THE SNM1B NUCLEASE PLAYS IMPORTANT ROLES IN MAINTAINING GENOME STABILITY

by

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Dedicated in loving memory

To my grandpa,

Domonic Barberis

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Table of Contents

List of Figures

Figure

List of Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and rad3 related
ATRIP	ATR interacting protein-
β-CASP	Beta-Csp1, Artemis, Snm1, Pso2
BLM	Bloom helicase
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
DCLRE1B	DNA crosslink repair enzyme 1B
DSB	double strand break
EME1	Essential meiotic endonuclease 1
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
FA	Fanconi anemia
FAAP24	Fanconi anemia-associated protein, 24 kDa-
FANCD2	Fanconi anemia complementation group D2
FANCI	Fanconi anemia complementation group I
FANCJ	Fanconi anemia complementation group J
FANCM	Fanconi anemia complementation group
HH	Hoyeraal-Hreidarsson syndrome
ID	FANCI and FANCD2 complex
IR	ionizing radiation
γH2AX	phosphorylated histone variant 2AX
MBL	metallo-β-lactamase
MMC	Mytomycin C
MRN	MRE11-RAD50-NBS1 complex
MUS81	MMS and UV sensitive 81
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NS	non-silencing siRNA
PCNA	proliferating cell nuclear antigen
PIKK	phosphoinositide 3-kinase-related
RPA	Replication protein A
SMC1	Structural maintenance of chromosome protein 1
Snm1B	Senstive to nitrogen mustard 1B
ssDNA	single stranded DNA
TLS	Translesion synthesis

TRF2	Telomeric repeat binding factor 2
UV	Ultraviolet light
WRN	Werners syndrome
XPF	Xeodermun pigmentosum complementation group

Abstract

Efficient repair of damaged DNA is of critical importance to maintain genome integrity. Inherited mutations in repair genes result in genome instability disorders characterized by developmental defects and predisposition to cancer. Understanding the mechanisms underlying repair of DNA lesions will provide key insights into how genome stability is maintained as well as how defective repair leads to human disease. This dissertation focuses on elucidating the roles of the Snm1B nuclease in the repair of spontaneous and induced DNA damage.

One of the most cytotoxic forms of DNA damage is the interstrand crosslink (ICL), which prevents strand separation and inhibits essential processes such as replication and transcription. ICL repair is complex and involves the coordination of many DNA repair pathways, including the Fanconia anemia (FA) pathway. FA is an inherited genome instability disorder characterized by cellular sensitivity to ICLs, developmental defects and cancer predisposition. There are 13 FA complementation groups; however, the functions of the FA factors as well as the mechanism of ICL repair remain poorly understood.

The studies described in this thesis provide insights into the regulation of cellular responses to ICLs by Snm1B. Previous studies suggest Snm1B promotes replication fork collapse. I demonstrate that Snm1B is critical for activation of ATR, the central ICL signal transduction protein kinase. I establish that Snm1B depletion reduces

phosphorylation of ATR and demonstrate that Snm1B functions epistatically to the FA factor, FANCD2 and promotes efficient FANCD2 localization. These studies indicate that Snm1B has key roles in checkpoint signaling as well as in processing of the ICL. In addition, I demonstrate that Snm1B functions in the response to replication stress downstream of ATR activation.

This work has established important roles for Snm1B in protecting the genome from spontaneous and induced DNA damage during replication. The evidence presented here has identified multiple roles for Snm1B in DNA repair processes at the replication fork, both upstream and downstream of the ATR kinase. Together, these findings provide evidence that Snm1B is critical for ATR-mediated signaling and the FA pathway, and suggest that mutations in Snm1B may underlie human genome instability diseases such as Fanconi anemia.

Chapter 1

Introduction

The efficient repair of spontaneous or induced DNA damage is of critical importance to maintain genome stability. If mis-repaired or unrepaired, DNA damage can lead to an accumulation of mutations and/or chromosomal instability including deletions, insertions, and translocations, which can result in a number of detrimental phenotypic outcomes including cell death, growth defects, developmental abnormalities and cancer predisposition. Understanding the cellular mechanisms that result in efficient repair of DNA damage is critical to our understanding of how genome integrity is maintained. Cells have evolved several different pathways to repair distinct types of damage. The biological importance of DNA repair pathways is highlighted by the numerous inherited genome instability disorders caused by mutations in one of the 150 known DNA repair genes. These patients exhibit a plethora of phenotypes including mental retardation, developmental defects, immunodeficiency, and predisposition to cancer. Therefore, a comprehensive understanding of the cellular responses to DNA damage is not only critical for our fundamental understanding of the maintenance of genome stability, but also for understanding how defects in DNA damage signaling results in human disease.

DNA damage response

To repair DNA lesions, cells activate a variety of cellular responses known collectively as the DNA damage response (Figure 1.1). Upon lesion recognition, cells activate cell cycle checkpoints that prevent the transmission of damage to daughter cells by halting cell cycle progression, allowing sufficient time to repair. In addition, the DNA damage response activates transcriptional programs, DNA repair pathways, and if the damage cannot be repaired, apoptosis to eliminate the cells from the population (1). Mutations in genes in the DNA damage response results in cellular sensitivity or decreased survival to DNA damaging agents. It is important to note that although the DNA damage response results in activation of distinct biological processes, they are intimately linked, as in a defect in one pathway often severely affects the function of the others. For example, deficiency in cell cycle checkpoints leads to repair defects because the cell does not have sufficient time to allow for repair to occur.

DNA repair pathways

In response to the wide array of DNA lesions, cells possess multiple pathways that repair distinct types of lesions (Figure 1.2). A subset of these pathways remove damaged bases or mismatches and involve repair of one strand of DNA. Chemical alterations of bases can occur due to by-products of normal metabolism and are repaired by base excision repair (2). Helix distorting lesions such as cyclobutane pyrimidine dimers that are induced by ultraviolet light are repair by nulceotide exision repair. Mismatch repair deals with mismatches and small insertions and deletions that arise during replication (2). Each pathway involves removal of the damaged bases,

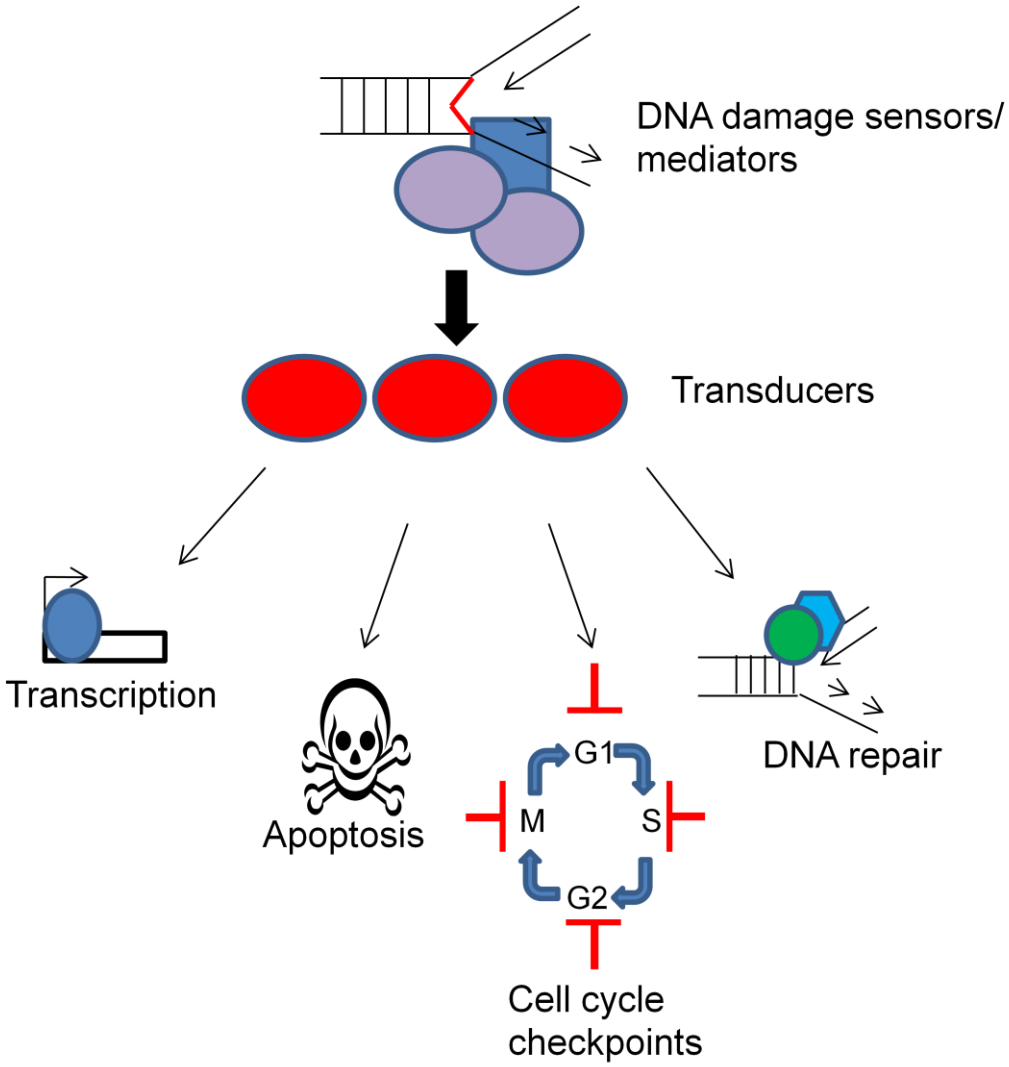


Figure 1.1. DNA damage response. Upon DNA damage recognition, a signaling transduction pathway involving sequential activation of proteins (sensors, mediators, transducers) is initiated leading to activation of multiple biological outcomes including transcriptional programs, apoptosis, cell cycle checkpoints, and DNA repair. Adapted from (3).

DNA repair mechanism	Lesions	Protein components	Human disease
Single strand lesion			
Mismatch repair	DNA mismatches, insertion/deletion loops	MSH2-MSH6, MSH2-MSH3, MLH1-PMS2, MLH1-PMS1	HNPCC
Nucleotide excision repair	Abnormal DNA bases, SSBs	XP family of proteins, CSA¸CSB	Xeroderma pigmentosum, Cockayne syndrome, RTS
Base excision repair	Lesions distorting helix, bulky base adducts, UV photo-products	DNA glycosylases, APEI endonucleases, FEN1	Adenomatous colorectal polyposis
Double strand lesions			
Non-homologous end joining	Double strand breaks	Ku70/80, DNAPKcs, Artemis, Ligase IV, XLF/Cerunnos, XRCC4	Lig4 syndrome RS-SCID
Homologous recombination	Double strand breaks	BRCA1, BRCA2, RAD51, RPA, WRN, BLM	Werners syndrome Bloom syndrome
Interstrand crosslink repair	Interstrand crosslinks	FA proteins TLS polymerases	Fanconi Anemia

Figure 1.2 DNA repair pathways. Cells possess multiple DNA repair pathways that respond to various types of DNA lesion. Summarized above are the major DNA repair pathways, the types of lesions repaired and major protein components of each pathway. Furthermore, human diseases resulting from defects in the different DNA repair pathways. Compiled from (4, 5).

followed by polymerase addition to fill in the resulting gap and ligation.

The remaining DNA repair pathways repair lesions affecting both strands of the double helix. Double strand breaks are considered one of the most cytotoxic lesions, with one double strand break being sufficient to kill a cell. DNA double strand breaks arise as a result of exogenous agents such as ionizing radiation or as a result of biological processes. Double strand breaks also can arise due to replication fork collapse. Furthermore, programmed double strand breaks are generated during biological processes such as V(D)J recombination and meiosis (2). If unrepaired, double strand breaks can lead to genomic rearrangements, including copy number variation that result from insertions and deletions and oncogenic translocations (6, 7). Double strand breaks are repaired through two main repair pathways: non-homologous end joining (NHEJ) and homologous recombination. NHEJ ligates the end of the DNA together without the use of homology. In contrast, homologous recombination utilizes homologous sequence to repair the double strand break and thus is largely restricted to S and G2 phase of the cell cycle (2).

