouse model for FANCM has also been generated (Bakker et al., 2009). *Fancm*$^{-/-}$ mice show gonadal anomalies, increased tumor formation (lymphoma, histiocytic sarcoma, hepatoma, lung cancer) and reduced life span. *Fancm*$^{-/-}$ MEF cells display ICL damage sensitivity, increased chromosome breakage, excessive G2 arrest, deficient FA pathway activation and increased SCE formation (Bakker et al., 2009). Although *Fancm*$^{-/-}$ MEF cells recapitulate typical FA cellular characteristics, the FANCM mouse model, as well as other FA mouse models, does not fully phenocopy FA clinical features, especially in terms of hematological pathology (Bakker et al., 2009; Parmar et al., 2009).

Since no genuine FANCM patient is available, an effective human genetic model for FANCM is lacking. On the other hand, genetic models for FAAP24 are unavailable. Generation of human cellular knockout mutants will therefore be important for further functional studies of FANCM and FAAP24.

In this dissertation, I constructed *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ human somatic knockout cell lines, and systematically investigated the function of FANCM and FAAP24 in chromosome stability, FA pathway, DNA damage checkpoint, and ICL repair. I found that FANCM and FAAP24 are not fully epistatic in response to ICL damage. Specifically, while FANCM and FAAP24 coordinately activate FA pathway and suppress SCE, they also possess non-overlapping functions - FANCM promotes recombination-independent ICL repair, and FAAP24 facilitates efficient ATR-mediated checkpoint

www.manaraa.com

activation in response to ICL damage. These genetic evidences support a model that FANCM and FAAP24 have cooperative as well as unique functions in maintaining genomic stability.

www.manaraa.com

CHAPTER II

Generation of *FANCM* and *FAAP24* somatic knockout cell lines

Introduction

Fanconi anemia (FA) is a rare recessive genetic disorder characterized by multiple developmental abnormalities, progressive bone marrow failure, and profoundly increased cancer susceptibility (Moldovan and D'Andrea, 2009). The genetic complexity of Fanconi anemia is remarkably high, with at least fifteen complementation groups identified to date, each caused by mutation(s) of a distinct gene. A hallmark of cells derived from FA patients is hypersensitivity to DNA interstrand crosslinking agents such mitomycin C (MMC) and cisplatin (German et al., 1987; Poll et al., 1985), suggesting that FA genes play important roles in DNA damage response and repair.

Accumulating evidences support that FA and FA-associated proteins constitute the Fanconi anemia pathway, which is important for repair of DNA interstrand crosslink (ICL) lesions and maintenance of genomic stability. In response to DNA damage, two groups of FA and FA-associated proteins coordinately execute a monoubiquitination reaction. Eight FA proteins (FANCA, B, C, E, F, G, L and M), together with three FA-associated proteins (FAAP100, FAAP24 and FAAP20) and two FANCM associated histone-fold proteins (MHF1 and MHF2), form a FA core complex (Ciccia et al., 2007; Ling et al., 2007). This complex functions as an E3 ligase, and monoubiquitinates a heterodimeric ID complex consisting of FANCD2 and FANCI (Meetei et al., 2004; Smogorzewska et al., 2007; Wang, 2007) upon DNA damage. This monoubiquitination reaction is the central event of FA pathway activation, and it triggers the recruitment of the FANCD2-associated nuclease

www.manaraa.com

FAN1 and the FANCP gene product SLX4, a scaffolding protein involved in a structure-specific nuclease complex (Crossan et al., 2011; Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Stoepker et al., 2011). The multiple nucleases recruited to the damage sites are important for lesion incision and therefore ICL repair. Four other FA proteins BRCA2/FANCD1, BRIP1/FANCJ, PALB2/FANCN, and Rad51C/FANCO, also known as breast cancer susceptibility proteins (Howlett et al., 2002; Levitus et al., 2005; Vaz et al., 2010; Wang, 2007; Xia et al., 2007), do not seem to be directly regulated by the FA core complex or involved in the ID complex monoubiquitination. Given their defined roles in homologous recombination (HR)-mediated repair, this group of FA proteins likely functions to repair DNA double strand breaks formed as a result of ICL processing and to reestablish collapsed replication forks.

FANCM is of particular interest among the FA core complex components. It contains a DEAH helicase domain and associated ATP-dependent DNA translocase activity, and also an ERCC4-like endonuclease domain albeit degenerate (Ciccia et al., 2007; Hoadley et al., 2012; Meetei et al., 2005). FANCM closely associates with a binding partner FAAP24 as well as the MHF1/MHF2 histone-fold complex, and cooperatively they bind to and stabilize/remodel stalled replication forks (Gari et al., 2008a; Gari et al., 2008b; Yan et al., 2010). The FANCM/FAAP24 complex constitutively associates with chromatin, and upon recognition of replication-fork blocking lesions, it targets the FA core complex to DNA damage sites to monoubiquitinate the ID complex (Kim et al., 2008). At the stalled replication fork, FANCM also coordinates FA pathway activation with other DNA damage responsive events (Kee and D'Andrea, 2010), including ATR-mediated checkpoint activation (Collis et al., 2008; Huang et al., 2010; Schwab et al., 2010), and recruitment of Bloom's

www.manaraa.com

complex which is important for suppression of sister chromatid exchange (SCE) formation (Deans and West, 2009).

While FANCM and FAAP24 have been suggested to play multiple roles in mammalian DNA damage response and repair, most of the supporting evidences were obtained from si-RNA based studies. The lack of genetic evidences resulted from the unavailability of ideal human genetic models for *FANCM* and *FAAP24*. The only FANCM patient reported to date also harbors bi-allelic mutations of *FANCA* (Singh et al., 2009), making the true classification of this patient controversial. On the other hand, *FAAP24* mutations have not been found yet in Fanconi anemia patients. Such a lack of genetic model system restricts substantiation of the functions of FANCM and FAAP24 in FA pathway activation, DNA damage checkpoint signaling, and suppression of crossover recombination. It also prevents in-depth studies of how these potential functions integrate together to maintain genomic stability. In addition, the lack of an isogenic cellular model system precludes epistatic analysis between FANCM and FAAP24, and renders exploration of additional functions of these two proteins difficult.

To precisely determine the functional significance of FANCM and FAAP24 in genomic integrity maintenance, I sought to establish isogenic human FANCM- and FAAP24-null mutants through homologous replacement targeting in HCT-116 cells. In this chapter, I describe the generation of *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ somatic knockout cell lines. This genetic model system will readily allow functional characterization of FANCM and FAAP24 in DNA damage response and repair, and epistatic analysis between these two proteins, which will be described in following chapters.

Materials and Methods

www.manaraa.com

Cell culture

Human colorectal carcinoma HCT-116 and human embryonic kidney 293T (HEK293T) cell lines were obtained from the American Type Culture Collection (ATCC). Wild type HCT-116, its somatic knockout derivatives, and HEK293T cells were all maintained in Dulbecco's Modification of Eagles Medium (DMEM) plus 10% fetal calf serum (FBS) and grown in a humidified 5% CO_2-containing atmosphere at 37°C.

Targeting construct

FANCM and *FAAP24* targeting vectors were constructed using the USER cloning technology (New England BioLabs). Briefly, ~1.5 kb homology arms were PCR amplified from HCT-116 genomic DNA, using the following primers (upper case letters indicate USER cloning sites, and lower case letters indicate specific genomic sequences):

FANCM Left arm forward: GGGAAAGUcacgttcacttccctttaaacaat

FANCM Left arm reverse: GGAGACAUcaaagctggcacttatctaggact

FANCM Right arm forward: GGTCCCAUttggggactgtaaacgttattgta

FANCM Right arm reverse: GGCATAGUgccagcatacctgtaatgtagtga

FAAP24 Left arm forward: GGGAAAGUgactcatcagcagatctatccaca

FAAP24 Left arm reverse: GGAGACAUggatgaaaagatggcaataatagg

FAAP24 Right arm forward: GGTCCCAUcgtatttttgttaagcatcagcac

FAAP24 Right arm reverse: GGCATAGUtcagcttgacttaagggaacaaat

The amplified homology arms were cloned into the pAAV-USER vector to flank a neomycin resistance gene (*neo*) cassette (Fig 2.1). The pAAV-USER vector differs from the originally described AAV-USER-3×FLAG-KI vector (Zhang et al., 2008) in that the triple FLAG tag has been removed.

www.manaraa.com

rAAV-mediated gene targeting

The targeting vector was co-transfected into HEK293T cells together with pAAV-RC and pHelper plasmids, and the virus produced was collected 72 hours post transfection by lysing cells with three cycles of freeze and thaw (each cycle consisted of 10 min freeze in a dry ice-ethanol bath, and 10 min thaw in a 37 °C water bath). The supernatants containing the rAAV virus was used to infect HCT-116 cells. After 48 hours of virus infection, cells were plated out in low density in G418 containing media to allow growth of G418-resistant colonies.

PCR screening

G418-resistant colonies were screened for desired gene targeting events using a PCR strategy (Fig 2.2). Genomic DNA was extracted using the Lyse and Go PCR Reagent (Thermo Scientific), and PCR was conducted using the following primers:

FMScrL1: aagccgcactcaggatgccacttgc

FMScrR3: ttccattgtctgggccacagttcagc

F24ScrL3: ttctcttgtgccgccccttgactg

F24ScrR1: ggcatggatctgctgtgccactgctt

neoS2: gcggcatcagagcagccgattgtct

3F1: gcgcatcgccttctatcgccttcttg

The *neo* cassettes in positively-targeted clones were excised by infecting the cells with Ade-Cre virus. After 24 hours of virus infection, cells were plated out in low density, and the colonies grown out were screened by PCR (Fig 2.3) to confirm the excision of *neo* cassette. The primers used were as follows:

FMLCreL: gcgtataccccatgggtaact

www.manaraa.com

FMRCreR: agctgtcagggctgtctggtt

F24ScrL2: gtcacagctggcgcaggagatgcaa

F24ScrR3: tccccatgctgggatcagaggcgta

The heterozygous clones were infected with the rAAV virus again to target the second allele, and the *neo* cassettes were again removed from positively-targeted null clones. Disruption of both wild type alleles were confirmed by PCR (Fig 2.3) using the following primers:

FMUpStrm: ctcaggatgccacttgctgatg

FME2R: ccttcctggtggaagcttgtgt

FME2F: tggtgcagtaagagagtgcttt

FMI2R: ctatggcctacaaaggctttca

F24UpStrm: gccccaacactttacccctaa

F24E3R: tttcctgccaccaaatcagctt

F24E3F: gatggcttgacaccagactttt

F24E4R: attccaaggtccagcacagtaa

Western blot

Whole cell lysates were prepared by lysing cells with NETN-SDS buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS) followed by brief sonication. Western blot was performed following standard protocol. Briefly, protein samples were mixed with 2×SDS buffer, boiled, separated with SDS-PAGE, and electrotransfered onto nitrocellulose membranes, and immunoblot was carried out with primary antibodies as indicated. After washing for three times, the membrane was incubated

www.manaraa.com

with secondary anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase. The blot was developed with enhanced chemiluminescence reagent (Amersham Biosciences).

Antibodies

Polyclonal antibodies against FAAP24 were raised in rabbits by using MBP-tagged full length FAAP24 protein as the antigen and affinity-purified with GST-FAAP24 fusion protein. Polyclonal rabbit antibodies against FANCM$^{1190\text{-}1273}$ were similarly raised and purified. FANCB antibody was described previously (Meetei et al., 2005). Sources of commercial antibodies are as follows: anti-β-actin (sc-69879, Santa Cruz); peroxidase-conjugated rabbit anti-mouse (315-035-048, Jackson ImmunoResearch); peroxidase-conjugated goat anti-rabbit (111-035-144, Jackson ImmunoResearch); and horseradish peroxidase (HRP)-conjugated protein A (NA-9120V, GE Healthcare).

Results

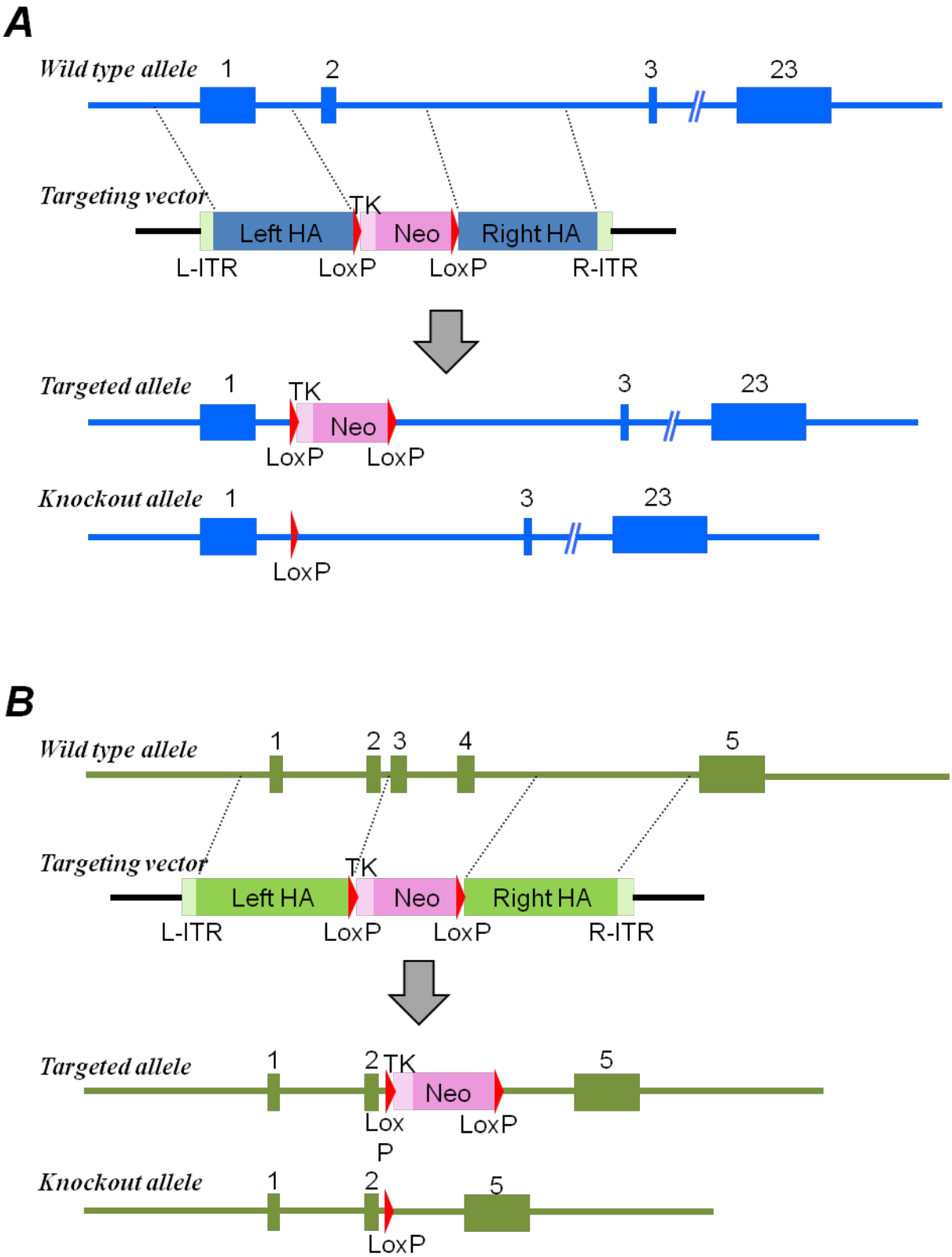
Strategy for *FANCM* and *FAAP24* loci targeting

To establish human genetic models for FANCM and FAAP24, homologous replacement-mediated gene targeting was performed in HCT-116 cells. Recombinant adeno-associated virus (rAAV) was used to deliver targeting vectors, which contained ~1.5 kb homology arms flanking a neomycin resistance gene (*neo*) cassette (Fig 2.1). Through gene targeting, exon 2 of *FANCM* was disrupted, leading to a frame-shift mutation and premature termination in exon 3 (Fig 2.1A). Similarly, exons 3 and 4 of *FAAP24* were ablated, resulting in a frame-shift mutation (Fig 2.1B). Sequential targeting was performed so that both alleles were inactivated, thereby generating the *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ knockout cell lines. A *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cell line was also generated by targeting *FAAP24* in the *FANCM*$^{-/-}$ knockout cells.

www.manaraa.com

Fig. 2.1 Homologous replacement mediated targeting of *FANCM* and *FAAP24* loci in HCT-116 cell line.
(A) Schematics of *FANCM* targeting strategy.
(B) Schematics of *FAAP24* targeting strategy.
Numbered boxes indicate relative locations of exons. HA: homology arm; TK: thymidine kinase gene promoter; Neo: neomycin resistance gene cassette; L(R)-ITR: left (right) inverted tandem repeats.

www.manaraa.com



www.manaraa.com

Generation of heterozygous *FANCM*$^{+/-}$ and *FAAP24*$^{+/-}$ cell lines

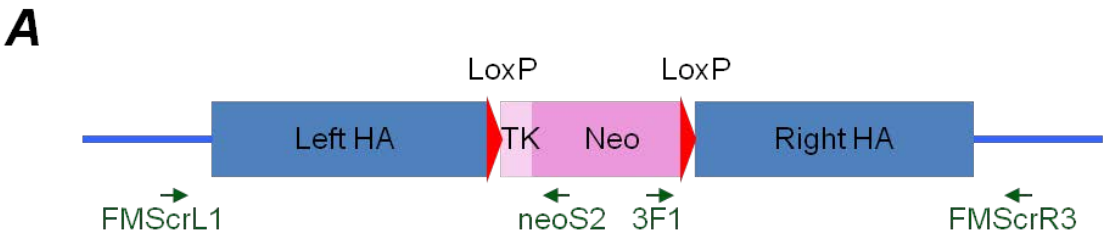
HCT-116 cells were infected with rAAV virus containing *FANCM* or *FAAP24* targeting vector, and G418-resistant clones were screened for correct targeting events using PCR. Each pair of screening primer includes one primer located outside of the homology arm and the other located within the *neo* cassette. For *FANCM* targeting, I screened ~200 G418-resistant colonies, and identified 4 positive (*+/n*) clones with correct targeting. For *FAAP24* targeting, 3 out of the ~400 colonies screened were positive for correct targeting. All *+/n* clones were expanded and confirmed by PCR using primers spanning both homology arms (Fig 2.2).

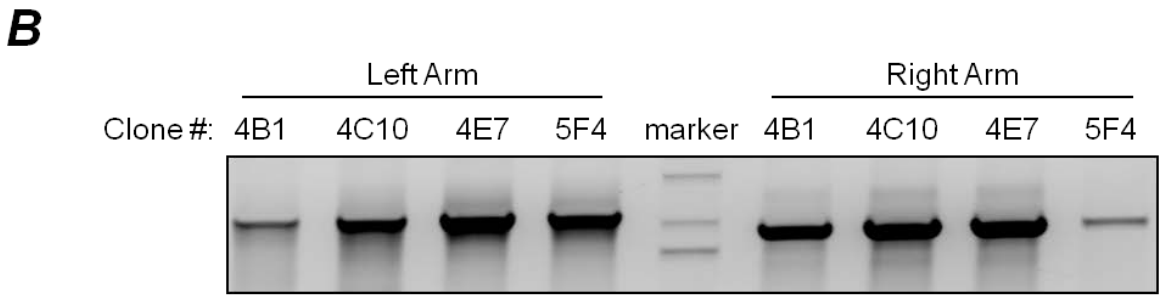
In order to remove the *neo* cassette, the *+/n* clones were exposed to Ad-Cre virus, and plated out in low density. The resulted cell colonies were screened by PCR to confirm the loss of *neo* cassette. Due to the high titer of virus used, most of the colonies screened were verified to be heterozygous *FANCM*$^{+/-}$ or *FAAP24*$^{+/-}$ clones (data not shown).

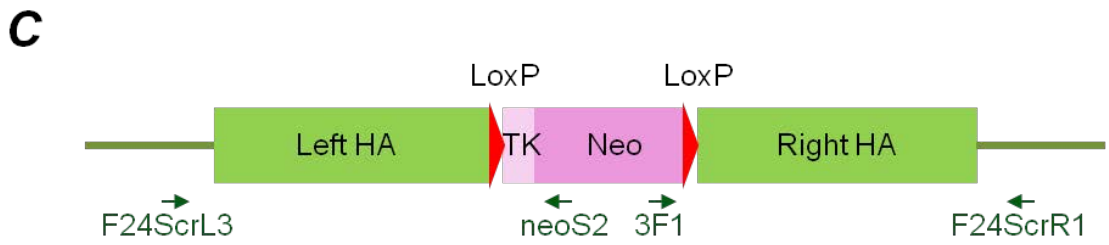
www.manaraa.com

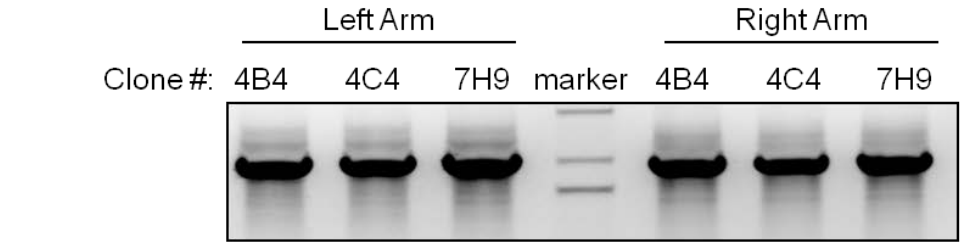
Fig. 2.2 Identification of *FANCM*$^{+/n}$ and *FAAP24*$^{+/n}$ clones.
(A) PCR screening strategy for *FANCM*$^{+/n}$ clone identification. HA: homology arm; TK: thymidine kinase gene promoter; Neo: neomycin resistance gene cassette.
(B) Confirmative PCR of the targeted allele in four *FANCM*$^{+/n}$ clones using both left arm (FMScrL1/neoS2) and right arm (3F1/FMScrR3) primers.
(C) PCR screening strategy for *FAAP24*$^{+/n}$ clone identification. HA: homology arm; TK: thymidine kinase gene promoter; Neo: neomycin resistance gene cassette.
(D) Confirmative PCR of the targeted allele in three *FAAP24*$^{+/n}$ clones using both left arm (F24ScrL3/neoS2) and right arm (3F1/F24ScrR1) primers.

www.manaraa.com









www.manaraa.com

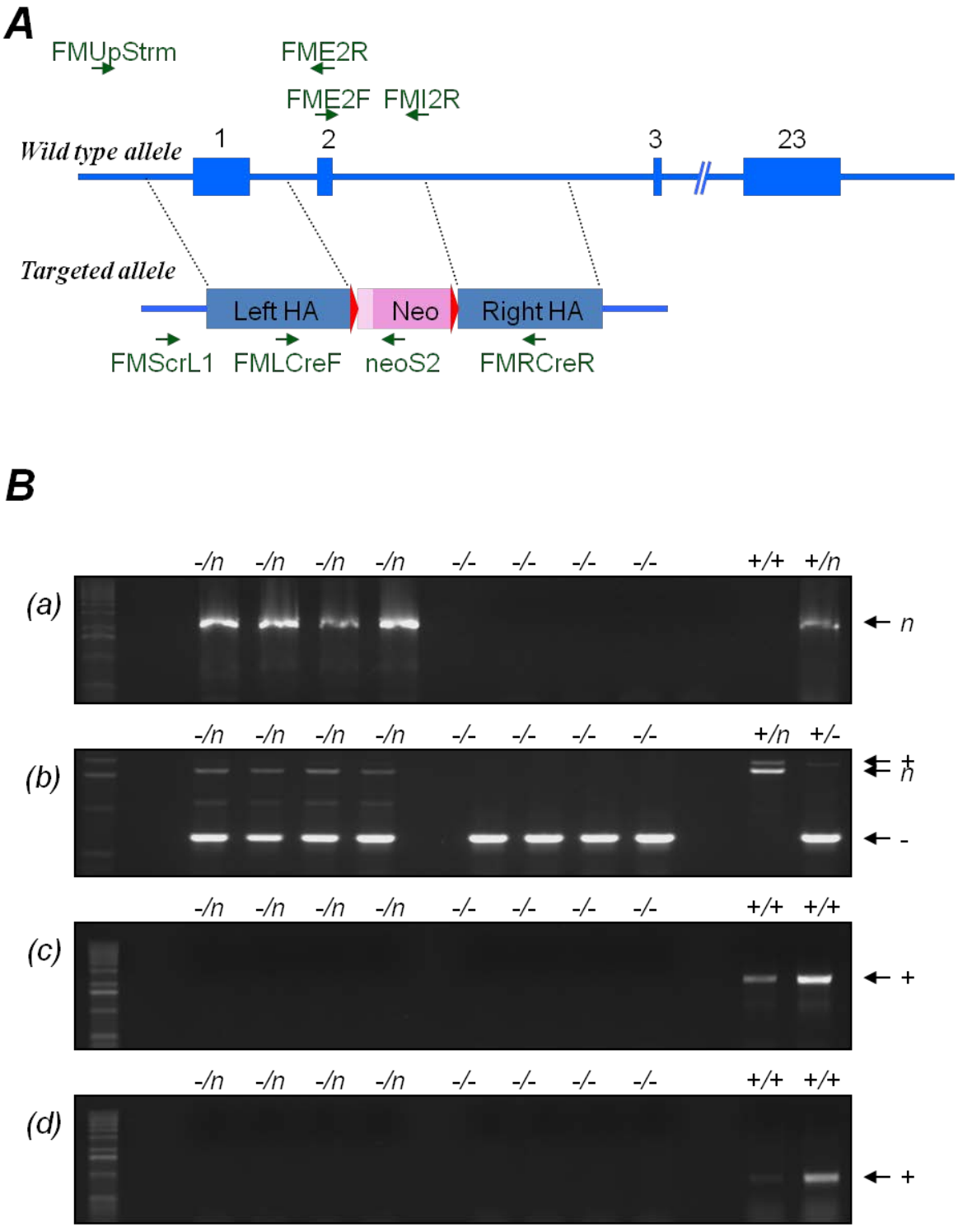
Generation of *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cell lines

In order to generate the *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cell lines, one heterozygous clone for each gene was used for second allele targeting. The *FANCM*$^{+/-}$ and *FAAP24*$^{+/-}$ cells were infected with rAAV virus containing corresponding targeting vector, and G418-resistant clones were again screened by PCR to identify correct targeting events. Since both the knockout allele and the remaining wild type allele may be targeted at equal frequencies, the positive clones were also screened for the presence of knockout allele as well as absence of wild type allele (Fig 2.3A & Fig 2.4A). For *FANCM* targeting, I screened ~700 G418-resistant colonies, and identified one positive (*-/n*) clone with correct targeting of the remaining wild type allele. For *FAAP24* targeting, one out of the ~1200 colonies screened was positive for correct targeting of the remaining wild type allele. The *FANCM*$^{-/n}$ and *FAAP24*$^{-/n}$ clones were expanded and confirmed by PCR for the presence of targeted allele and knockout allele, as well as the absence of wild type allele (Fig 2.3B & Fig 2.4B).

www.manaraa.com

Fig. 2.3 Identification of the *FANCM*$^{-/-}$ clone.
(A) PCR screening strategy for *FANCM*$^{-/n}$ and *FANCM*$^{-/-}$ clone identification. HA: homology arm; Neo: neomycin resistance gene cassette.
(B) PCR genotyping of the *FANCM*$^{-/n}$ clones and *FANCM*$^{-/-}$ clones. +: wild type allele; *n*: targeted allele; -: knockout allele.
(*a*) PCR of targeted allele using left arm primers (FMScrL1/neoS2).
(*b*) PCR of knockout allele using FMLCreL/FMRCreR primers.
(*c*) PCR of wild type allele using FMUpStrm/FME2R primers.
(*d*) PCR of wild type allele using FME2F/FMI2R primers.

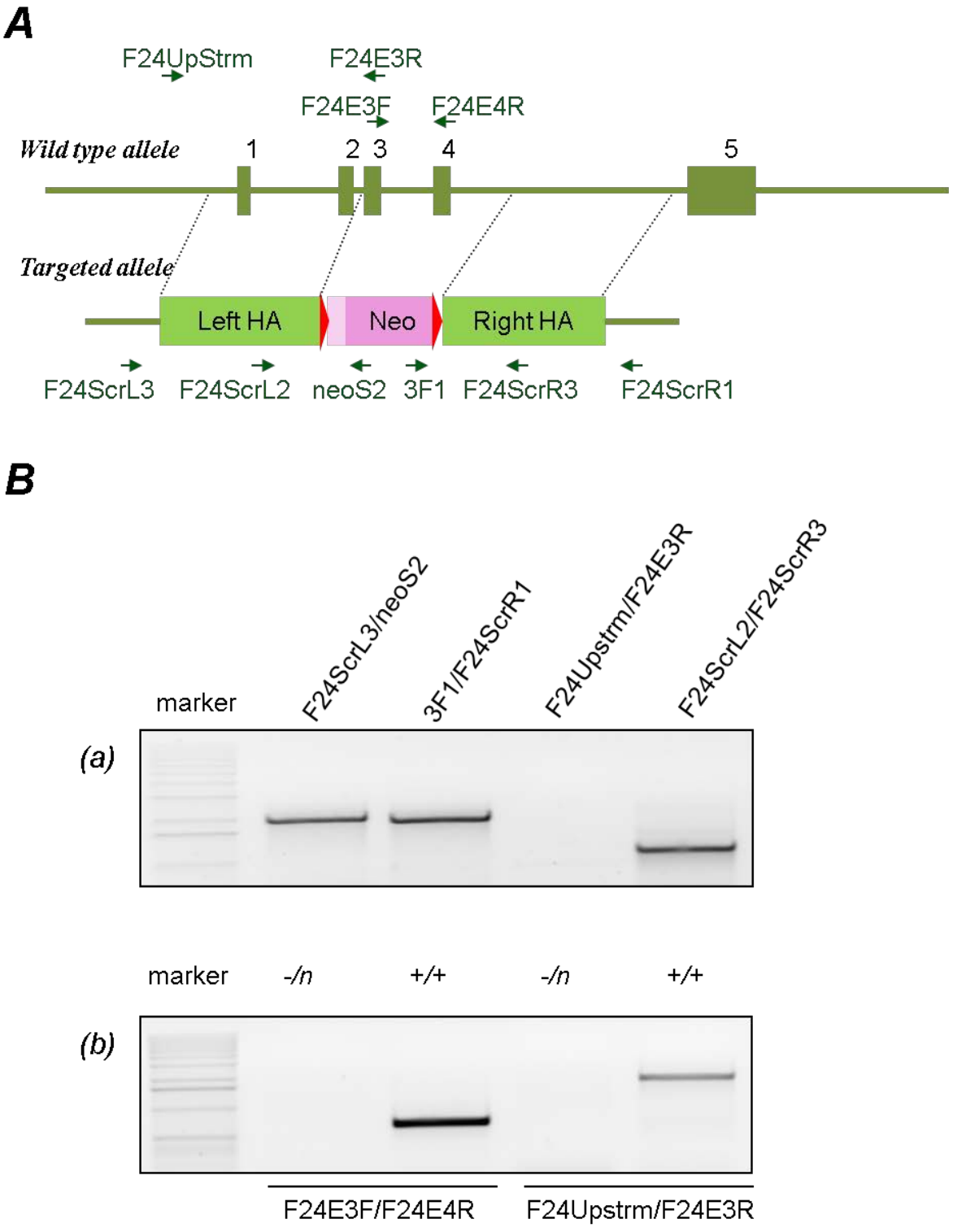
www.manaraa.com



www.manaraa.com

Fig. 2.4 Identification of the *FAAP24*$^{-/n}$ clone.
(A) PCR screening strategy for *FAAP24*$^{-/n}$ clone identification. HA: homology arm; Neo: neomycin resistance gene cassette.
(B) PCR genotyping of the *FAAP24*$^{-/n}$.
(*a*) PCR of targeted allele, knockout allele and wild type allele using indicated primers.
(*b*) PCR of wild type allele using both F24UpStrm/F24E3R and F24E3F/F24E4R primers.

www.manaraa.com



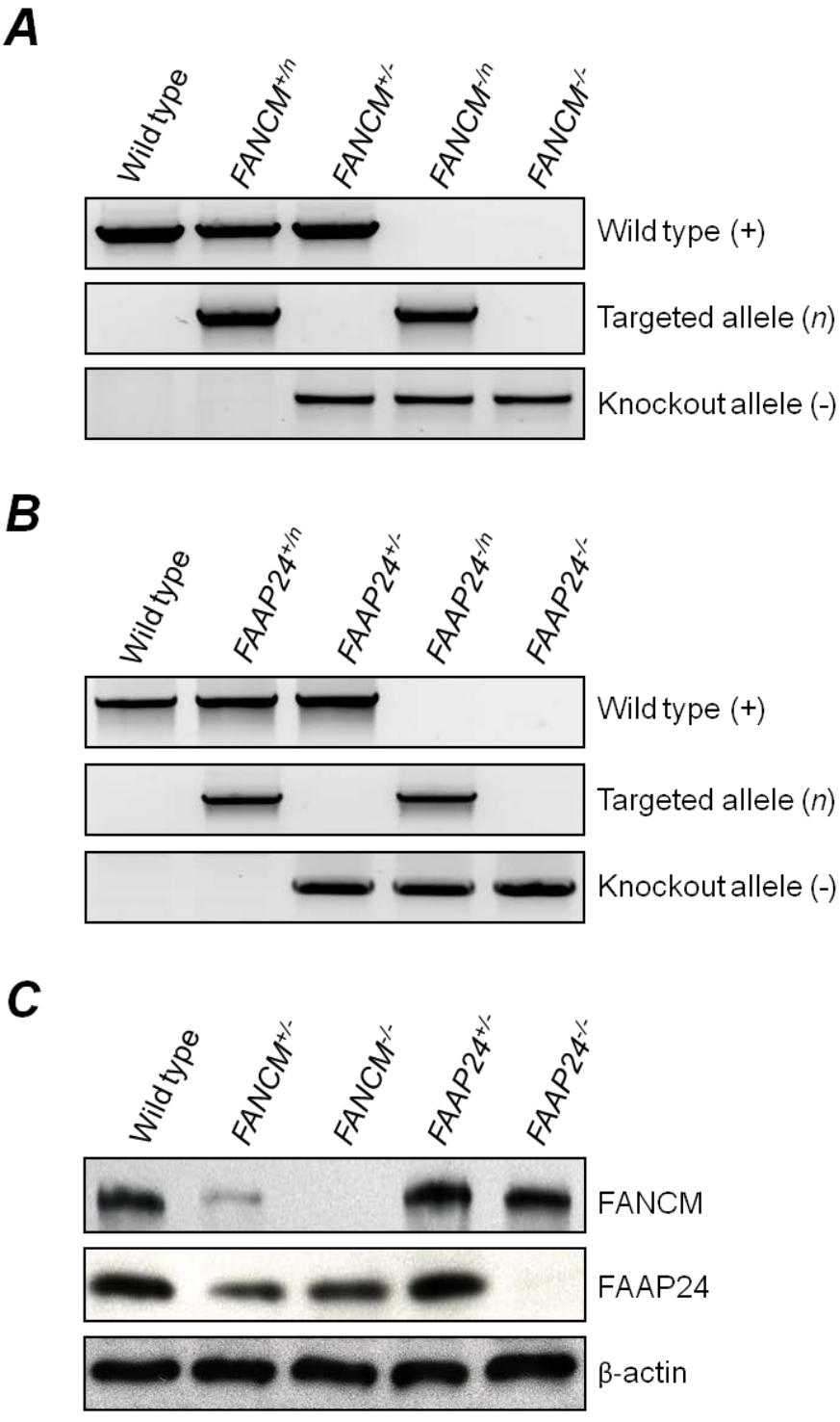
www.manaraa.com

Finally, the *neo* cassettes were removed from the *FANCM*$^{-/n}$ and *FAAP24*$^{-/n}$ clones to generate the *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cell lines. Genotyping of representative clones for each genotype was shown in Figs 2.5A&B. Complete loss of gene expression in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ knockout cell lines was verified by western blot (Fig 2.5C).