Interstrand crosslink repair

One of the main goals of my thesis is to gain a better understanding into the mechanisms underlying repair of one of the most cytotoxic lesions that can arise in a cell, the interstrand crosslink. Interstrand crosslinks covalently link the two strands of the double helix together, thereby preventing essential processes such as transcription and replication. It is estimated that 20-40 unrepaired interstrand crosslinks are sufficient to kill a mammalian cell (8). The replication block induced by interstrand crosslinks is the cause of cytotoxicity (9). Cancer cells are highly proliferative, thus interstrand crosslink

inducing agents such as mitomycin C (MMC) are potent chemotherapeutic agents. However, tumors develop resistance to interstrand crosslink agents presumably by up regulation of repair pathways, but the mechanism of resistance is unclear (10). Therefore, understanding interstrand crosslink repair has implications for both cancer progression as well as therapeutics.

Interstrand crosslinks form as a result of both exogenous and endogenous agents. Interstrand crosslinks can form as byproducts of metabolic processes such as lipid peroxidation, or as a result of compounds such as formaldehyde (11). Furthermore, interstrand crosslinks are formed by chemotherapeutics such as cisplatin and MMC. These drugs are commonly used as reagents to study into the cellular response to interstrand crosslinks.

The repair of interstrand crosslinks is poorly understood partly due to the complexity of the drugs used in biological studies. Crosslinking agents form a wide variety of monoadducts, with interstrand crosslinks only representing a small fraction of the lesions generated (*e.g.*, 10% of lesions induced MMC), but evidence suggests that interstrand crosslinks are the cytotoxic lesions (10). Therefore, activation of a DNA repair pathway does not always reflect a role in the repair of interstrand crosslinks, but may be due to a role in repair of monoadducts. Furthermore, different crosslinking agents have varying effects on the DNA helix (Figure 1.3). For instance, cisplatin and psoralen cause high degrees of helix distortion. In contrast, interstrand crosslinks formed by MMC cause minimal distortion and do not affect base pairing (8). Therefore, although interstrand crosslink repair may occur by similar mechanisms, there may be

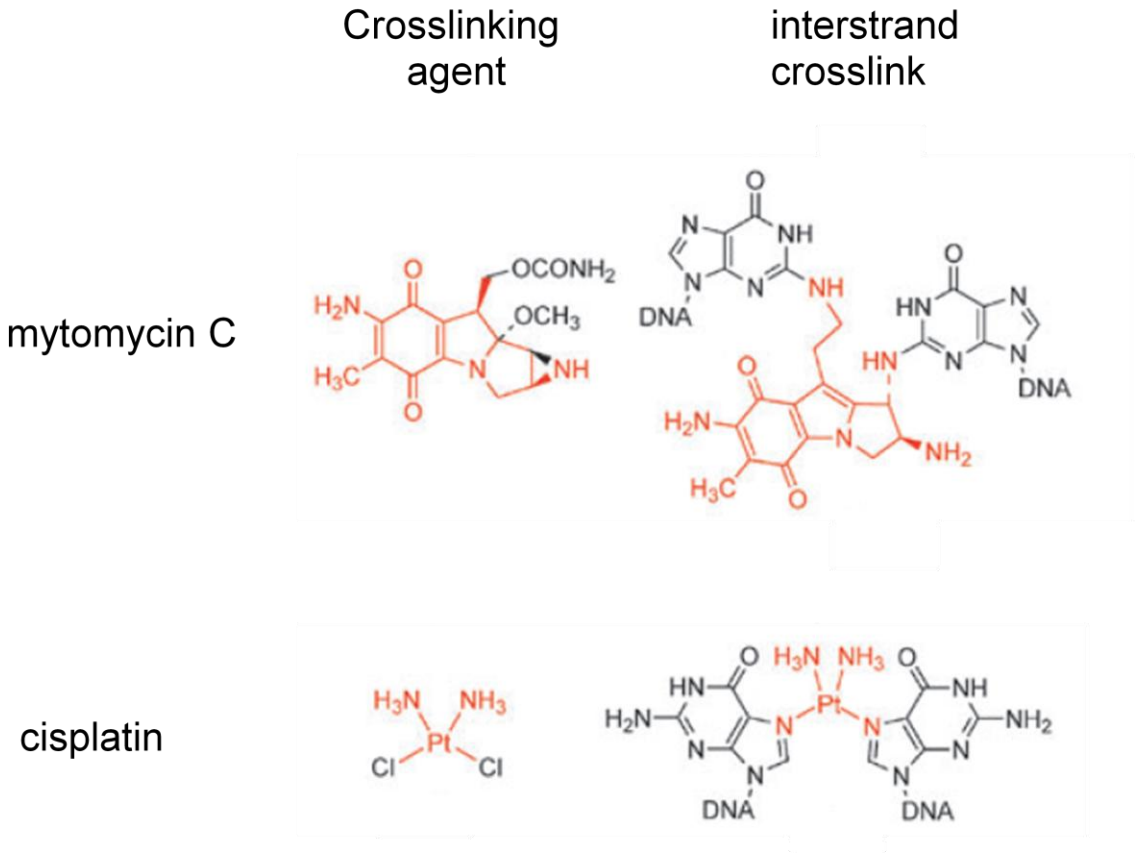


Figure 1.3. Diagrams of interstrand crosslinks. Top. Mytomycin C induced interstrand crosslink. Bottom. Cisplatin induced interstrand crosslink . The portion of the interstrand crosslink that interacts with DNA is depicted in red. Adapted from (11).

different modes of recognition of DNA structures and activation of cellular responses to one crosslinking drug may differ from another.

Replication-dependent repair of interstrand crosslinks

Although recent advances have been made, the molecular events leading to repair of interstrand crosslinks are poorly understood. Interstrand crosslinks present a unique challenge to cells because covalent linkage of the strands eliminates the possibility of using the opposing strand as a template and requires repair of both strands. Therefore, it is not surprising that cells have evolved an elaborate system to deal with interstrand crosslinks by utilizing components from DNA repair pathways including nucleotide excision repair (NER), homologous recombination, and translesion synthesis (TLS) (5).

Interstrand crosslink repair can be divided into three main steps: recognition, removal, and repair. Evidence suggests that recombination-independent and recombination-dependent interstrand crosslink repair pathways exist in mammalian cells (Figure 1.4). In the absence of replication, interstrand crosslinks can be recognized by either helical distortion by the NER protein XPC or during transcription (8) (Figure 1.4A). In contrast, lesion recognition during replication-dependent repair occurs when the replication fork collides with an interstrand crosslink, resulting in fork stalling.

One critical step in repair is "unhooking" of the interstrand crosslink by nucleolytic incision. In replication-independent repair, dual incision flanking the crosslink on one of the two covalently linked strands occurs by the NER endonuclease ERCC1-XPF, thereby "unhooking" the lesion. For replication-dependent repair, "unhooking" is achieved by two endonucleases. The replication fork is cleaved by the

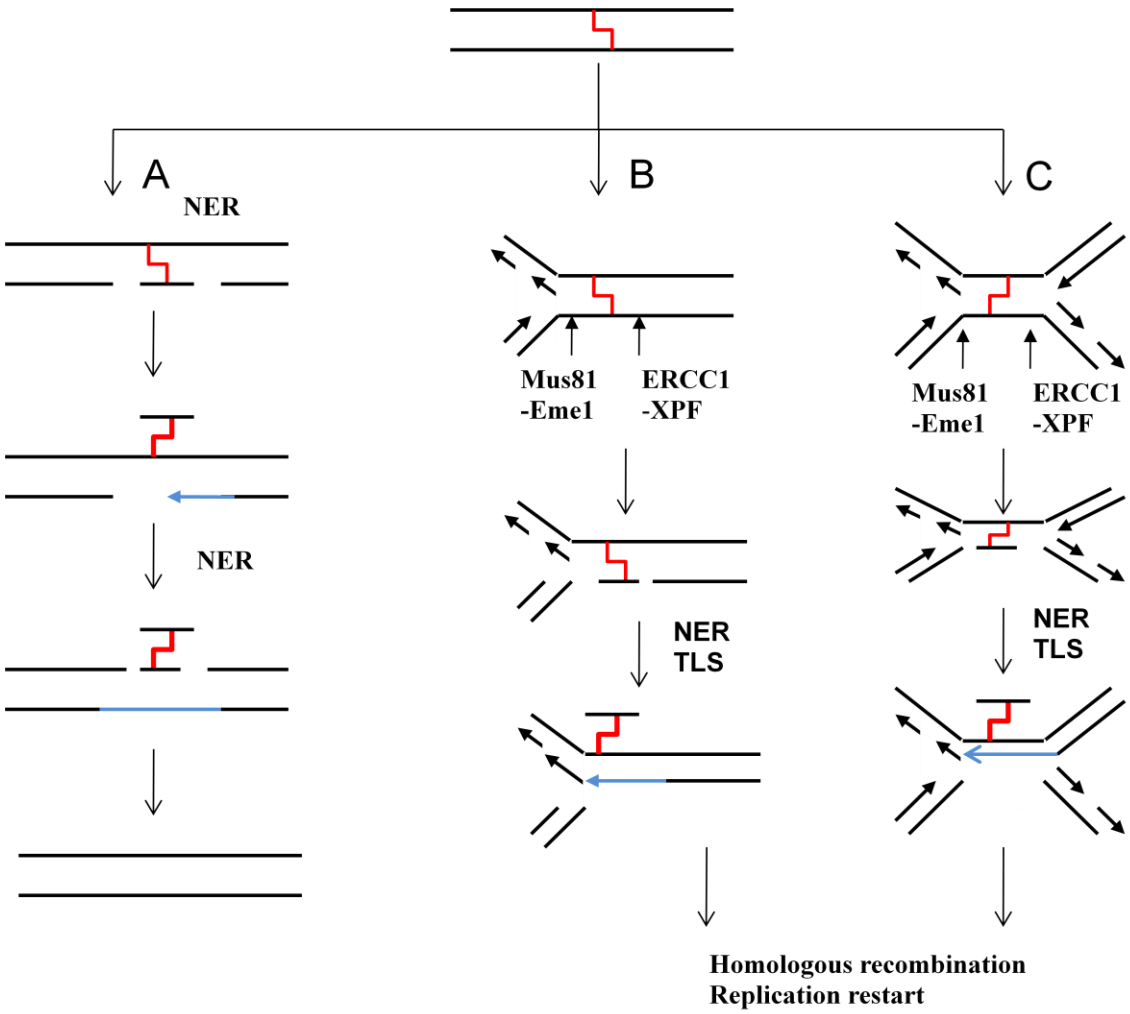


Figure 1.4. Replication-independent and replication-dependent repair pathways. (A) Replication-independent repair. Dual incision of the interstrand crosslink occurs resulting in unhooking. DNA synthesis occurs past the unhooked interstrand crosslink, followed by removal of the interstrand crosslink and a second round of DNA synthesis. (B) Replication-dependent repair. The interstrand crosslink is recognized when the replication machinery collides with the interstrand crosslink. The endonucleases MUS81-EME1 and ERCC1-XPF make incisions on the leading strand resulting in unhooking. DNA synthesis occurs past the unhooked interstrand crosslink. The interstrand crosslink is removed and the resulting double strand break is repaired by homologous recombination. (C) Double collision model. Repair is similar to (B), but replication forks converge on the interstrand crosslink. Red: interstrand crosslink. Blue; DNA synthesis. Adapted from (8).

structure-specific endonuclease MUS81-EME1 resulting in a double strand break intermediate (12) (Figure1.4B). The NER nuclease, ERCC1-XPF cleaves 5' of the interstrand crosslink resulting in "unhooking" (13). Gap synthesis occurs past the unhooked interstrand crosslink by a process known translesion DNA synthesis (TLS) (8). TLS utilizes translesion polymerases, which are recruited to bypass DNA lesions that would otherwise stall high-fidelity polymerases. The precise mechanism of removal of the "unhooked" interstrand crosslink is currently unknown, but is thought to occur by the classical NER pathway. Once removed, the resulting double strand break is repaired by homologous recombination (8).

Double collision model of interstrand crosslink repair

Until recently, repair models consisted of one replication fork encountering the interstrand crosslink. Using a *Xenopus laevis* cell free extracts, Raschle *et al.* (2008) monitored repair of a plasmid containing a synthetic interstrand crosslink by restriction enzyme digestion at discrete time points following initiation of replication (14). This study found that replication forks converge 20-24 base pairs away from the interstrand crosslink. One fork approaches the crosslink followed by dual incision, leading to unhooking and double strand break formation. A nucleotide is inserted directly across from the unhooked interstrand crosslink and DNA synthesis occurs off the nascent strand. Once the interstrand crosslink is removed, the resulting double strand break is repaired presumably by homologous recombination. Using this plasmid based assay, many of the steps of the current model were verified. However, whether all interstrand crosslinks are repaired by the double collision *vs.* a single replication fork remains unknown.