www.manaraa.com

Fig. 2.5 Generation of *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ knockout cell lines.
(A) PCR genotyping of *FANCM* in representative clones.
(B) PCR genotyping of *FAAP24* in representative clones.
(C) Western blot showing complete FANCM and FAAP24 protein loss in corresponding somatic knockout cell lines.

www.manaraa.com



www.manaraa.com

Generation of *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cell line

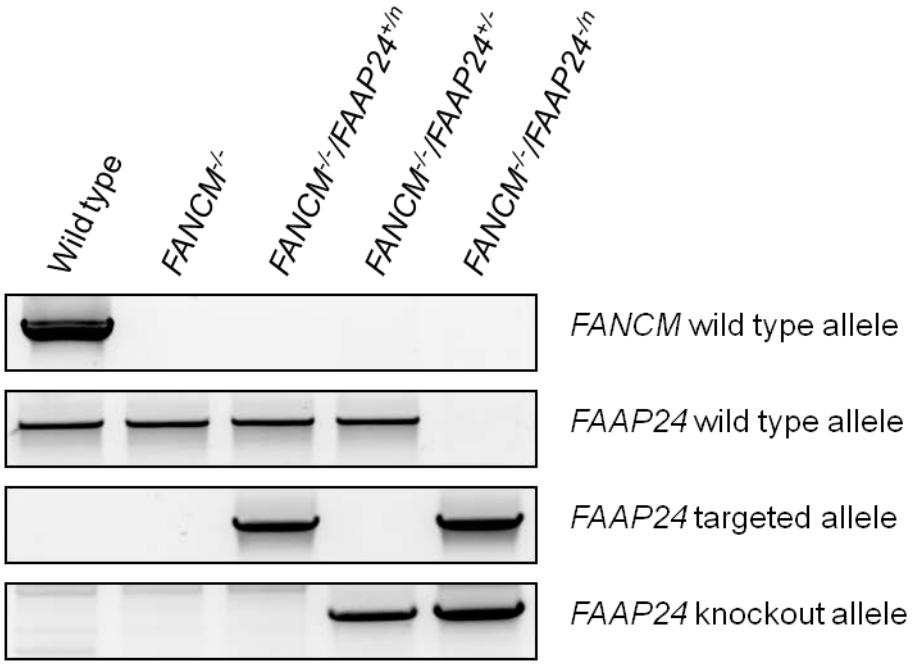
To generate the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cell line, gene targeting of *FAAP24* was performed in *FANCM*$^{-/-}$ cells. First allele targeting resulted in six *FANCM*$^{-/-}$/ *FAAP24*$^{+/n}$ clones out of ~1000 colonies screened. For the second allele targeting, one out of ~1300 colonies screened was positive for correct targeting of the remaining wild type *FAAP24* allele. This clone was expanded and the genotype was verified by PCR. Genotyping of representative clones for each genotype was shown in Fig 2.6A. Finally, the *neo* cassette was removed to generate the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cell line. Complete loss of gene expression was confirmed by western blot (Fig 2.6B).

www.manaraa.com

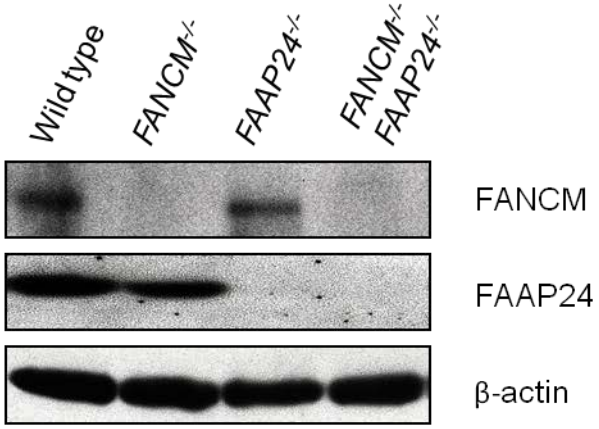
Fig. 2.6 Generation of *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cell line.
(A) PCR genotyping of *FANCM* and *FAAP24* in representative clones.
(B) Western blot showing complete FANCM and FAAP24 protein loss in corresponding somatic knockout cell lines.

www.manaraa.com

A



B



www.manaraa.com

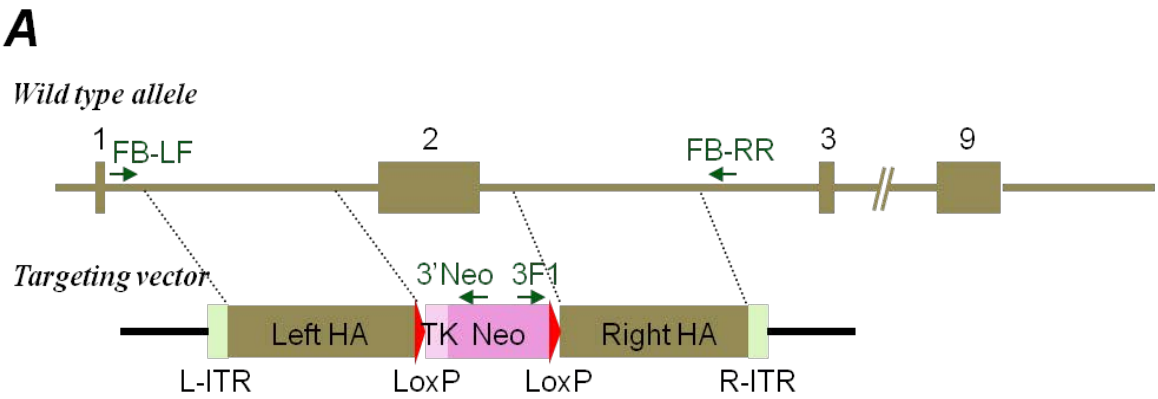
Generation of *FANCB*$^{-}$ knockout cell line

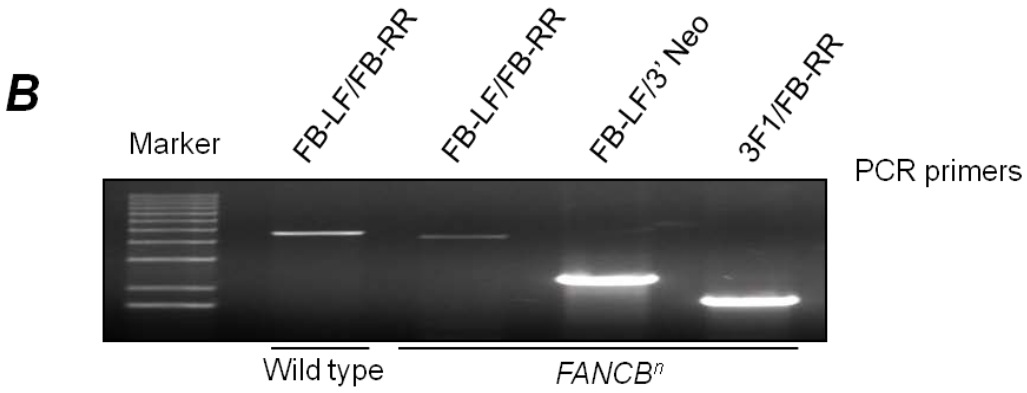
Gene targeting of *FANCB* was done by Dr. Yingjun Jiang (a former postdoctoral fellow in Dr. Lei Li's group). Since FANCB gene is located in X chromosome, only one round of gene targeting was necessary to generate the null mutant. As shown in Fig 2.7A, exon 2 was deleted through homologous replacement-mediated targeting. One *FANCB*^{n} clone was obtained and the genotype was confirmed by PCR (Fig 2.7B). I removed the *neo* cassettes from this *FANCB*^{n} clone and generated the *FANCB*$^{-}$ somatic knockout cell line. Finally I did an IP-western and confirmed the loss of FANCB expression in the *FANCB*$^{-}$ knockout cells (Fig 2.7C).

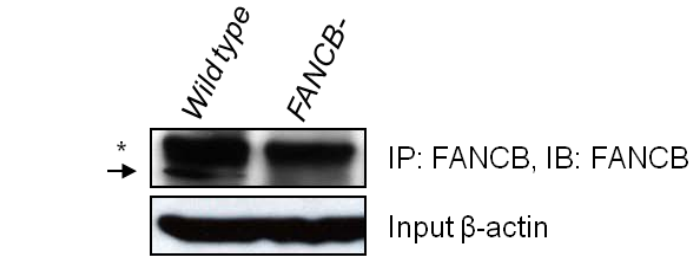
www.manaraa.com

Fig. 2.7 Generation of *FANCB*$^{-}$ knockout cell line.
(A) Schematics of *FANCB* targeting strategy. Numbered boxes indicate relative locations of exons. HA: homology arm; TK: thymidine kinase gene promoter; Neo: neomycin resistance gene cassette; L(R)-ITR: left (right) inverted tandem repeats. Locations of PCR screening primers were also indicated.
(B) PCR genotyping of the *FANCB*^{n} clone.
(C) IP-Western blot showing complete FANCB protein loss in the somatic knockout cell line. Asterisk indicates nonspecific bands.

www.manaraa.com







www.manaraa.com

Discussion

FANCM and FAAP24 functions have been implicated in the activation of FA pathway, DNA damage checkpoint signaling, and suppression of crossover recombination. However, lack of human genetic model systems restricted in-depth studies of how these functions integrate to protect cells from DNA damage. While no patient mutations have been identified for *FAAP24*, the only FANCM patient also harbors bi-allelic *FANCA* mutations (Singh et al., 2009), rendering the true classification of this patient difficult. In this study, I created isogenic human FANCM- and FAAP24-null mutants, which allow the functional impact of FANCM and FAAP24 on DNA damage response to be precisely determined.

The fact that the FANCM patient harbors bi-allelic mutations of both *FANCM* and *FANCA* makes whether *FANCM* is a *bona fide* Fanconi anemia gene controversial. In the present study, the *FANCM*$^{-/-}$ somatic knockout cells are viable, and recapitulate cellular hallmarks of Fanconi anemia (described in Chapter III). These results indicate the potential existence of genuine FANCM patient(s). Similarly, the viability and the typical cellular FA characteristics of *FAAP24*$^{-/-}$ somatic knockout cells (Chapter III) suggest the possibility of FAAP24 patients. However, since the FA pathway activation is only partially impaired in the absence of FANCM or FAAP24 (Chapter III), the clinical phenotypes of FANCM and FAAP24 patients, if any, might be less severe compared with typical FA patients.

Generation of *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single mutants as well as *FANCM*$^{-/-}$*/FAAP24*$^{-/-}$ double mutant allows the epistatic functions of FANCM and FAAP24 in DNA damage response and repair to be determined. Although FANCM can form a heterodimer with FAAP24 through its C-terminal ERCC-4 like endonuclease domain, it also contains other domains including the translocase domain associated with ATP-dependent enzymatic activity,

www.manaraa.com

MM1 motif required for FA core complex interaction, and MM2 motif responsible for Bloom's complex association (Deans and West, 2009; Meetei et al., 2005). It is therefore likely that FANCM may carry out additional functions independent of FAAP24. In fact, while FANCM has been implicated in replication fork remodeling and SCE suppression, whether FAAP24 is also involved in these functions remains unclear. On the other hand, the apparent differential preference for DNA substrates of FANCM and FAAP24 (Ciccia et al., 2007) indicates the possibility that FAAP24 may also possess FANCM-independent functions. A systematic epistatic analysis will elucidate whether FANCM and FAAP24 are interdependent for their functions in maintaining genomic stability.

In this study, a *FANCB*$^{-}$ somatic knockout cell line is also generated and included in a number of functional studies of FANCM and FAAP24. This allows the dissection of both FA core complex-associated and FA core complex-independent functions of FANCM and FAAP24 in DNA damage response and repair.

www.manaraa.com

CHAPTER III

FANCM and FAAP24 Coordinately Activate the Fanconi Anemia Pathway and Suppress Sister Chromatid Exchange

Introduction

FANCM was initially identified as FAAP250, a FANCA interacting protein in a tandem affinity purification experiment. FAAP250 associates with known FA core complex components and functions to promote FANCD2 monoubiquitination (Meetei et al., 2005). *FAAP250* mutations were found in a previously unclassified Fanconi anemia patient, and the gene was therefore named *FANCM* (Meetei et al., 2005). However, biallelic mutations of *FANCA* were later discovered in the same patient. To date, this patient remains to be the only FA patient harboring *FANCM* mutations (Singh et al., 2009). Consequently, whether FANCM is a *bona fide* FA gene appears controversial. Nevertheless, lymphoblasts from this patient complemented with FANCA remains sensitive to MMC and display attenuated FANCD2 monoubiquitination (Singh et al., 2009), consistent with si-RNA based knockdown studies (Meetei et al., 2005), suggesting that FANCM functions to activate FA pathway.

FAAP24 was identified as a FANCM interacting partner (Ciccia et al., 2007), and FANCM-FAAP24 complex structurally resembles that of the XPF-ERCC1 and Mus81-Eme1/2 endonuclease complexes, suggesting that FAAP24 may function with FANCM in a similar heterodimer. Consistently, like FANCM, FAAP24 also associates with FA core complex, and cells devoid of FAAP24 display MMC sensitivity and defective FANCD2 monoubiquitiantion (Ciccia et al., 2007). Collectively, these studies suggest that FANCM and FAAP24 are important for the activation of FA pathway.

www.manaraa.com

Unlike typical FA core complex components, FANCM and FAAP24 have DNA interacting domains and activities. FANCM contains a DEAH helicase domain and associated ATP-dependent translocase activity (Meetei et al., 2005). Both FANCM and FAAP24 contain ERCC4-like nuclease domain, which are important for their in *vitro* DNA binding (Ciccia et al., 2007). These properties of FANCM and FAAP24 may allow them to function independently of the FA pathway. Indeed, FANCM has been shown to be required for resistance to UV and campothecin (Singh et al., 2009). In addition, cells deficient for FANCM display increased sister chromatid exchange, a phenotype seemingly not shared with other FA core complex mutant cells (Bakker et al., 2009). Furthermore, FANCM and FAAP24 have been implicated in the activation of ATR-mediated DNA damage checkpoint (Collis et al., 2008; Huang et al., 2010; Luke-Glaser et al., 2009; Schwab et al., 2010). More recently, FANCM has also been demonstrated to function in nucleotide excision repair and in recombination-mediated processes (Crismani et al., 2012; Kelsall et al., 2012; Knoll et al., 2012; Lorenz et al., 2012).

In this study, I sought to systematically study the functions of FANCM and FAAP24 in cellular response to ICL damage using an isogenic human genetic model system established earlier (Chapter II). Unexpectedly, I found that *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cells were much more sensitive to crosslinking agents than *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single knockout cells, suggesting that FANCM and FAAP24 functions are not fully epistatic in response to ICL damage. This prompted me to define shared as well as unique functions of FANCM and FAAP24 in DNA damage response and repair. I show in this Chapter that FANCM and FAAP24 work in concert to activate the FA pathway and to suppress crossover recombination. An extended study of the functions of FANCM and

www.manaraa.com

FAAP24 in DNA damage checkpoint signaling and actual ICL repair processes has also been carried out, and the results will be described in following Chapters.

Materials and Methods

Antibodies

FANCL antibody was described previously (Meetei et al., 2003). Sources of commercial antibodies not described in previous chapters are as follows: anti-β-tubulin (T4026, Sigma-Aldrich); anti-FANCD2 (ab2187, Abcam; sc-20022, Santa Cruz; and NB100-182, Novus); anti-FANCA (A301-980A, Bethyl); anti-FANCG (NB100-2566, Novus); anti-histone H3 (05-928, Millipore); anti-BLM (A300-110A, Bethyl); and rhodamine Red-X-conjugated goat anti-rabbit (111-295-144, Jackson ImmunoResearch).

Clonogenic survival assay

Cells were seeded at a density of 1-3×10^5 cells per 100 mm culture plate and allowed to attach and recover for overnight. Cells were then exposed to MMC or cisplatin at escalating dosages for 1 hr. After drug treatment, cells were seeded at appropriate densities in triplicates for each dosage group, and grown in drug-free medium for approximately 14 days. Cell colonies were then fixed with 6% glutaraldehyde (v/v) and stained with 0.5% crystal violet (w/v). Colonies containing more than 50 cells were counted, and relative survival was calculated as follows: % survival = # colonies counted / (# cells plated × plating efficiency) × 100.

Mitotic spread and SCE assay

For MMC-induced chromosomal abnormality analysis, cells were treated with 40 ng/ml MMC for 18 hrs, and mitotic spread was prepared as previously described (Wang et al.,

www.manaraa.com

2006). Slides were stained with 4% Giemsa and chromosomal abnormalities were quantified by scoring 40-80 metaphases.

For SCE assay, cells were cultured in the presence of 100 mM of 5-bromodeoxyuridine for approximately 48 hrs to allow for twice of doubling. For MMC-induced SCE analysis, 20 ng/ml MMC were added 18 hrs before harvesting cells. Mitotic spread preparation and slide staining were performed as previously described (Yin et al., 2005). About 40 metaphases were scored for each sample.

Immunofluorescence staining

Cells were cultured on cover slips and treated with desired dosage of MMC for 24 hrs. Cells were then washed with PBS, fixed with 3% formaldehyde for 15 min and permeabilized with 0.5% Triton buffer (20 mM HEPES, pH7.4, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, 0.5% Triton-X-100) for 5 min at room temperature. Samples were incubated sequentially with desired primary and secondary antibodies for 30 min, and finally stained with 4', 6-diamidino-2-phenylindole (DAPI) for 2 min in dark, all at room temperature. Cover slips were mounted on slides with 1, 4-phenylenediamine based anti-fade solution, and visualized under Nikon Eclipse 90i fluorescence microscopy. Nuclear foci were counted in at least 100 nuclei at 5 different locations for each sample.

Cell fractionation

Cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0,100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 2 μg/ml aprotinin, and 1μg /ml pepstatin) at 4 °C for 30 min and centrifuged at 14,000 rpm for 10 min. The soluble fraction contains the cytosol and soluble nuclear proteins. The insoluble chromatin enriched pellet was washed with phosphate buffered saline (PBS) for three times

www.manaraa.com

and extracted with 0.2 N HCl for 30 min on ice, and the extract was neutralized with equal volume of 1 M Tris-HCl (pH 8.0).

Results

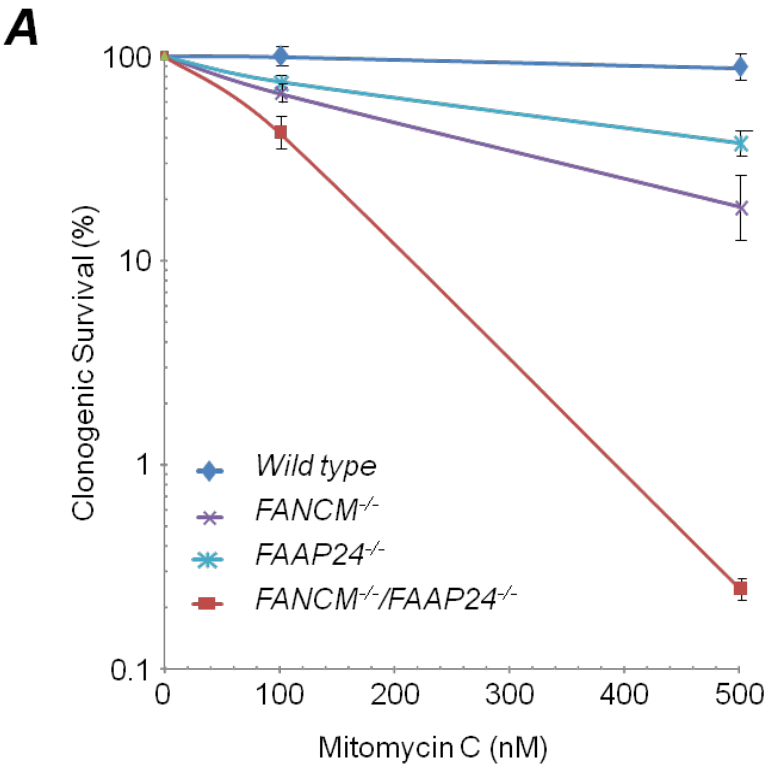
FANCM$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cells are hypersensitive to interstrand crosslinking agents

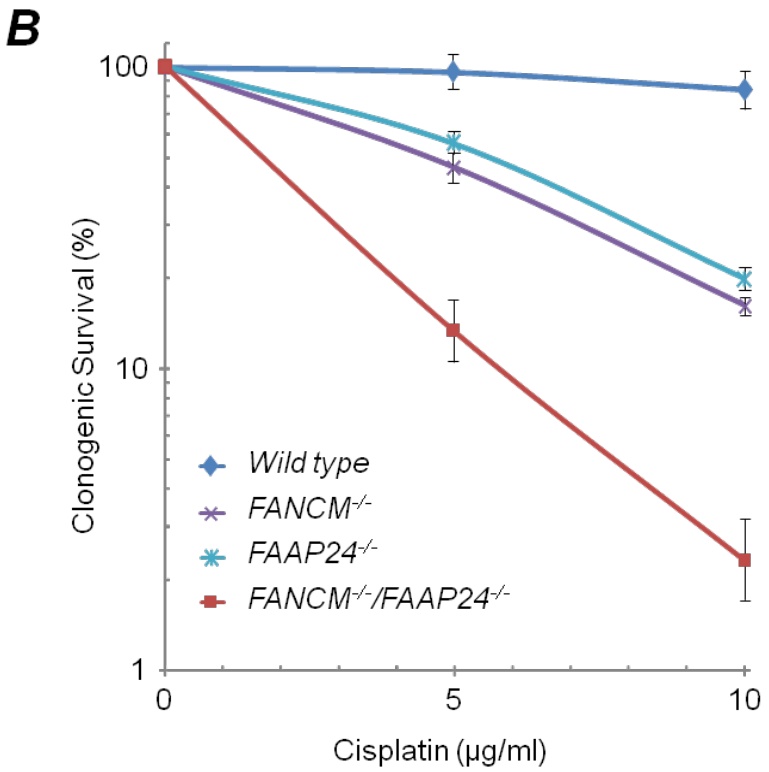
A characteristic cellular hallmark of FA is hypersensitivity to DNA interstrand crosslinking agents such as mitomycin C (MMC), which manifests as reduced survival and increased chromosomal abnormalities (Moldovan and D'Andrea, 2009). To test whether the *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cells display typical FA cellular phenotypes, I first exposed them to different concentrations of MMC and compared their survival to wild type HCT-116 cells. As shown in Fig 3.1A, cells lacking FANCM or FAAP24 showed significantly reduced cellular survival in response to MMC. Similar results were also obtained when exposing cells to another interstrand crosslinking agent cisplatin (Fig 3.1B).

www.manaraa.com

Fig. 3.1 FANCM and FAAP24 deficient cells are hypersensitive to ICL agents.
(A) Clonogenic survival of *FANCM* and *FAAP24* knockout cell lines treated with MMC. Error-bars represent standard deviations from four independent experiments with triplicated plates.
(B) Clonogenic survival of *FANCM* and *FAAP24* knockout cell lines treated with cisplatin. Error-bars represent standard deviations from four independent experiments with triplicated plates.

www.manaraa.com





www.manaraa.com

I then exposed *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cells to a low concentration of MMC, and examined metaphase cells for chromosome abnormalities. Cells deficient for FANCM or FAAP24 displayed increased levels of chromosome breakage compared to wild type cells (Fig 3.2), reflecting another typical FA cellular feature.

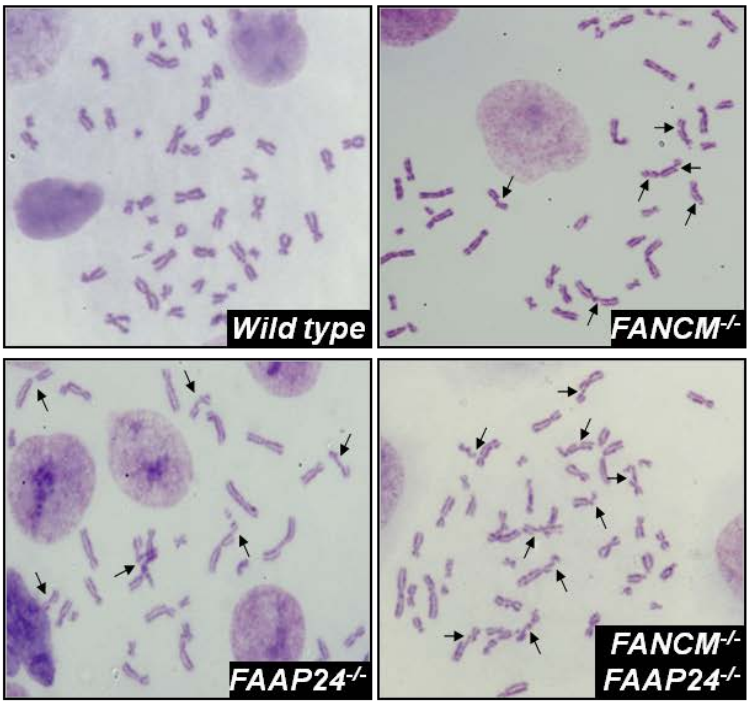
Collectively, these data demonstrate that *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ somatic knockout cells recapitulate characteristic FA cellular hallmarks, and suggest that FANCM and FAAP24 are important for cellular resistance to interstrand crosslink damage.

www.manaraa.com

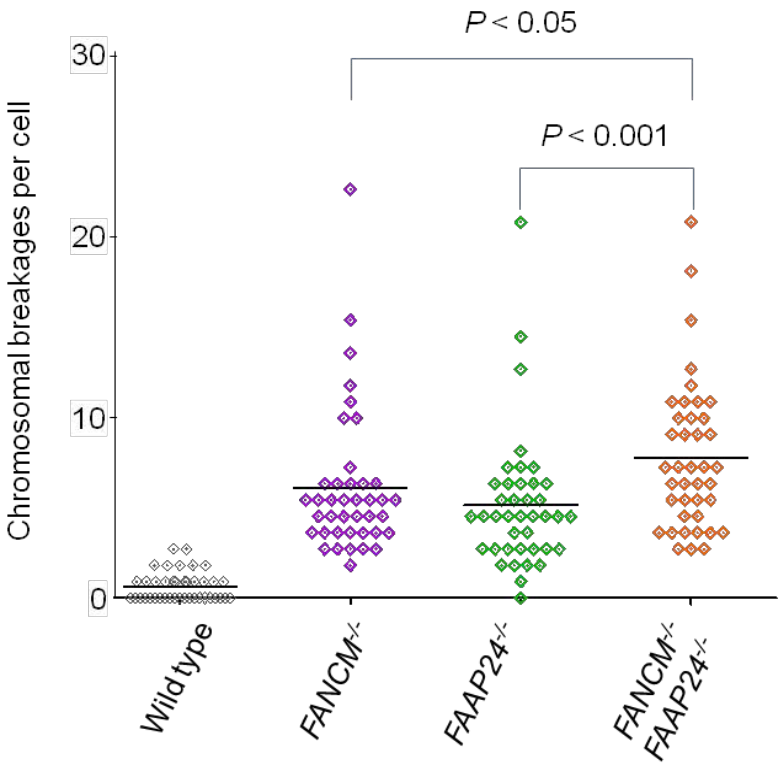
Fig. 3.2 FANCM and FAAP24 deficient cells display increased chromosome breakage in response to ICL damage.
(***A***) Chromosome breakage in *FANCM* and *FAAP24* knockout cell lines exposed to MMC (40 ng/ml for 18 hr). Arrows indicate visible chromosome breaks.
(***B***) Quantification of chromosomal abnormality in *FANCM* and *FAAP24* knockout cell lines. 40 metaphase spreads were scored for each cell line. Bars represent average chromosomal breakage per spread.

www.manaraa.com

A



B



www.manaraa.com

FANCM and FAAP24 possess non-overlapping functions in cellular resistance to ICL damage

The isogenic property of the FANCM and FAAP24 mutants allowed direct comparison of their hypersensitivities to crosslinking agents. Compared with *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single knockout cells, the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cells were much more sensitive to both MMC and cisplatin in terms of cellular survival (Figs 3.1A&B). Consistently, the double mutant also had significantly higher frequency of MMC-induced chromosomal breakages than either single mutant (Fig 3.2). These data strongly indicate that FANCM and FAAP24 may possess non-overlapping functions in response to DNA interstrand crosslinking damage.

FANCM and FAAP24 cooperatively support activation of the Fanconi anemia pathway

Compared to *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells, the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cells displayed more chromosome breaks and lower survival when treated with MMC (Fig 3.1A & Fig 3.2). One plausible explanation could be that FANCM and FAAP24 are redundant in FA pathway activation. To test this hypothesis, I examined FANCD2 monoubiquitination, a marker of FA pathway activation, in cells lacking FANCM and/or FAAP24. As shown in Figs 3.3A&B, MMC-induced FANCD2 monoubiquitination in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells was significantly reduced compared with that in wild type cells, and the defects could be rescued by re-expressing wild type FANCM or FAAP24 proteins (Fig 3.4). These data suggest that FANCM and FAAP24 promote the FA pathway activation. In contrary to my hypothesis however, MMC-induced FANCD2 monoubiquitination in *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cells was at a comparable level

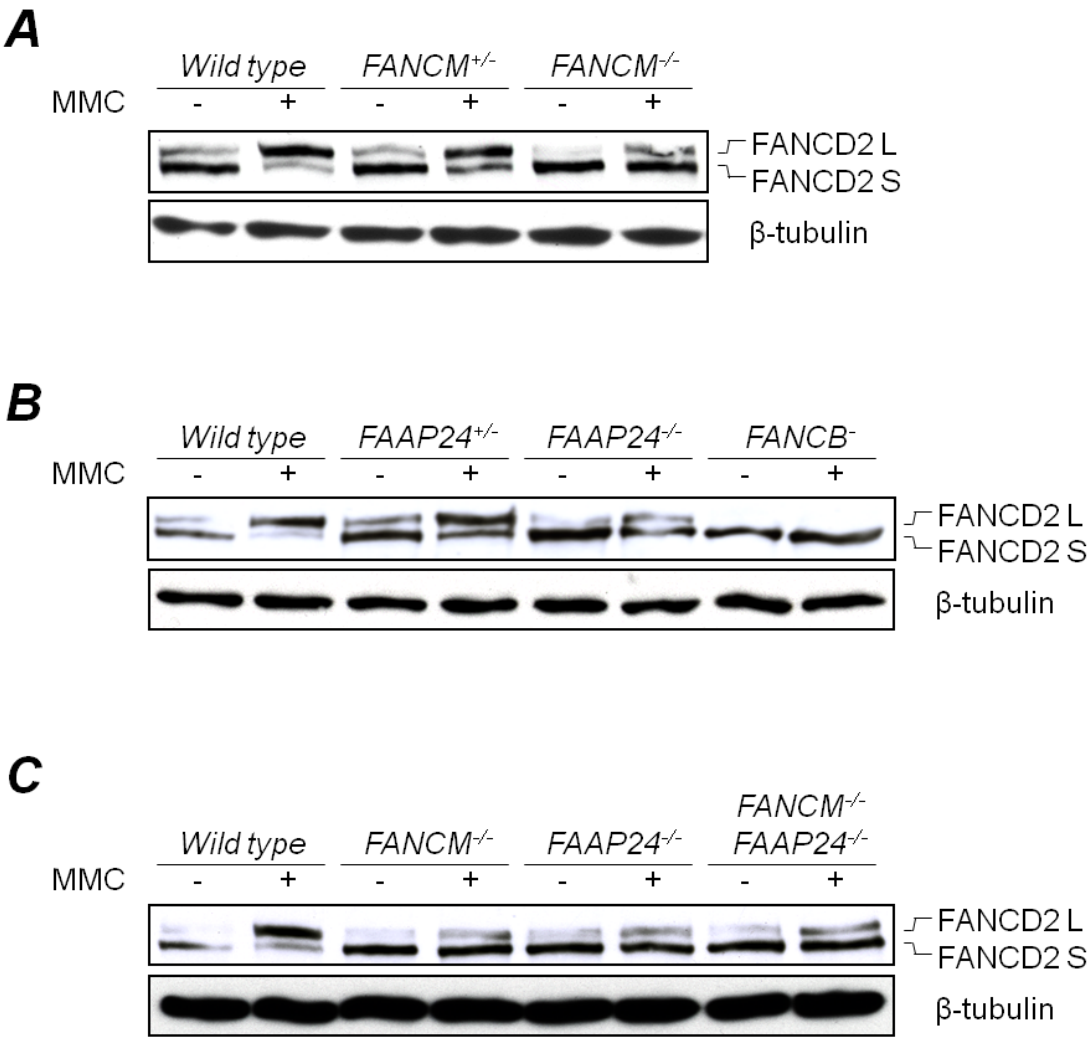
www.manaraa.com

to those in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells (Fig 3.3C), suggesting that FANCM and FAAP24 work together to promote the activation of FA pathway.

www.manaraa.com

Fig. 3.3 FANCM and FAAP24 promote FANCD2 monoubiquitination.
(***A-C***) Immunoblots detecting FANCD2 monoubiquitination in cells with indicated genotypes, treated or mock-treated with MMC (200 ng/ml, 16 hrs). L and S represent monoubiquitinated and native forms of FANCD2, respectively.