Furthermore, at what step many of the known DNA repair proteins functions in the context of this system has not been tested.

DNA damage response activation and cell cycle checkpoint signaling

Besides direct removal and of lesions, DNA damage responses involve a complex network of proteins involved in activation of cellular processes including cell cycle checkpoints. In mammalian cells, DNA damage responses are activated by the PIKK protein kinases ATM and ATR. Both are large kinases and prefer to phosphorylate serine and threonine residues that are followed by glutamine (15). ATM is mutated in patients with the genomic instability disorder ataxia telangiectasia (AT) and is characterized by phenotypes such as neurodegeneration, premature aging, and a predisposition to cancer (16). ATM is activated in response to double strand breaks (17). Ataxia telangiectasia and Rad3 related (ATR) is mutated in a subset of patients with Seckel syndrome (18). Patients exhibit severe microcephaly, developmental defects, and mental retardation (19). ATR is activated in response to ssDNA regions that are generated at stalled replication forks as well as resected ends that occur at double strand breaks (20).

The role of ATM in response to interstrand crosslinks is unclear. Previous studies have indicated that ATM does signal at collapsed replication forks. ATR deficiency leads to the accumulation of DSBs, γ-H2AX foci, and ATM activation (21). Furthermore, cell lines derived from AT patients are not sensitive to the interstrand crosslinking drug MMC indicating that ATM is not critical for the repair of interstrand crosslinks (22-24). In contrast, work performed using Xenopus extracts determined that ATM has important roles in preventing double strand break formation at replication

forks, but this may be due to a difference in experimental systems (25, 26). Taken together, this data suggests that the role of ATM in interstrand crosslink repair most likely reflects a role in the repair of the double strand break intermediate.

Activation of ATR dependent S phase checkpoint

In contrast to ATM, it is well established that activation of ATR is critical to the repair of interstrand crosslinks. In response to interstrand crosslinks, cells activate the intra-S phase checkpoint to allow cells time to repair the DNA lesions encountered during replication. Central to the S phase checkpoint is the ATR kinase (20). Stalled replication forks lead to the formation of ssDNA that is likely the result of uncoupling of the replication polymerases from the helicases or by nucleolytic processing of the replication fork (20). Replication protein A (RPA) binds to ssDNA and the ssDNA-RPA complex functions to recruit ATR along with its binding partner, ATRIP (27). Another critical function of ssDNA-RPA is recruitment of RAD17, which is phosphorylated by ATR and is also important for checkpoint signaling (28-33). ATR is then activated by an unknown mechanism, and phosphorylates downstream targets that have critical roles in cell cycle checkpoint activation as well as DNA repair.

In response to MMC, evidence indicates that ATR activates two parallel pathways of the S phase checkpoint (34). One branch involves the major effector substrate of ATR, the CHK1 kinase. CHK1 then phosphorylates several targets including CDC25A. CDC25 phosphorylation inhibits its activity, therefore preventing activation of cyclin dependent kinases and halting S phase progression (35). Simultaneously, ATR activates a second branch involving NBS1 and FANCD2 (34). NBS1 is the product of the gene

mutated in Nijmegan break syndrome and along with Mre11 and Rad50 forms the DNA damaging sensing complex, MRN, which has critical roles in checkpoint signaling as well as double strand break repair (36). FANCD2 is a downstream component of the Fanconi anemia pathway that is discussed in detail below (5). Besides S phase progression, ATR functions to inhibit firing of replication origins and to reduce DNA synthesis, but the mechanism is currently unknown (20).

In addition to proteins involved in cell cycle checkpoint activation, ATR activates proteins with roles in direct repair. Amongst these are proteins involved in homologous recombination including BRCA1, WRN, and BLM (5). In addition, ATR phosphorylates the FA proteins, FANCD2 and FANCI (37, 38). The role of the FA proteins in interstrand crosslink repair will be discussed in detail below.

Fanconi anemia pathway

Fanconi anemia (FA) is an inherited chromosomal instability disorder consisting of 13 known complementation groups (A,B,C,D1,D2,E,F,G,I,J,K,L,M,N) (39). The FA pathway is critical in the repair of interstrand crosslinks. FA patients are clinically heterogeneous, but hallmarks of this disease include bone marrow failure, skeletal defects, and predisposition to cancer. Cells derived from FA patients are hypersensitive to interstrand crosslinking drugs, including MMC. Although the primary function of the FA pathway is in repair of interstrand crosslinks, FA patient cell lines are sensitive to ionizing radiation and UV, as well as replication inhibitors such as aphidicolin and hydroxyurea (39). In response to interstrand crosslinking agents, FA cells have increased incidence of genomic instability, especially radial structures which are observed as

aberrant associations of chromosomal arms from multiple chromosomes. In fact, induction of radials MMC is used as a diagnostic tool for FA. Although FA has been intensely studied, many questions regarding the roles of the FA proteins in DNA repair remain unanswered.

Molecular functions of FA proteins

The FA pathway is believed to promote replication-mediated repair of interstrand crosslinks. Activation of the FA pathway in interstrand crosslink repair can be divided into four steps: recognition of DNA damage, monoubiquitination of FANCD2 and FANCI by the FANC core complex, chromatin localization of FANCD2 and FANCI, and recruitment of protein factors to repair foci (Figure 1.5). The FA pathway promotes repair of the interstrand crosslink, but the exact mechanism of action is unclear.

The FA pathway has been implicated as a possible mechanism for DNA damage recognition at the site of the stalled replication fork. FANCM and FAAP24 recognize ssDNA-dsDNA structures (40). FAAP24 is a protein that associates with the core complex, but mutations in FA patients have not been identified (39). FANCM and FAAP24 recruit the FANC core complex to the site of the stalled replication fork through its DNA translocase activity (40, 41). The FANC core complex is a large multi-subunit protein complex consisting of eight FANC proteins (A,B,C,E,F,G,K,L,N) (39).

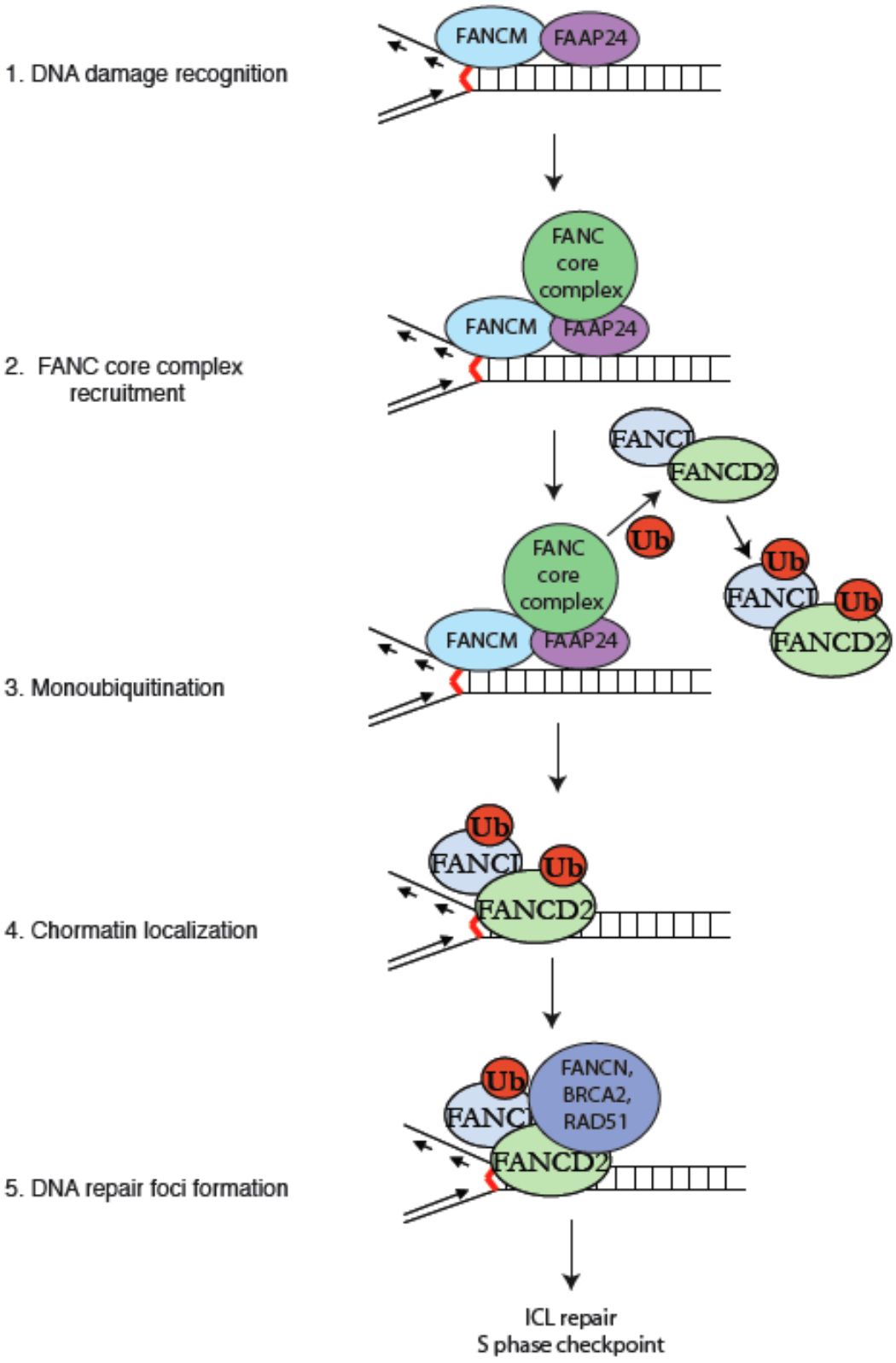
Once localized to chromatin, one member of the FANC complex, FANCL, is an E3 ubiquitin ligase that functions to monoubiquitinate two FA proteins, FANCD2 and FANCI (ID complex) on K561 and K523, respectively (24, 42-45). Efficient monoubiquitination requires all the core complex components. Thus, FANCD2

modification is a convenient tool to determine if FANC proteins are components of the core complex. Ubiquitination of FANCD2 is considered the critical step in FA pathway activation (39). However, evidence suggests that the core complex may have additional undiscovered roles outside of ID ubiquitination. In the DT40 avian B cell line, a $FANCD2^{K561R}$ mutant fused to ubiquitin was able to complement phenotypes of FANCD2 deficiency, but not mutations in the core complex (46). Monoubiquitination of FANCI is not essential for DNA repair, but instead FANCI through ATR-dependent phosphorylation acts as a regulator of the FA pathway, and this role may be independent of its monoubiquitination (37).

Monoubiquitination of the ID complex acts as a chromatin localization signal. The mechanism that mediates localization to chromatin is currently unknown. However, recent evidence indicates that chromatin localization of FANCD2 is reduced in ERCC1 and XPF deficient cells. This indicates that the interstrand crosslink has to be "unhooked" for FANCD2 to associate with chromatin (47).

Once localized to chromatin, the ID complex localizes to DNA repair foci with many other repair factors including RAD51, NBS1, BRCA1, BRCA2 and PCNA (22, 45, 48, 49). However, the function of monoubiquitinated ID remains unknown. Recent studies using *Xenopus laevis* extracts, indicates that the role of the FA pathway may be to promote TLS and homology mediated repair of interstrand crosslinks (50). One possibility is that the ID complex acts as scaffold proteins stabilizing DNA repair proteins at the site of DNA damage. Another hypothesis is that ID is required for recruitment of DNA repair proteins. Finally, it is possible that the ID complex is involved in direct repair of DNA lesions, but the proteins lack any known enzymatic

Figure 1.5. FA pathway. The FA pathway can be subdivided to 5 main steps: 1) DNA damage recognition by FANCM and FAAP24, 2) recruitment of the FANC core complex (A, B, C, E, F, G, K, L, N) to the site of the interstrand crosslink, 3) monoubuquitination of FANCD2 and FANCI by the FANC core complex, 4) chromatin localization of Ub-FANCD2 and Ub-FANCI, 5) and formation of large protein complexes containing Ub-FANCD2 and Ub-FANCI known as DNA repair complexes. The FA pathway promotes direct repair of the interstrand crosslink repair and S phase checkpoint activation. Adapted from (5).