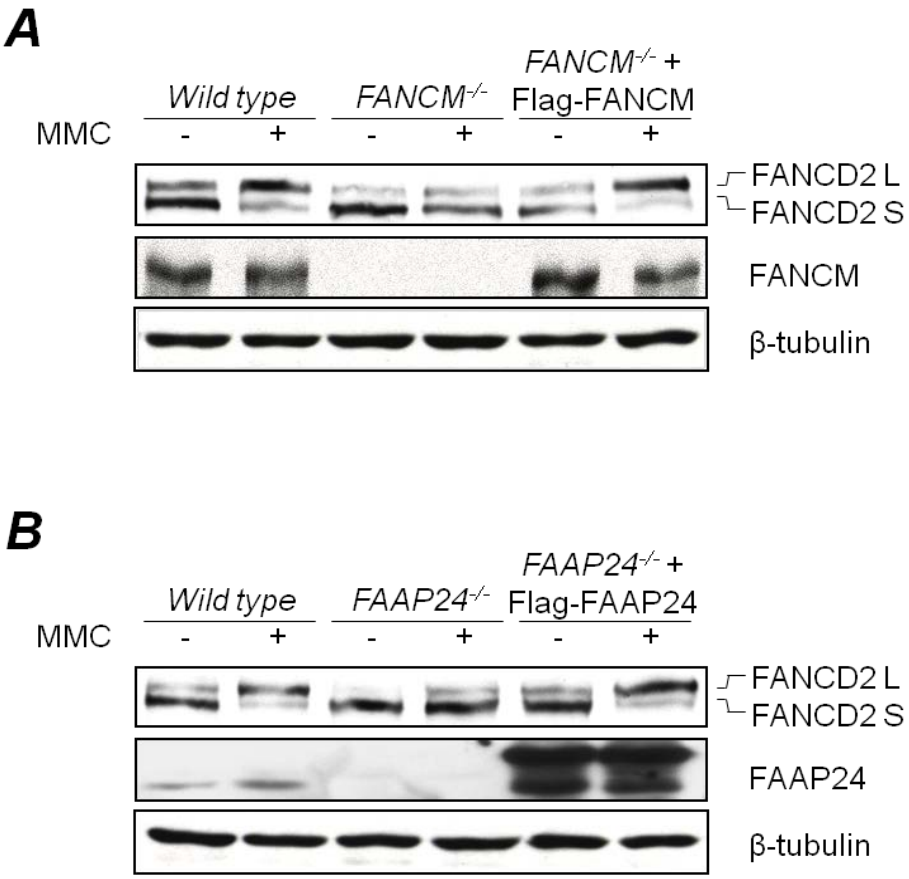
www.manaraa.com



www.manaraa.com

Fig. 3.4 Re-expression of FANCM and FAAP24 restores FANCD2 monoubiquitination in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ knockout cell lines.
(***A***) Immunoblot detecting MMC-induced monoubiquitination of FANCD2 in *FANCM*$^{-/-}$ cells complemented with wild-type Flag-FANCM. L and S represent monoubiquitinated and native forms of FANCD2, respectively.
(***B***) Immunoblot detecting MMC-induced monoubiquitination of FANCD2 in *FAAP24*$^{-/-}$ cells complemented with wild-type Flag-FAAP24. L and S represent monoubiquitinated and native forms of FANCD2, respectively.

www.manaraa.com



www.manaraa.com

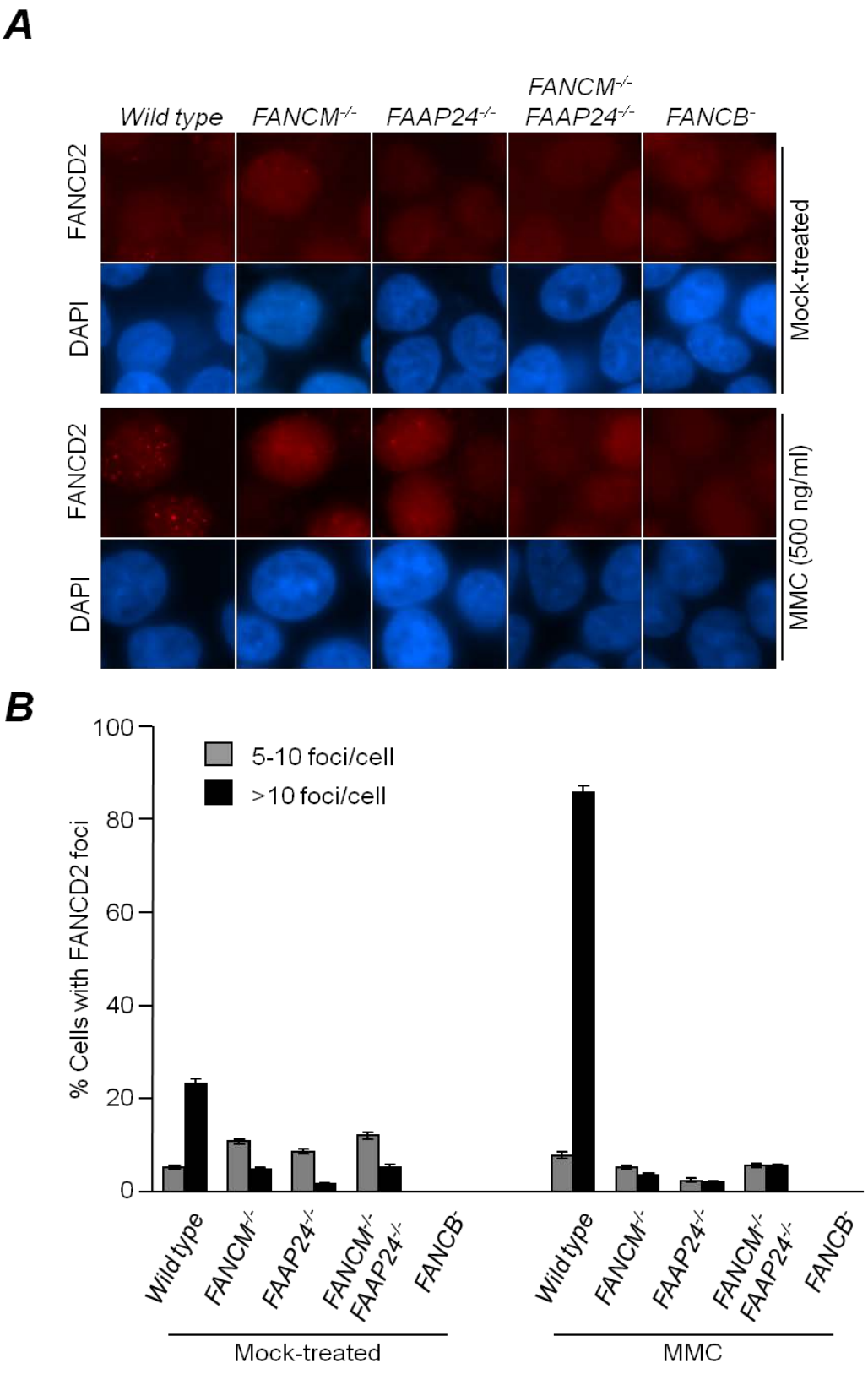
In addition, MMC-induced FANCD2 nuclear foci formation was remarkably diminished in *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$ and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ cells, and the levels of reduction in all three mutants were comparable (Fig 3.5). The defective FANCD2 foci formation in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells could be corrected by re-expressing the wild type proteins (Fig 3.6). These data further support that FANCM and FAAP24 promote FA pathway activation in a non-redundant manner.

Notably, in *FANCB*$^{-}$ knockout cells, FANCD2 monoubiquitination and nuclear foci formation were completely abolished (Fig 3.3B & Fig 3.5). This result suggests that unlike other FA proteins within the core complex, FANCM and FAAP24 are not indispensable for FA pathway activation, although they strongly promote this process.

www.manaraa.com

Fig. 3.5 FANCM and FAAP24 promote FANCD2 nuclear foci formation.
(***A***) Formation of MMC-induced (500 ng/ml) FANCD2 nuclear foci in cells with indicated genotypes.
(***B***) Quantification of FANCD2 nuclear foci formation in cells with indicated genotypes. Data represent three independent experiments and error bars depict standard deviation derived from 5 data sets.

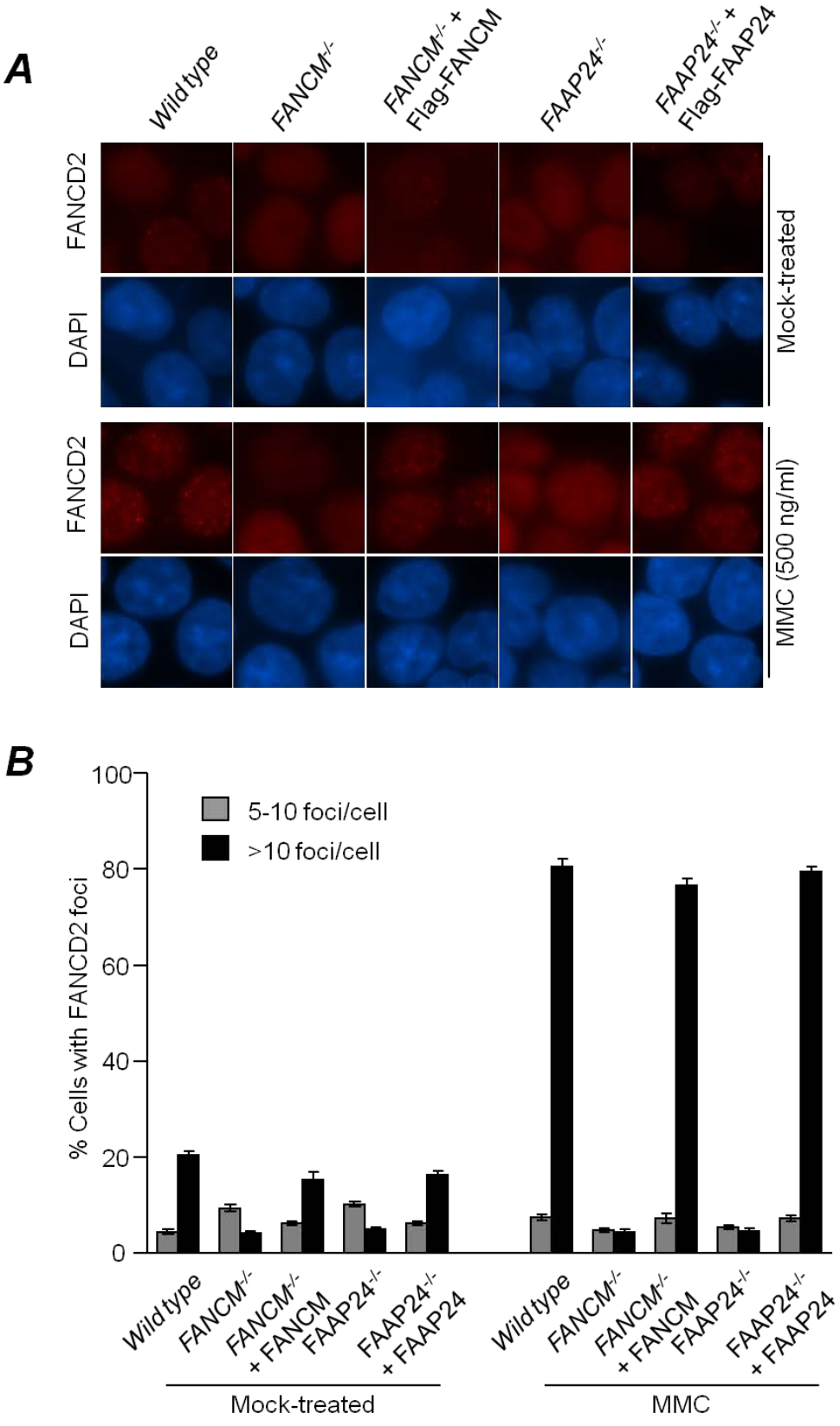
www.manaraa.com



www.manaraa.com

Fig. 3.6 Re-expression of FANCM and FAAP24 restores FANCD2 nuclear foci formation in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ knockout cell lines.
(***A***) Formation of MMC-induced (500 ng/ml) FANCD2 nuclear foci in *FANCM* and *FAAP24* knockout cells stably complemented with wild type proteins.
(***B***) Quantification of FANCD2 nuclear foci formation in *FANCM* and *FAAP24* knockout cells stably complemented with wild type proteins. Data represent three independent experiments and error bars depict standard deviation derived from 5 data sets.

www.manaraa.com



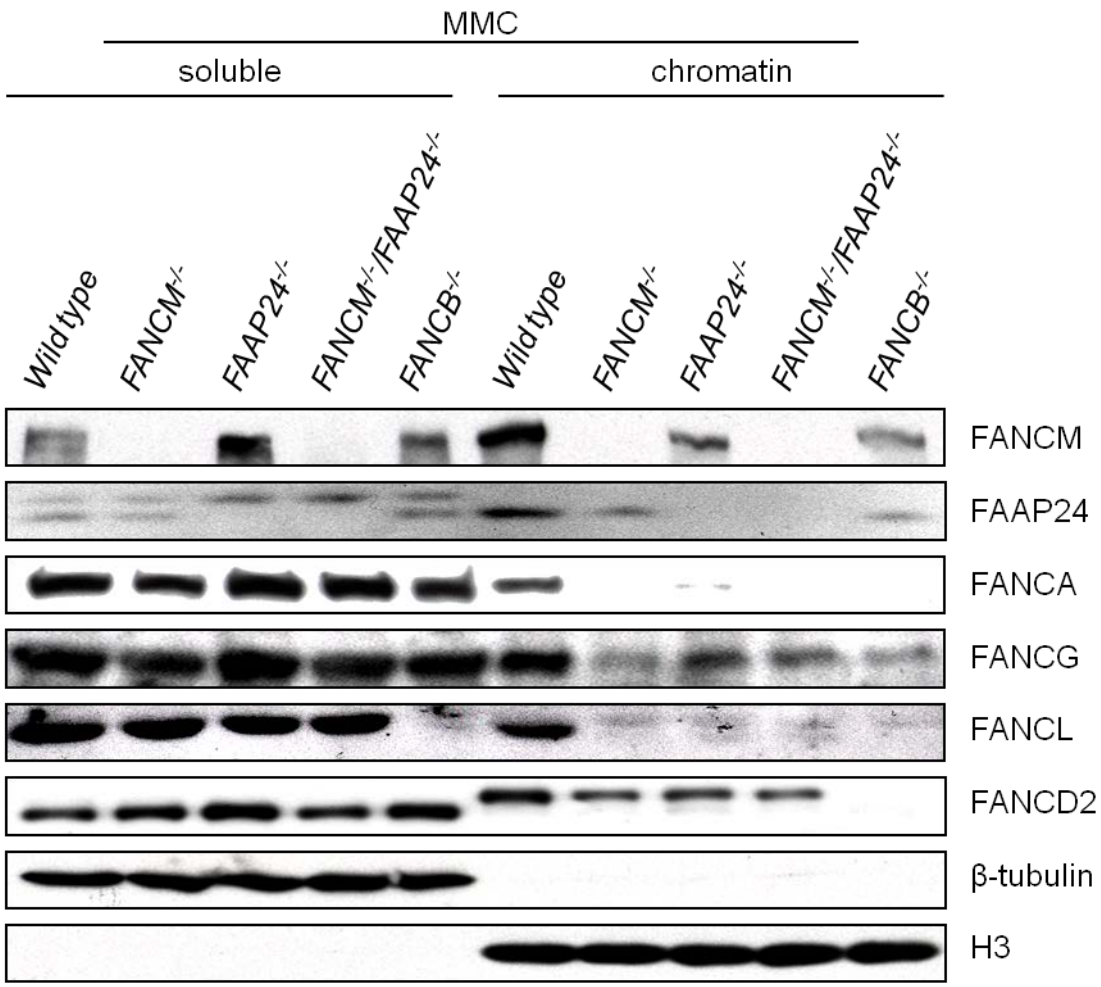
www.manaraa.com

To investigate how FANCM and FAAP24 promote FA pathway activation, I examined whether FANCM and FAAP24 facilitate FANCD2 monoubiquitination by recruiting the FA core E3 ligase complex. To this end, I examined the chromatin loading of FA core complex and FANCD2 in response to MMC. As is shown in Fig 3.7, the recruitment of FANCA, FANCG, and FANCL to chromatin was remarkably reduced in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells compared with that in wild type cells. The chromatin association of FA core complex proteins were identically reduced in the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ cells (Fig 3.7), indicating that FANCM and FAAP24 cooperatively promote the chromatin loading of FA core complex to facilitate FACND2 monoubiquitination and its chromatin localization. In addition, in the absence of FAAP24, a larger amount of FANCM proteins were retained in the soluble fraction, leading to decreased amount of chromatin-associated FANCM (Fig 3.7). Similarly, the chromatin association of FAAP24 was greatly reduced in *FANCM*$^{-/-}$ cells (Fig 3.7). These data suggest that FANCM and FAAP24 facilitate the chromatin association of each other, and they work in concert to promote the FA pathway activation.

www.manaraa.com

Fig. 3.7 FANCM and FAAP24 promote chromatin loading of FA core complex. Chromatin association of FANCM, FAAP24, FANCA, FANCG, FANCL and FANCD2 in response to MMC treatment (200 ng/ml, 6 hrs) was detected by Western blot.

www.manaraa.com



www.manaraa.com

FANCM and FAAP24 cooperatively suppress sister chromatid exchange

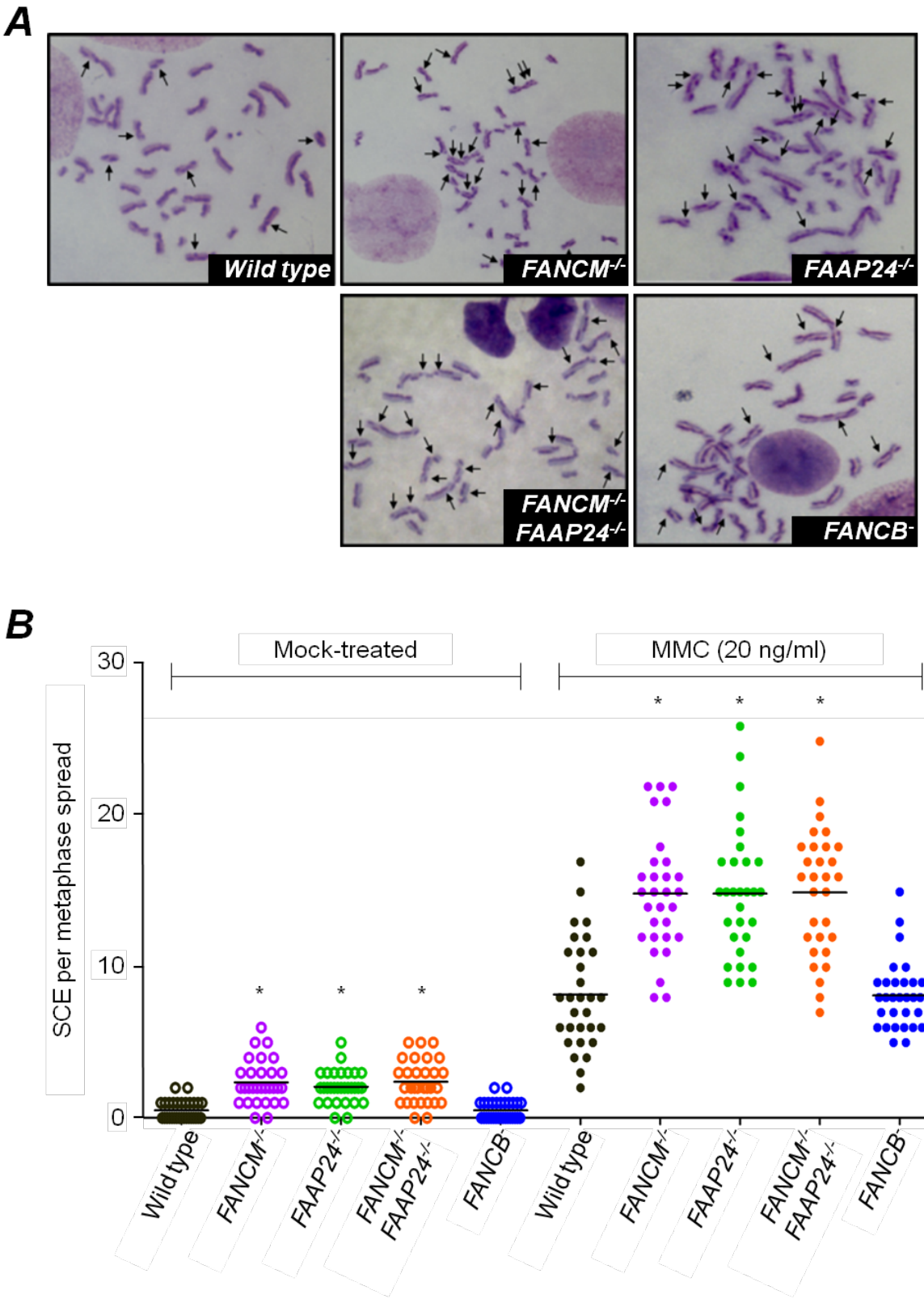
Increased levels of sister chromatid exchanges (SCEs) have been reported in chicken DT40 cells, mouse embryonic fibroblasts (MEFs) as well as human HEK293 cells that are deficient for FANCM (Bakker et al., 2009; Deans and West, 2009; Rosado et al., 2009). However, it is unclear whether FAAP24 also works to suppress SCE. To address this question, I examined the somatic knockout cells lacking FANCM and/or FAAP24 for SCEs. As shown in Fig 3.8, both the basal and MMC-induced SCE levels were significantly higher in *FANCM*$^{-/-}$ cells compared with those in wild type cells, consistent with previous studies (Bakker et al., 2009; Deans and West, 2009; Rosado et al., 2009). In addition, *FAAP24*$^{-/-}$ cells also displayed markedly increased SCE levels (Fig 3.8), suggesting FAAP24 is also required to suppress SCE formation. Notably, *FANCM*$^{-/-}$*/FAAP24*$^{-/-}$ double knockout cells did not produce any additional SCE phenotype than each single mutant alone, strongly indicating that FANCM and FAAP24 work in a same pathway to suppress SCE.

Interestingly, cells deficient for FANCB did not have increased SCE levels compared with wild type cells (Fig 3.8), consistent with data derived from MEFs lacking FANCA (Bakker et al., 2009), suggesting that the function of FANCM-FAAP24 in suppressing SCE formation is likely independent of the FA core complex.

www.manaraa.com

Fig. 3.8 FANCM and FAAP24 suppress sister chromatid exchange.
(A) Representative metaphase chromosome spreads showing MMC-induced SCEs (arrows) in *FANCM* and *FAAP24* knockout cells.
(B) Quantification of basal level (mock-treated) and MMC-induced (20 ng/ml for 18 hrs) SCEs in *FANCM* and *FAAP24* knockout cells. 30 metaphases were scored for each sample and bars represent averages. The asterisks (*) denote $P < 0.01$ vs. wild type.

www.manaraa.com



www.manaraa.com

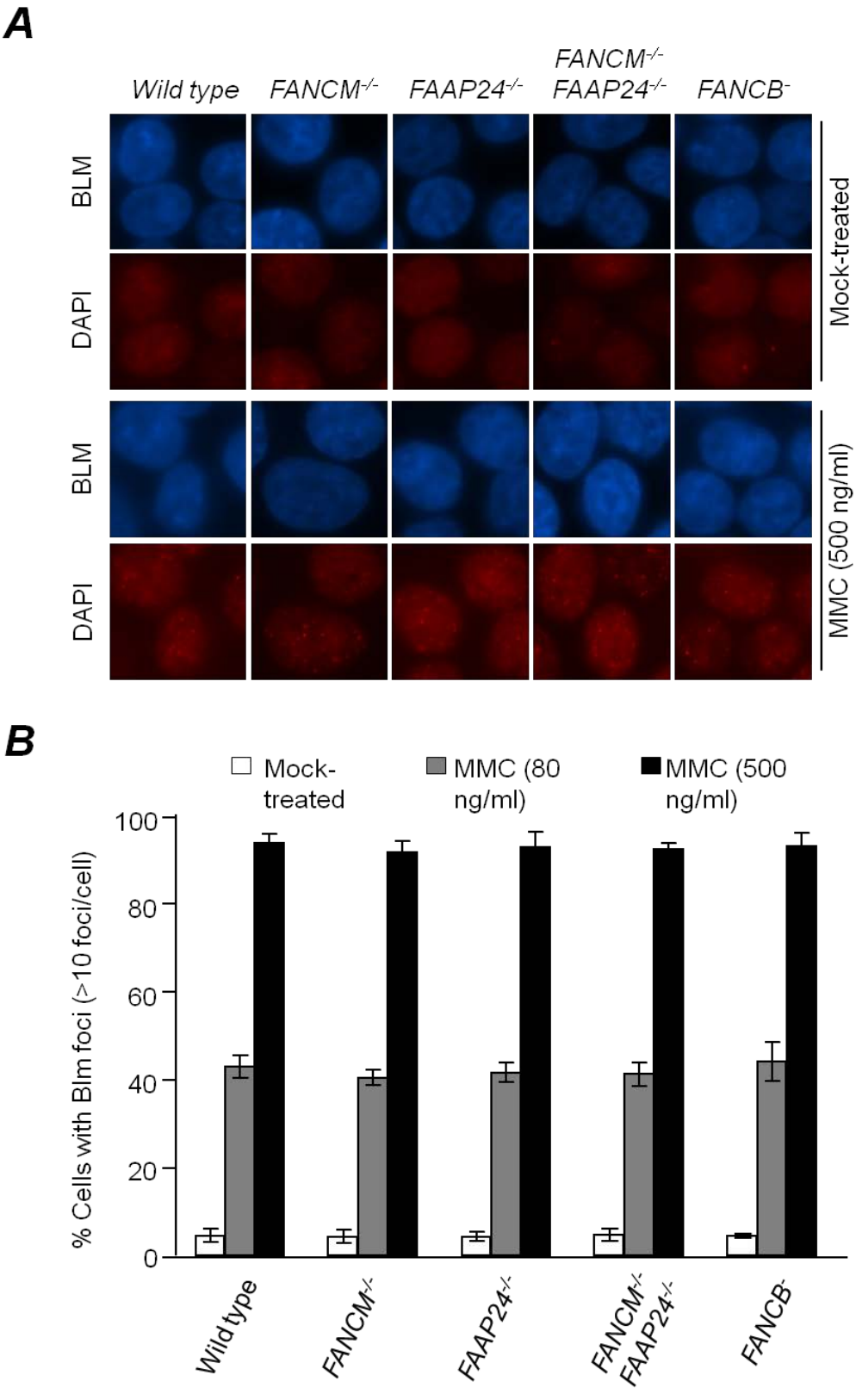
Recent studies suggested that elevated SCE levels in FANCM deficient cells is likely due to the loss of FANCM's interaction with and recruitment of the BLM helicase (Deans and West, 2009; Hoadley et al., 2012). I therefore tested whether the increased SCE levels in cell lacking FANCM and/or FAAP24 were due to compromised recruitment of the Bloom's complex. To this end, I examined BLM foci formation in wild type and the knockout cell lines. Surprisingly, the basal level as well as MMC-induced BLM foci formation was not visibly affected in the absence of FANCM, FAAP24, or both (Fig 3.9). This indicates that there exists a FANCM- and FAAP24-independent mechanism for BLM recruitment. It also suggests that FANCM and FAAP24 may have BLM-independent functions in suppressing SCE formation.

The increased SCE levels observed in FANCM and FAAP24 deficient cell lines indicate a possibility that FANCM and FAAP24 act to suppress crossovers during recombination-mediated repair. Consistently, I found that *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells displayed significantly increased formation of tetra-radial chromosome structures when exposed to MMC (Fig 3.10). The *FANCM*$^{-/-}$*/FAAP24*$^{-/-}$ cells had comparable amounts of tetra-radial chromosomes compared with *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells, further suggesting that the suppression of crossover formation is cooperatively achieved by FANCM and FAAP24, most likely through function of the heterodimer formed between the two proteins.

www.manaraa.com

Fig. 3.9 Loss of FANCM and FAAP24 does not affect MMC-induced BLM nuclear foci formation.
(A) Immunofluorescent staining of BLM foci in *FANCM* and *FAAP24* knockout cells treated or mock treated with MMC (80 ng/ml or 500 ng/ml for 24 hr).
(B) Quantification of BLM nuclear foci in mock-treated and MMC-treated cells with indicated genotypes. Data are representative of two independent experiments, and error bars represent standard deviation of 5 datum points.

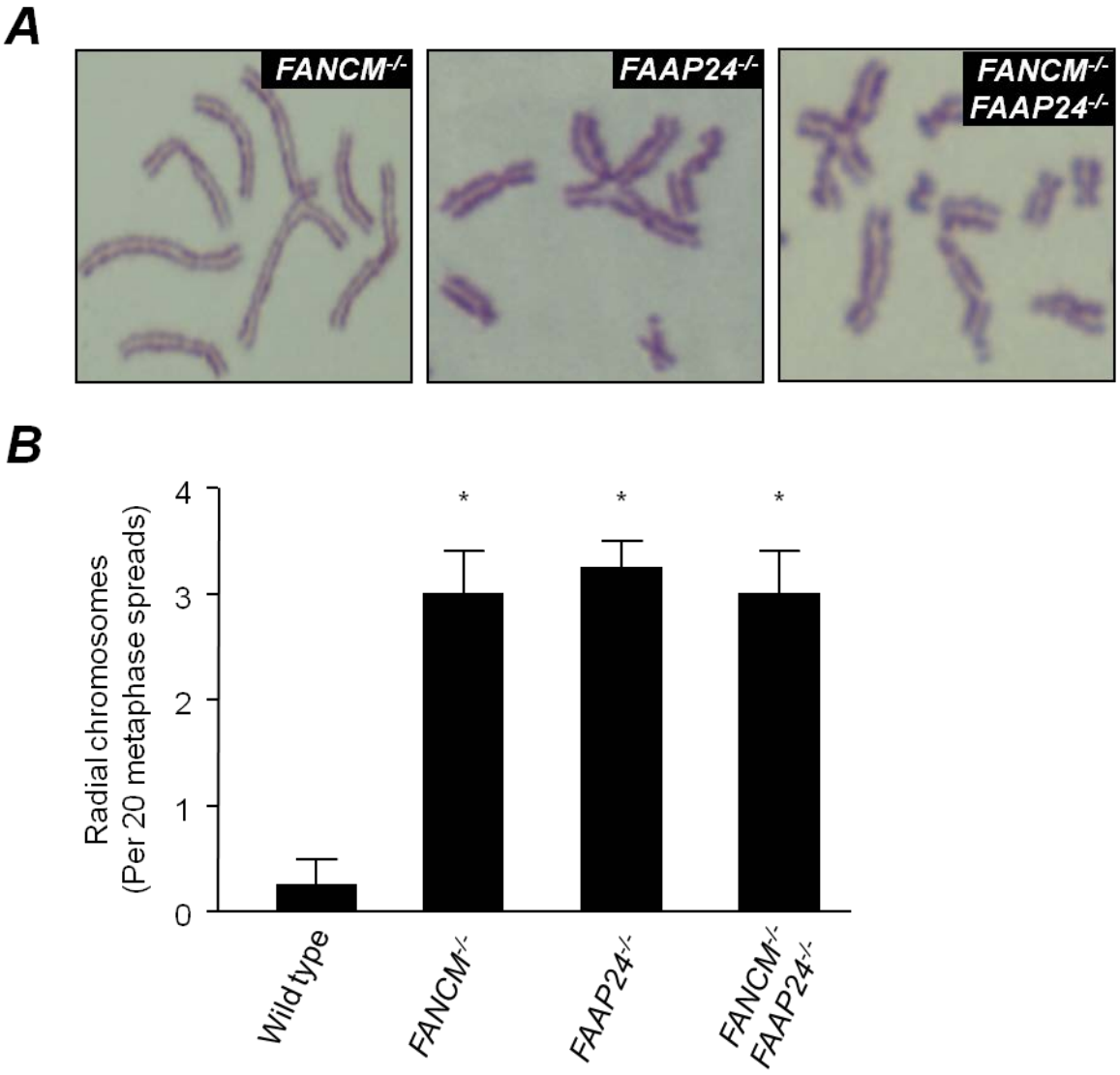
www.manaraa.com



www.manaraa.com

Fig. 3.10 FANCM and FAAP24 suppress radial chromosome formation.
(***A***) Representative picture of tetra-radial chromosome formation in MMC-treated (20 ng/ml for 18 hr) cells with indicated genotypes.
(***B***) Quantification of radial chromosome formation in cells with indicated genotypes. 80 metaphase spreads were scored for each genotype. Error bars represent standard deviation of 4 datum points. The asterisks (*) denote $P < 0.01$ vs. wild type.

www.manaraa.com



www.manaraa.com

Discussion

FANCM and FAAP24 have been assumed to function together as a heterodimer due to their structural resemblance to the heterodimeric endonucleases XPF/ERCC1 and Mus81/Eme1, their co-purification, and their coordinated biochemical activities. In the present study however, I found that the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout cell line was much more sensitive to ICL damage than either *FANCM*$^{-/-}$ or *FAAP24*$^{-/-}$ single knockout cell lines (Fig 3.1 & Fig 3.2), suggesting that FANCM and FAAP24 are not fully epistatic in mediating ICL resistance.

This result may not be surprising for a number of reasons. First, Western blot showed that FANCM and FAAP24 are not obligated heterodimers (Fig 2.5C & Fig 2.6B), suggesting that each protein may have important cellular functions independent of the other. Second, there exist protein complexes which contain only one of the FANCM and FAAP24 proteins. For example, FANCM can form either homodimers or FANCM-MHF complex independent of FAAP24 (Xue et al., 2008; Yan et al., 2010), while a significant fraction of FAAP24 was observed not to co-migrate with FANCM in gel filtration experiments (Collis et al., 2008). Third, FANCM and FAAP24 can each independently bind DNA substrates *in vitro*, and they have preferences to different substrates such that FANCM binds branched DNA structures while FAAP24 has high affinity towards ssDNA (Ciccia et al., 2007; Gari et al., 2008b). Fourth, the different architectures of these two proteins may also confer diverged biological functions. FANCM possesses an ATP-dependent DNA translocase activity, which has been shown to be independent of FAAP24 *in vitro* (Gari et al., 2008b). In addition, FANCM contains domains that mediate additional physical and functional protein interactions not requiring FAAP24 (Deans and West, 2009). On the other hand, FAAP24 has a more

www.manaraa.com

conserved $(HhH)_2$ domain, especially the first HhH motif which contains a canonical glycine-hydrophobic residue-glycine (GhG) hairpin critical for DNA binding (Ciccia et al., 2007; Ciccia et al., 2008).

Nevertheless, the fact that the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double mutant gives rise to significantly increased damage sensitivity compared to each single mutant alone prompted me to identify both epistatic and non-epistatic functions of FANCM and FAAP24 in response to ICL damage.

The epistatic function between FANCM and FAAP24 are reflected by the comparable phenotypes of the *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single mutants and the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double mutant. I found that FANCD2 monoubiquitination and nuclear foci formation in response to ICL damage were affected to the same extent in the single and double mutants. I also showed that MMC-induced tetraradial chromosome formation and SCEs occur with equal frequencies among all three mutants. These data suggest that FANCM and FAAP24 function cooperatively to activate the FA pathway and to suppress crossover recombination and SCE formation. The non-epistatic functions of FANCM and FAAP24 are described in following chapters.

It has been shown that the recruitment of FA core complex upon DNA damage is mediated by the MM1 motif in FANCM (Deans and West, 2009). However, my results show that FAAP24 deletion also impairs damage-induced chromatin association of the core complex, identically to the FANCM mutant (Fig 3.7), suggesting that recruitment of FA core complex require both FANCM and FAAP24. Consistently, the *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells showed comparably defective FANCD2 monoubiquitination and nuclear foci formation. Furthermore, *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double mutant showed no further defects in FANCD2

www.manaraa.com

monoubiquitination and nuclear foci formation, suggesting that FANCM and FAAP24 work together to promote the activation of FA pathway. This is most likely attributed to the cooperative binding of stalled DNA replication fork by FANCM and FAAP24. The preferred substrates for FANCM and FAAP24 are fork structure and ssDNA, respectively. On the other hand, the FANCM associated MHF complex prefers dsDNA substrates. Therefore, it is plausible that these proteins bind to different parts of a stalled replication fork, such that FANCM binds the branch point, and MHF complex and FAAP24 binds dsDNA and ssDNA regions, respectively (Yan et al., 2010). Cooperatively, they protect the replication fork and recruit the FA core complex.

Consistent with previous studies using various model systems (Bakker et al., 2009; Deans and West, 2009; Rosado et al., 2009), I found that cells lacking FANCM showed increased levels of SCE (Fig 3.8). Interestingly, I demonstrated that FAAP24 was also required to suppress SCE formation in response to ICL damage, similar to a previous study showing MHF1 functions with FANCM to suppress SCE (Yan et al., 2010). These results suggest that FANCM and its binding partners function coordinately in SCE repression. On the other hand, while it has been reported that the coordinated recruitment of FA core complex and Bloom's complex by FANCM is important for suppression of SCE formation (Deans and West, 2009), my data indicate that the function of FANCM and FAAP24 in SCE suppression is likely independent of FA core complex or BLM (Fig 3.8 & Fig 3.9). Possibly, there exists a FANCM-independent mechanism to support BLM recruitment to ICL sites, where damage-induced homologous recombination occurs between sister chromatids.

Mechanistically, the role of FANCM and FAAP24 in limiting crossover and suppressing SCE formation in response to ICL damage may be attributed to the replication-

www.manaraa.com

fork remodeling activities. FANCM has the ability to convert a replication-fork structure to a four-way junction *in vitro*, causing a replication fork regression (Gari et al., 2008a). Therefore, it is plausible that FANCM mediates fork regression to generate a movable four-way junction in response to replication stress, and facilitates the re-start of blocked forks through a mechanism that avoids crossover recombination and SCE. Since such functions are associated with intrinsic enzymatic activity of FANCM, it is likely that FANCM can suppress SCE formation independent of FA core complex and Bloom's complex. Alternatively, FANCM may function to limit crossovers in homologous recombination. In fact, FANCM could promote branch migration of Holliday junction structures *in vitro* (Gari et al., 2008b; Xue et al., 2008). In addition, like its yeast orthologs Fml1 (*S. Pombe*) and Mph1 (*S. cerevisiae*), recombinant human FANCM protein is able to dissociate D-loop structures *in vitro* (Gari et al., 2008a; Prakash et al., 2009; Sun et al., 2008). Furthermore, FANCM orthologs in fission yeast and plants are important for limiting crossovers during meiosis (Crismani et al., 2012; Knoll et al., 2012; Lorenz et al., 2012). It is therefore possible that human FANCM plays an important role in limiting crossover and suppressing SCE formation via yet to defined functions in homologous recombination.

www.manaraa.com

CHAPTER IV

FAAP24 Promotes ATR-mediated Checkpoint Activation in Response to Interstrand Crosslink Damage

Introduction

The DNA interacting activities set FANCM and FAAP24 apart from other FA core complex components. The coordinated DNA binding activity of the FANCM-FAAP24 heterodimer and the translocase activity of FANCM likely allow them to act as DNA damage sensing components. Indeed, FANCM and FAAP24 have been shown to be constantly associated with chromatin and are believed to be important for the loading of FA core complex in response to DNA damage (Kim et al., 2008). While the activation of FA pathway in response to ICL damage is important for the crosslinking damage repair, this pathway has to coordinate with other damage-responsive pathways to ensure ICL repair carried out in an orderly manner. Presumably functioning in the initiation of FA pathway activation, FANCM and FAAP24 are plausible candidates that convey signals to DNA damage checkpoint signaling pathways.

Whether the potential function of FANCM and FAAP24 as DNA damage sensors is specifically linked to ICL damage is uncertain. However, given their FA core complex-independent DNA binding and translocating activities, it is likely they also function in response to other types of DNA damage. In fact, FANCM mutant has been shown to be sensitive to UV light and replication stress inducers (Singh et al., 2009). Therefore, it will be interesting to dissect whether and how FANCM and FAAP24 function in cellular response to DNA damage other than ICL, such as UV and HU.

www.manaraa.com

A number of studies have addressed the potential function of FANCM and FAAP24 in ATR-mediated checkpoint signaling (Collis et al., 2008; Huang et al., 2010; Luke-Glaser et al., 2009; Schwab et al., 2010). Using siRNA based studies, Collis *et al* and others have shown that FANCM and FAAP24 are required for optimal activation of ATR-mediated checkpoint signaling in response to UV and HU (Collis et al., 2008; Luke-Glaser et al., 2009; Schwab et al., 2010). A more recent study, however, demonstrated that the function and FANCM and FAAP24 in checkpoint activation seems to be specifically associated with response to ICL damage (Huang et al., 2010).

In this Chapter, I describe genetic studies that investigate the function of FANCM and FAAP24 in DNA damage checkpoint signaling, particularly the ATR-mediated checkpoint pathway. I provide genetic evidence demonstrating FAAP24, but not FANCM, is required for activation of the ATR-dependent checkpoint signaling in response to ICL damage, and neither protein seems to be essential for ATR-mediated checkpoint activation in response to UV and HU. Discussions addressing discrepancies among studies will be presented, which help to better understand the FANCM and FAAP24 function in ATR-dependent checkpoint signaling in response to different types of DNA damage.

Materials and Methods

Antibodies

Sources of antibodies (that were not described in previous chapters) are as follows: anti-Chk1 (sc-8408, Santa Cruz); anti-phospho-Chk1 Ser317 (2344, Cell Signaling); anti-phospho-Chk1 Ser345 (2348, Cell Signaling); anti-phospho-histone H3 Ser10 (9706, Cell Signaling); anti-Myc (sc-40, Santa Cruz); and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse.

www.manaraa.com

Co-immunoprecipitation

Constructs expressing Flag-tagged FANCM and Myc-tagged FAAP24 (wild type or V198A mutant) were co-transfected into HEK293T cells. 18 hours later, cells were lystated in NETN buffer supplemented with Benzonase nuclease for 1 hour at 4 °C. After centrifugation, supernatants were collected and subjected to immunoprecipitation with anti-Myc antibodies for overnight at 4 °C. Proteins bound with the anti-Myc antibodies were then recovered by incubating the lysates with protein G beads, and detected by western blot.

Cell cycle checkpoint analysis

G2/M checkpoint analysis was performed as described earlier (Wang et al., 2003). Briefly, cells were treated with MMC (1000 ng/ml for 6 or 16 hrs) or IR (6 Gy for 90 min or 10 Gy for 2 hrs), harvested, and fixed with 70% ethanol at 20 °C overnight. Fixed cells were permeabilized with 0.25% Triton X-100/ PBS buffer on ice for 15 min and then sequentially incubated with anti-phospho-histone H3 antibody (1:30 in 1% BSA/PBS) at room temperature for 3 h and FITC-conjugated secondary antibody (1:50 in 1% BSA/PBS) in the dark at room temperature for 30 min. Cells were finally stained with propidium iodide (PI) buffer (0.2 mg/ml RNase A and 5 μg/ml PI in PBS) in the dark at room temperature for 30 min, and percentage of cells with positive phospho-histone H3 staining was determined by flow cytometry analysis.

Results

FANAM and FAAP24 are not required for ATR-mediated checkpoint activation in response to HU or UV

Unlike other FA proteins among the FA core complex, FANCM and FAAP24 both possess DNA-interacting domains. The associated DNA interacting activities might be

www.manaraa.com

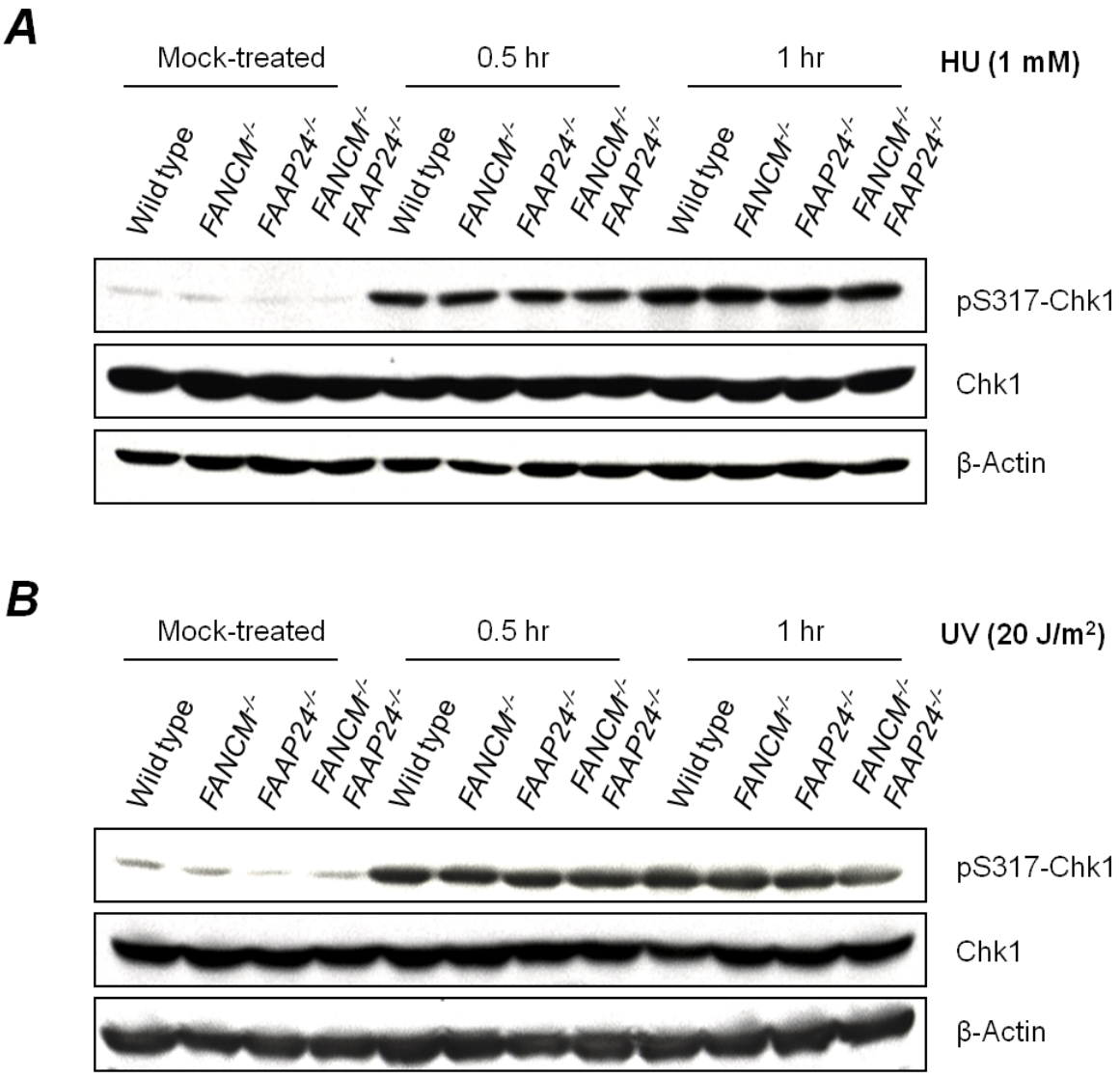
important for FANCM and FAAP24 to function independently of the FA core complex in DNA damage response pathways, therefore connecting the FA pathway to DNA damage response signaling network. Particularly, the potential functions of FANCM and FAAP24 in ATR-mediated checkpoint and G2/M checkpoint activation have been explored by a few studies (Collis et al., 2008; Huang et al., 2010; Luke-Glaser et al., 2009; Schwab et al., 2010). However, the results reported were not fully consistent.

To determine whether FANCM and FAAP24 are important for the activation of ATR-mediated checkpoint, I exposed *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells to various DNA damage agents, and assessed ATR-mediated checkpoint activation by measuring Chk1 phosphorylation. As shown in Fig 4.1A, when exposed to hydroxyurea (HU), *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells did not display any visible defect in Chk1 phosphorylation at Ser317. Similarly, when exposed to UV, cells lacking FANCM and/or FAAP24 did not show any apparent defect in activating the ATR/Chk1 signaling (Fig 4.1B). These experiments were repeated numerous times using different doses of HU and UV, with Chk1 phosphorylation examined at multiple time points. In all experimental conditions tested, lack of FANCM and FAAP24 did not result in visible effects on ATR-dependent checkpoint activation, both in terms of the initial onset and dose/time-dependent accumulation of Chk1 phosphorylation (data not shown).

www.manaraa.com

Fig. 4.1 FANCM and FAAP24 are not required for ATR-mediated checkpoint activation in response to HU or UV.
(***A***) Ser317 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed to HU (1 mM) and harvested at indicated time points. Chk1 and β-Actin serve as loading controls.
(***B***) Ser317 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed to UV (20 J/m^2) and harvested at indicated time points. Chk1 and β-Actin serve as loading controls.

www.manaraa.com



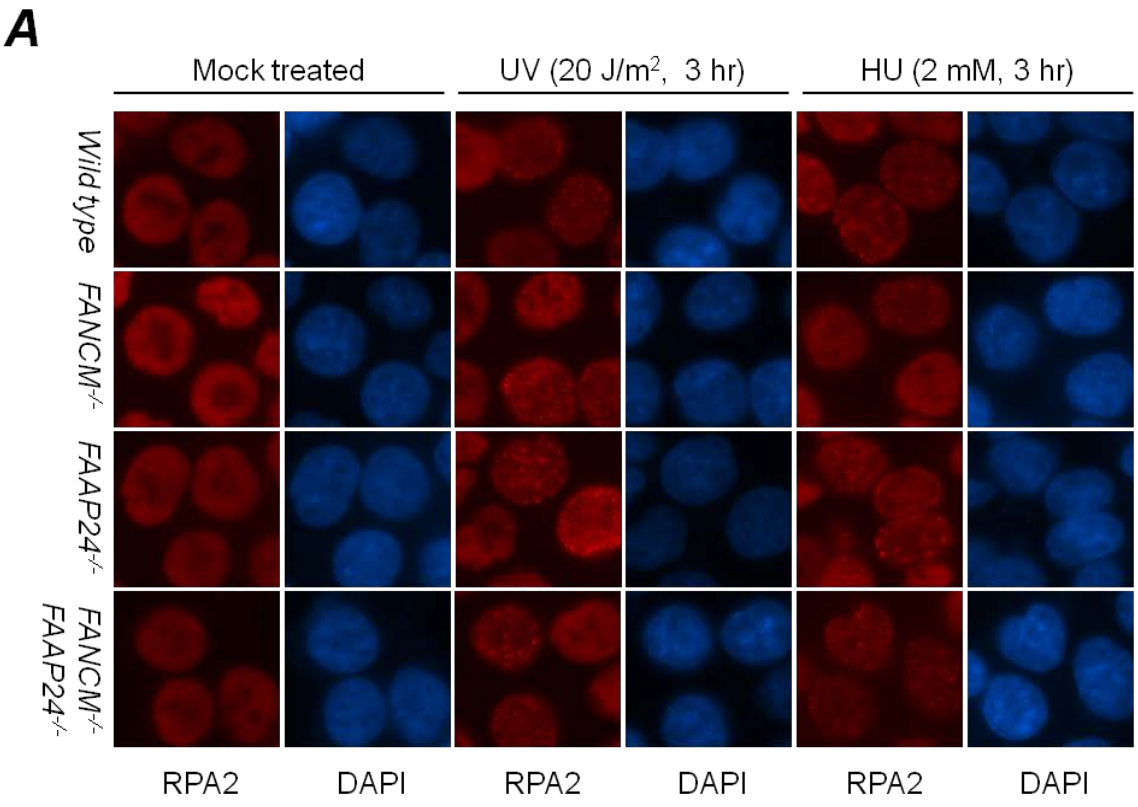
www.manaraa.com

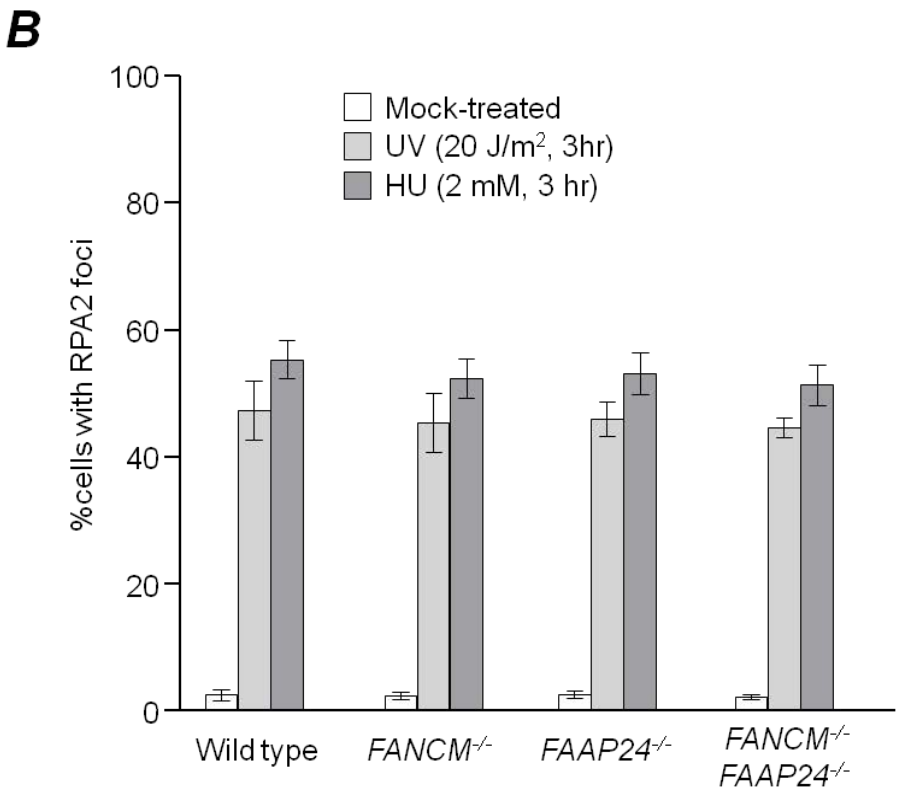
When cells are treated with HU or UV, the replication stress will lead to uncoupling of the replicative helicase and polymerase. Unwinding of DNA by the helicase ahead of the stalled replication fork will efficiently generate a long stretch of ssDNA, which will be coated rapidly by ssDNA binding protein RPA. RPA will recruit the ATR binding partner ATRIP, and initiate the ATR/Chk1 signaling, thereby activating the ATR-mediated checkpoint. I examined HU and UV induced RPA foci formation in *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells, and found that lack of FANCM and/or FAAP24 did not result in reduced RPA foci assembly (Fig 4.2), consistent with the intact checkpoint activation. Collectively, these results suggest that FANCM and FAAP24 are dispensable for ATR-mediated checkpoint activation in response to HU and UV.

www.manaraa.com

Fig. 4.2 FANCM and FAAP24 are not required for RPA foci formation in response to HU or UV.
(***A***) Immunofluorescent staining of RPA2 foci in FANCM and FAAP24 mutant cells treated with UV or HU.
(B) Quantification of RPA2 foci in UV or HU treated cells with indicated genotypes. Data are representative of two independent experiments, and error bars represent standard deviation of 5 datum points.

www.manaraa.com





www.manaraa.com

FAAP24 promotes ATR-mediated checkpoint activation in response to MMC

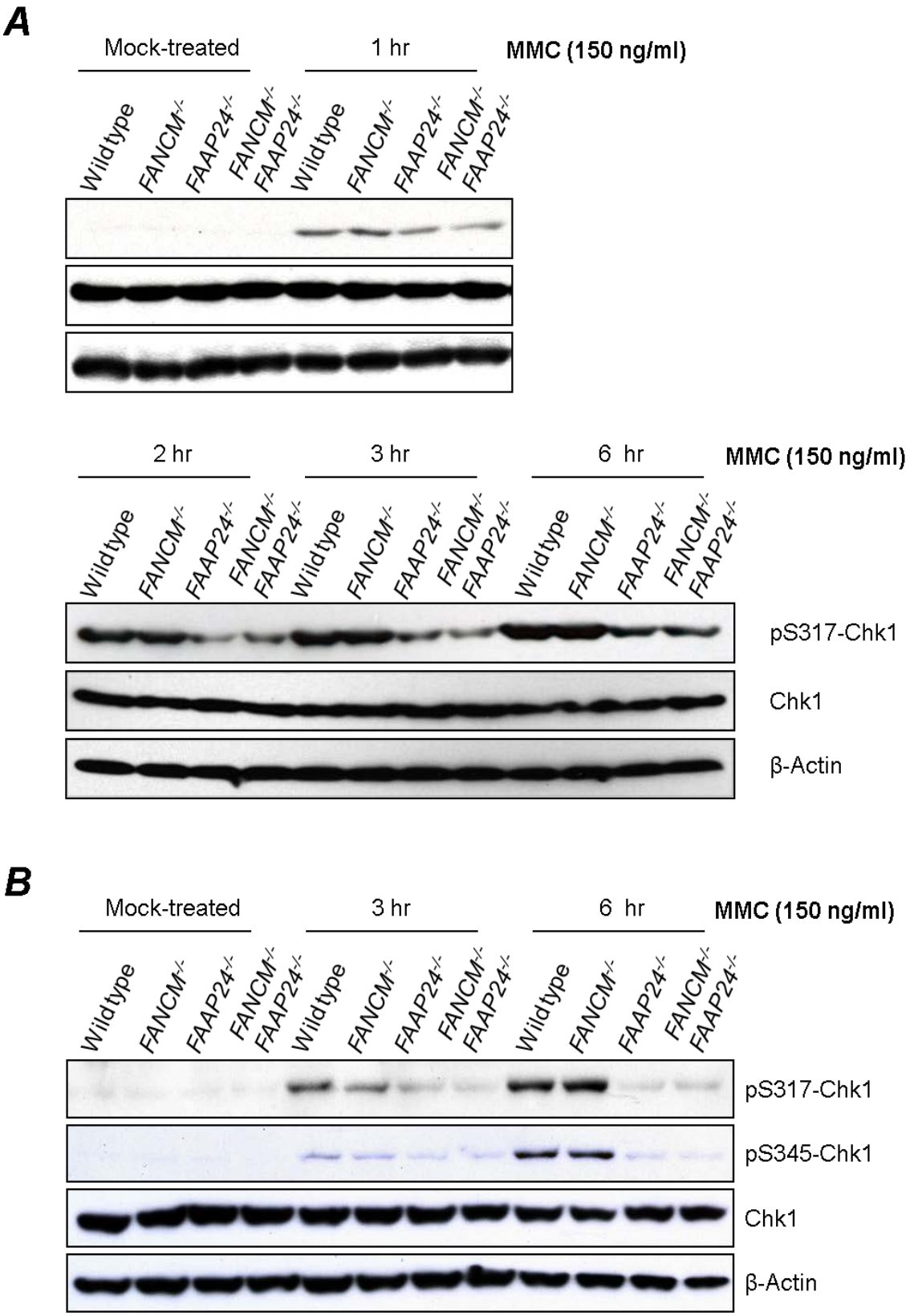
In contrast to the HU and UV treatment, when cells are exposed to interstrand crosslinking agents, both the replicative helicase and polymerase will be stalled. As a result, the generation of ssDNA will be less efficient. How ATR-mediated checkpoint gets activated in this scenario is not well understood.

To examine whether FANCM and FAAP24 are required for ATR-mediated checkpoint activation in response to ICL damage, I exposed *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells to MMC, and examined Chk1 phosphorylation. As shown in Fig 4.3, FAAP24 but not FANCM deficiency, resulted in compromised activation of ATR-mediated checkpoint in response to MMC, as demonstrated by decreased phosphorylation of Chk1 at both Ser317 and Ser345. Collectively, these results suggest that FANCM is not essential for ATR-mediated checkpoint activation, and FAAP24 is specifically required for activating ATR-Chk1 signaling in response to ICL damage.

www.manaraa.com

Fig. 4.3 FAAP24 promotes ATR-mediated checkpoint activation in response to MMC.
(***A***) Ser317 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed to MMC (150 ng/ml) and harvested at indicated time points. Chk1 and β-Actin serve as loading controls.
(B) Ser317 and Ser345 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed exposed to MMC (150 ng/ml) and harvested at indicated time points. Chk1 and β-Actin serve as loading controls.

www.manaraa.com



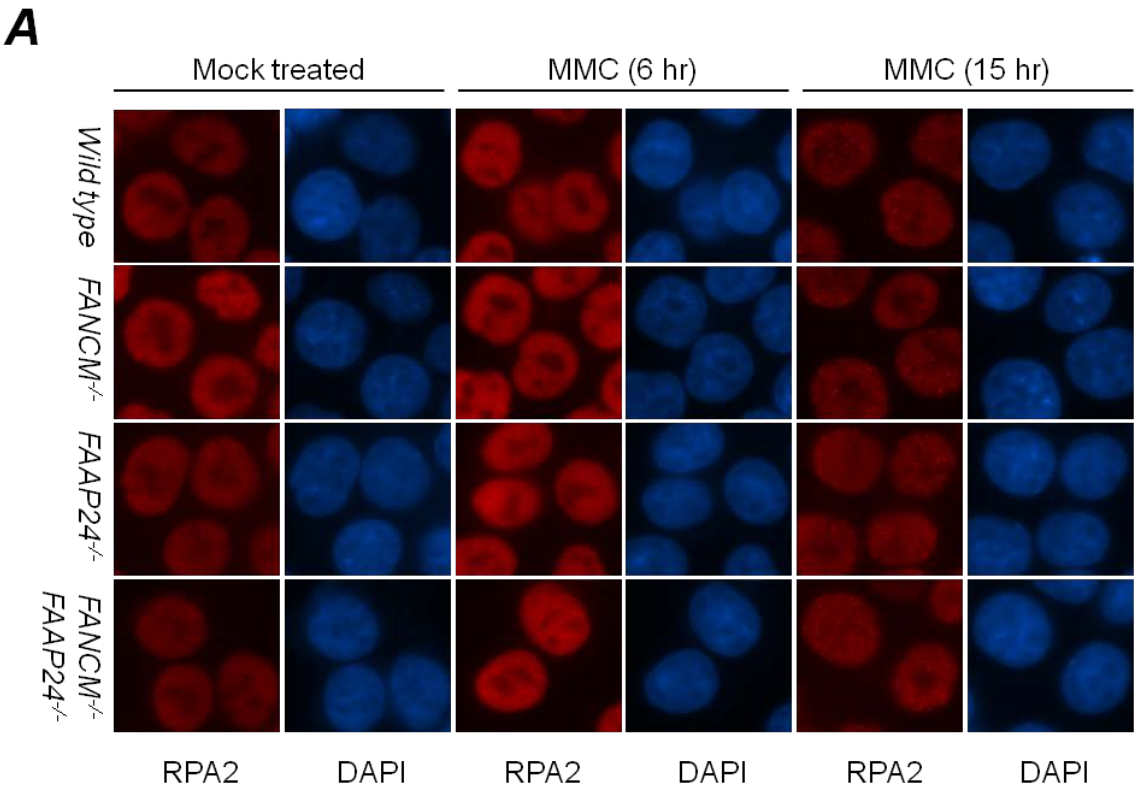
www.manaraa.com

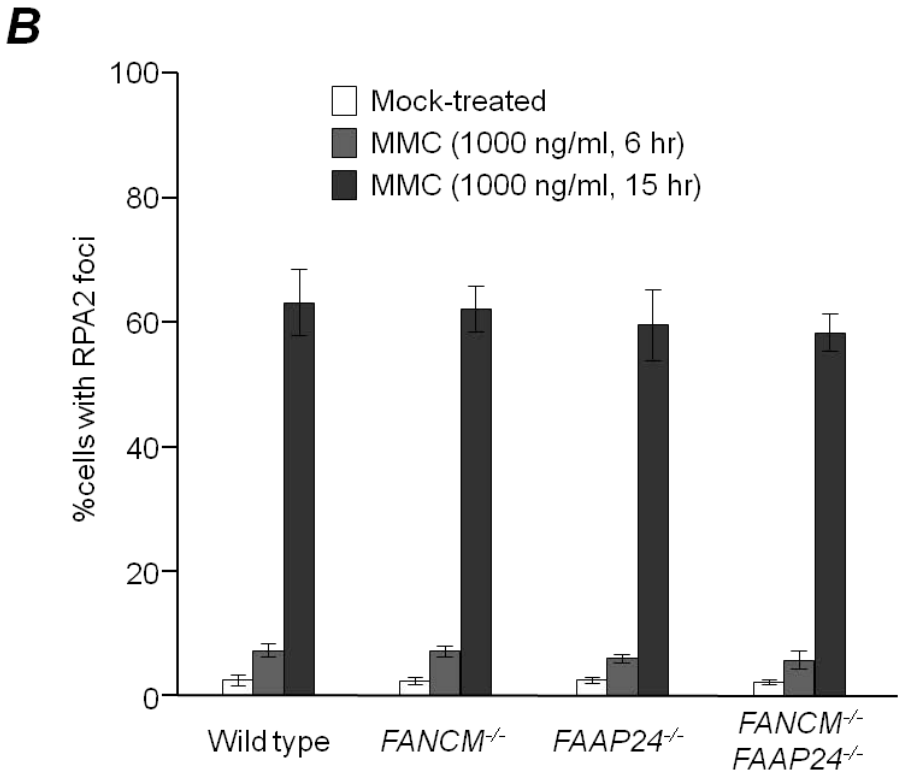
To determine whether the ATR-mediated checkpoint activation defect in cells lacking FAAP24 was due to deficient RPA recruitment, I examined MMC-induced RPA nuclear foci formation in the knockout cell lines. As shown in Fig 4.4, although RPA foci assembly in response to MMC treatment was inefficient in cells lacking FANCM and/or FAAP24, the *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ cells did not display reduced RPA foci formation compared with wild type cells. At later time points, RPA foci were efficiently assembled in both wild type cells and cells deficient for FANCM and/or FAAP24 (Fig 4.4), probably reflecting robust RPA recruitment in response to double strand break formation caused by prolonged replication fork stall. These data suggest that FAAP24 is important for optimal ATR-mediated checkpoint activation in response to ICL damage, when RPA recruitment was relatively inefficient. The function of FAAP24 in this context is mostly likely attributed to the intrinsic affinity of FAAP24 to ICL DNA (Huang et al., 2010), which might be important for recruitment of ATR co-activators and therefore activation of the ATR-mediated checkpoint signaling pathway.

www.manaraa.com

Fig. 4.4 FANCM and FAAP24 are not required for RPA foci formation in response to MMC. (***A***) Immunofluorescent staining of RPA2 foci in FANCM and FAAP24 mutant cells treated with MMC.
(B) Quantification of RPA2 foci in MMC treated cells with indicated genotypes. Data are representative of two independent experiments, and error bars represent standard deviation of 5 datum points.

www.manaraa.com





www.manaraa.com

The role of FAAP24 in ICL-induced ATR-mediated checkpoint activation is independent of its interaction with FANCM

In response to ICL damage, cells deficient for FAAP24 were defective in activating the ATR-mediated checkpoint. However, the ICL-induced ATR-mediated checkpoint activation was not affected by the loss of FANCM. These results suggest that FAAP24 is able to mediate checkpoint signal activation without forming the heterodimeric complex with FANCM. To test this notion, I sought to determine whether the interaction with FANCM is required for the function of FAAP24 in activating the ATR-mediated checkpoint signaling in response to DNA interstrand crosslinking agents.

FAAP24 interacts with FANCM through its $(HhH)_2$ domain (see Chapter VI for detail). I therefore disrupted a few amino acids within this domain in order to generate a FAAP24 mutant that is defective for FANCM binding. Specifically, I substituted the Val198, Val199 and Gly200 residues with alanines and generated the FAAP24-V198A mutant. The interaction of wild type and mutant FAAP24 with FANCM was examined by Co-IP experiment. As shown in Fig 4.5A, the FAAP24-V198A mutant failed to interact with FANCM.