1. DNA damage recognition
FANCM
FAAP24
2. FANC core complex recruitment
FANC core complex
FANCM
FAAP24
FANCI
FANCD2
Ub
3. Monoubiquitination
4. Chormatin localization
FANCN, BRCA2, RAD51
5. DNA repair foci formation
ICL repair
S phase checkpoint

domains, and so no known DNA repair activity is known (39). Consistent with a role in direct repair, FANCD1 was identified as the breast cancer susceptibility gene, BRCA2 (51). BRCA2/FANCD1 is critical for homologous recombination and is important for the localization of RAD51 to ssDNA at resected DNA ends (52, 53). Furthermore, it was shown that FANCD2 is important for BRCA2 to localize to DNA repair proteins suggesting that one function for the FA pathway may be to promote homologous recombination (48, 49). However, BRCA2 is essential for homologous, whereas the other FANC proteins only have a minor role.

The remaining FANC proteins, FANCJ and FANCN also function downstream of FANCD2 monoubiquitination (39). FANCJ/BRIP1/BACH1 is a 5' to 3' helicase that interacts at DNA repair structures containing RPA and BRCA2. Its current function in interstrand crosslink repair is unknown, but one proposed role is that FANCJ may remodel DNA, enabling repair (54-56). Another potential role for FANCJ is resolution of D loop formation during homologous recombination mediated repair of the double strand break intermediate (55). FANCN was initially described as a BRCA2 interacting partner, but its role in the FA pathway and interstrand crosslink repair is unknown (57). Thus, although the precise roles of the 13 identified FA factors and FA associated proteins remain to be defined, the FA/BRCA network plays a central role in signaling and repair of interstrand crosslink damage. There is evidence that additional factors that function with the FA pathway have yet to be identified (Coriell cell repository, unidentified complementation groups). In this thesis, I test the hypothesis that Snm1B functions within the FA/BRCA network.

Snm1B, a novel interstrand crosslink repair factor

Snm1B (sensitivity to nitrogen mustard 1B) was identified based on homology to the *Saccharomyces cerevisae* gene, PS02, which was identified n a screen for yeast mutants sensitive to the interstrand crosslinking agent, psoralen (58). In vertebrates, there are three functional orthologs: DCLRE1A (Snm1A), DCLRE1B (Snm1B/ Apollo), and DCLRE1C (Snm1C /Artemis) (59). All the PS02-related proteins are members of the metallo-β-lactamase (MBL) super family of proteins that share a common, conserved catalytic domain. Specifically, PS02 and Snm1 family members belong to the β-CASP subfamily of nucleic acid processing enzymes (59). Growing evidence indicates that the mammalian Snm1 members have critical roles as DNA nucleases during DNA repair. Snm1A has been implicated in interstrand crosslink repair. Artemis is involved in double strand break repair and in processing intermediates during V(D)J recombination (59). Snm1B has been implicated in repair of interstrand crosslinks, double strand breaks as well as telomere maintenance, but the functions of Snm1B within these repair processes remain poorly understood.

Like other Snm1 family members, Snm1B contains MBL and βCASP motifs characteristic of a DNA processing enzymes (Figure 1.5). The MBL consists of 4 motifs which are critical for binding of metal zinc ions (61), and growing evidence indicates that HxHxDH in MBL motif 1 constitutes the active site of the Snm1 family as mutation of these residues in Artemis abolishes nuclease activities *in vitro (62)*. The β-CASP domain contains three motifs that harbor conserved amino acids: aspartate or glutamate (motif A), histidine (motif B), and valine (motif C). Valine in motif C identifies Snm1B as a DNA nuclease, whereas a histidine is present in RNA processing enzymes (61).

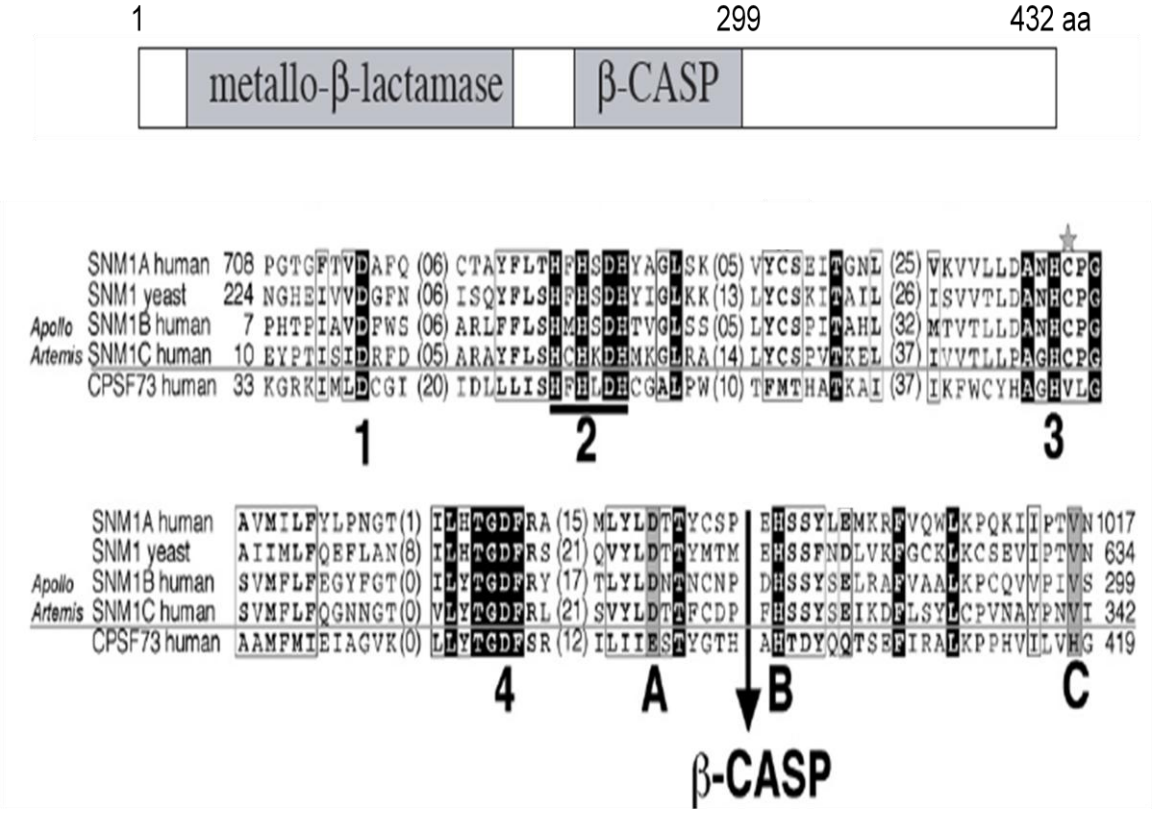


Figure 1.6. Snm1B is a member of the β-CASP family of proteins. Top. Schematic of Snm1B with locations of the conserved metallo-β-lactamase (MBL) and β-CASP domains shown in gray. Bottom. Alignment of Snm1 proteins with conserved motifs. Motifs 1-3 are conserved within the MBL superfamily. Motif 2 is the nuclease active site. Motifs A-C are specific to the β-CASP family. Conserved amino acids are shown in black, similar amino acids are boxed. Adapted from (60).

Interestingly, the conservation between Snm1 family members is restricted to the nuclease domains.

Both Snm1A and Artemis have been implicated as tumors suppressors that are critical for the maintenance of genomic integrity. Patients with hypomorphic mutations in Artemis develop aggressive B cell lymphomas indicating a role for this gene in suppressing tumorigenesis (63). Furthermore, both Artemis- and Snm1-deficient mice exhibit a predisposition to B cell lymphomas and thymic tumors respectively when crossed onto a p53-deficent background. Although no direct link between Snm1B and tumorigenesis has been identified, Snm1B was recently found to be mutated in almost half of primary mediastinal B cell lymphomas indicating that Snm1B may also function as a tumor suppressor and highlights the importance of elucidating the role of Snm1B in DNA repair processes (64).

Known roles of Snm1B in DNA repair processes

The roles of Snm1B in DNA repair processes only recently have begun to be elucidated. Initial studies characterizing HeLa cells depleted of Snm1B indicated that Snm1B functions in the repair of double strand breaks and interstrand crosslinks. Snm1B depleted cells exhibit decreased survival when treated with ionizing radiation, cisplatin, or MMC (58). In addition, Snm1B depletion increased chromosomal anomalies, but only upon MMC treatment. Although this study identified repair processes requiring Snm1B activity, how Snm1B contributed to the repair of these lesions was not determined. Thus, these initial studies raised many questions about the functions of Snm1B during DNA repair.

Roles for Snm1B in double strand break repair

The ATM protein kinase plays a central role in initiating the repair of DNA double strand breaks. In human fibroblasts, Snm1B depletion resulted in a moderate decrease in ATM signaling following ionizing radiation as indicated by a reduction in phosphorylation of ATM and downstream targets such as p53 and SMC1. Furthermore, it was shown that Snm1B can localize to the sites of double strand breaks within seconds after induction of breaks by micro-irradiation (65). Together, these findings suggest that Snm1B localizes to the sites of double strand breaks early to promote ATM-dependent signaling. Recently, it was shown that Snm1B interacts with the DNA damaging signaling complex, Mre11-Nbs1-Rad50 (MRN) through an interaction with Mre11 (66). The MRN complex is critical for ATM signaling so one function of Snm1B may be to recruit MRN to the sites of IR induced DNA breaks (67). Although the precise role of Snm1B in double strand break repair is not yet clear, these studies provide evidence that Snm1B is involved in the early response to DNA damage.

Roles for Snm1B in replication-mediated DNA repair

It has recently emerged that Snm1B likely plays multiple roles in interstrand crosslink repair. In response to interstrand crosslinks, Snm1B depleted cells exhibit impaired formation of the double strand break. Snm1B interacts with the endonuclease, Mus81, which cleaves the stalled replication fork, thus leading to the hypothesis that Snm1B functions coordinately with Mus81 to generate the double strand break intermediate (66). The defect in double strand break formation led to impaired ATM

dependent signaling as indicated by a reduction in phosphorylation of ATM and its downstream target CHK2 (66). Interestingly, this group observed a defect in the interstrand crosslink induced S phase checkpoint in Snm1B depleted cells upon MMC treatment presumably as a result of attenuated ATM signaling. However, previous studies in ATM null cells have demonstrated that interstrand crosslink induced S phase checkpoint activation is ATM-independent (34, 68). Therefore, I hypothesize that Snm1B has additional roles in interstrand crosslink repair independent of ATM signaling.

One of the most striking aspects of the phenotypes of Snm1B depleted cells is how closely they resemble those of cell lines derived from FA patients indicating that Snm1B may function within the FA/BRCA pathway of interstrand crosslink repair (58). Snm1B depletion results in cellular sensitivity to crosslinking agents and ionizing radiation. Snm1B deficiency results in an increase in frequency of chromosomal anomalies after MMC treatment including the formation of radial structures, a hallmark of FA (58, 66). Monoubiquitination of FANCD2 is intact in Snm1B depleted cells indicating Snm1B may function downstream of activation of the FA pathway (58, 66). Recently, it was shown that FANCD2 and Snm1B co-immunopreciptate but do not directly interact indicating that they are factors of the same protein complex (66). However, the functional relevance of this interaction has not been investigated. I hypothesize that Snm1B plays critical roles in the FA/BRCA pathway of interstrand crosslink repair.

Does Snm1B have other functions in DNA repair processes that occur during replication? Snm1B plays a role in processing of telomere ends during replication. Snm1B interacts with the TRF2, a component of the shelterin complex that protects

telomeres from being recognized as DNA breaks (69-71). Depletion of Snm1B leads to a growth defect in U20S cells as well as activation of a DNA damage response at telomeres as indicated by the formation of 53BP1 and γ-H2AX foci indicating that Snm1B deficiency results in telomere dysfunction (69, 70). These foci were activated specifically in S phase indicating Snm1B may be required for telomere processing during replication. Interestingly, only 60% of the DNA damage foci were localized to telomeres indicating that Snm1B may be required for repair of repair of spontaneous lesions that occur elsewhere in the genome during replication (70). One possibility is that Snm1B is required for resolution of stalled/collapsed replication forks that occur naturally during replication. Indeed, many components of the interstrand crosslink repair pathway such as ATR, CHK1 and the FA pathway are critical for the response to replication stress (72-75). I hypothesize that Snm1B is required for the cellular response to replication stress.