To test whether the FAAP24-V198A mutant can correct the ATR-mediated checkpoint activation defect caused by FAAP24 loss, I stably expressed the mutant in the *FAAP24*$^{-/-}$ knockout cell line (Fig 4.5B). A wild type FAAP24 complemented *FAAP24*$^{-/-}$ cell line was also established as a control. When exposed to MMC, Chk1 phosphorylation at Ser317 in both wild type FAAP24 and V198A mutant complemented *FAAP24*$^{-/-}$ cells was comparable to that in wild type HCT-116 cells, suggesting that the FAAP24-V198A mutant was able to rescue the ATR-mediated checkpoint deficiency associated with lack of the

www.manaraa.com

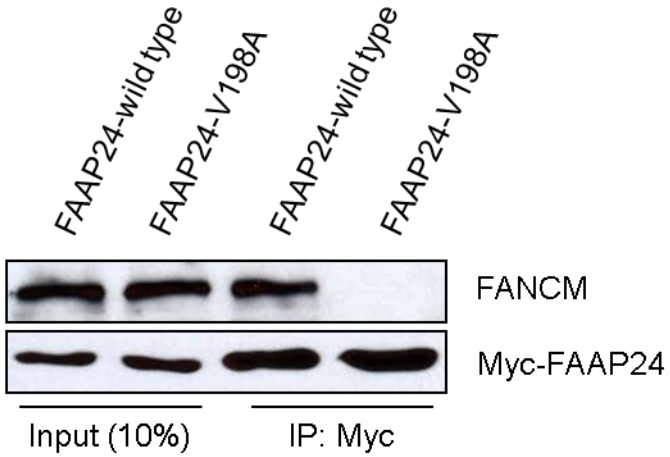
FAAP24 gene. This result supports that the checkpoint activation function of FAAP24 is independent of its interaction with FANCM.

www.manaraa.com

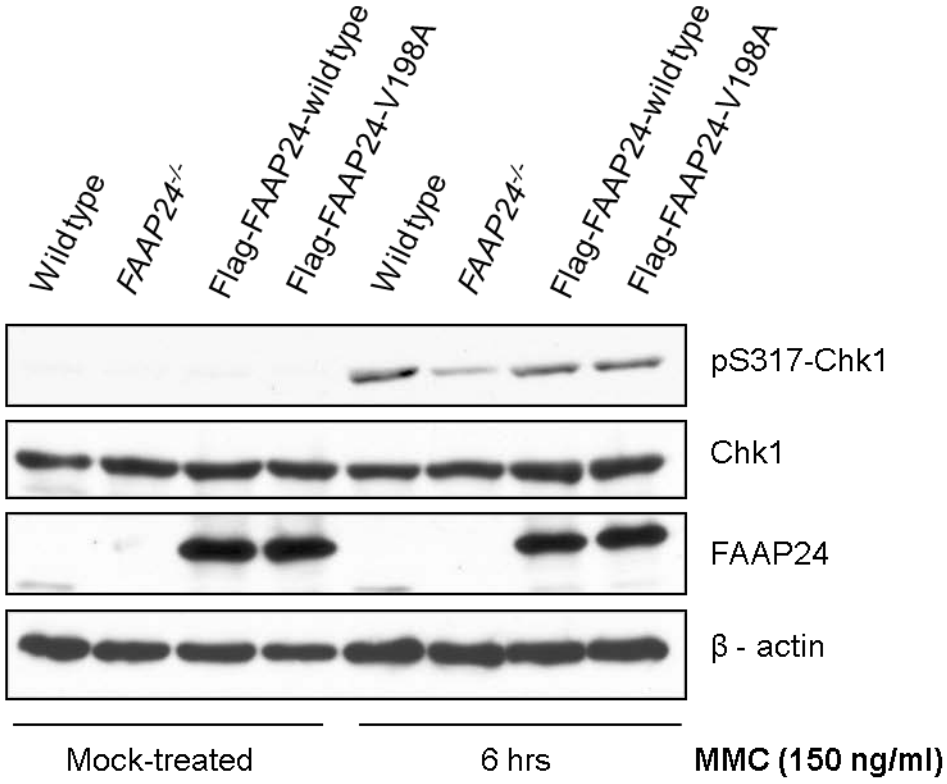
Fig. 4.5 The ATR-mediated checkpoint activation function of FAAP24 is independent of its interaction with FANCM.
(***A***) Co-immunoprecipitation of wild-type FAAP24 and the FAAP24-V198A mutant with FANCM.
(***B***) MMC-induced Chk1 activation in *FAAP24*$^{-/-}$ cells complemented with Flag-FAAP24-wild type or Flag-FAAP24-V198A mutant.

www.manaraa.com

A



B



www.manaraa.com

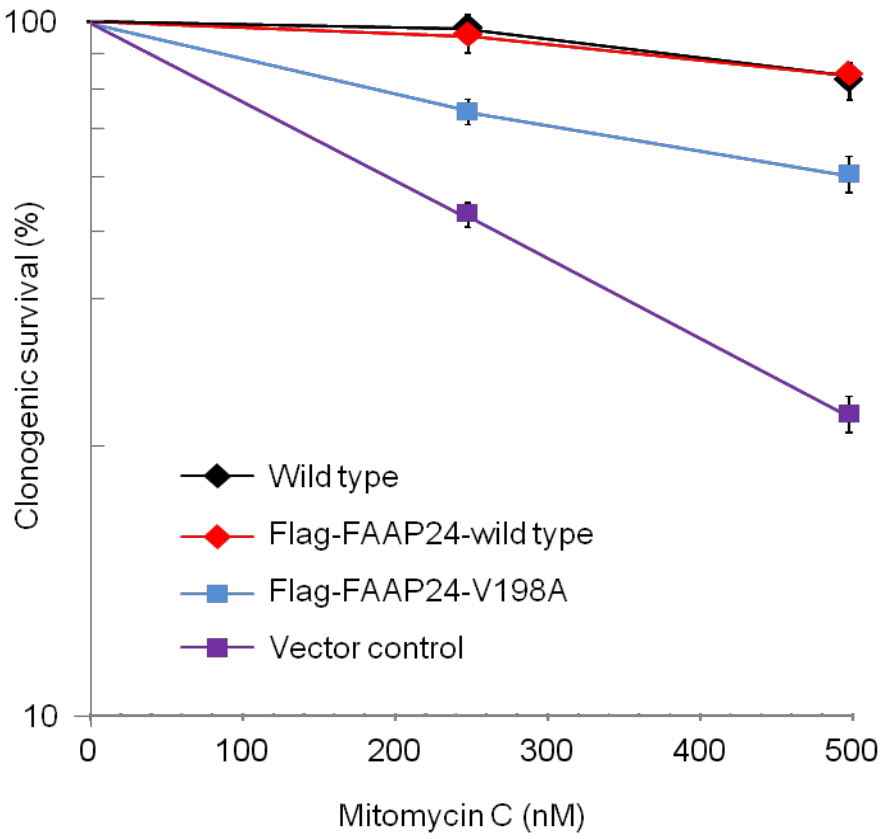
FANCM-associated FA pathway activation function and FANCM-independent ATR-mediated checkpoint activation function of FAAP24 are both required for resistance to ICL damage

While FAAP24 act together with FANCM to activate the FA pathway, its function in ATR-mediated checkpoint activation in response to ICL is independent of FANCM. This suggests that the function of FAAP24 in FA pathway activation and its function in ATR-mediated checkpoint activation can be segregated. To determine whether both functions are important for cellular resistance to ICL damage, I tested wild type FAAP24 and V198A mutant complemented *FAAP24*$^{-/-}$ cells for cellular survival in response to MMC damage. As shown in Fig 4.6, while wild type FAAP24 protein fully rescued the hypersensitivity of *FAAP24*$^{-/-}$ cells to MMC, the FAAP24-V198A mutant was only able to partially rescue the MMC sensitivity of *FAAP24*$^{-/-}$ knockout cells. These results suggest that both the FANCM-associated FA pathway activation function and the FANCM-independent ATR-mediated checkpoint activation function of FAAP24 are required for protecting cells against ICL damage.

www.manaraa.com

Fig. 4.6 Clonogenic survival of *FAAP24*$^{-/-}$ cells complemented with Flag-FAAP24-wild type or Flag-FAAP24-V198A. Error-bars represent standard deviations from three independent experiments with triplicated plates.

www.manaraa.com



www.manaraa.com

FANCM and FAAP24 are not required for G2/M checkpoint activation in response to MMC or IR

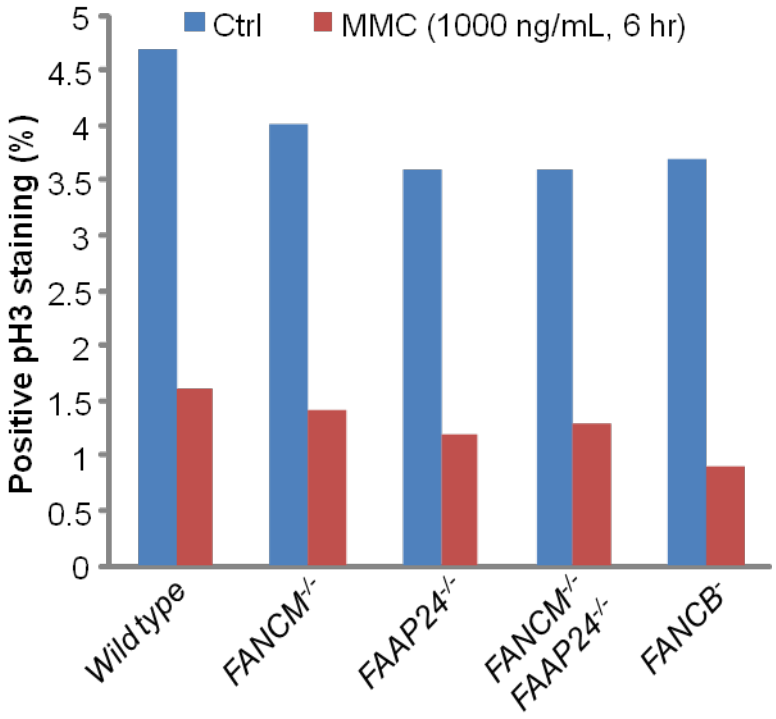
To determine whether FANCM and FAAP24 are involved in the activation of G2/M checkpoint in response to DNA damage, I first exposed the *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells to MMC, and examined mitotic entry by phosphorylated histone H3 staining based flow cytometry analysis. As shown in Fig 4.7, cells lacking FANCM and/or FAAP24 were proficient in activating G2/M checkpoint in response to different doses of MMC. When treated with ionizing radiation (IR), the *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells were also competent in the activation of G2/M checkpoint (Fig 4.8). These data suggest that FANCM and FAAP24 are dispensable for efficient G2/M checkpoint activation in response to DNA damage.

www.manaraa.com

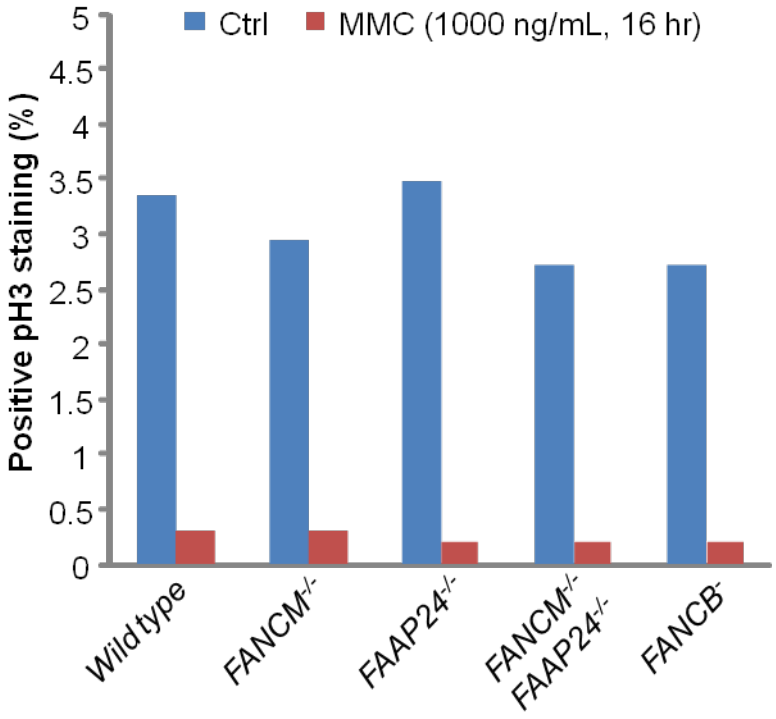
Fig. 4.7 Loss of FANCM and FAAP24 does not affect G2/M checkpoint activation in response to MMC. Cells were treated with MMC, and phospho-histone H3 staining was determined by flow cytometry analysis.

www.manaraa.com

A



B

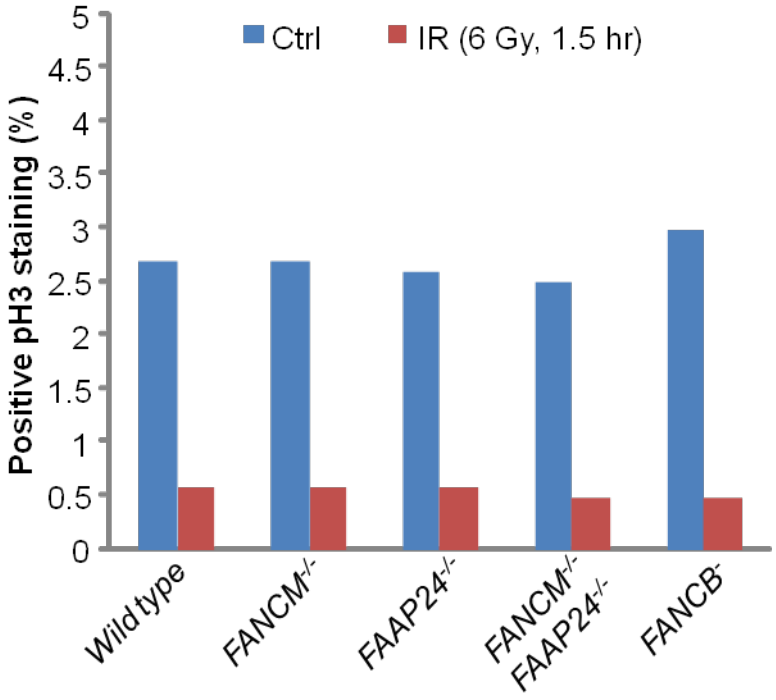


www.manaraa.com

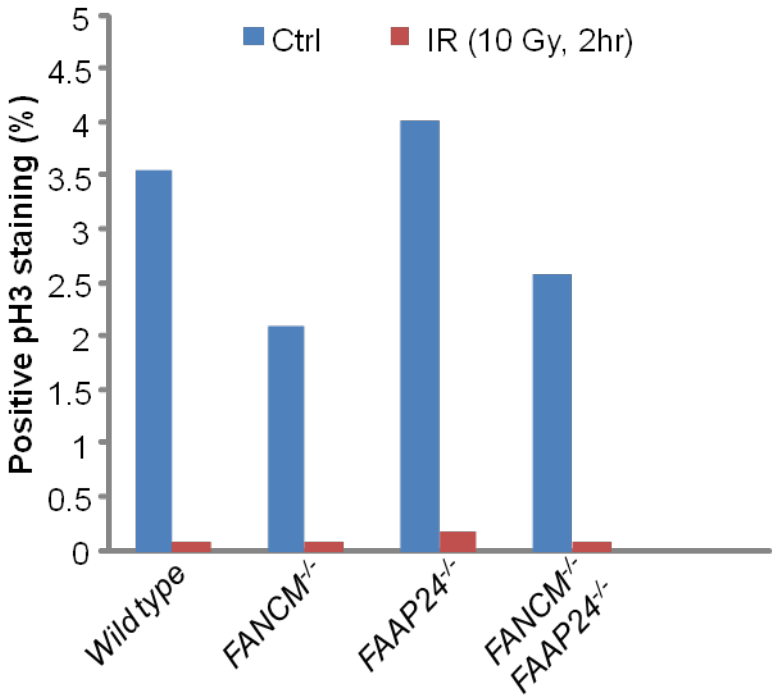
Fig. 4.8 Loss of FANCM and FAAP24 does not affect G2/M checkpoint activation in response to IR. Cells were treated with IR, and phospho-histone H3 staining was determined by flow cytometry analysis.

www.manaraa.com

A



B



www.manaraa.com

Discussion

FANCM and FAAP24 have been implicated in ATR-mediated DNA damage checkpoint signaling by separate studies (Collis et al., 2008; Huang et al., 2010; Luke-Glaser et al., 2009; Schwab et al., 2010), although the results reported were not fully consistent. In the present study, I found that FANCM and FAAP24 are not important for efficient ATR activation in response to UV or HU (Fig 4.1). This appears to be in agreement with studies by Huang *et al* (Huang et al., 2010), but is contradictory to a few other studies (Collis et al., 2008; Luke-Glaser et al., 2009; Schwab et al., 2010). I also found that FAAP24 but not FANCM promotes ATR-mediated checkpoint activation in response to DNA crosslinking agents (Fig 4.3), which is partially consistent with a previous report (Huang et al., 2010).

A key step in ATR-mediated checkpoint activation is extensive exposure of ssDNA and its coating by RPA, which recruits the ATR binding partner ATPIP and the ATR kinase itself. ATR activation then follows recruitment of RFC-like Rad17/Rfc2-5 clamp-loader, PCNA-like Rad9-Rad1-Hus1 (9-1-1) clamp, and additional co-activators such as TopBP1 (Burrows and Elledge, 2008). In response to UV or HU damage, while the DNA polymerase is stalled by damaged DNA, the replicative helicase can proceed and therefore excessively expose ssDNA. This polymerase-helicase uncoupling allows the RPA coated ssDNA to promptly accumulate, which in turn leads to efficient ATR recruitment and robust activation of ATR and the DNA damage checkpoint signaling. FANCM and FAAP24 have been shown to interact with HCLK2, which also associates with ATR-ATRIP complex, and have therefore been speculated to be important for ATR recruitment and activation (Collis et al., 2008; Horejsi et al., 2009). It is likely that previous studies (Collis et al., 2008; Luke-Glaser et al., 2009; Schwab et al., 2010) employing siRNA depletion reflect a more acute

www.manaraa.com

consequence of FANCM and FAAP24 loss. In contrast, the knockout cell lines used in this study may have adapted to the loss of FANCM and FAAP24 and have evolved alternative mechanisms to compensate any deficiency of ATR recruitment caused by absence of FANCM or FAAP24. However, it is still possible that the function of FANCM and FAAP24 in UV- and HU-induced ATR activation is not essential in the first place, since the RPA accumulation was not affected by loss of FANCM or FAAP24 in these contexts (Huang et al., 2010).

In response to ICL damage however, both replicative helicase and DNA polymerase are stalled by the DNA lesions. Consequently, the accumulation of RPA coated ssDNA is less efficient. My result (Fig 4.3) showed that defects in MMC-induced ATR activation is manifested specifically by loss of FAAP24. Furthermore, I showed that the function of FAAP24 in ICL-induced ATR activation is independent of its interaction with FANCM (Fig 4.5). Why FAAP24 is uniquely required for ICL-induced checkpoint activation is unclear. Given the importance of ssDNA structures for ATR activation, the fact that FAAP24 has high affinity towards ssDNA while FANCM prefers branched DNA structures (Ciccia et al., 2007; Gari et al., 2008b) may be one explanation of selective requirement for FAAP24 in ICL-induced activation of ATR-mediated DNA damage checkpoint. In addition, the intrinsic ICL binding activity of FAAP24 also underscores the importance of FAAP24 in activating ATR-mediated checkpoint in response to crosslinking agents (Huang et al., 2010). It is plausible that the FANCM-independent ICL binding activity of FAAP24 is an important alternative mechanism for enhanced recruitment of ATR-ATRIP kinase in the context of insufficient RPA-ssDNA accumulation in response to ICL damage.

www.manaraa.com

CHAPTER V

FANCM Promotes Recombination-independent Repair of Interstrand Crosslinks

Introduction

In previous Chapters, I demonstrated that the *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double knockout mutant was much more sensitive to DNA crosslinking agents than *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single mutants, suggesting that FANCM and FAAP24 have cooperative as well as unique functions in response to ICL damage. I also showed that FAAP24 has a major function in activating ATR-mediated DNA damage checkpoint in response to ICL damage, which is independent of FANCM. Lack of checkpoint activation defect in the *FANCM*$^{-/-}$ knockout cells prompted me to explore checkpoint-independent function of FANCM in response to ICL damage. One possibility is that FANCM may be involved in the actual removal of ICL lesions.

In mammalian cells, ICLs are primarily repaired in S phase cells when the ICL damage is encountered by DNA replication forks. This repair process involves multiple coordinated steps including ICL unhooking, translesion synthesis, homologous recombination, and nucleotide excision repair (Deans and West, 2011). The function of FANCM and FAAP24 in this pathway is likely tethered together for two reasons. First, a functional FA pathway is required for the replication-coupled repair of ICL (Knipscheer et al., 2009; Long et al., 2011). Second, the replication fork remodeling-stabilization activity *in vivo* is likely achieved by the FANCM-FAAP24 heterodimer since they bind stalled fork synergistically (Yan et al., 2010).

www.manaraa.com

There exists another pathway for ICL repair in mammalian cells that is not coupled with DNA replication. This pathway mainly consists of ICL unhooking, translesion synthesis and nucleotide excision repair (Deans and West, 2011; Wang et al., 2001). It is considered to be a minor pathway that primarily operates in G1 phase. Since no DNA replication is involved, the processing or unhooking of ICL will not lead to double strand break generation. Therefore, homologous recombination mediated repair is not involved in this repair pathway. Consequently, this recombination-independent ICL repair pathway tends to be error prone.

Whether FA and FA-associated proteins are involved in recombination-independent ICL repair remains unclear. In this Chapter, I investigated the impact of FANCM, FAAP24 and FANCB loss on recombination-independent ICL repair efficiency, and I found that FANCM, but not FAAP24 or the FA core complex component FANCB, is required for efficient ICL removal through the recombination-independent repair pathway. I provide further evidence demonstrating that FANCM functions to recruit NER factors and lesion bypass factors to damage sites for efficient ICL repair. These functions rely on the translocase activity of FANCM, which likely allows FANCM to detect ICL lesions and act to create lesion accessibility for repair factors.

Materials and Methods

Cell culture

ERCC1$^{+/+}$ and *ERCC1*$^{-/-}$ Chinese hamster ovary (CHO) cells were cultured in Minimum Essential Medium (MEM) plus 10% FBS, and *XPA*$^{-/-}$ and XPA complemented *XPA*$^{-/-}$ fibroblasts were cultured in MEM plus 15% FBS. All cells were grown in a humidified 5% CO_2-containing atmosphere at 37°C.

Antibodies

www.manaraa.com

FAAP100 antibody was described previously (Ling et al., 2007). Sources of antibodies (that were not described in previous chapters) are as follows: anti-PCNA (P8825, Sigma-Aldrich); anti-ERCC1 (MS-671-P0, Neomarker); anti-RPA (ab2175, Abcam); anti-phospho-RPA Ser10 (MA1-26418, Thermo Scientific); anti-Rev1 (sc-48806X, Santa Cruz); anti-XPA (MS-650-P1ABX, Neomarker).

Cell cycle analysis

To determine G1/S transition in response to DNA damage, cells were treated with MMC for 6 or 16 hrs, pulse labeled with 10 μM BrdU for 30 min, harvested and fixed as above. Fixed cells were resuspended in 2 N HCl containing 0.2 mg/ml pepsin and incubated at 37 °C for 20 min, after which 0.1 M sodim tetraborate (pH 8.5) was added for neutralization. Cells were then washed with 1% BSA/PBS containing 0.5% Tween 20, incubated with Alexa Fluor 488-conjugated anti-BrdU (1:20 in 1% BSA/PBS containing 0.5% Tween 20) in the dark at room temperature for 30 min, and stained with propidium iodide (PI) buffer (0.2 mg/ml RNase A and 5 μg/ml PI in PBS) in the dark at room temperature for 30 min. Percentages of cells in G1, S and G2/M phases were quantified by flow cytometry analysis.

Recombination-independent ICL repair assay

To assess recombination-independent ICL repair efficiency, the luciferase reporter reactivation assay was performed as descried previously (Shen et al., 2009). Briefly, 1 ng of reporter substrate with a transcription blocking ICL, or 1 ng of an undamaged control reporter, was transfected into cells together with 50 ng β-galactosidase plasmid. The relative ICL repair efficiencies were calculated as the percentage of luciferase activity of the crosslinked reporter normalized to that of the undamaged reporter, after individual

www.manaraa.com

normalization against the β-galactosidase internal control. Experiments were performed with duplicate transfections.

eChIP assay

eChIP assay was carried out according to protocols described earlier (Shen et al., 2009; Wang and Li, 2011).

Results

FANCM is required for recombination-independent repair of ICLs

The convergence of the FA pathway and the breast cancer susceptibility pathway strongly indicates that FA proteins play important roles in homologous recombination mediated repair of ICLs. In mammalian cells, there exists another ICL repair mechanism which is recombination-independent and error-prone (Deans and West, 2011; Wang et al., 2001). Whether FA proteins are involved in the recombination-independent repair of ICLs has not been well studied.

To determine whether FANCM and FAAP24 are required for recombination-independent ICL repair, I examined the ICL repair efficiency in *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$ and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ somatic knockout cell lines using a luciferase reporter assay previously established by our group (Wang et al., 2001). Briefly, a single psoralen ICL was introduced at a defined position between the CMV promoter and the luciferase coding region in the reporter plasmid. Presence of this site-specific ICL blocks gene transcription and luciferase expression, and repair of the ICL *in vivo* results in reactivation of lucidersase expression (Fig 5.1A). Due to unavailability of undamaged homologous sequences in chromosomal DNA, removal of this psoralen ICL *in vivo* is expected to be achieved by recombination-independent repair mechanisms. When the reporter plasmids were introduced into cells

www.manaraa.com

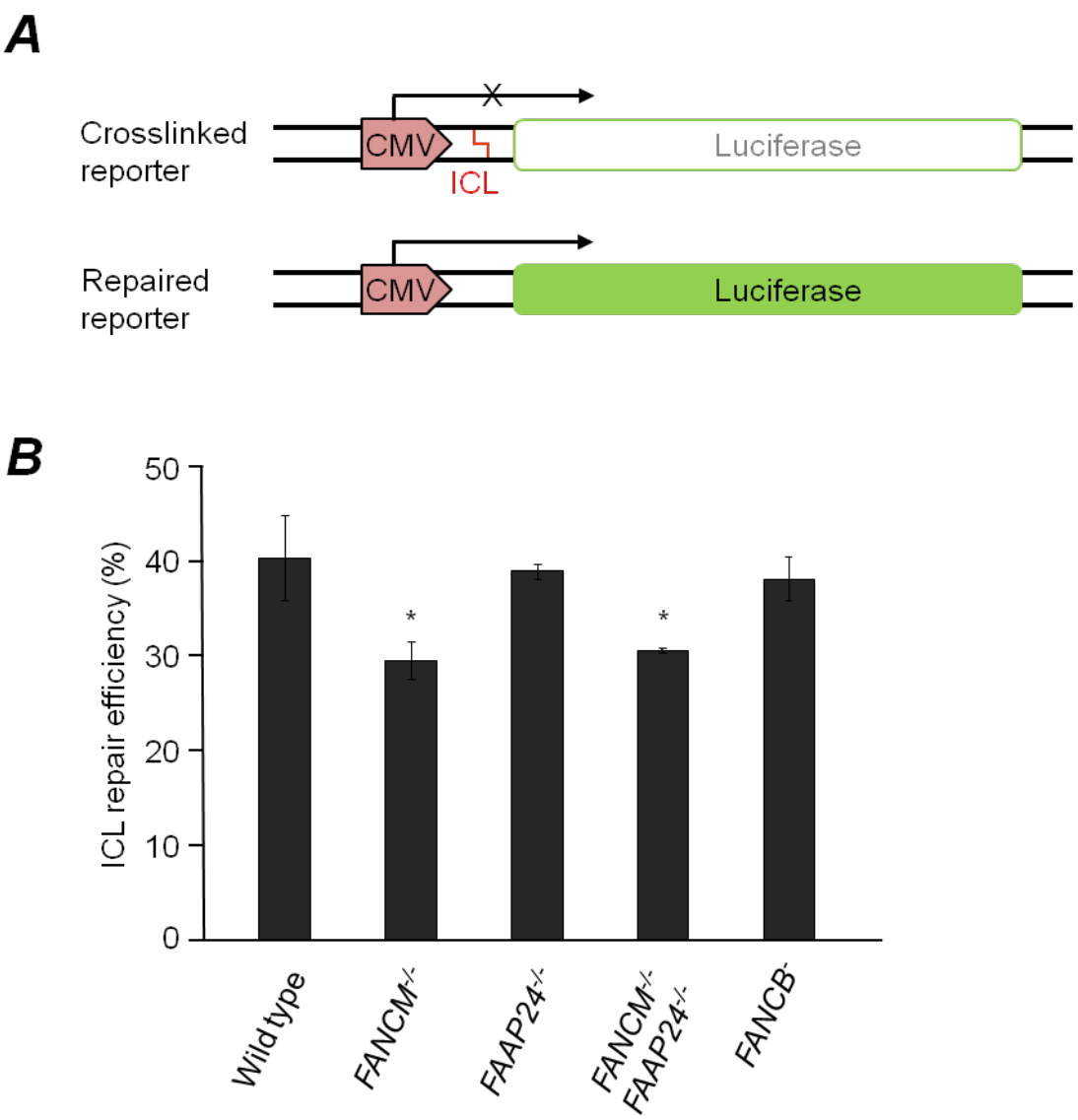
together with internal control vectors expressing beta-galactosidase, the recombination-independent ICL repair efficiency can be quantified by normalizing luciferase activity against beta-galactosidase activity.

As shown in Fig 5.1B, *FANCM*$^{-/-}$ and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells showed reduced recombination-independent ICL repair by ~30% compared with wild type HCT-116 cells, suggesting that FANCM is required for efficient removal of ICLs through the recombination-independent repair pathway. In contrast, the ICL repair in *FAAP24*$^{-/-}$ and *FANCB*$^{-}$ cells was as efficient as that in wild type cells, suggesting that the function of FANCM in recombination-independent ICL repair is likely FAAP24- and FA core complex-independent.

www.manaraa.com

Fig. 5.1 FANCM promotes recombination-independent ICL repair.
(A) Schematics of the reporter reactivation-based recombinant-independent ICL repair assay. Control and crosslinked reporter represent the unmodified and site-specifically crosslinked plasmid substrates, respectively. CMV: CMV promoter.
(B) Recombination-independent ICL repair efficiencies in FANCM and FAAP24 mutant cell lines. Error bars represent standard deviation of 6 independent experiments (* $P < 0.05$ vs. wild type).

www.manaraa.com



www.manaraa.com

Reduced recombination-independent ICL repair leads to delayed G1/S transition

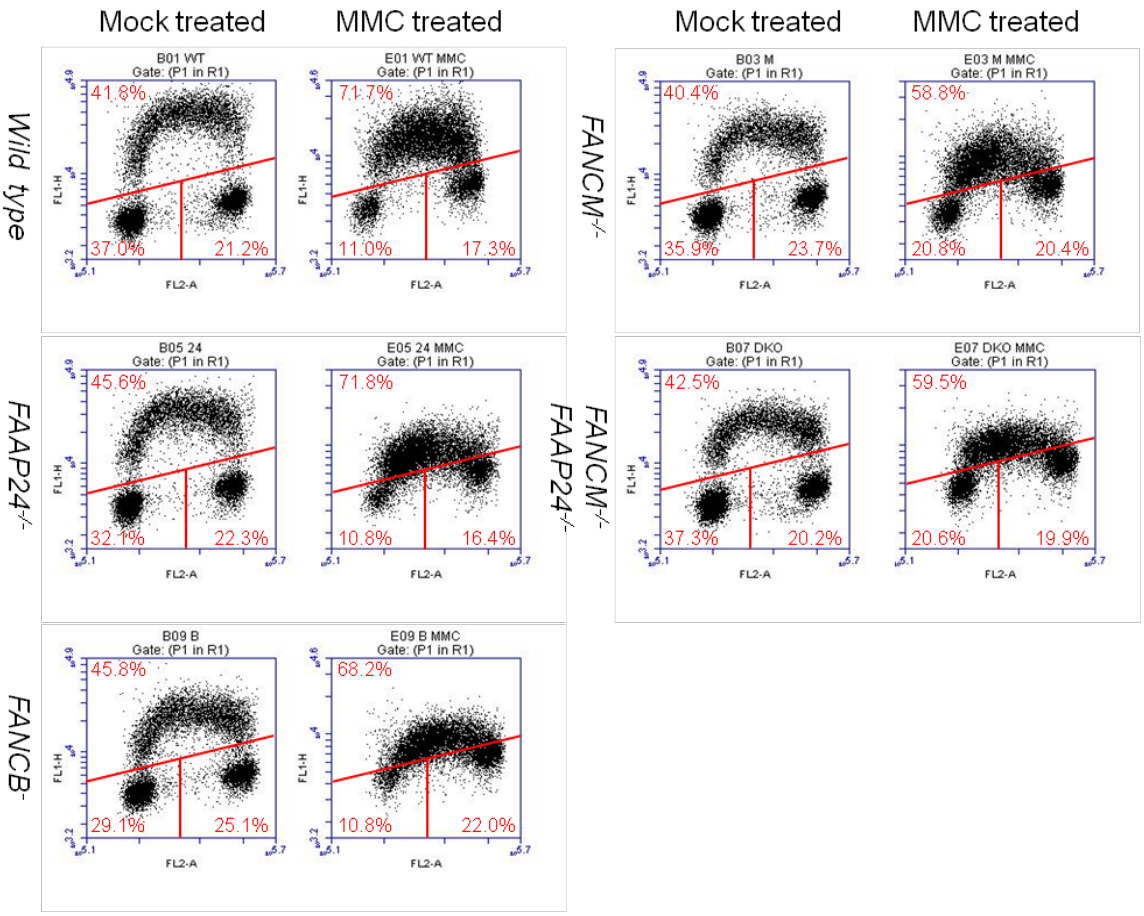
Recombination-independent ICL repair primarily operates during the G1/G0 phase of cell cycle when the lesion is not encountered by DNA replication and when homologous recombination mediated repair is dormant. Therefore, failure in efficient ICL removal through the recombination-independent repair mechanism is expected to impact primarily G1/G0 phase cells. Most likely, insufficient ICL repair will cause cell accumulation at G1/G0 phase or the G1/S boundary. To validate this prediction, I analyzed cell cycle progression in *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$ and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells that were exposed to MMC. The cell cycle profile in untreated wild type cells and knockout cells were identical, suggesting that loss of FANCM, FAAP24 or FANCB did not alter cell cycle progression in unperturbed growth conditions (Fig 5.2). However, when exposed to MMC, there was a prominent increase of G1/G0 phase cells in *FANCM*$^{-/-}$ and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ knockout cells, but not in the *FAAP24*$^{-/-}$ or *FANCB*$^{-}$ mutants (Fig 5.2). These results suggest that impaired recombination-independent ICL repair associated with FANCM loss leads to delayed G1/S cell cycle transition.

www.manaraa.com

Fig. 5.2 Loss of FANCM leads to delayed G1/S cell cycle transition in response to MMC.
(***A***) Cells with indicated genotypes were exposed to 1000 ng/ml MMC for 16 hours and pulse-labeled with BrdU before harvesting. Cell cycle distribution was determined by bivariate flow cytometry with anti-BrdU and PI staining.
(***B-C***) Quantification of cell cycle distribution in cells with indicated genotypes exposed to MMC (1000 ng/ml). Represented results of several independent experiments are shown.

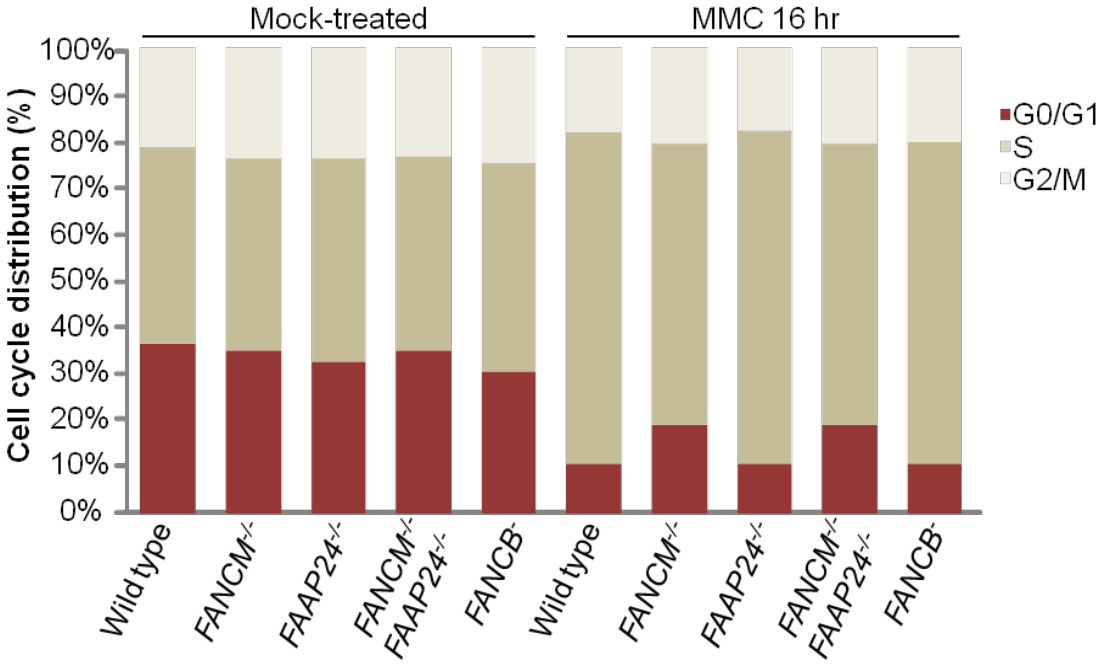
www.manaraa.com

A

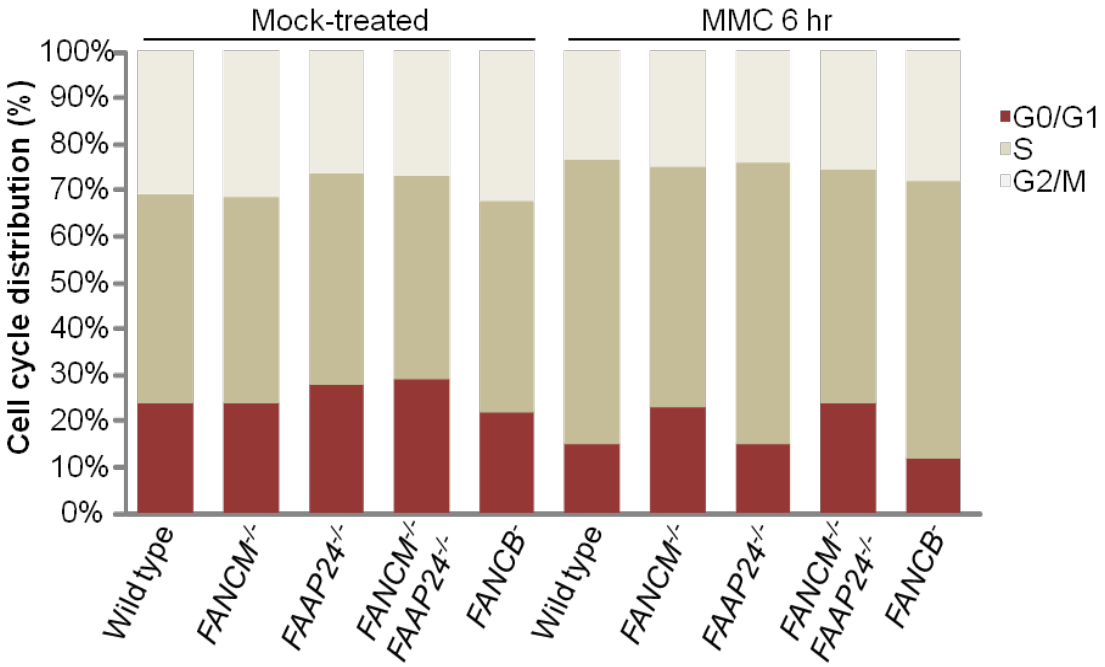


www.manaraa.com

B



C



www.manaraa.com

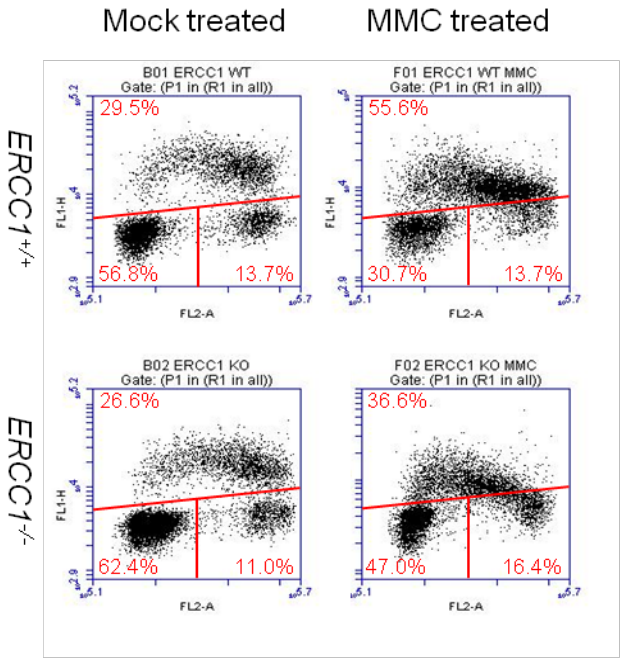
To further validate the impact of recombination-independent ICL repair on G1/S cell cycle transition, I tested cell cycle progression in cells lacking ERCC1 or XPA, which were shown to be defective in recombination-independent ICL repair (Wang et al., 2001; Zheng et al., 2003). As shown in Fig 5.3, compared to wild type cells, *ERCC1*$^{-/-}$ CHO cells exhibited significant increases in G1/G0 phase cells when treated with MMC. Similarly, compared with XPA corrected cells, *XPA*$^{-/-}$ fibroblasts showed significant accumulation of G1/G0 phase cells when identically exposed to MMC (Fig 5.4). Collectively, these results suggest that impaired recombination-independent ICL repair leads to blocked transition of cell cycle form G1/G0 phase to S phase.

www.manaraa.com

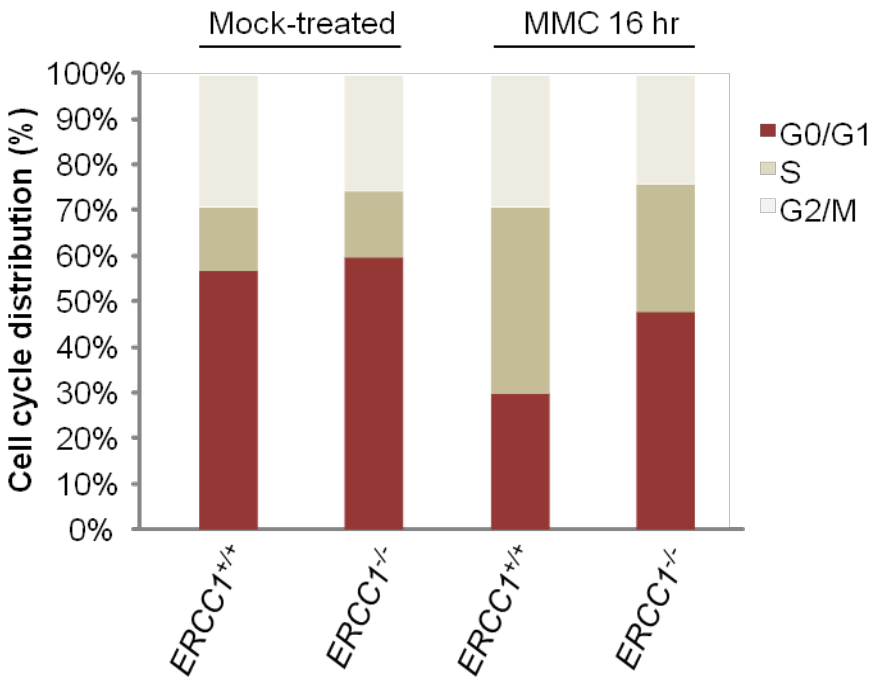
Fig. 5.3 Loss of ERCC1 leads to delayed G1/S cell cycle transition in response to MMC.
(***A***) Cells with indicated genotypes were exposed to 1000 ng/ml MMC for 16 hours and pulse-labeled with BrdU before harvesting. Cell cycle distribution was determined by bivariate flow cytometry with anti-BrdU and PI staining.
(***B***) Quantification of cell cycle distribution in cells with indicated genotypes exposed to MMC (1000 ng/ml). Represented results of several independent experiments are shown.

www.manaraa.com

A



B

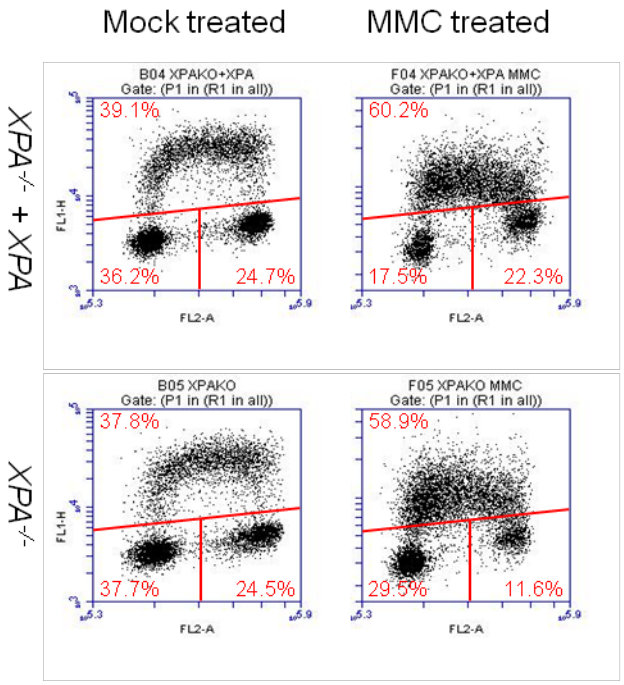


www.manaraa.com

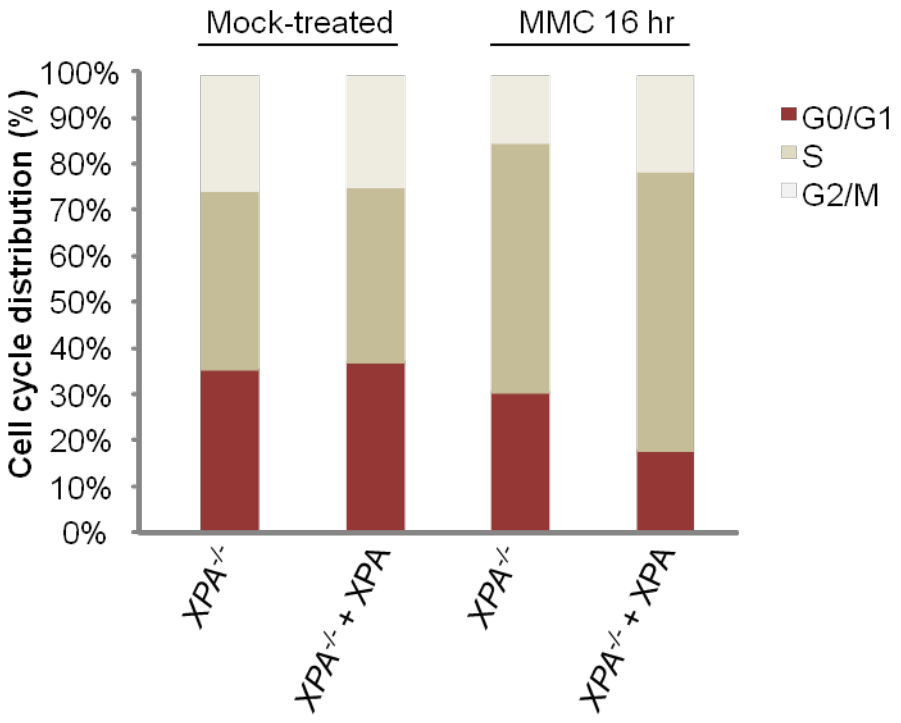
Fig. 5.4 Loss of XPA leads to delayed G1/S cell cycle transition in response to MMC.
(***A***) Cells with indicated genotypes were exposed to 1000 ng/ml MMC for 16 hours and pulse-labeled with BrdU before harvesting. Cell cycle distribution was determined by bivariate flow cytometry with anti-BrdU and PI staining.
(***B***) Quantification of cell cycle distribution in cells with indicated genotypes exposed to MMC (1000 ng/ml). Represented results of several independent experiments are shown.

www.manaraa.com

A



B



www.manaraa.com

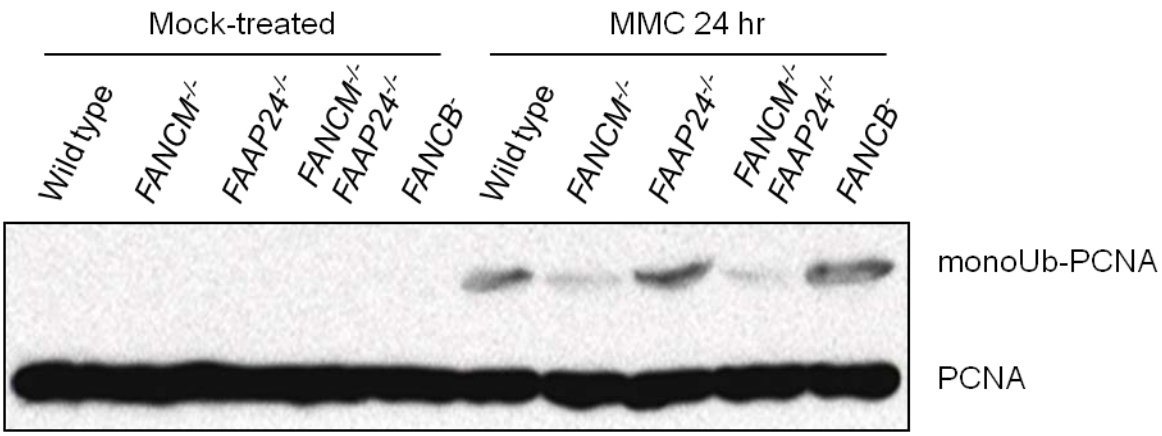
FANCM promotes PCNA monoubiquitination and lesion bypass factor recruitment during recombination-independent ICL repair

Next I sought to investigate how FANCM functions to promote the recombination-independent repair of ICL. A key step in this particular ICL repair pathway is PCNA monoubiquitination. Ubiquitinated PCNA recruits the lesion bypass polymerase to replace the normal replicative polymerase and to enable translesion DNA synthesis (Kelsall et al., 2012; Shen et al., 2006). Therefore, I first examined whether the absence of FANCM will affect PCNA monoubiquitination in response to MMC. To eliminate potential interference from PCNA-monoubiquitination in response to replication fork stall, cells were grown to over-confluency to maximally enrich for cells in G1/G0 phase, during which the recombination-independent ICL repair is the predominant ICL removal mechanism. As shown in Fig 5.5, loss of FANCM caused a significant reduction of PCNA monoubiquitination in response to MMC, whereas cells lacking FAAP24 or FANCB exhibited wild-type levels of PCNA monoubiquitination. These results indicate that FANCM, but not FAAP24 or the FA core complex, functions in the recombination-independent repair of ICLs.

www.manaraa.com

Fig. 5.5 Immunoblot of PCNA monoubiquitination in confluent FANCM and FAAP24 mutant cells exposed to MMC. Upper and lower bands represent monoubiquitinated and native forms of PCNA respectively.

www.manaraa.com



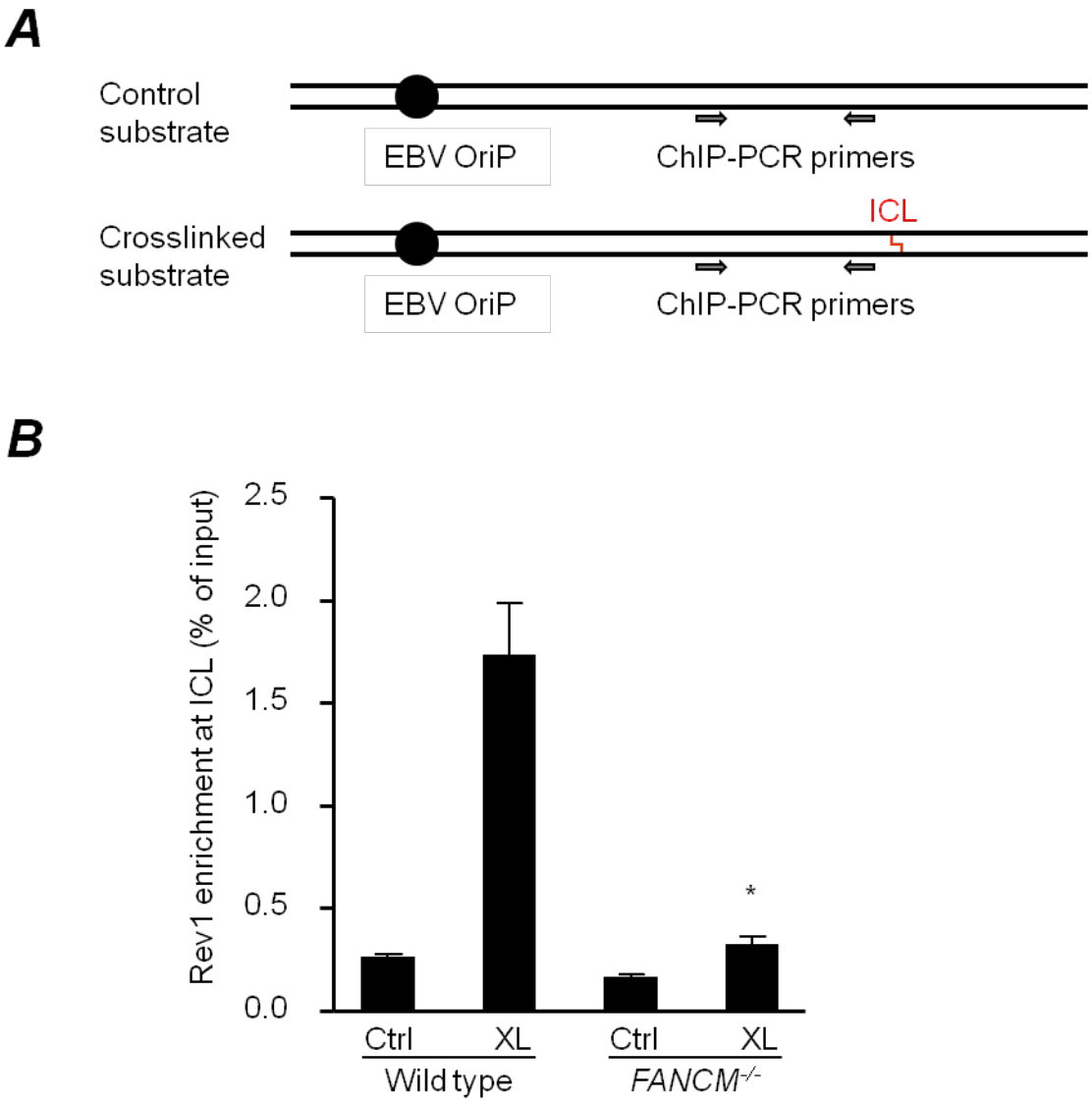
www.manaraa.com

To determine whether impaired PCNA monoubiquitination observed in cells lacking FANCM leads to decreased recruitment of lesion bypass factors, I examined Rev1 recruitment in response to ICL damage, using the eChIP assay established by our group (Shen et al., 2009). This assay (Fig 5.6A) allows protein recruitment at site-specific ICLs on an episomal plasmid to be directly examined by chromatin immunoprecipitaion followed by quantitative PCR (ChIP-QPCR). The plasmid substrate contains an Epstein-Barr virus (EBV) replication origin (OriP), which allows its unidirectional replication only in the presence of Epstein-Barr nuclear antigen 1 (EBNA1). I introduced the control substrate and the ICL substrate in parallel into wild type HCT-116 and *FANCM*$^{-/-}$ cells that were both EBNA1-negative, and measured Rev1 enrichment at the defined ICL site, which was reflective of *in vivo* Rev1 recruitment during recombination-independent ICL repair. As shown in Fig 5.6B, Rev1 was significantly enriched at the ICL sites in wild type cells. However, lack of FANCM greatly impaired Rev1 recruitment to site-specific ICLs, suggesting that decreased PCNA monoubiquitination resulted in compromised recruitment of lesion bypass factor.

www.manaraa.com

Fig. 5.6 Loss of FANCM impairs Rev1 recruitment at ICL sites.
(***A***) Schematics of the eChIP assay. Control and crosslinked substrate represent the unmodified and site-specifically crosslinked plasmid substrates, respectively. EBV OriP: EBV replication origin.
(***B***) eChIP assay for Rev1 recruitment to a site-specific ICL in wild type and *FANCM*$^{-/-}$ mutant cells. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisk (*) denotes $P < 0.01$ vs. wild type.

www.manaraa.com



www.manaraa.com

FANCM is required for the recruitment of nucleotide excision repair factors during recombination-independent ICL repair

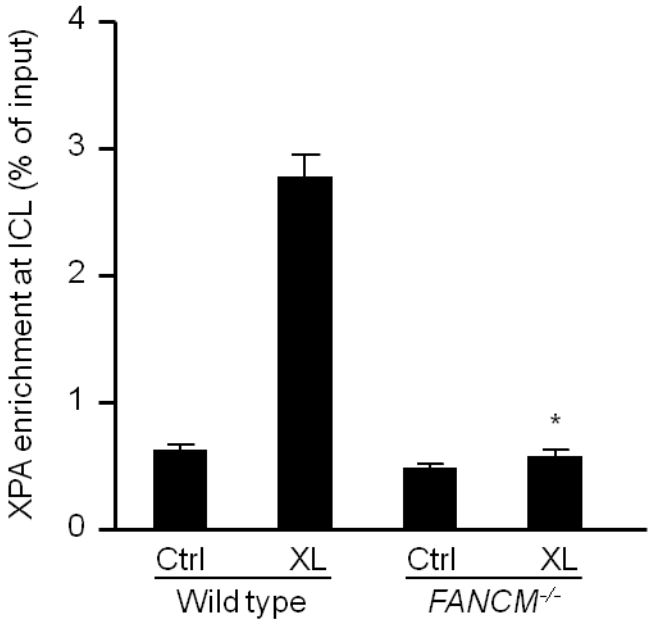
Reduced PCNA monoubiquitination in FANCM deficient cells may reflect insufficient upstream incision of crosslinked DNA by the nucleotide excision repair (NER) proteins, since the NER-mediated ICL unhooking creates a repair intermediate necessary for the onset of PCNA monoubiquitination. I therefore investigated whether lack of FANCM affects the recruitment of NER factors to ICL damage sites. To this end, I examined recruitment of XPA and ERCC1 to site-specific ICLs using the eChIP assay. As shown in Fig 5.7, XPA and ERCC1 were significantly enriched at the defined ICL site in wild type cells. In contrast, in *FANCM*$^{-/-}$ knockout cells, the recruitment of XPA and ERCC1 were significantly reduced. These results suggest that FANCM promotes ICL unhooking by facilitating localization of incision activities in the absence of DNA replication. Consistent with its proficient ICL repair efficiency (Fig 5.1B), the *FAAP24*$^{-/-}$ mutant did not show visible defect of ERCC1 recruitment (Fig 5.7B). This result further suggests that the function of FANCM in recombination-independent ICL repair is independent of FAAP24.

www.manaraa.com

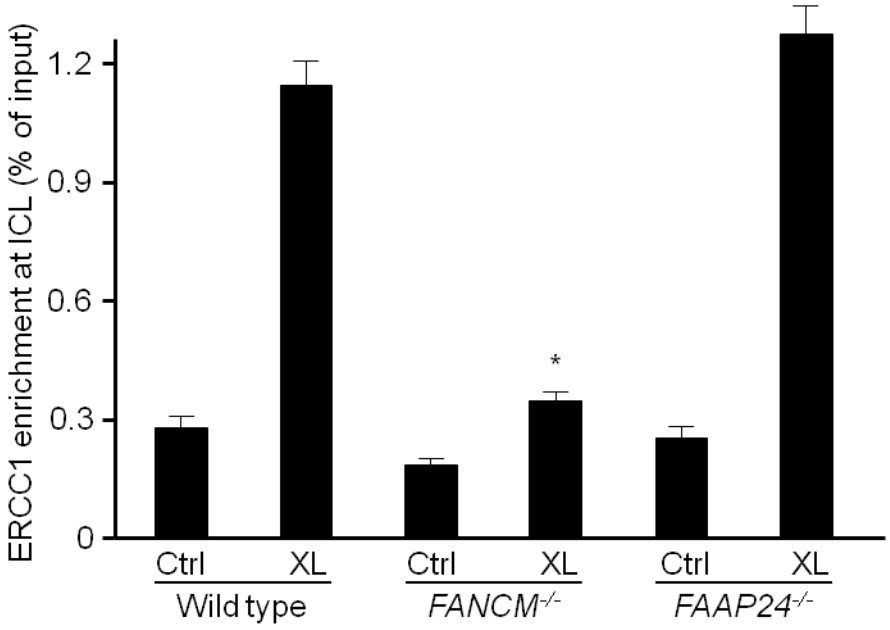
Fig. 5.7 Loss of FANCM impairs XPA and ERCC1 recruitment at ICL sites.
(***A***) eChIP assay for XPA recruitment to a site-specific ICL in wild type and *FANCM*$^{-/-}$ mutant cells. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisk (*) denotes $P < 0.01$ vs. wild type.
(***B***) eChIP assay for ERCC1 recruitment to a site-specific ICL in wild type, *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutant cells. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisk (*) denotes $P < 0.01$ vs. wild type.

www.manaraa.com

A



B



www.manaraa.com

The function of FANCM in recombination-independent ICL repair requires its translocase activity

FANCM-FAAP24 displays structural resemblance with the XPF-ERCC1 and Mus81-Eme1/2 structure-specific nucleases. It is possible that FANCM recruits ERCC1 and other NER proteins through protein-protein interaction. However, when tested by Co-IP experiments, FANCM was not found to interact with ERCC1, or other NER proteins including XPA, XPC, XPD and XPG (data not shown). Therefore, the function of FANCM in facilitating the localiztion of NER proteins is likely through mechanisms other than direct protein recruitment.

FANCM possesses a DEAH helicase domain and associated translocase activity. This allows FANCM to translocate on DNA. It is plausible that the FAAP24-independent translocase activity functions in ICL damage recognition, or acts to create lesion access, thereby allowing the recruitment of NER factors and other downstream repair proteins. To test this premise, I stably complemented the *FANCM*$^{-/-}$ cells with a FANCM translocase mutant (K117R) and examined its function in recombination-independent ICL repair. Wild type FANCM complemented *FANCM*$^{-/-}$ cell lines were also established as controls (Fig 5.8A).

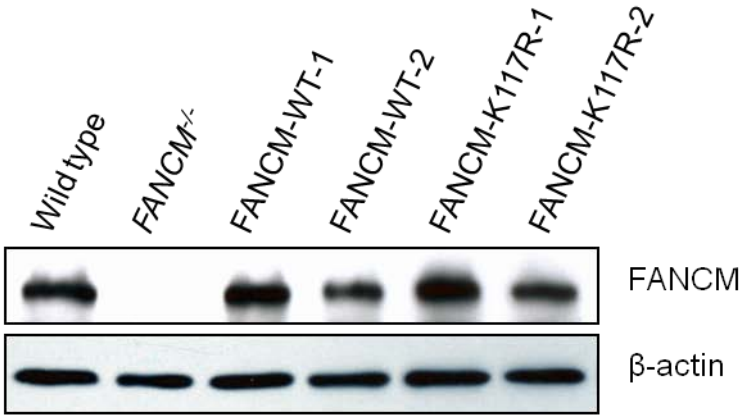
As shown in Fig 5.8B, while wild type FANCM protein rescued the ICL repair deficiency of the *FANCM*$^{-/-}$ knockout cell line, the K117R translocase mutant complemented *FANCM*$^{-/-}$ cells remained deficient in recombination-independent ICL repair. This result strongly suggests that the translocase activity is important for FANCM to function in ICL repair.

www.manaraa.com

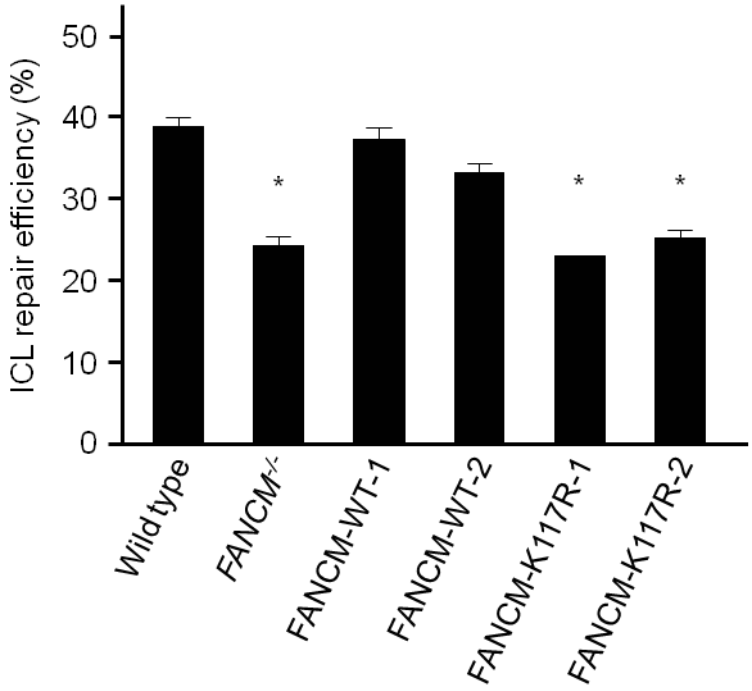
Fig. 5.8 FANCM translocase activity is required for efficient recombination-independent ICL repair.
(***A***) Immunoblot detecting expression of wild type FANCM and the K117R translocase mutant in two independent stable clones.
(***B***) Recombination-independent ICL repair efficiencies in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM. Error bars represent standard deviation of 6 independent experiments (* $P < 0.05$ vs. wild type).

www.manaraa.com

A



B



www.manaraa.com

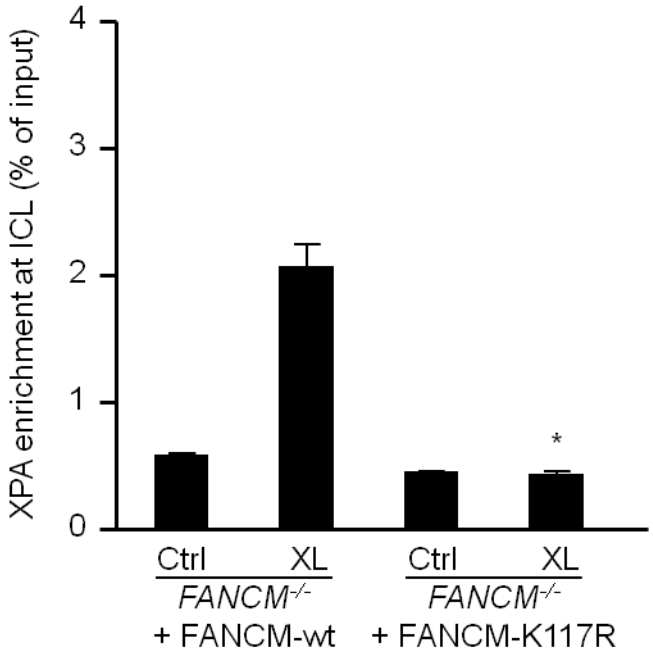
To further understand the function of FANCM in ICL repair, I examined the K117R translocase mutant for its ability to recruit NER and lesion bypass factors. As shown in Fig 5.9, recruitments of the lesion binding protein XPA and lesion incision protein ERCC1 were significantly decreased in cells lacking the FANCM translocase activity. In addition, the recruitment of lesion bypass factor Rev1 was also markedly reduced in the absence of the FANCM translocase activity (Fig 5.10). Taken together, these results suggest that the translocase activity of FANCM promotes the recruitment of multiple repair factors, and it is therefore crucial for recombination-independent ICL repair.

www.manaraa.com

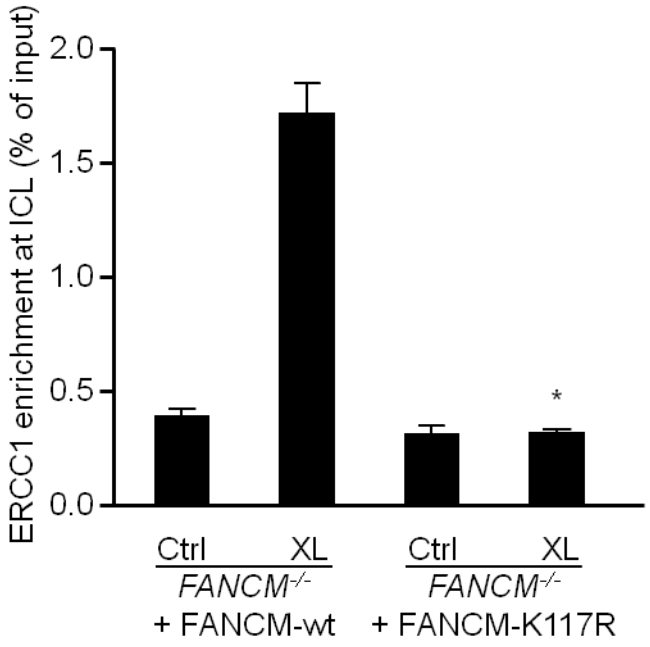
Fig. 5.9 FANCM translocase activity is required for XPA and ERCC1 recruitment at ICL sites.
(***A***) eChIP assay for XPA recruitment to a site-specific ICL in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisk (*) denotes $P < 0.01$ vs. wild type complemented cells.
(***B***) eChIP assay for ERCC1 recruitment to a site-specific ICL in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisk (*) denotes $P < 0.01$ vs. wild type complemented cells.

www.manaraa.com

A



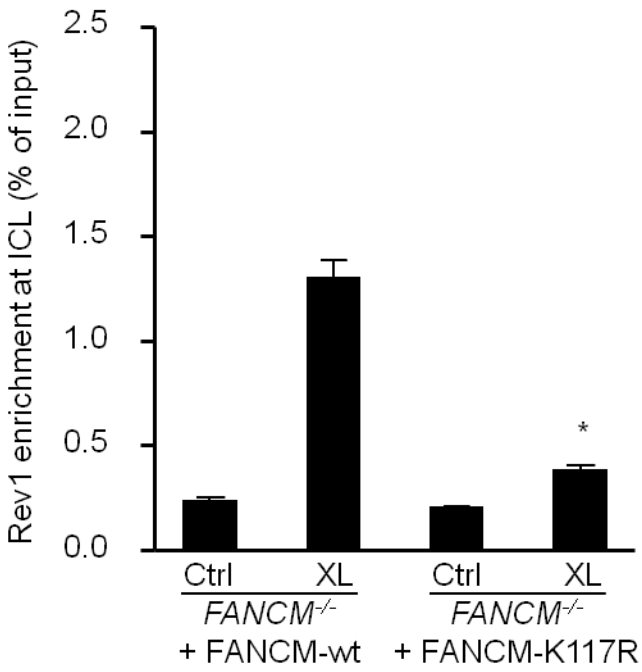
B



www.manaraa.com

Fig. 5.10 eChIP assay for Rev1 recruitment to a site-specific ICL in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisk (*) denotes $P < 0.01$ vs. wild type complemented cells.

www.manaraa.com



www.manaraa.com

The translocase activity of FANCM is dispensable for FA pathway activation

The fact that FAAP24 and FANCB are not required for the recombination-independent repair of ICL (Fig 5.1B & Fig 5.7B) suggests that the function of FANCM in this particular ICL repair pathway may be independent on its function in FA pathway activation. To test this hypothesis, I examined the FA pathway function in the K117R translocase mutant complemented *FANCM*$^{-/-}$ knockout cells.

As shown in Fig 5.11A, the K117R translocase mutant of FANCM was competent in interacting with FA core complex components including FAAP100, FANCG and FANCL. Therefore, the recruitment of FA core complex in response to DNA damage most likely does not require the translocase activity of FANCM. Consistent with this notion, K117R translocase mutant complemented *FANCM*$^{-/-}$ cells showed wild type levels of FANCD2 monoubiquitination (Fig 5.11B) and FANCD2 nuclear foci formation in response to MMC (Fig 5.12). These results suggest that the translocase activity of FANCM is not required for efficient activation of the FA pathway.