Summary

In this thesis, I have investigated the role of Snm1B within the repair of interstrand crosslinks and in the cellular response to replication stress. In Chapter 2, I demonstrate that Snm1B is critical for ATR-dependent signaling indicating that Snm1B functions in the earliest stages of interstrand crosslink repair. In Chapter 3, I show that Snm1B is epistatic with the FA gene, FANCD2, in interstrand crosslink repair and is required for FANCD2 foci formation that mutations in Snm1B may result in FA. In chapter 4, I demonstrate that Snm1B has critical roles in the cellular response to

replication stress. Finally, conclusions and implications of this work and its contributions to the DNA repair field will be discussed in Chapter 5.

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Chapter 2

Snm1B is critical for ATR-dependent signaling in response to interstrand crosslinks

Summary

In response to DNA interstrand crosslinks, cells activate an S phase checkpoint allowing time to repair the lesion. Snm1B, a member of metallo-β-lactamase/βCASP family of nucleases, has been demonstrated to function in interstrand crosslink repair; however, its precise roles are not understood. Here we identify a key function for Snm1B in activating the ATR-dependent S phase cell cycle checkpoint in response to interstrand crosslink-induced damage. ATR is the central protein kinase that mediates the interstrand crosslink checkpoint, and we demonstrate that Snm1B deficiency leads to impaired phosphorylation of ATR substrates and defective S phase arrest. Furthermore, we find that Snm1B is important for localization of key interstrand crosslink proteins: RPA, γ-H2AX, and BRCA1. Our findings establish that Snm1B promotes efficient activation of the ATR-dependent signaling cascade and indicates that Snm1B functions early in the cellular response to interstrand crosslinks.

Introduction

Interstrand crosslinks cause covalent linkage of the two strands of the DNA, preventing strand separation. Therefore, collision of the replication machinery with an interstrand crosslink leads to fork stalling activating the S phase checkpoint. The ATR serine/threonine protein kinase is activated in response to stalled replication forks and phosphorylates downstream targets, including the CHK1 protein kinase (1). CHK1 is the major effector substrate of ATR and functions to induce the S phase checkpoint, thereby allowing time for interstrand crosslink repair. Removal of the interstrand crosslink blocking lesion requires the concerted activities of the Mus81-Eme1 and XPF-ERCC1 endonucleases which excise, or "unhook", the interstrand crosslink (2, 3). The resulting single stranded gaps can serve as substrates for translesion synthesis by error-prone polymerases. During the processing of interstrand crosslinks, double strand break intermediates are generated which can be repaired via homologous recombination to restore replication (4). While the precise molecular interactions involved in these processes have not been fully elucidated, it is established that the ATR and CHK1 protein kinases play central roles in regulating both cell cycle arrest and direct DNA repair of interstrand crosslink damage.

In yeast, the Snm1/Pso2 gene plays critical roles in interstrand crosslink repair (5). There are three Snm1 orthologs in mammalian cells, Dclre1A (Snm1A), Dclre1B (Snm1B/Apollo), and Dclre1C (Artemis), which share a highly conserved metallo-β-lactamase/βCASP domain (6, 7). Snm1A functions in interstrand crosslink repair in a pathway distinct from the FA/BRCA pathway (8), and Dclre1C/Artemis functions within

the non-homologous end-joining pathway of DNA DSB repair (9-11). Snm1B, a 60 kDa protein, possesses intrinsic 5' to 3' single strand exonuclease activity and functions in the repair of interstrand crosslinks and IR-induced DNA damage (12-15). Previous studies have demonstrated that Snm1B deficiency results in hypersensitivity to interstrand crosslink damage, moderate sensitivity to ionizing radiation, chromosomal instability and increased interstrand crosslink-induced quadriradial chromosome formation (12, 14). However, the precise role of Snm1B in these pathways remains largely unknown.

Previous studies have shown that in response to interstrand crosslinks and ionizing radiation, Snm1B depletion results in attenuation of ATM signaling as indicated by reduction in phosphorylation of ATM targets p53, SMC1, and CHK2 (12, 13). This indicated that Snm1B is important for ATM-mediated signaling. Interestingly, in response to MMC, Snm1B depletion resulted in reduction of interstrand crosslink-induced NBS1 phosphorylation, which is an established ATR-dependent event (1, 12). This finding raised the possibility that Snm1B may also modulate the major ATR-mediated signaling pathway in response to interstrand crosslinks. However, a role for Snm1B in ATR dependent signaling in response to interstrand crosslink damage has not been investigated.

In this chapter, I examine the role of Snm1B in the ATR-mediated S phase checkpoint in response to the interstrand crosslinking drug, MMC. Using an siRNA approach in human cell lines, we establish that Snm1B plays important roles in activation of the ATR-dependent interstrand crosslink checkpoint. Furthermore, I show that Snm1B deficiency results in reduced localization of key interstrand crosslink factors including RPA and BRCA1. This chapter provides the first evidence that Snm1B in important for

ATR mediated signaling in response to interstrand crosslinks and establishes that Snm1B functions early in the response to interstrand crosslink damage.

Materials and Methods

Silencing RNAs. The Snm1B target sequences are: siSnm1B-1: 5'CCTCTTGCATCGTCACCTACATT 3'and siSnm1B-2: 5' AGTGCTGATCAATCTCAAGTT 3' (MWG, Qiagen). A non-silencing siRNA (Qiagen All Stars negative control) was used as the NS siRNA control.

Knockdown of Snm1B expression by siRNA. The HCT116 colon cancer, HeLa cervical cancer, and FANCD2 mutant and control fibroblast human cell lines were cultured as previously described (16, 17). HCT116 or HeLa cells were plated at a density of $1x10^5$ cells per well of 6 well dish 24 hours prior to siRNA transfection. All siRNAs (25-50 nM) were transfected using Liptofectamine 2000 (Invitrogen) as per manufacturer's instructions. PD20F and control fibroblasts were transfected with 150 nM siRNA as previously described (18). Snm1B mRNA levels were determined via RT-PCR in every experiment to verify the extent of siRNA knockdown.

HCT116 cells stably expressing V5-tagged Snm1B protein. HCT116 cells were transfected with a human Snm1B cDNA in pEF6 vector with C-terminal V5 and 6X His tags. Stable transfectants were selected in media containing 15 μg/ml blasticidin. Well-formed colonies were expanded and PCR was used to screen for clones harboring the V5-

Snm1B pEF6 expression construct. V5-Snm1B expression in positive clones was verified by immunoprecipitation with the V5 antibody followed by Western blot analysis.

Semi-quantitative RT-PCR analysis of Snm1B mRNA levels. Total RNA (1 μg) was reverse transcribed using a poly-dT(20) primer and MLV-reverse transcriptase (Invitrogen). cDNA was amplified with Snm1B-specific primers to exon 3 (5'GCAATCCAGCCCTGGTTCTTCCTTCCC 3') and exon 4 (5'GGCTTGTGGGAAGGATAGCAATCG 3') and GAPDH for normalization of cDNA levels. Bands were quantitated using AlphaImager 2200 (AlphaInnotech).

Cell cycle analyses. HeLa or HCT116 cells ($1.0x10^5$ cells/well of 6 well dish) were transfected with siSnm1B-1 or -2 then continuously exposed to 100 ng/ml MMC at 48 hours post-transfection. Cells were harvested, fixed in 70% EtOH, then stained with propidium iodide (50 μg/ml) in PBS containing RNase A (50μg/ml) and 0.1% TX-100. Cells were analyzed by flow cytometry (Becton Dickenson FC500) and cell cycle distributions were determined using Flow-Jo Software. All experiments in both cell lines were repeated at least three independent times.

Western blot analyses. Cells ($1-2x10^6$) were harvested and resuspended in lysis buffer (25 mM HEPES, pH 7.4, 10% glycerol, 200mM KCL, 0.1% NP40, 1mM DTT) containing phosphatase (Roche PhosSTOP) and protease inhibitors (Roche Complete Mini EDTA free). Soluble and chromatin bound FANCD2 fractions were separated as previously described (19). Protein concentration was determined using the Bradford

assay (BioRad). Lysates (50-100 μg) were analyzed by Western blotting with the appropriate primary antibodies and IRdye 800 CW secondary antibodies (Li-Cor). Bands were visualized and quantitated using the Li-Cor scanner and Odyssey 2.1 software. All experiments were performed three times from at least two independent transfections.

Quantification of Westerns. Western blots were quantitated using Odyssey 2.1 software. Bands were quantified by drawing a box around the entire signal. Signal intensity was calculated by taking the signal intensity of the band minus the signal intensity of background within the same lane. Each sample was then normalized to a loading control such as Tubulin or GAPDH. Fold induction was calculated by determing the increase of the signal intensity as compared to the untreated sample. Representative quantifications for each experiment are shown.

Immunofluorescence of subnuclear foci. HeLa cells (4×10^4) were seeded on coverslips in 12 well dishes 24 hours prior to transfection. At 48 hours post-transfection, cells were continuously exposed to MMC (330 ng/ml) for 8 hours, then incubated in cold pre-extraction buffer (20mM HEPES, 50mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% TX-100) for 5 minutes followed by fixation in 3.7% p-formaldehyde, 2% sucrose, 0.5% TX100 for 20 minutes at room temperature and washed 3X with PBS. Cells were incubated with the appropriate primary antibody for 45 minutes then stained with Alexa Fluor 488 or 594 secondary antibodies (Invitrogen Molecular Probes). Coverslips were mounted on slides using ProLong Gold anti-fade reagent containing DAPI (Invitrogen). Foci were visualized using an Olympus BX61 microscope and FISHview software

(Applied Spectral Imaging). All foci experiments were performed blinded. Each experiment was performed at least three independent times.

Antibodies. α-GAPDH (clone # FL-335) was from Santa Cruz. α–p53 (clone Ab-6), α-RPA (clone #Ab-3). α–BRCA1 (clone # Ab-4) were from CalBiochem. α–pChk1ser317 (Cat # 2334), α–pRAD17(ser645)(Cat#3421) were from Cell signaling. α–γ-H2AX (Cat #05-636) was from Upstate. α–FANCD2 (Cat # NB100-182) was from Novus Biologicals. α–V5 was from Invitrogen.

Mitomycin C survival assay. Cellular survival upon treatment with MMC was determined using a colorimetric assay, as previously described (20). Average percent survival was obtained from at least three independent experiments.

Results

Depletion of Snm1B in human cells.

To elucidate the roles of Snm1B in cellular responses to DNA damage, we used an siRNA approach to knockdown expression in human cell lines. We designed two specific siRNAs, siSnm1B-1 which targets mRNA sequences in a unique region of the conserved metallo-β-lactamase/β-CASP domain (nt 339-359) and siSnm1B-2 which targets a sequence within the non-conserved C-terminus (nt 1248-1266) (Supplementary Figure 2.1A). To validate the siRNA approach, siSnm1B-1 and -2 were transfected into HeLa cells, and *Snm1B* transcript levels were assessed by semi-quantitative RT-PCR. We observed that both siRNAs markedly reduced *Snm1B* mRNA levels at 48 post

transfection compared to a nonspecific (NS) siRNA control (Supplementary Figure 2.1B, C). Due to sequence conservation between members of the β-CASP superfamily, we also performed RT-PCR to examine transcript levels of the related *Snm1A* and *Artemis* genes and confirmed that knockdown is specific to *Snm1B* (Supplementary Figure 2.1B). We also verified that Snm1B protein levels were correspondingly decreased upon siRNA transfection in HCT116 cells expressing a V5-epitope tagged Snm1B protein to approximately 10% of controls (Supplementary Figure 2.1B). Thus, transient transfection of the siRNAs efficiently depleted *Snm1B* mRNA and significantly reduced protein levels.

Defective interstrand crosslink induced ATR-dependent cellular responses in Snm1B deficient cells.

ATR is the primary checkpoint kinase that becomes activated in response to interstrand crosslink induced damage and functions to elicit DNA damage responses during S phase (1, 21). ATR deficient cells exhibit hypersensitivity to interstrand crosslink damage (22, 23), and ATR deficiency or inhibition of kinase activity leads to impaired interstrand crosslink-induced S phase arrest, which is evidenced by markedly decreased accumulation of S phase cells (21, 22). We examined the impact of Snm1B deficiency on the ATR-dependent S-phase checkpoint. Exponentially growing Snm1B depleted and control HeLa cells were continuously treated with MMC (100 ng/ml), then the cell cycle profiles were examined by flow cytometry at 4, 16 and 24 hours. We observed that HeLa cells transfected with the NS siRNA accumulated in S phase after 16 and 24 hours of MMC treatment (Figure 2.1A), consistent with previous reports (21, 22,

24). In contrast, Snm1B depleted HeLa cells exhibited a substantial reduction in S phase accumulation at 16 and 24 hours post MMC treatment (Figure 2.1A). We observed similar results in HCT116 cells depleted of Snm1B, which also exhibited significantly, decreased accumulation S phase cells upon exposure to MMC (Supplementary Figure 2.2).