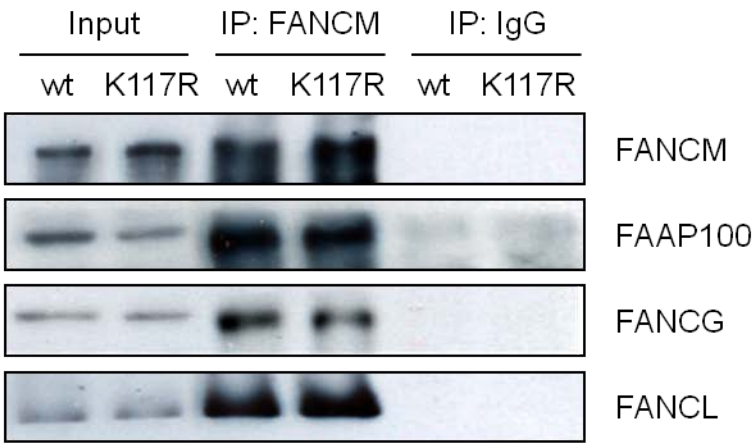
Previous studies have reported that the FANCM translocase activity contributes to normal resistance to MMC but not FANCD2 monoubiquitination (Gari et al., 2008b; Xue et al., 2008), indicating that FANCM had additional translocase-associated function outside the FA pathway. This is consistent with my finding that FANCM promotes recombination-independent ICL repair through its translocase activity.

www.manaraa.com

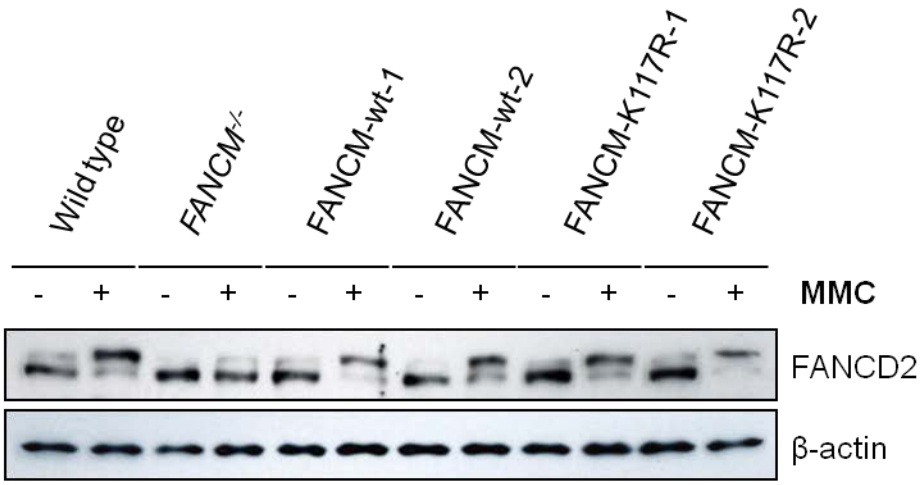
Fig. 5.11 The translocase activity of FANCM is not required for its interaction with FA core complex and FANCD2 monoubiquitination.
(***A***) Co-immunoprecipitation detecting interactions between indicated FA core components and wild type FANCM or K117R mutant FANCM.
(***B***) Immunoblot detecting MMC-induced monoubiquitination of FANCD2 in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM. L and S represent monoubiquitinated and native forms of FANCD2, respectively.

www.manaraa.com

A



B

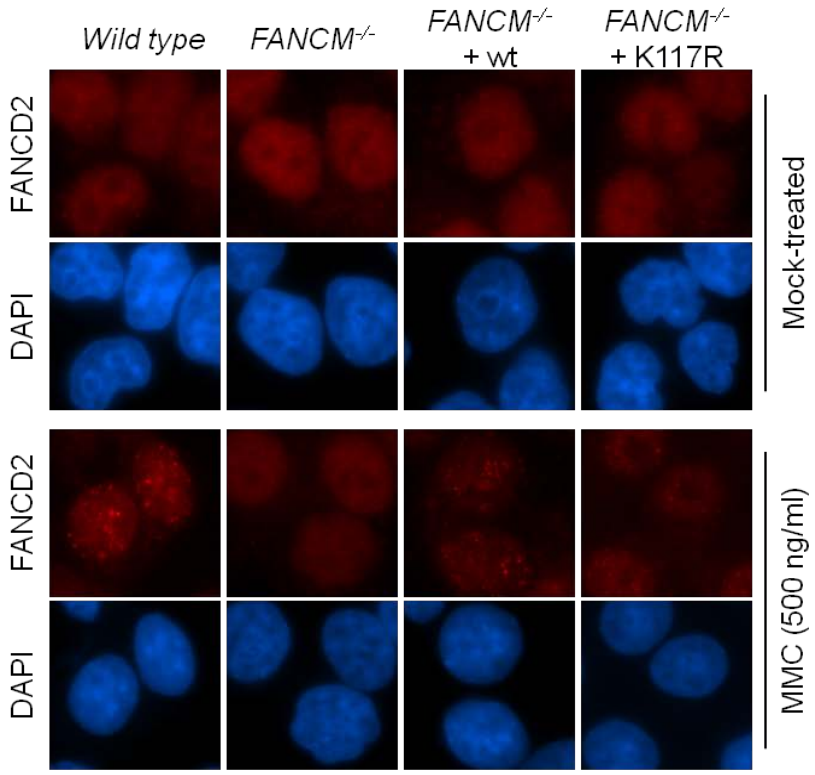


www.manaraa.com

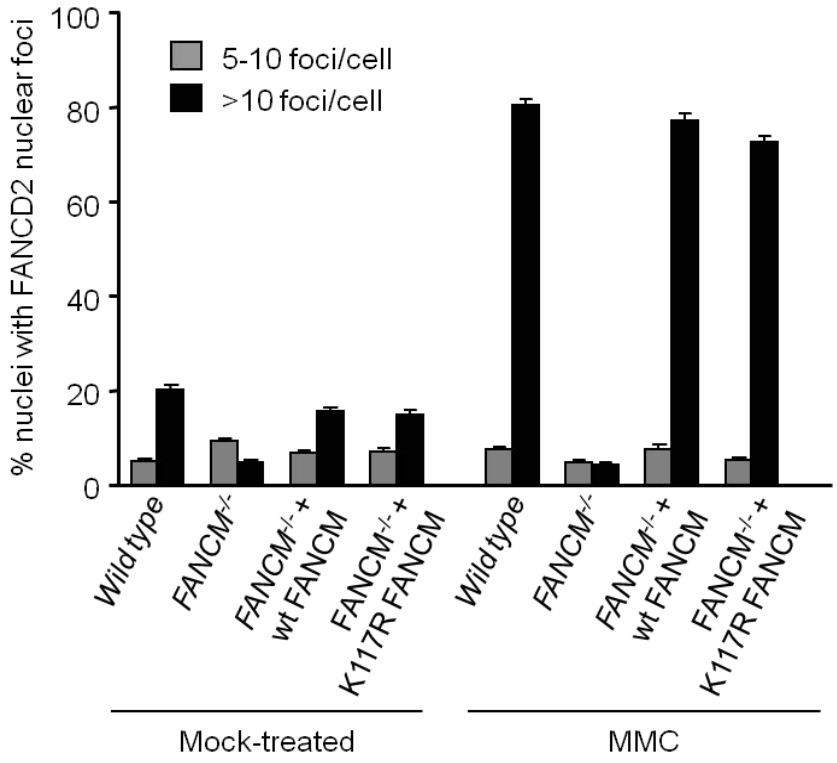
Fig. 5.12 FANCM translocase activity is not required for FANCD2 nuclear foci formation.
(***A***) Formation of MMC-induced (500 ng/ml) FANCD2 nuclear foci in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM.
(***B***) Quantification of FANCD2 nuclear foci formation in *FANCM*$^{-/-}$ cells complemented with wild type or K117R FANCM. Data represent three independent experiments and error bars depict standard deviation derived from 5 data sets.

www.manaraa.com

A



B



www.manaraa.com

Discussion

The functional impacts of FA proteins on DNA damage response and ICL repair have largely been studied in the context of DNA replication. In mammalian cells, there exists an alternative recombination-independent mechanism for ICL repair which primarily operates in the G1/G0 phase of cell cycle. However, it remains unclear whether FA proteins function in the recombination-independent ICL repair. In this study, I found that FANCM, but not FAAP24 or the FA core complex component FANCB, is required for efficient ICL repair through the recombination-independent pathway. I also demonstrated that the function of FANCM in recombination-independent ICL repair requires its tranlocase activity. My study therefore uncovers a previously unappreciated role of FANCM in recombination-independent ICL repair through its translocase activity.

Repair of ICL by this recombination-independent pathway involves a few major steps, including ICL unhooking via lesion incision by nucleotide excision repair (NER) endonucleases, PCNA-mediated polymerase switch, translesion synthesis across the lesion, and lesion removal by NER factors (Shen et al., 2006). The data I presented here demonstrated that FANCM deficiency leads to compromised ERCC1 recruitment to ICL sites, reduced PCNA monoubiquitination, and impaired recruitment of lesion bypass factor REV1. Therefore, it is very likely that FANCM functions at an early step to facilitate ICL processing by endonucleases.

The recruitment of structural-specific endonuclease complex XPF/ERCC1 is less likely to be through direct FANCM association, since the interactions among XPF family members (XPF/ERCC1, Mus81/Eme1 or Eme2, FANCM/FAAP24) are quite specific (Ciccia et al., 2007). Indeed, I did not detect any direct interaction between FANCM and ERCC1. In

www.manaraa.com

addition, FANCM was not found to interact with other NER factors including XPA, XPC, XPD and XPG. Collectively, these results suggest that FANCM promotes the recruitment of NER factors through a mechanism other than protein-protein interaction.

Since FANCM possesses ATP-dependent DNA translocase activity, it may slide on DNA and act as a DNA damage sensor that facilitates the recruitment of XPF/ERCC1 and other repair components upon lesion recognition. Consistent with this model, I found that *FANCM*$^{-/-}$ cells carrying a FANCM K117R translocase mutant showed defective recruitment of repair factors including XPA, ERCC1 and Rev1, and consequently displayed impaired recombination-independent ICL repair.

How FANCM promotes the recruitment of downstream repair components upon DNA damage recognition remains unclear. FANCM translocase activity may function in creating lesion accessibility for incision activities and subsequent repair factors. NER incision requires a significant span of nucleosome-free duplex DNA (Hara et al., 2000). A feasible role for FANCM is therefore to release DNA flanking the ICL lesion from nucleosome contact via its translocation actions, thus allow access of NER factors. Consistently, recent observations by Kelsall *et al* show that FANCM participates in the NER process (Kelsall et al., 2012). Alternatively, FANCM may promote the relaxation of chromatin structures and exposure of DNA flanking the ICL lesions. DNA is highly compacted in G1/G0 phase, and chromatin remodeling processes are likely required for efficient ICL repair through the recombination-independent pathway. It remains a possibility that FANCM recruits certain chromatin remodeling factors upon ICL recognition, and subsequent DNA exposure will allow better access of NER factors, particularly the XPF/ERCC1 endonuclease complex which mediates the ICL unhooking.

www.manaraa.com

CHAPTER VI

Conclusions and Future Directions

Conclusions

The purpose of this study was to generate human genetic models for FANCM and FAAP24, and to systematically investigate the functions of FANCM and FAAP24 in DNA damage response and repair.

I found that while *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ single mutants displayed readily detectable hypersensitivity to ICL damage, *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double-knockout cells exhibited much more severe hypersensitivity. Consistently, I found that MMC-induced chromosomal breakage occurred at a higher frequency in *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double mutant than in either single mutant. These results suggest that FANCM and FAAP24 are not fully epistatic in cellular response to ICL damage, and prompted me to explore cooperative as well as unique functions of FANCM and FAAP24 in DNA damage response.

I found that by facilitating chromatin loading of the FA core complex, FANCM and FAAP24 promote FANCD2 monoubiquitination and FANCD2 nuclear foci formation in response to ICL damage, which are central events in FA pathway activation. Notably, *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ double mutant showed no further decrease in FANCD2 monoubiquitination compared with either single mutant, suggesting that FANCM and FAAP24 work in concert to promote FA pathway activation. In addition, I found that *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$ and *FANCM*$^{-/-}$/*FAAP24*$^{-/-}$ cells displayed comparable amount of tetra-radial chromosomes and sister chromatid exchanges in response to ICL damage, suggesting

www.manaraa.com

that FANCM and FAAP24 cooperatively suppress crossover recombination during ICL repair.

Besides their cooperative functions, I discovered unique functions of FANCM and FAAP24 in DNA damage response that is independent of the FA pathway. I found that FAAP24 but not FANCM promotes ATR-mediated checkpoint activation specifically in response to ICL damage. A V198A FAAP24 mutant which failed to interact with FANCM could restore the ATR-mediated checkpoint activation and partially rescue ICL hypersensitivity of *FAAP24*$^{-/-}$ cells, further indicating a FANCM-independent function of FAAP24 in DNA damage checkpoint activation. Conversely, I found that FANCM but not FAAP24 promotes recombination-independent repair of ICL during G1/G0 phase, leading to delayed G1/S transition. Mechanistically, I established that FANCM exerts its function in recombination-independent ICL repair through its FAAP24-independent translocase activity. In the absence of FANCM translocase activity, the recruitments of NER factors including XPF/ERCC1 which are crucial for ICL incision, as well as the Rev1-containing lesion bypass complex that is important for translesion DNA synthesis, were greatly impaired. In addition, the FANCM translocase mutant was capable of supporting FA core complex interaction and FANCD2 monoubiquitination, further suggesting that FANCM functions independently of the FA pathway to promote efficient ICL repair through the recombination-independent mechanism.

Collectively, my studies defined that FANCM and FAAP24 maintain genomic stability via intercalated mechanisms - facilitating FA pathway activation, activating DNA damage checkpoint, and promoting recombination-independent ICL repair. These findings

www.manaraa.com

reveal novel mechanistic insights into how genome integrity is both coordinately and independently protected by FANCM and FAAP24.

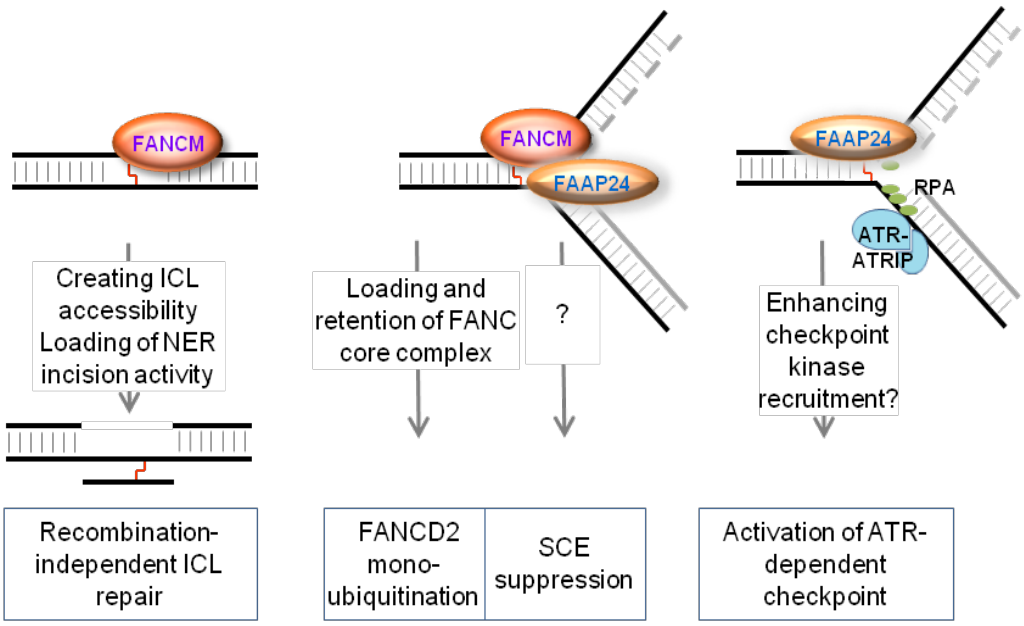
Working Model

Based on the above findings, I propose a model that depicts refined understanding of FANCM and FAAP24 in the maintenance of genomic stability (Fig 6.1). FANCM and FAAP24 protect cells from crosslinking damage via intercalated mechanisms - facilitating FA pathway activation, suppressing crossover recombination, activating ATR-mediated DNA damage checkpoint, and promoting recombination-independent ICL repair. When cells are individually devoid of FANCM or FAAP24, the FA pathway activation is partially disabled and the SCE phenotype manifests, accompanied by impaired recombination-independent ICL repair or DNA damage checkpoint control, respectively, resulting in intermediate sensitivities to ICL damage. When FANCM and FAAP24 are both absent, the compounded defects in FA pathway activation, DNA damage checkpoint control and recombination-independent ICL repair is expected to cause significantly increased sensitivity.

www.manaraa.com

Fig. 6.1 Coordinated and non-epistatic functions of FANCM and FAAP24 in response to DNA interstrand crosslink damage.

www.manaraa.com



Wild type	✓	✓	✓	Normal resistance
FANCM$^{-/-}$	×	×	✓	Intermediate sensitivity
FAAP24$^{-/-}$	✓	×	×	Intermediate sensitivity
FANCM$^{-/-}$ *FAAP24*$^{-/-}$	×	×	×	Severe sensitivity

www.manaraa.com

Future Studies

Structure-function analysis of FAAP24

As was described in Chapter IV, I found that the function of FAAP24 in ICL-induced ATR-mediated checkpoint activation was independent of its interaction with FANCM (Fig 4.5). It would be interesting to determine whether and how the FANCM-associated FA pathway activation function and the FANCM-independent checkpoint activation function of FAAP24 are segregated. To this end, I initiated a structure-function analysis of FAAP24.

FAAP24 contains an ERCC4 domain and an $(HhH)_2$ domain. The conserved ERKXXXD motif in the ERCC4 domain is degenerated to LYVTEAD in FAAP24, and consequently FAAP24 was not found to possess any nuclease activity. However, whether the degenerated ERCC4 domain remains important for FAAP24 function is not clear. $(HhH)_2$ motifs are present in many DNA-binding proteins, and are found to be important for dimerization of XPF-ERCC1. Whether the $(HhH)_2$ domain of FAAP24 mediates FANCM interaction and confers DNA binding activities needs to be determined.

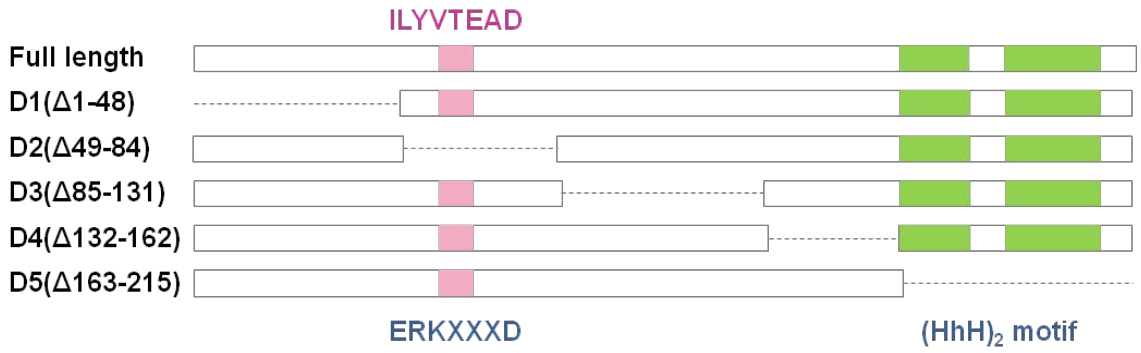
I first generated a series of FAAP24 truncation mutants (Fig 6.2A) to determine which domain of FAAP24 was responsible for FANCM interaction. As shown by Co-IP experiments (Fig 6.2B), the $(HhH)_2$ domain of FAAP24 is required for its interaction with FANCM. Importantly, the $(HhH)_2$ domain was also required for its *in vitro* ssDNA binding activity (Biochemical experiments were performed by our collaborators and data were not shown here). These results suggest that the C-terminal $(HhH)_2$ domain of FAAP24 is important for both dimerization with FANCM and DNA binding.

www.manaraa.com

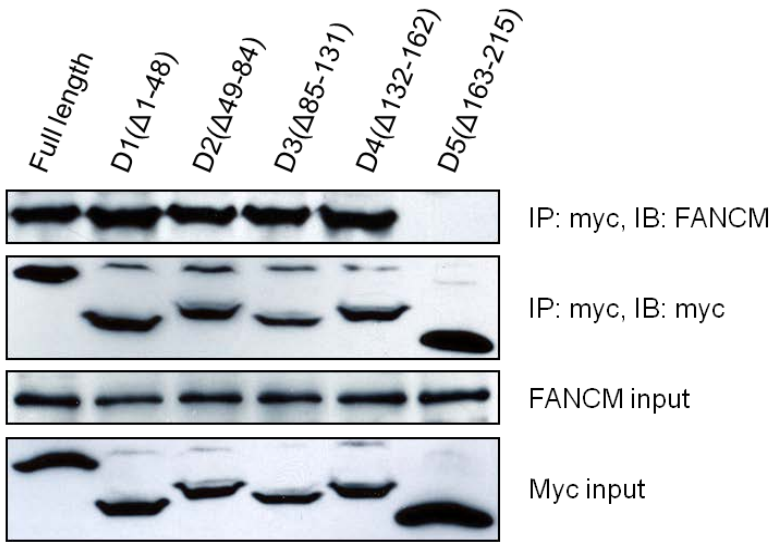
Fig. 6.2 The $(HhH)_2$ domain of FAAP24 is required for FANCM interaction.
(***A***) Schematics of FAAP24 truncation mutants. The conserved ERKXXXD motif in ERCC4-like domain critical for nuclease activity is degenerated to ILYVTEAD, as indicated in pink. The HhH motifs are indicated in green.
(***B***) Co-immunoprecipitation of Myc-tagged full length FAAP24 and the truncation mutants with FANCM.

www.manaraa.com

A



B



www.manaraa.com

To better understand the function of the $(HhH)_2$ domain of FAAP24, we collaborated with structural biologists and determined the C-terminal structure of FAAP24 (data not shown). I also generated a series of FAAP24 point mutants according to insights from sequence alignment (Fig 6.3) and structural data. We tested the FAAP24 mutants for FANCM interaction and DNA binding activity. I co-expressed the FAAP24 mutants along with wild type FANCM in HEK293T cells, and examined their interaction. As shown in Fig 6.4, replacement of the GVG, LLL, VVG and FF residues of FAAP24 resulted in severe disruption of its FANCM interaction. *In vitro* DNA binding assay performed by our collaborators showed that disruption of K171 and K173 residues significantly reduced the DNA binding activity of FAAP24 (data not shown).

www.manaraa.com

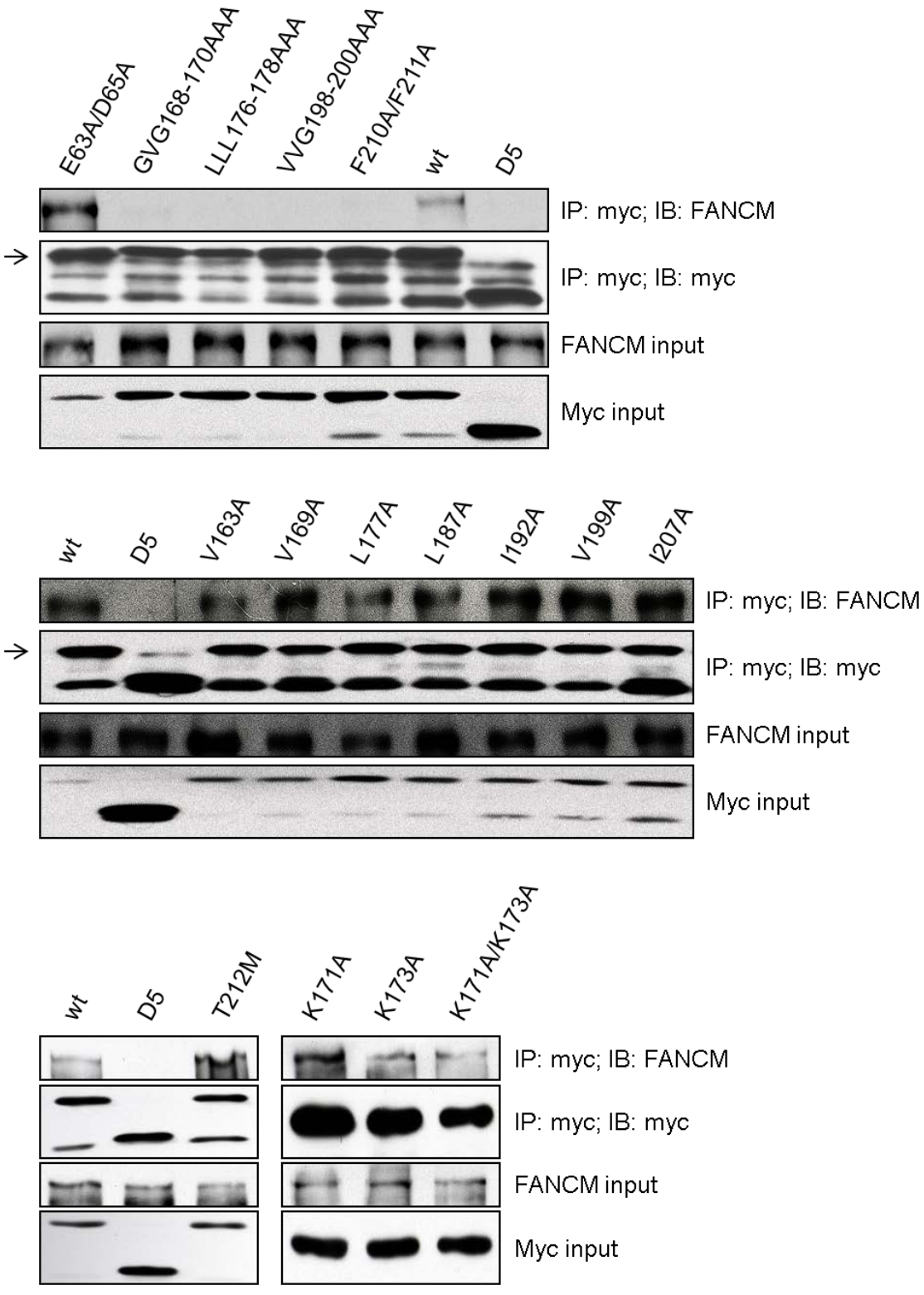
Fig. 6.3 Sequence alignment of the core ERCC4-like domain and $(HhH)_2$ domain across species. Locations of the degenerated ERKXXXD motif in ERCC4-like domain critical for nuclease activity and the conserved HhH motifs are indicated in blue. Mutation sites are indicated in red.

www.manaraa.com

www.manaraa.com

Fig. 6.4 Co-immunoprecipitation of Myc-tagged wild type FAAP24 and point mutants with FANCM.

www.manaraa.com



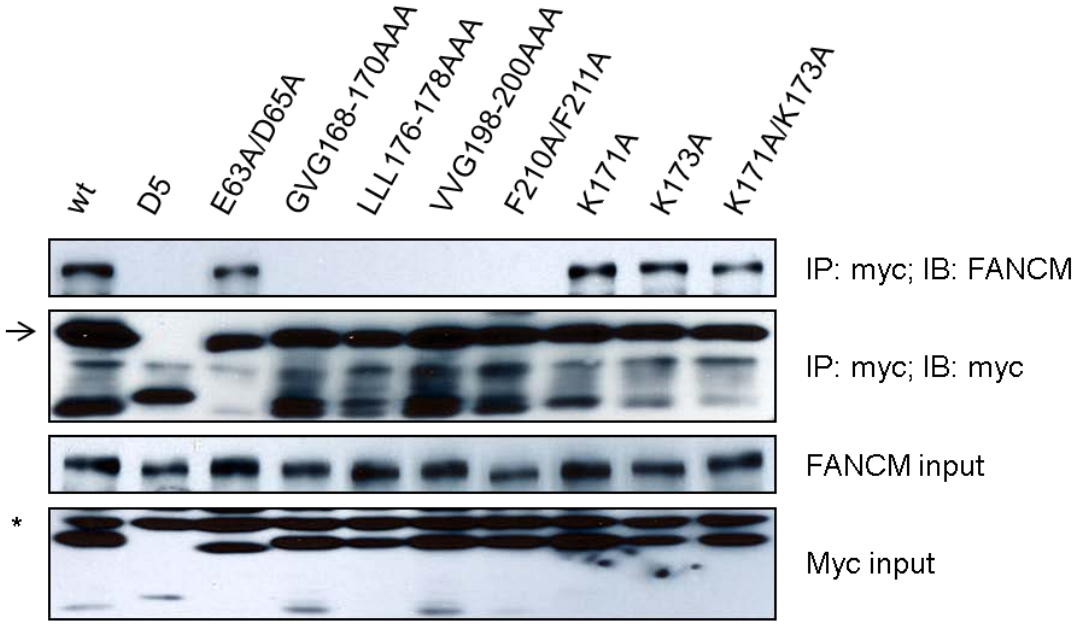
www.manaraa.com

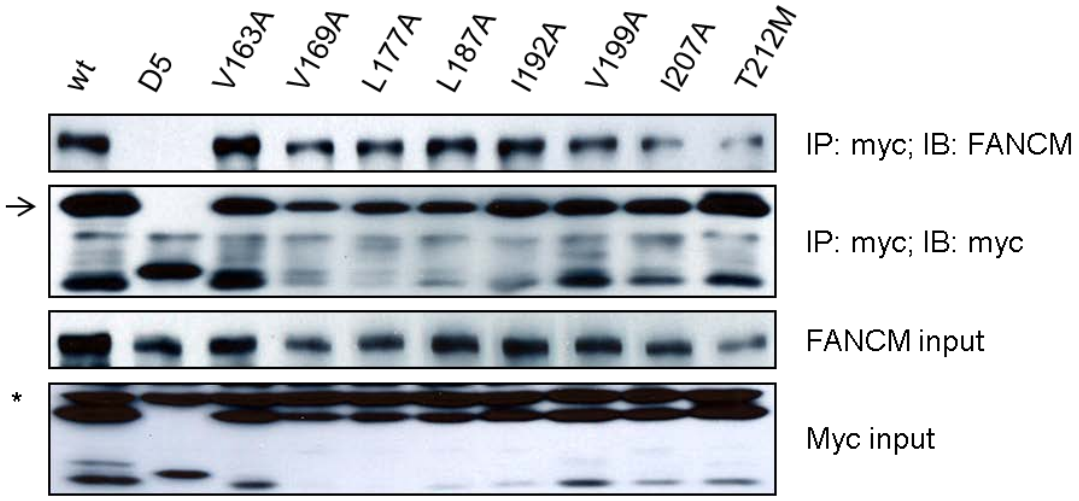
To allow *in vivo* functional studies, I introduced the FAAP24 mutants into the *FAAP24*$^{-/-}$ cells and generated a series of stable cell lines each carrying a unique FAAP24 mutation. Co-IP experiment using endogenous proteins confirmed that the GVG, LLL, VVG and FF residues are important for FAAP24 to dimerize with FANCM (Fig 6.5). I also tested the *in vivo* DNA binding activity of FAAP24 mutants by examining their chromatin association. As shown in Fig 6.6, chromatin fractionation experiments confirmed that the K171 and K173 residues are crucial for the DNA binding activity of FAAP24.

www.manaraa.com

Fig. 6.5 Co-immunoprecipitation of FAAP24 with FANCM in *FAAP24*$^{-/-}$ cells stably expressing Myc-tagged wild type FAAP24 and point mutants.

www.manaraa.com

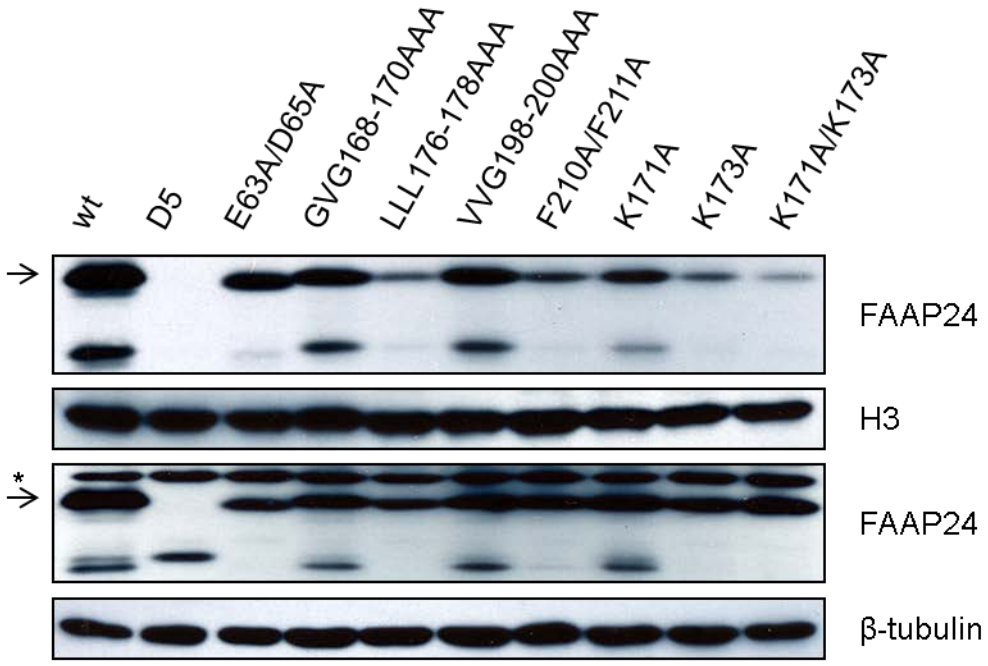


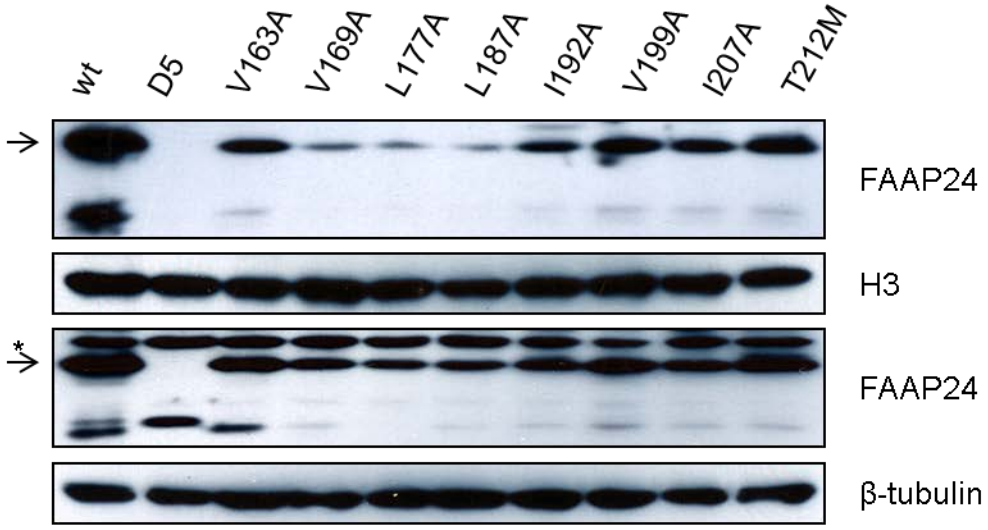


www.manaraa.com

Fig. 6.6 Chromatin association of FAAP24 in *FAAP24*$^{-/-}$ cells stably expressing Myc-tagged wild type FAAP24 and point mutants.

www.manaraa.com





www.manaraa.com

Future studies will be carried out to further understand how the FANCM interaction and DNA binding activity of FAAP24 contribute to ICL resistance and genomic stability maintenance. To this end, I will examine the stable cell lines carrying various FAAP24 mutations for their MMC sensitivity, chromosomal stability, FA pathway activation, SCE formation, and ATR-mediated checkpoint signaling. In addition, efforts will be taken to work with collaborators to determine the co-crystal structure of full length FAAP24 bound with FANCM C-terminal domain. Attempts will be made to include DNA molecules in the structural work. These studies are anticipated to provide additional valuable insights into the FAAP24 function on both structural and molecular/cellular levels.