The defect in interstrand crosslink induced accumulation of S phase cells in Snm1B depleted cells could be due to a prolonged G1 arrest or impaired activation of the S phase checkpoint. To distinguish these two possibilities, we examined levels of cyclin A by Western blot analysis. Cyclin A is required for progression of cells through S phase through its interaction with the cell cycle dependent kinase, CDK2 (26). Expression of cyclin A begins at the onset of S phase (27) and defective entry into S phase leads to markedly decreased cyclin A levels (28-30). Thus, we examined cyclin A levels in HeLa cells transfected with siSnm1B-1 or NS siRNA at 4, 8, 16 hours post MMC treatment. NS siRNA controls exhibited a substantial increase in cyclin A protein levels at 16 hours post MMC treatment compared to untreated cells (Figure 2.1B). In contrast, we did not observe a significant increase or reduction in cyclin A levels in Snm1B depleted HeLa cells at any of the time points examined (Figure 2.1B). These results indicate that Snm1B deficiency does not significantly affect entry into S phase, but impairs the interstrand crosslink induced S phase checkpoint.

One critical substrate of ATR is the CHK1 protein kinase, which is phosphorylated and activated in response to the presence of interstrand crosslink damage (24, 31). We assessed the impact of Snm1B deficiency on phosphorylation of CHK1 on S317 in MMC treated cells by Western blotting. We observed that exposure of control

cells to MMC induced phosphorylation of CHK1 to levels approximately 40-50-fold higher than untreated cells by 16 hours post treatment (Figure 2.2B; Supplementary Figure 2.3A). In contrast, induction of CHK1 phosphorylation was markedly compromised in cells transfected with siSnm1B-1, which exhibited only a 3 to 5 fold increase at 16 hours post MMC treatment. We observed a similar dramatic reduction in interstrand crosslink-induced CHK1 phosphorylation in siSnm1B-2 transfected HeLa cells (Supplementary Figure 2.3B). To further confirm these findings, we depleted Snm1B in the HCT116 cell line and exposed the knockdown and control cells to two concentrations of MMC (100 ng/ml and 10 μg/ml). Snm1B deficient HCT116 cells also exhibited a significant impairment in interstrand crosslink induced CHK1 phosphorylation at 4 and 24 hours post MMC treatment at both drug concentrations compared to controls (Supplementary Figure 2.3C, data not shown). Therefore, we conclude that Snm1B is required for efficient interstrand crosslink induced CHK1 phosphorylation.

A previous study found that Snm1B depletion did not significantly impair CHK1 phosphorylation upon MMC exposure (12). It is possible that differences in the experimental approaches may have impacted observed phenotypes. In the previous study, one stable shRNA Snm1B knockdown cell line was used, whereas we transiently transfected siRNAs to knockdown Snm1B mRNA and protein levels. The high efficiency of Snm1B transient knockdown used in our analyses may result in more severe cellular phenotypes in response to interstrand crosslinks. In this regard, we consistently observed that transient transfection of Snm1B siRNA-1 and -2 in unperturbed cells induced a robust DNA damage response, including p53 stabilization, γH2AX induction,

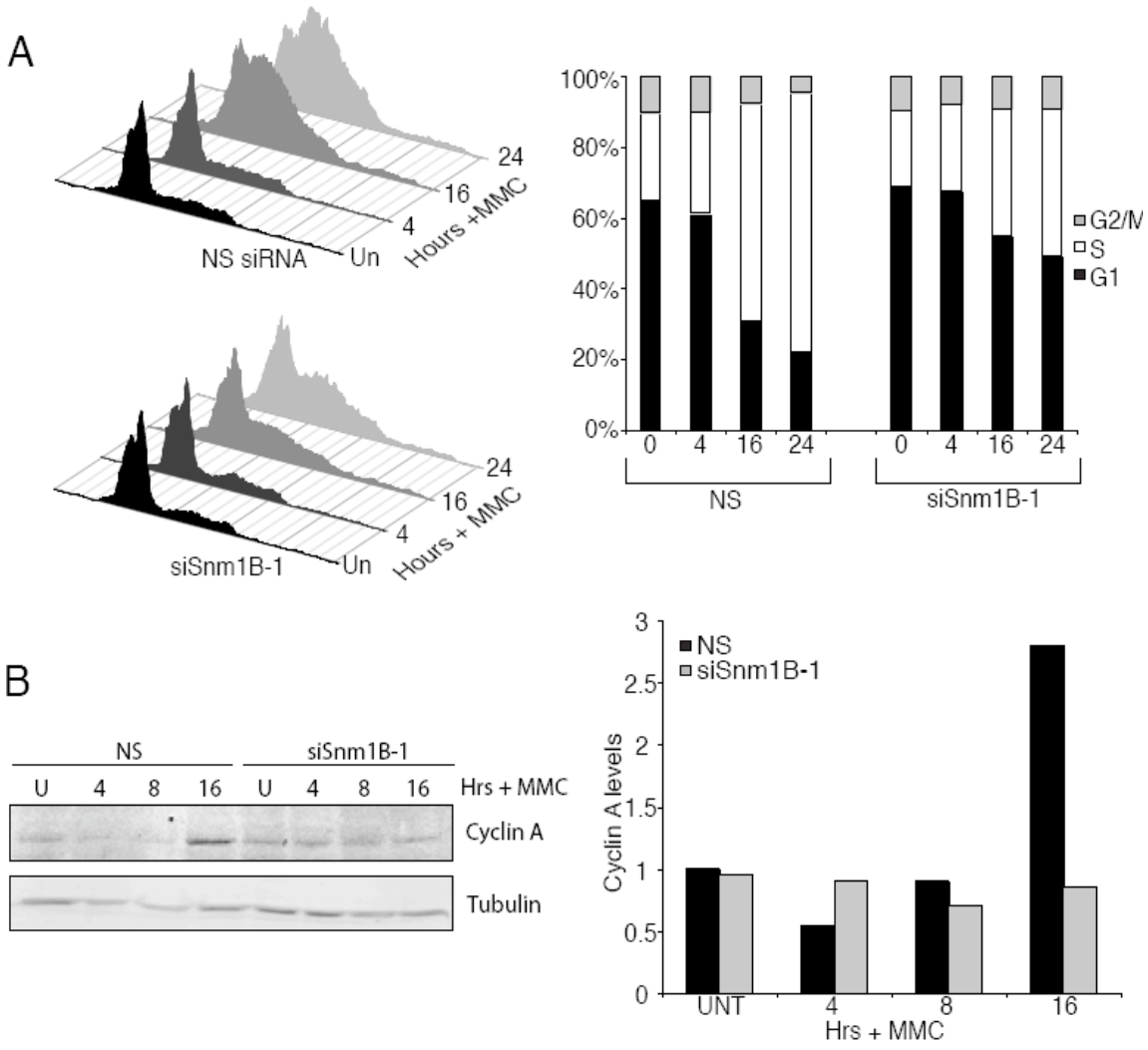
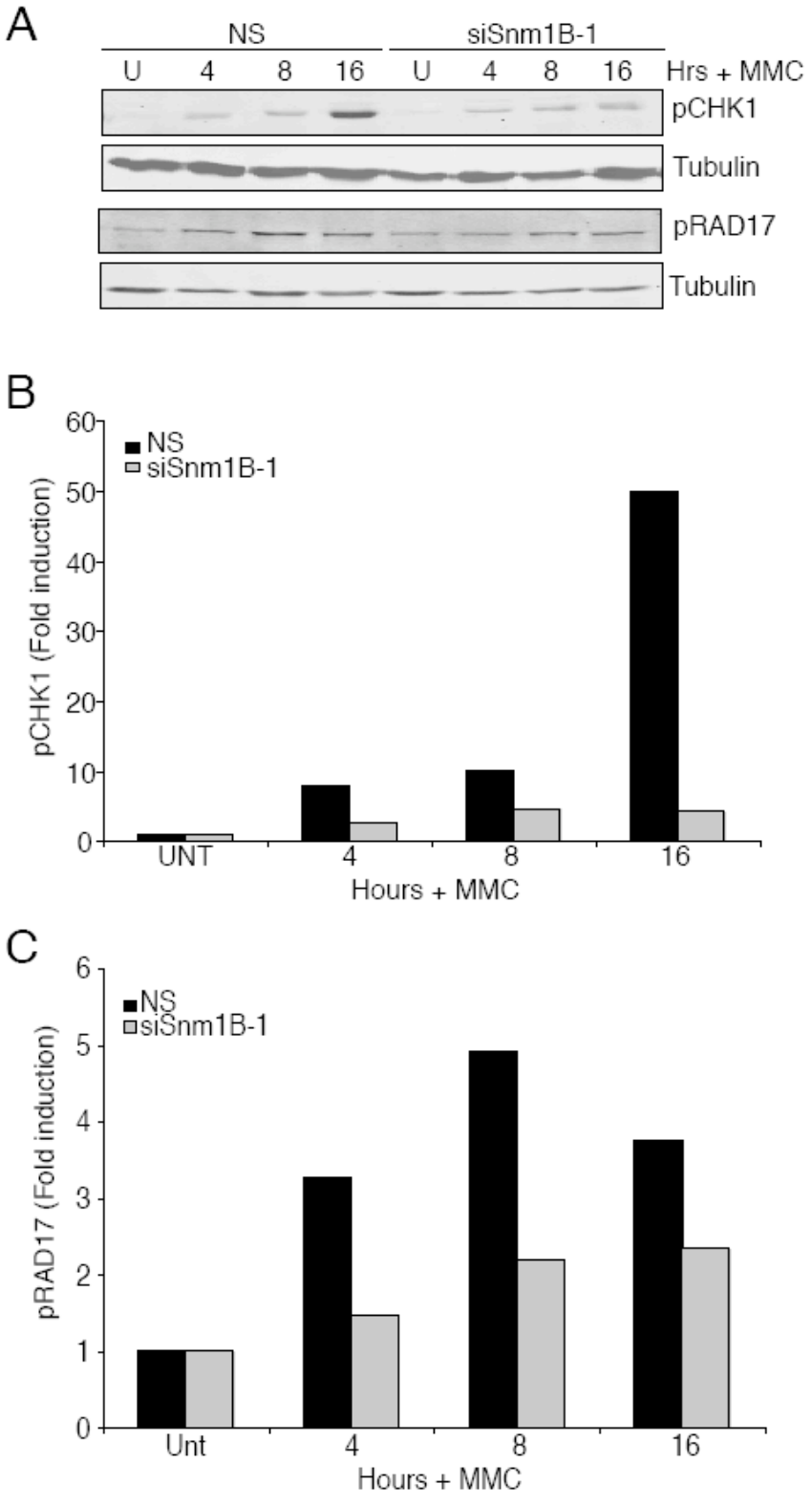


Figure 2.1. Impact of Snm1B depletion on cell cycle distribution upon MMC exposure. (A) S phase accumulation in response to interstrand crosslinks. HeLa cells were continuously treated with MMC (100 ng/ml) for indicated times at 48 hours post transfection with NS or siSnm1B-1. Cell cycle distributions were determined by propidium iodide staining. Experiment was repeated at least three independent times. Representative plots are shown**. (B)** Cyclin A levels in HeLa cells. HeLa cells transfected with NS or siSnm1B-1 were treated with MMC (1 μg/ml) for the indicated times at 48 hr post transfection. Cyclin A levels were determined by Western blotting. Representative results are shown. Western blots were repeated at least three times from two independent transfection experiments.

growth defects and increased apoptosis (Chapter 4, Figures 4.1, 4.2), and other studies have reported that Snm1B knockdown also induces 53BP1 repair foci and increased phospho-p53 levels as well as defects in telomere maintenance (13, 15, 32). Thus, given the importance of Snm1B in repair of spontaneous DNA damage, it is possible that the clonal Snm1B shRNA line used in previous studies may have accumulated genomic damage which could likewise influence the observed phenotypes. The transient transfection approach circumvents the potential impact of accumulated chromosomal instability and permits analyses of isogenic control and knockdown cell lines. Using this method, we demonstrate that Snm1B depletion impairs interstrand crosslink-induced CHK1 phosphorylation in two different cell lines with two distinct siRNAs at several MMC concentrations.