Further investigation of the FANCM function in recombination-independent ICL repair

I demonstrated in Chapter V that the translocase activity of FANCM is important for its function in recombination-independent ICL repair (Fig 5.8B). How this DNA interacting activity allows FANCM to promote efficient repair of ICL through the recombination-independent pathway warrants further investigation. In the absence of FANCM translocase activity, recruitment of NER factors ERCC1 and XPA as well as lesion bypass factor Rev1 was severely impaired (Fig 5.9 & Fig 5.10). These results indicate the possibility that FANCM acts as an ICL damage sensor, and upon lesion detection it functions to create lesion accessibility to allow the recruitment of repair factors. Since in G1/G0 phase the highly packed chromatin structure presents obstacles for DNA access, it is likely that FANCM alters the chromatin/DNA structure, directly or otherwise, to allow efficient recruitment of other repair proteins.

www.manaraa.com

Interestingly, tandem affinity purification of FANCM identified that RuvbL1 and RuvbL2, two integral components of the INO80 chromatin remodeling complex, strongly associate with FANCM (Fig 6.7). Co-immunoprecipitation experiment using tagged proteins verified that FANCM, but not FAAP24, interacts with RuvbL1 and RuvbL2 (Figs 6.8A&B). This specific interaction was also verified by Co-IP of endogenous proteins (Fig 6.8C). Since RuvbL1 and RuvbL2 belong to the INO80 chromatin remodeling complex, it is likely that FANCM recruits the INO80 complex to remodel chromatin/DNA structure and therefore allows access of ICL repair proteins.

www.manaraa.com

Fig. 6.7 IP-mass spec analysis of FANCM-interacting proteins via tandem affinity purification. Numbers of peptides recovered from mass spectrometry analysis are presented.

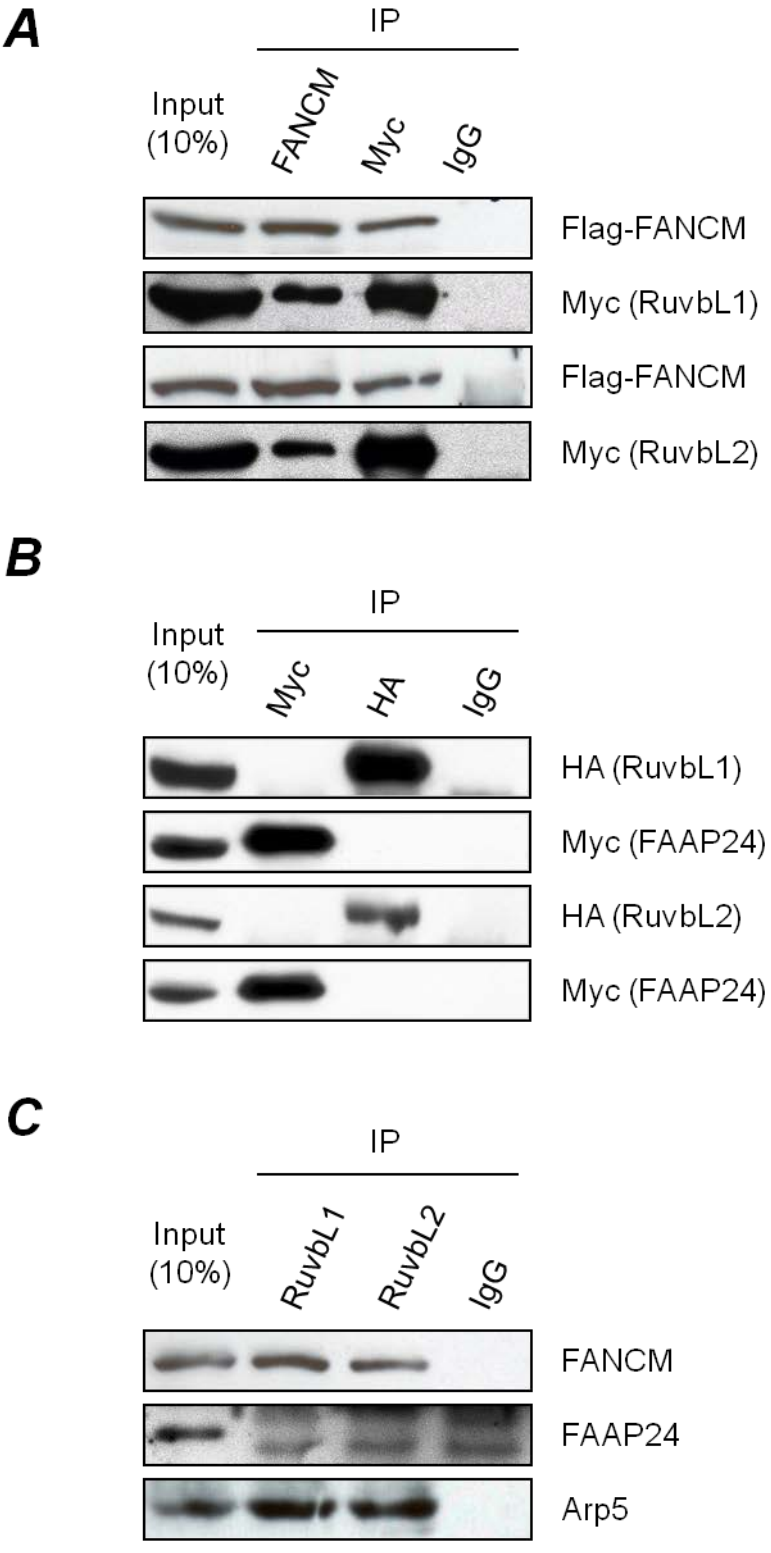
www.manaraa.com

Protein	# of peptide
FANCM	91
RUVBL2	18
RUVBL1	13
TOP3A	13
RMI1	11
BLM	9
FAAP16	8
FAAP24	7
FAAP10	3
RMI2	3
MSH6	2
FBXW11	2
POLDIP2	2
FANCC	1
MCM7	1
USP7	1
CEP55	1

www.manaraa.com

Fig. 6.8 FANCM but not FAAP24 interacts with RuvbL1 and RuvbL2.
(***A***) Co-immunoprecipition of Flag-FANCM with Myc-RuvbL1 or Myc-RuvbL2 in HEK293T cells.
(***B***) Co-immunoprecipition of Myc-FAAP24 with HA-RuvbL1 or HA-RuvbL2 in HEK 293T cells.
(***C***) Immunoprecipitation of endogenous RuvbL1 or RuvbL2 and immunoblot with antibodies against FANCM, FAAP24 and Arp5.

www.manaraa.com



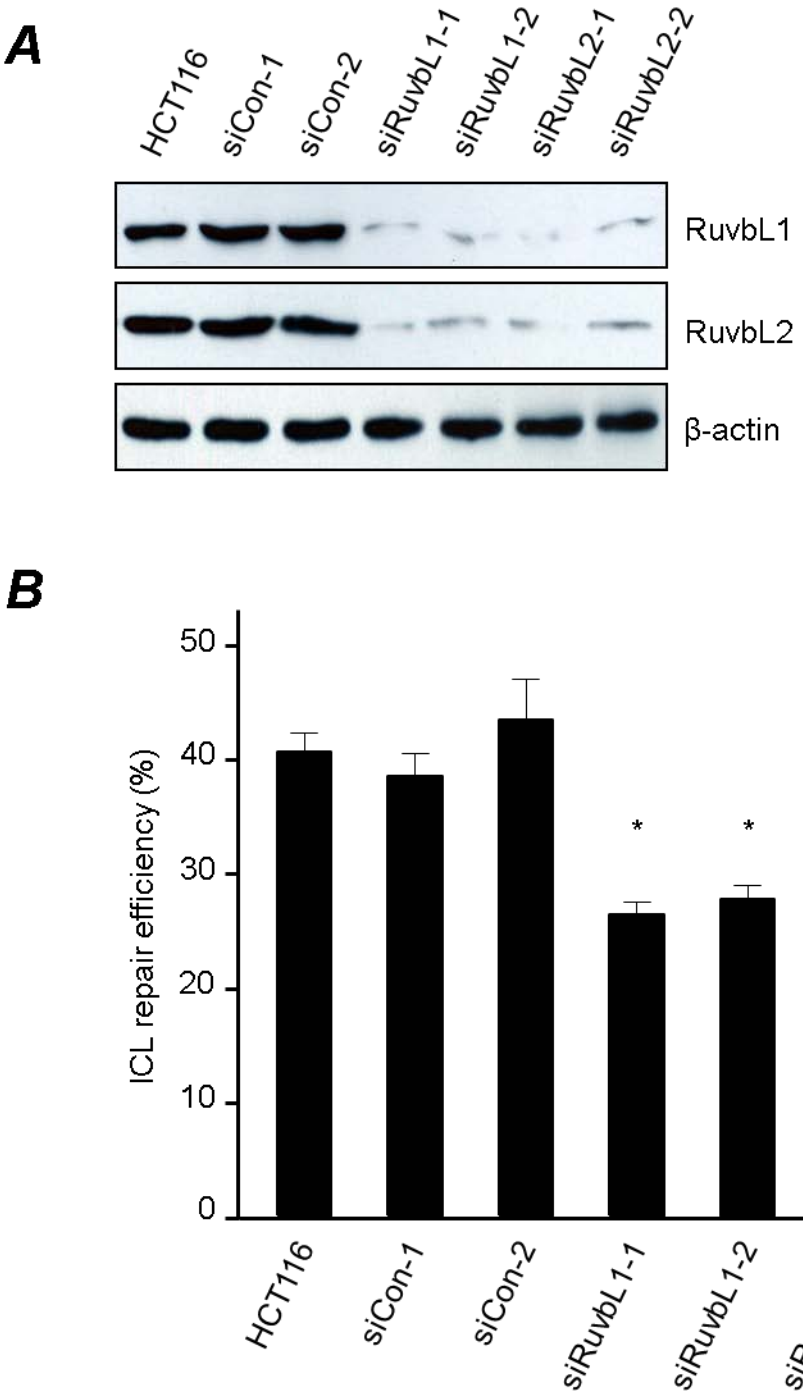
www.manaraa.com

To determine whether RuvbL1 and RuvbL2 indeed act in the recombination-independent ICL repair pathway, I established RuvbL1- and RuvbL2- shRNA stable knockdown cell lines (Fig 6.9A) to study their functional outcome in ICL repair. As shown in Fig 6.9B, lack of RuvbL1 or RuvbL2 rendered cells unable to efficiently remove ICL in a recombination-independent manner. In addition, loss of RuvbL1 or RuvbL2 significantly impaired the recruitment of ERCC1 to ICL sites (Fig 6.10).

www.manaraa.com

Fig. 6.9 RuvbL1 and RuvbL2 are required for recombination-independent ICL repair.
(***A***) Immunoblotting of RuvbL1 and RuvbL2 in HCT-116 cell clones stably transfected with constructs expressing control shRNA (siCon), RuvbL1 shRNA (siRuvbL1), or RuvbL2 shRNA (siRuvbL2).
(***B***) ICL repair efficiencies in RuvbL1- and RuvbL2- knockdown cells. Error bars represent standard deviation of 6 independent experiments (* $P < 0.05$ vs. wild type).

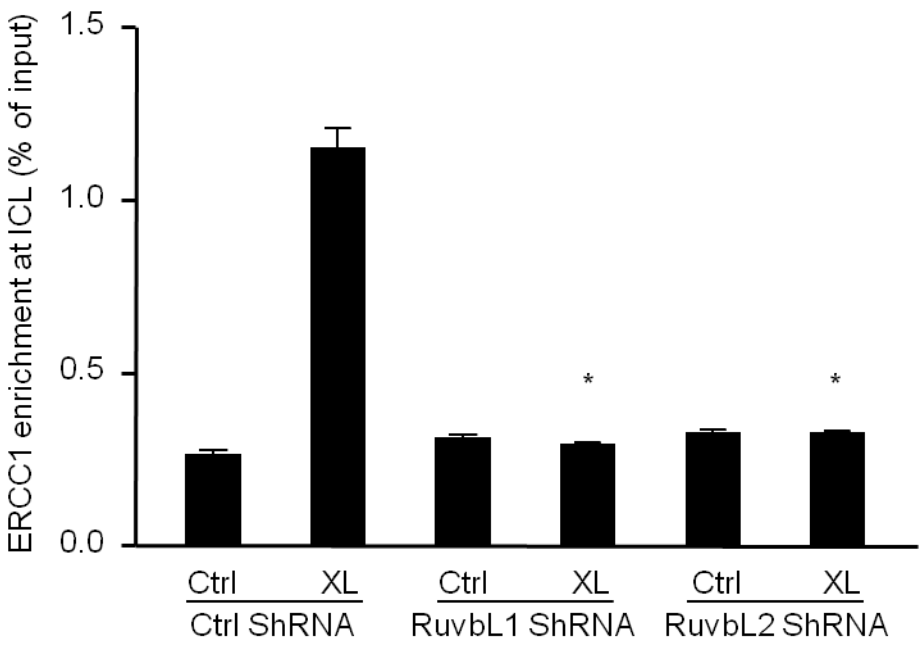
www.manaraa.com



www.manaraa.com

Fig. 6.10 eChIP assay for ERCC1 recruitment to a site-specific ICL in RuvbL1- and RuvbL2-knockdown cells. Ctrl: unmodified substrate. XL: crosslinked substrate. Percentages of relative enrichment were calculated by normalizing comparative concentration of each sample against that of its input. Error bars represent stand deviation of triplicates. The asterisks (*) denote $P < 0.01$ vs. Ctrl ShRNA.

www.manaraa.com



www.manaraa.com

While largely preliminary, these results strongly indicate that FANCM recruits chromatin remodeling activities upon ICL lesion detection, which will create DNA accessibility to allow recruitment of NER factors to initiate the ICL repair. Future studies will be conducted to provide further evidences to substantiate such a promising working model. Specifically, the following experiment will be performed: 1) to test whether RuvbL1/2 are indeed recruited to ICL sites using the eChIP assay, and to examine whether FANCM loss impairs the RuvbL1/2 recruitment; 2) to generate FANCM mutant(s) defective for RuvbL1/2 interaction, and to test whether such mutant(s) could restore ERCC1 recruitment and ICL repair deficiency of *FANCM*$^{-/-}$ cells; 3) to investigate whether other components of the INO80 complex are also involved in the recombination-independent repair of ICL. Collectively, these results will potentially uncover novel functions of FANCM and the INO80 complex in ICL repair.

Role of FANCM and FAAP24 in tumorigenesis

Since the knockout models I generated are on the HCT-116 cell platform, a further functional investigation of FANCM and FAAP24 in genomic stability maintenance can be achieved by examining their impact on tumorigenesis. To this end, I will use xenograft models to study the tumorigenicity of FANCM and FAAP24 knockout cells along with wild type HCT-116 cells. While a short term assay of tumor formation can be potentially affected by the impact of FANCM and/or FAAP24 loss on cell proliferation and/or survival, it may still provide valuable information in terms of the roles of FANCM and FAAP24 in tumor development.

The FA pathway is important for ICL repair. Therefore, platinum based therapeutics will be another emphasis in this study. Whether loss of FANCM and FAAP24 affects the

www.manaraa.com

sensitivity of tumor cells to platinum treatment is of clinical interest. If dysfunction of FANCM or FAAP24 leads to increased response rate of the colon cancer cells to platinum treatment, two implications will be obtained. First, although FANCM and FAAP24 mutations have not been identified in FA patients, minor functional alterations of FANCM and FAAP24 may be possible in colon cancer patients. In these patients, platinum based chemotherapy may be emphasized. Second, small molecule inhibitors against FANCM and FAAP24 will then be of clinical value in colon cancer treatment.

Given the roles of FANCM and FAAP24 in maintaining genome stability, it would also be interesting to ask whether loss of FANCM and FAAP24 will affect the genomic stability of cancer cells during tumor development. To this end, a long term follow up of xenograft tumors will be carried out. By dissecting tumors at later stages during development and looking for secondary genetic changes in the cells, new information of FANCM and FAAP24 function in genomic stability maintenance may be discovered.

In summary, my current and extending studies provide novel mechanistic insights into how FANCM and FAAP24 maintain genomic stability through cooperative as well as unique functions.

www.manaraa.com

BIBLIOGRAPHY

Ali, A.M., Pradhan, A., Singh, T.R., Du, C., Li, J., Wahengbam, K., Grassman, E., Auerbach, A.D., Pang, Q., and Meetei, A.R. (2012). FAAP20: a novel ubiquitin-binding FA nuclear core-complex protein required for functional integrity of the FA-BRCA DNA repair pathway. Blood *119*, 3285-3294.

Alter, B.P., Greene, M.H., Velazquez, I., and Rosenberg, P.S. (2003). Cancer in Fanconi anemia. Blood *101*, 2072.

Auerbach, A.D. (1993). Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Exp Hematol *21*, 731-733.

Bakker, S.T., van de Vrugt, H.J., Rooimans, M.A., Oostra, A.B., Steltenpool, J., Delzenne-Goette, E., van der Wal, A., van der Valk, M., Joenje, H., te Riele, H., and de Winter, J.P. (2009). Fancm-deficient mice reveal unique features of Fanconi anemia complementation group M. Hum Mol Genet *18*, 3484-3495.

Blackford, A.N., Schwab, R.A., Nieminuszczy, J., Deans, A.J., West, S.C., and Niedzwiedz, W. (2012). The DNA translocase activity of FANCM protects stalled replication forks. Hum Mol Genet *21*, 2005-2016.

www.manaraa.com

Burrows, A.E., and Elledge, S.J. (2008). How ATR turns on: TopBP1 goes on ATRIP with ATR. Genes & development *22*, 1416-1421.

Ciccia, A., Ling, C., Coulthard, R., Yan, Z., Xue, Y., Meetei, A.R., Laghmani el, H., Joenje, H., McDonald, N., de Winter, J.P., *et al.* (2007). Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. Mol Cell *25*, 331-343.

Ciccia, A., McDonald, N., and West, S.C. (2008). Structural and functional relationships of the XPF/MUS81 family of proteins. Annu Rev Biochem *77*, 259-287.

Collis, S.J., Ciccia, A., Deans, A.J., Horejsi, Z., Martin, J.S., Maslen, S.L., Skehel, J.M., Elledge, S.J., West, S.C., and Boulton, S.J. (2008). FANCM and FAAP24 function in ATR-mediated checkpoint signaling independently of the Fanconi anemia core complex. Mol Cell *32*, 313-324.

Crismani, W., Girard, C., Froger, N., Pradillo, M., Santos, J.L., Chelysheva, L., Copenhaver, G.P., Horlow, C., and Mercier, R. (2012). FANCM limits meiotic crossovers. Science *336*, 1588-1590.

Crossan, G.P., and Patel, K.J. (2012). The Fanconi anaemia pathway orchestrates incisions at sites of crosslinked DNA. J Pathol *226*, 326-337.

www.manaraa.com

Crossan, G.P., van der Weyden, L., Rosado, I.V., Langevin, F., Gaillard, P.H., McIntyre, R.E., Gallagher, F., Kettunen, M.I., Lewis, D.Y., Brindle, K., *et al.* (2011). Disruption of mouse Slx4, a regulator of structure-specific nucleases, phenocopies Fanconi anemia. Nat Genet *43*, 147-152.

D'Andrea, A.D. (2010). Susceptibility pathways in Fanconi's anemia and breast cancer. N Engl J Med *362*, 1909-1919.

Deans, A.J., and West, S.C. (2009). FANCM connects the genome instability disorders Bloom's Syndrome and Fanconi Anemia. Mol Cell *36*, 943-953.

Deans, A.J., and West, S.C. (2011). DNA interstrand crosslink repair and cancer. Nat Rev Cancer *11*, 467-480.

Gari, K., Decaillet, C., Delannoy, M., Wu, L., and Constantinou, A. (2008a). Remodeling of DNA replication structures by the branch point translocase FANCM. Proc Natl Acad Sci U S A *105*, 16107-16112.

Gari, K., Decaillet, C., Stasiak, A.Z., Stasiak, A., and Constantinou, A. (2008b). The Fanconi anemia protein FANCM can promote branch migration of Holliday junctions and replication forks. Mol Cell *29*, 141-148.

www.manaraa.com

German, J., Schonberg, S., Caskie, S., Warburton, D., Falk, C., and Ray, J.H. (1987). A test for Fanconi's anemia. Blood *69*, 1637-1641.

Hara, R., Mo, J., and Sancar, A. (2000). DNA Damage in the Nucleosome Core Is Refractory to Repair by Human Excision Nuclease. Mol. Cell. Biol. *20*, 9173-9181.

Hoadley, K.A., Xue, Y., Ling, C., Takata, M., Wang, W., and Keck, J.L. (2012). Defining the molecular interface that connects the Fanconi anemia protein FANCM to the Bloom syndrome dissolvasome. Proc Natl Acad Sci U S A *109*, 4437-4442.

Horejsi, Z., Collis, S.J., and Boulton, S.J. (2009). FANCM-FAAP24 and HCLK2: roles in ATR signalling and the Fanconi anemia pathway. Cell Cycle *8*, 1133-1137.

Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., *et al.* (2002). Biallelic inactivation of BRCA2 in Fanconi anemia. Science *297*, 606-609.

Huang, M., Kim, J.M., Shiotani, B., Yang, K., Zou, L., and D'Andrea, A.D. (2010). The FANCM/FAAP24 complex is required for the DNA interstrand crosslink-induced checkpoint response. Mol Cell *39*, 259-268.

Kee, Y., and D'Andrea, A.D. (2010). Expanded roles of the Fanconi anemia pathway in preserving genomic stability. Genes Dev *24*, 1680-1694.

www.manaraa.com

Kee, Y., Kim, J.M., and D'Andrea, A.D. (2009). Regulated degradation of FANCM in the Fanconi anemia pathway during mitosis. Genes Dev *23*, 555-560.

Kelsall, I.R., Langenick, J., MacKay, C., Patel, K.J., and Alpi, A.F. (2012). The Fanconi anaemia components UBE2T and FANCM are functionally linked to nucleotide excision repair. PLoS One *7*, e36970.

Kim, H., Yang, K., Dejsuphong, D., and D'Andrea, A.D. (2012). Regulation of Rev1 by the Fanconi anemia core complex. Nat Struct Mol Biol *19*, 164-170.

Kim, J.M., Kee, Y., Gurtan, A., and D'Andrea, A.D. (2008). Cell cycle-dependent chromatin loading of the Fanconi anemia core complex by FANCM/FAAP24. Blood *111*, 5215-5222.

Kim, Y., Lach, F.P., Desetty, R., Hanenberg, H., Auerbach, A.D., and Smogorzewska, A. (2011). Mutations of the SLX4 gene in Fanconi anemia. Nat Genet *43*, 142-146.

Kitao, H., and Takata, M. (2011). Fanconi anemia: a disorder defective in the DNA damage response. Int J Hematol *93*, 417-424.

Knipscheer, P., Raschle, M., Smogorzewska, A., Enoiu, M., Ho, T.V., Scharer, O.D., Elledge, S.J., and Walter, J.C. (2009). The Fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. Science *326*, 1698-1701.

www.manaraa.com

Knoll, A., Higgins, J.D., Seeliger, K., Reha, S.J., Dangel, N.J., Bauknecht, M., Schropfer, S., Franklin, F.C., and Puchta, H. (2012). The Fanconi anemia ortholog FANCM ensures ordered homologous recombination in both somatic and meiotic cells in Arabidopsis. Plant Cell *24*, 1448-1464.

Kratz, K., Schopf, B., Kaden, S., Sendoel, A., Eberhard, R., Lademann, C., Cannavo, E., Sartori, A.A., Hengartner, M.O., and Jiricny, J. (2010). Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. Cell *142*, 77-88.

Kutler, D.I., Singh, B., Satagopan, J., Batish, S.D., Berwick, M., Giampietro, P.F., Hanenberg, H., and Auerbach, A.D. (2003). A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood *101*, 1249-1256.

Leung, J.W., Wang, Y., Fong, K.W., Huen, M.S., Li, L., and Chen, J. (2012). Fanconi anemia (FA) binding protein FAAP20 stabilizes FA complementation group A (FANCA) and participates in interstrand cross-link repair. Proc Natl Acad Sci U S A *109*, 4491-4496.

Levitus, M., Waisfisz, Q., Godthelp, B.C., de Vries, Y., Hussain, S., Wiegant, W.W., Elghalbzouri-Maghrani, E., Steltenpool, J., Rooimans, M.A., Pals, G., *et al.* (2005). The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet *37*, 934-935.

www.manaraa.com

Ling, C., Ishiai, M., Ali, A.M., Medhurst, A.L., Neveling, K., Kalb, R., Yan, Z., Xue, Y., Oostra, A.B., Auerbach, A.D., *et al.* (2007). FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. Embo J *26*, 2104-2114.

Litman, R., Peng, M., Jin, Z., Zhang, F., Zhang, J., Powell, S., Andreassen, P.R., and Cantor, S.B. (2005). BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. Cancer Cell *8*, 255-265.

Liu, T., Ghosal, G., Yuan, J., Chen, J., and Huang, J. (2010). FAN1 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. Science *329*, 693-696.

Long, D.T., Raschle, M., Joukov, V., and Walter, J.C. (2011). Mechanism of RAD51-dependent DNA interstrand cross-link repair. Science *333*, 84-87.

Lorenz, A., Osman, F., Sun, W., Nandi, S., Steinacher, R., and Whitby, M.C. (2012). The fission yeast FANCM ortholog directs non-crossover recombination during meiosis. Science *336*, 1585-1588.

Luke-Glaser, S., Luke, B., Grossi, S., and Constantinou, A. (2009). FANCM regulates DNA chain elongation and is stabilized by S-phase checkpoint signalling. EMBO J *29*, 795-805.

MacKay, C., Declais, A.C., Lundin, C., Agostinho, A., Deans, A.J., MacArtney, T.J., Hofmann, K., Gartner, A., West, S.C., Helleday, T., *et al.* (2010). Identification of

www.manaraa.com

KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. Cell *142*, 65-76.

Meetei, A.R., de Winter, J.P., Medhurst, A.L., Wallisch, M., Waisfisz, Q., van de Vrugt, H.J., Oostra, A.B., Yan, Z., Ling, C., Bishop, C.E., *et al.* (2003). A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet *35*, 165-170.

Meetei, A.R., Medhurst, A.L., Ling, C., Xue, Y., Singh, T.R., Bier, P., Steltenpool, J., Stone, S., Dokal, I., Mathew, C.G., *et al.* (2005). A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet *37*, 958-963.

Meetei, A.R., Yan, Z., and Wang, W. (2004). FANCL replaces BRCA1 as the likely ubiquitin ligase responsible for FANCD2 monoubiquitination. Cell Cycle *3*, 179-181.

Moldovan, G.L., and D'Andrea, A.D. (2009). How the fanconi anemia pathway guards the genome. Annu Rev Genet *43*, 223-249.

Mosedale, G., Niedzwiedz, W., Alpi, A., Perrina, F., Pereira-Leal, J.B., Johnson, M., Langevin, F., Pace, P., and Patel, K.J. (2005). The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. Nat Struct Mol Biol *12*, 763-771.

Parmar, K., D'Andrea, A., and Niedernhofer, L.J. (2009). Mouse models of Fanconi anemia. Mutat Res *668*, 133-140.

www.manaraa.com

Poll, E.H., Arwert, F., Joenje, H., and Wanamarta, A.H. (1985). Differential sensitivity of Fanconi anaemia lymphocytes to the clastogenic action of cis-diamminedichloroplatinum (II) and trans-diamminedichloroplatinum (II). Hum Genet *71*, 206-210.

Prakash, R., Satory, D., Dray, E., Papusha, A., Scheller, J., Kramer, W., Krejci, L., Klein, H., Haber, J.E., Sung, P., and Ira, G. (2009). Yeast Mph1 helicase dissociates Rad51-made D-loops: implications for crossover control in mitotic recombination. Genes Dev *23*, 67-79.

Reid, S., Schindler, D., Hanenberg, H., Barker, K., Hanks, S., Kalb, R., Neveling, K., Kelly, P., Seal, S., Freund, M., *et al.* (2007). Biallelic mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet *39*, 162-164.

Rosado, I.V., Niedzwiedz, W., Alpi, A.F., and Patel, K.J. (2009). The Walker B motif in avian FANCM is required to limit sister chromatid exchanges but is dispensable for DNA crosslink repair. Nucleic Acids Res *37*, 4360-4370.

Schwab, R.A., Blackford, A.N., and Niedzwiedz, W. (2010). ATR activation and replication fork restart are defective in FANCM-deficient cells. EMBO J *29*, 806-818.

Shen, X., Do, H., Li, Y., Chung, W.H., Tomasz, M., de Winter, J.P., Xia, B., Elledge, S.J., Wang, W., and Li, L. (2009). Recruitment of fanconi anemia and breast cancer proteins to DNA damage sites is differentially governed by replication. Mol Cell *35*, 716-723.

www.manaraa.com

Shen, X., Jun, S., O'Neal, L.E., Sonoda, E., Bemark, M., Sale, J.E., and Li, L. (2006). REV3 and REV1 play major roles in recombination-independent repair of DNA interstrand cross-links mediated by monoubiquitinated proliferating cell nuclear antigen (PCNA). J Biol Chem *281*, 13869-13872.

Singh, T.R., Bakker, S.T., Agarwal, S., Jansen, M., Grassman, E., Godthelp, B.C., Ali, A.M., Du, C.H., Rooimans, M.A., Fan, Q., *et al.* (2009). Impaired FANCD2 monoubiquitination and hypersensitivity to camptothecin uniquely characterize Fanconi anemia complementation group M. Blood *114*, 174-180.

Singh, T.R., Saro, D., Ali, A.M., Zheng, X.F., Du, C.H., Killen, M.W., Sachpatzidis, A., Wahengbam, K., Pierce, A.J., Xiong, Y., *et al.* (2010). MHF1-MHF2, a histone-fold-containing protein complex, participates in the Fanconi anemia pathway via FANCM. Mol Cell *37*, 879-886.

Smogorzewska, A., Desetty, R., Saito, T.T., Schlabach, M., Lach, F.P., Sowa, M.E., Clark, A.B., Kunkel, T.A., Harper, J.W., Colaiacovo, M.P., and Elledge, S.J. (2010). A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. Mol Cell *39*, 36-47.

Smogorzewska, A., Matsuoka, S., Vinciguerra, P., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Ballif, B.A., Gygi, S.P., Hofmann, K., D'Andrea, A.D., and Elledge, S.J. (2007).

www.manaraa.com

Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. Cell *129*, 289-301.

Stoepker, C., Hain, K., Schuster, B., Hilhorst-Hofstee, Y., Rooimans, M.A., Steltenpool, J., Oostra, A.B., Eirich, K., Korthof, E.T., Nieuwint, A.W., *et al.* (2011). SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. Nat Genet *43*, 138-141.

Sun, W., Nandi, S., Osman, F., Ahn, J.S., Jakovleska, J., Lorenz, A., and Whitby, M.C. (2008). The FANCM ortholog Fml1 promotes recombination at stalled replication forks and limits crossing over during DNA double-strand break repair. Mol Cell *32*, 118-128.

Thompson, L.H., and Hinz, J.M. (2009). Cellular and molecular consequences of defective Fanconi anemia proteins in replication-coupled DNA repair: mechanistic insights. Mutat Res *668*, 54-72.

Vaz, F., Hanenberg, H., Schuster, B., Barker, K., Wiek, C., Erven, V., Neveling, K., Endt, D., Kesterton, I., Autore, F., *et al.* (2010). Mutation of the RAD51C gene in a Fanconi anemia-like disorder. Nat Genet *42*, 406-409.

Wang, W. (2007). Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet *8*, 735-748.

www.manaraa.com

Wang, X., Peterson, C.A., Zheng, H., Nairn, R.S., Legerski, R.J., and Li, L. (2001). Involvement of nucleotide excision repair in a recombination-independent and error-prone pathway of DNA interstrand cross-link repair. Mol Cell Biol *21*, 713-720.

Wang, X., Zou, L., Lu, T., Bao, S., Hurov, K.E., Hittelman, W.N., Elledge, S.J., and Li, L. (2006). Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. Mol Cell *23*, 331-341.

Wang, X., Zou, L., Zheng, H., Wei, Q., Elledge, S.J., and Li, L. (2003). Genomic instability and endoreduplication triggered by RAD17 deletion. Genes Dev *17*, 965-970.

Wang, Y., and Li, L. (2011). Detecting recruitment of DNA damage response factors through the eChIP approach. Methods Mol Biol *782*, 245-255.

Xia, B., Dorsman, J.C., Ameziane, N., de Vries, Y., Rooimans, M.A., Sheng, Q., Pals, G., Errami, A., Gluckman, E., Llera, J., *et al.* (2007). Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nature genetics *39*, 159-161.

Xue, Y., Li, Y., Guo, R., Ling, C., and Wang, W. (2008). FANCM of the Fanconi anemia core complex is required for both monoubiquitination and DNA repair. Hum Mol Genet *17*, 1641-1652.

www.manaraa.com

Yan, Z., Delannoy, M., Ling, C., Daee, D., Osman, F., Muniandy, P.A., Shen, X., Oostra, A.B., Du, H., Steltenpool, J., *et al.* (2010). A histone-fold complex and FANCM form a conserved DNA-remodeling complex to maintain genome stability. Mol Cell *37*, 865-878.

Yan, Z., Guo, R., Paramasivam, M., Shen, W., Ling, C., Fox, D., 3rd, Wang, Y., Oostra, A.B., Kuehl, J., Lee, D.Y., *et al.* (2012). A ubiquitin-binding protein, FAAP20, links RNF8-mediated ubiquitination to the Fanconi anemia DNA repair network. Mol Cell *47*, 61-75.

Yin, J., Sobeck, A., Xu, C., Meetei, A.R., Hoatlin, M., Li, L., and Wang, W. (2005). BLAP75, an essential component of Bloom's syndrome protein complexes that maintain genome integrity. EMBO J *24*, 1465-1476.

Zhang, X., Guo, C., Chen, Y., Shulha, H.P., Schnetz, M.P., LaFramboise, T., Bartels, C.F., Markowitz, S., Weng, Z., Scacheri, P.C., and Wang, Z. (2008). Epitope tagging of endogenous proteins for genome-wide ChIP-chip studies. Nat Methods *5*, 163-165.

Zheng, H., Wang, X., Warren, A.J., Legerski, R.J., Nairn, R.S., Hamilton, J.W., and Li, L. (2003). Nucleotide excision repair- and polymerase eta-mediated error-prone removal of mitomycin C interstrand cross-links. Mol Cell Biol *23*, 754-761.

www.manaraa.com

VITA

Yucai Wang was born in Taizhou, Jiangsu, People's Republic of China on August 21st, 1981. After attending Taizhou High School, he entered Nanjing University in Jiangsu, P. R. China in September 2000. He studied in Medical School, and received a degree of Bachelor of Medicine in June 2005 and a degree of Master of Medicine in June 2007. He then came to the United States and became a graduate student in The University of Texas Graduate School of Biomedical Sciences in Houston in August 2007. He joined Dr. Lei Li's laboratory in The University of Texas M. D. Anderson Cancer center in June 2008 for PhD studies.

www.manaraa.com