To further examine the impact of Snm1B deficiency on ATR-dependent phosphorylation events, we examined levels of phospho-RAD17 upon MMC treatment. Rad17 is an established ATR substrate (33, 34) and undergoes phosphorylation at serine 645 in response to interstrand crosslink damage (35). Rad17 plays critical roles in ATR-dependent checkpoint signaling and is required for cellular survival in response to interstrand crosslink damage (33-37). We observed the Snm1B depletion significantly attenuated induction of phospho-Rad17 in response to MMC treatment in comparison to controls (Figure 2.2A, C, Supplementary Figure 2.3D). Thus, Snm1B is required for efficient phosphorylation of ATR substrates in response to interstrand crosslink damage.

Figure 2.2. Snm1B is required for activation of the ATR-dependent S phase checkpoint. (A) Activation of ATR-dependent S phase checkpoint. HeLa cells transfected with NS or siSnm1B-1 were exposed to MMC (1 μg/ml) for indicated times at 48 hr post transfection. Cells were harvested and membranes were probed with α-pCHK1ser317 and α-pRAD17ser645. Protein levels were normalized to tubulin levels. Representative results are shown. Western blots were repeated three times from at least two independent transfection experiments. **(B)** Graphical representation of pCHK1 induction in (A). Fold induction of pCHK1 was determined compared to the untreated control. Representative graph is shown. **(C)** Graphical representation of pRAD17 induction in (A). Fold induction of pRAD17 was determined compared to the untreated control. Representative graph is shown.


A
NS
siSnm1B-1
U
4
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U
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8
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Hrs + MMC
pCHK1
Tubulin
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Tubulin
B
NS
siSnm1B-1
pCHK1 (Fold induction)
60
50
40
30
20
10
0
UNT
4
8
16
Hours + MMC
C
NS
siSnm1B-1
pRAD17 (Fold induction)
6
5
4
3
2
1
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4
8
16
Hours + MMC

Impact of Snm1B deficiency on localization of key interstrand crosslink factors to repair foci.

To further investigate the molecular defects in interstrand crosslink repair caused by Snm1B deficiency, we examined the localization of repair factors to subnuclear foci in response to MMC treatment. Since we observed a significant decrease in S phase cells in Snm1B depleted cells exposed to MMC for 16-24 hours (Figure 2.1A), we initially sought to identify a time point prior to when the defect in S phase accumulation is observed. To this end, we examined the cell cycle distributions of control and Snm1B depleted cells at 8 hours post MMC exposure. Importantly, we found no significant difference in the proportion of S phase cells in Snm1B deficiency compared to controls at this time point (Supplementary Figure 2.4). Therefore, we performed the foci formation analyses at 8 hours post MMC addition.

We assessed whether Snm1B is required for interstrand crosslink-induced RPA foci formation. RPA is a heterotrimeric protein complex that binds ssDNA with high affinity (38). During interstrand crosslink repair, regions of ssDNA can be generated during S phase upon replication fork stalling or resection of DSB intermediates that result from fork collapse. The ssDNA-RPA intermediates can be observed as foci in response to interstrand crosslink damage (24, 39) and are thought to recruit ATR in association with its interacting factor, ATRIP, thereby leading to its activation (40, 41). We used immunofluorescence microscopy using α-RPA34 antibodies to examine RPA foci formation in MMC treated Snm1B depleted and control cells. Untreated Snm1B deficient and control cells exhibited similar levels of RPA foci formation. However,

upon MMC exposure, Snm1B depletion significantly attenuated interstrand crosslink-induced RPA foci formation to 50% of controls (Figure 2.3). These results indicate that Snm1B is required for efficient localization of RPA to interstrand crosslink induced nuclear foci.

The histone variant, H2AX, undergoes phosphorylation by ATR in response to interstrand crosslink damage to form γ-H2AX (21, 42). γ-H2AX localizes to foci upon exposure to MMC (21, 43, 44) and is involved in the recruitment of repair factors, including FANCD2 and BRCA1, to sites of damage (45). Thus, we addressed whether depletion of Snm1B would impact interstrand crosslink-induced γ-H2AX foci formation. HeLa cells transfected with NS or siSnm1B-1 were exposed to MMC for 8 hours, then the percentage of cells containing interstrand crosslink induced γ-H2AX foci was quantitated. We observed that untreated Snm1B depleted cells exhibited a significantly higher percentage of cells containing γ-H2AX foci compared to control NS transfected cells (Figure 2.4A), perhaps due to telomere dysfunction and unrepaired spontaneous chromosomal damage (15, 32). Exposure of control cells to MMC significantly increased the percentage of cells containing γ-H2AX foci (Figure 2.4A). In Snm1B depleted cells, interstrand crosslink damage did not result in further induction of γ-H2AX foci formation (Figure 2.4A). We also performed Western blot analyses and found that the levels of γ-H2AX were elevated in untreated Snm1B depleted cells, but were not further increased upon MMC exposure (Figure 2.4B). Thus, Snm1B deficiency does not lead to a significant increase in interstrand crosslink induced H2AX phosphorylation and γ-H2AX foci formation beyond the levels observed in untreated cells.

BRCA1 plays a key role in HR repair of DSB intermediates formed during

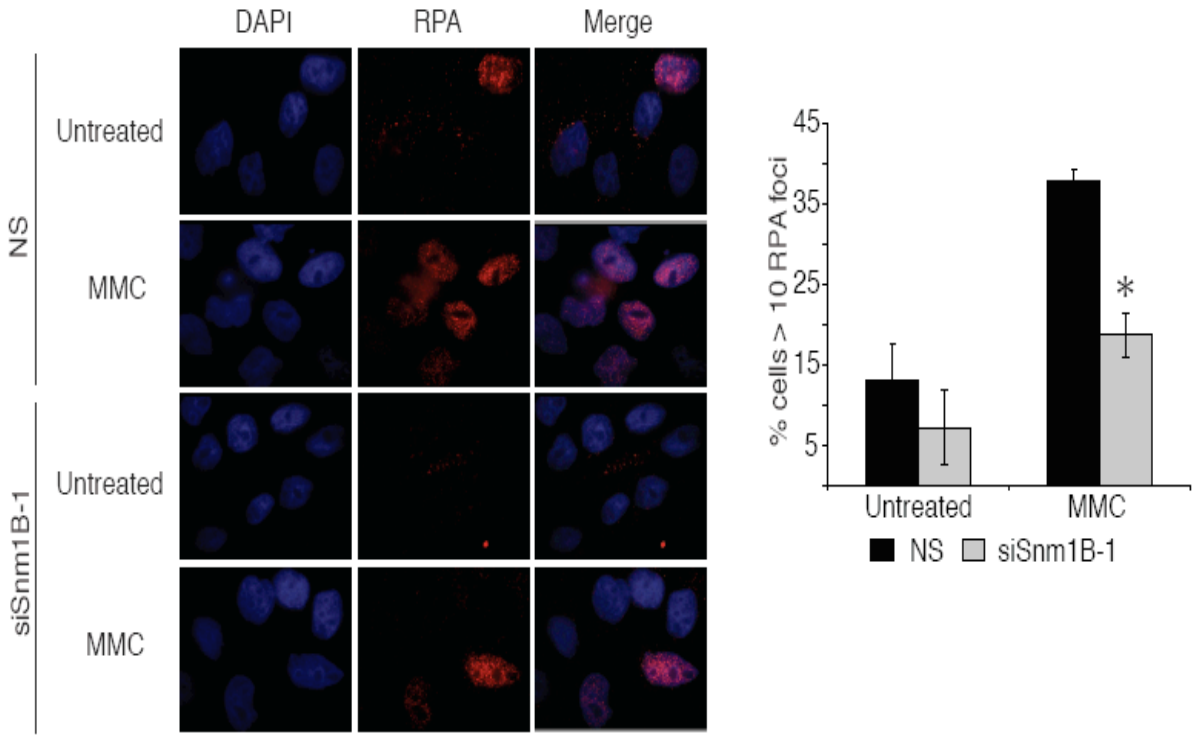

Figure 2.3. RPA foci formation is impaired in Snm1B depleted HeLa cells in response to interstrand crosslink damage. HeLa cells transfected with NS or siSnm1B-1 were treated with MMC (330 ng/ml) for 8 hours. Cells were fixed in p-formaldehyde and stained with α–RPA (red). Nuclei were stained with DAPI (blue). Magnification, x100. The percentage of cells containing greater than 10 foci was determined. Graph represents average of three independent experiments with standard deviation. *P< 0.05, Student's two-tailed t-test.

interstrand crosslink repair (46, 47). In response to stalled replication forks, BRCA1 is phosphorylated by ATR and relocalized to DNA repair foci (48). The interstrand crosslink-induced defect of ATR-dependent signaling we observed in Snm1B depleted cells led us to examine BRCA1 foci formation in response to interstrand crosslink damage. We observed that MMC exposure of control cells resulted in a 3 to 4-fold increase in the percentage of cells containing interstrand crosslink-induced BRCA1 foci (Figure 2.5). However, Snm1B depleted cells exhibited a substantial impairment in MMC-induced BRCA1 foci formation (Figure 2.5, Supplementary Figure 2.5). Thus, we conclude that Snm1B is required for efficient localization of BRCA1 to interstrand crosslink induced repair foci.

Discussion

Snm1B functions in activation of the ATR-dependent interstrand crosslink induced S phase checkpoint.

Our findings reveal an important role for Snm1B in mediating the activation of ATR-dependent cellular responses to interstrand crosslink damage. Two parallel ATR-dependent pathways that elicit S phase checkpoint arrest in response to interstrand crosslink damage have been described, the ATR-CHK1 and ATR-NBS1-FANCD2 pathways (1). In the current study, we provide evidence that Snm1B is required for optimal activation of the ATR-CHK1 branch of the interstrand crosslink induced S phase checkpoint. We demonstrate that Snm1B depletion significantly reduces phosphorylation of CHK1, the major effector target of ATR, as well as the ATR substrates, Rad17 and H2AX, in response to interstrand crosslink damage. The impact of Snm1B deficiency on

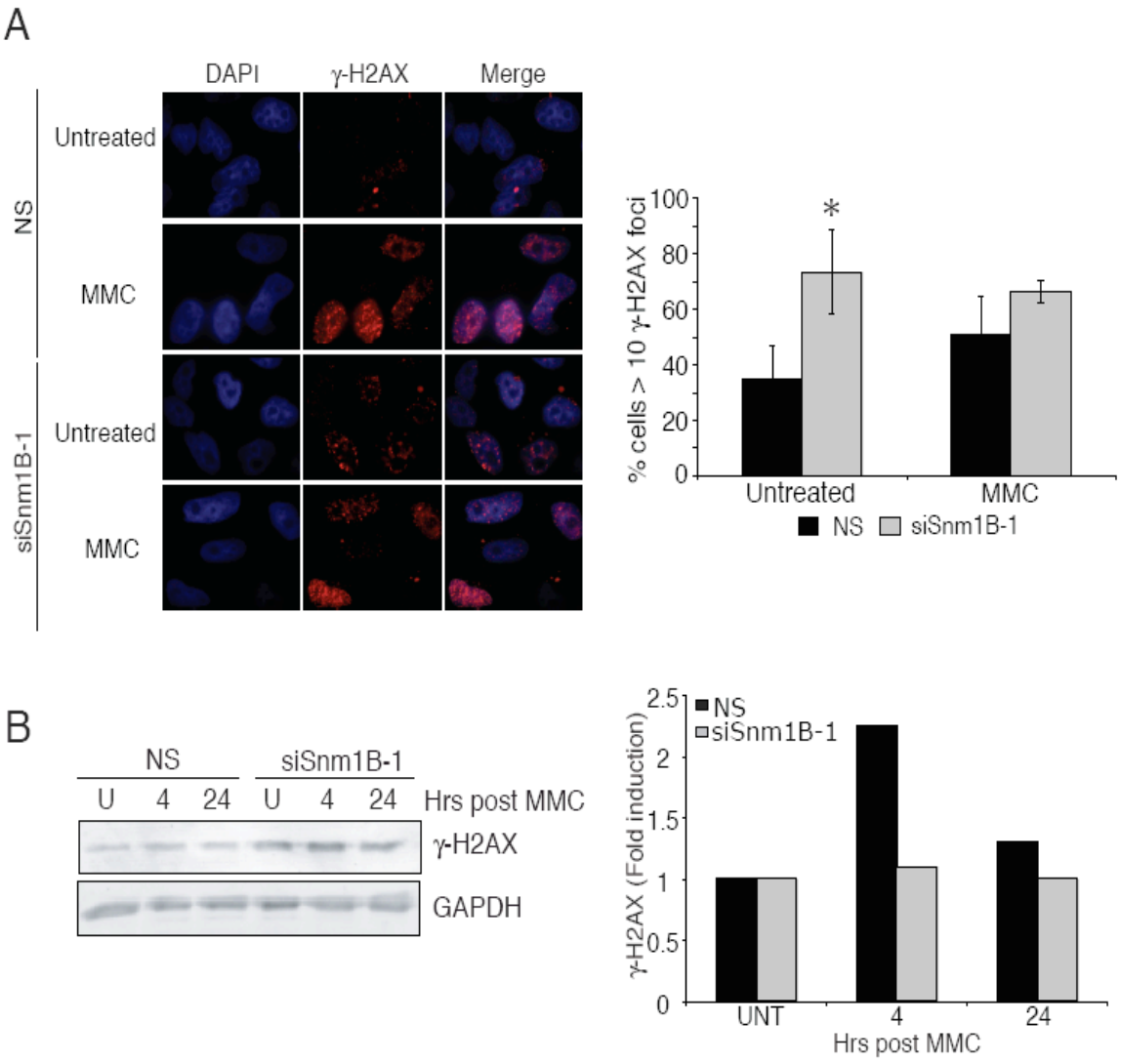

Figure2.4. γ-H2AX induction is impaired in Snm1B depleted cells in response to MMC treatment. (A) HeLa cells transfected with NS or siSnm1B-1 were treated with MMC (330 ng/ml) for 8 hours. Cells were fixed with p-formaldehyde and stained with α–γ-H2AX antibodies (red). Nuclei were stained with DAPI (Blue). Magnification, x100. Graph represents at least three independent experiments. Error bars, SD; *P<0.05. **(B)** γ-H2AX levels in HCT116 cells. HCT116 cells transfected with NS or siSnm1B-1 were treated with MMC (10 μg/ml) for 1 hour. MMC was removed and cells were harvested at indicated times. γ-H2AX levels were analyzed by Western blot analyses with α-γ-H2AX antibodies. Levels were determined in two independent experiments. GAPDH was used as a normalization control. Graph represents γ-H2AX induction as compared to untreated control.

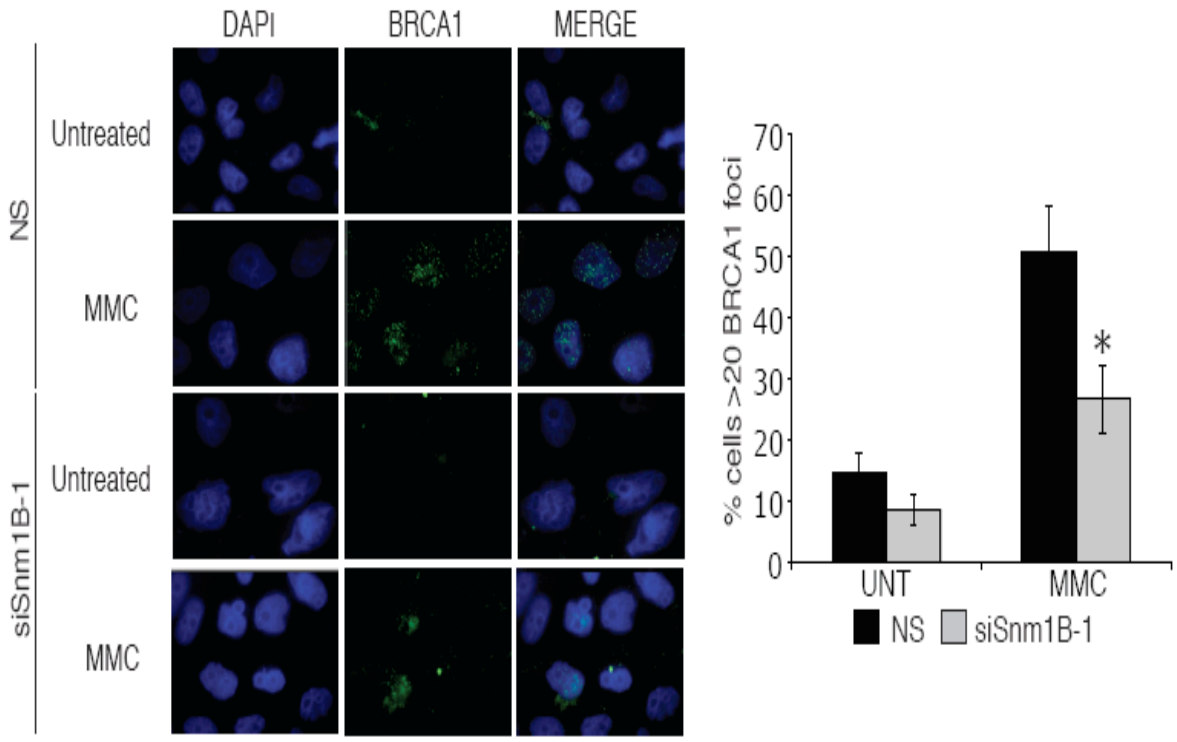

Figure 2.5. BRCA1 foci formation is impaired in Snm1B deficient cells. HeLa cells transfected with NS or siSnm1B-1 were treated with MMC (330 ng/ml) for 8 hr at 48 hr post transfection. Cells were fixed with p-formaldehyde and stained with α-BRCA1 antibodies (green). Nuclei were stained with DAPI (Blue). The percentage of cells containing greater than 20 foci was determined. Graph represents average of three independent experiments with standard deviation. *P<0.05, Students two-tailed t-test.

ATR-dependent signaling manifests as defective interstrand crosslink checkpoint activation which results in decreased accumulation of cells in S phase, a phenotype also observed in ATR deficiency (21, 22). Snm1B depleted cells were also previously observed to fail to arrest DNA synthesis in response to interstrand crosslink damage (12). These findings indicate that Snm1B acts early during interstrand crosslink repair, at the stage of ATR signaling, to facilitate efficient activation of the DNA damage response.

Snm1B functions within direct repair of the interstrand crosslink

The direct repair of the interstrand crosslinks during S-phase arrest occurs in a complex process involving translesion synthesis, nucleotide excision repair, homologous recombination and the FA/BRCA pathway (42, 47). Although there is not yet a consensus regarding the precise molecular details of interstrand crosslink repair, one early event that inevitably occurs is the excision, or unhooking, of the interstrand crosslink which involves the activities of the ERCC1/XPF and Mus81/Eme1 endonucleases (16, 24). These cleavage events result in formation of a single-stranded gap that is filled in by translesion DNA polymerases (42). Breakage at the replication fork also generates a one-sided DSB intermediate which then undergoes nucleolytic resection. RPA coats ssDNA that forms at the stalled replication forks and as well as after resection of the double strand break (38). The ssDNA then engages in homologous recombination with the sister chromatid mediated by BRCA1, BRCA2 (FANCD1) and to ultimately restart replication (1, 37).

In this chapter, I have shown that Snm1B is required for efficient formation of MMC-induced RPA and BRCA1 foci, indicating that it plays important roles in

processing of interstrand crosslink damage. I hypothesize that the Snm1B nuclease participates in the processing of stalled replication forks to generate the ssDNA intermediates required for the recruitment of RPA and downstream homologous recombination factors. In support of this notion, Snm1B interacts with Mus81-Eme1 and is involved with replication fork collapse during interstrand crosslink repair (12). Thus, the nucleases may cooperate to coordinate processing of the DNA ends for efficient repair.

Acknowlegements

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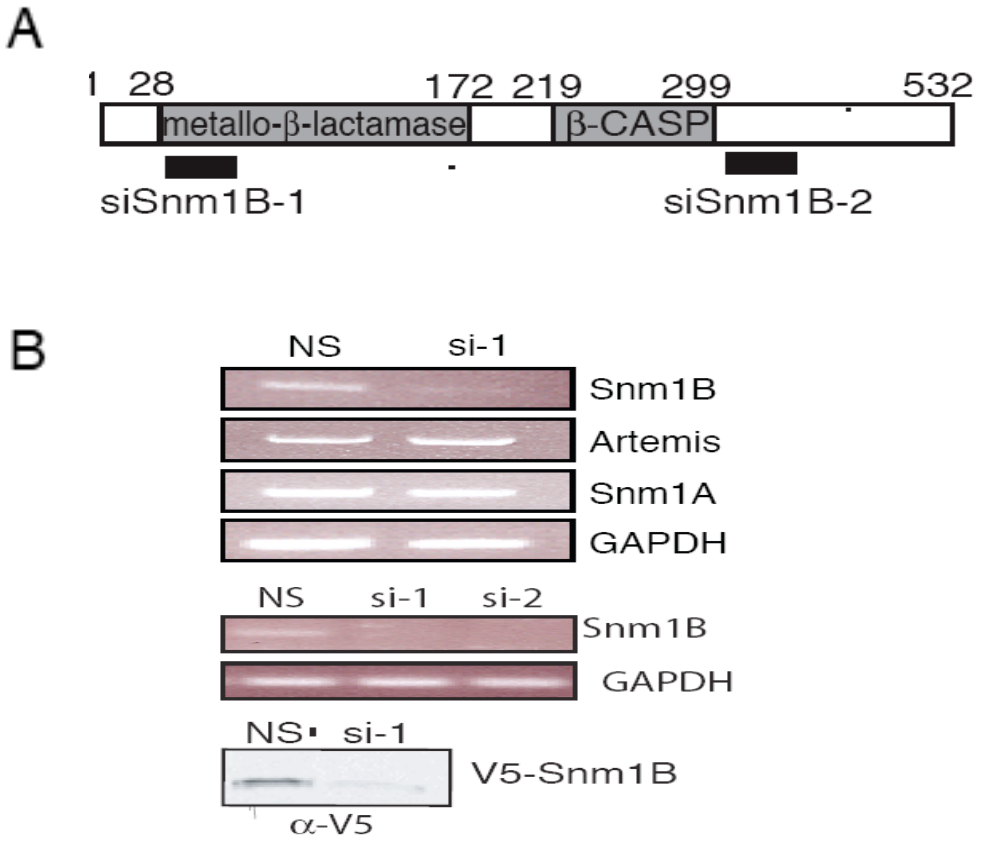
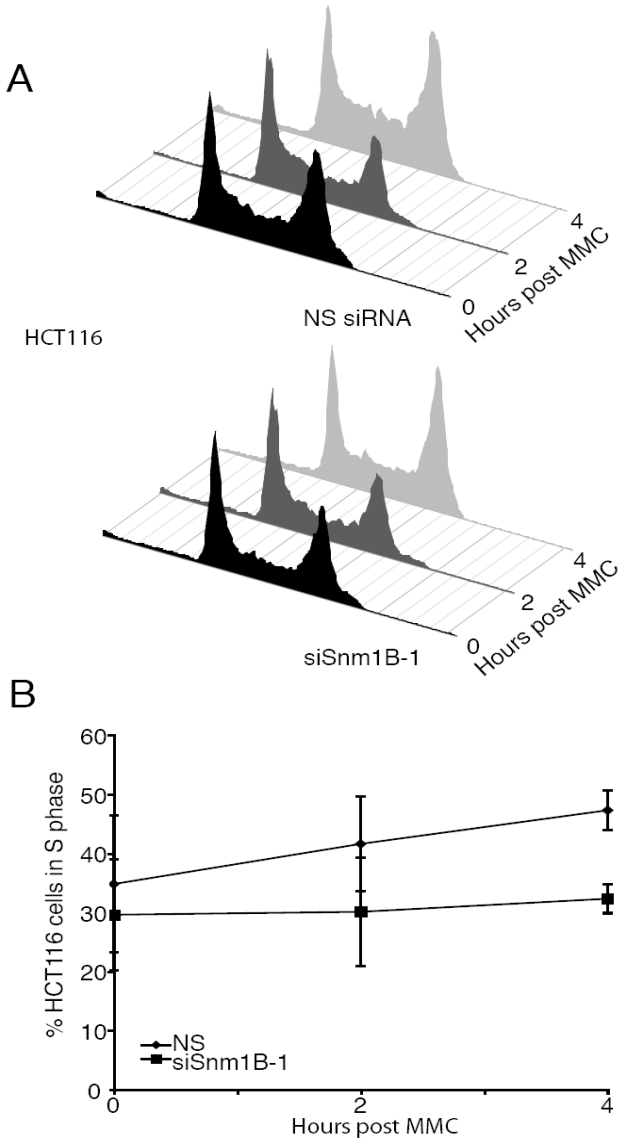


C

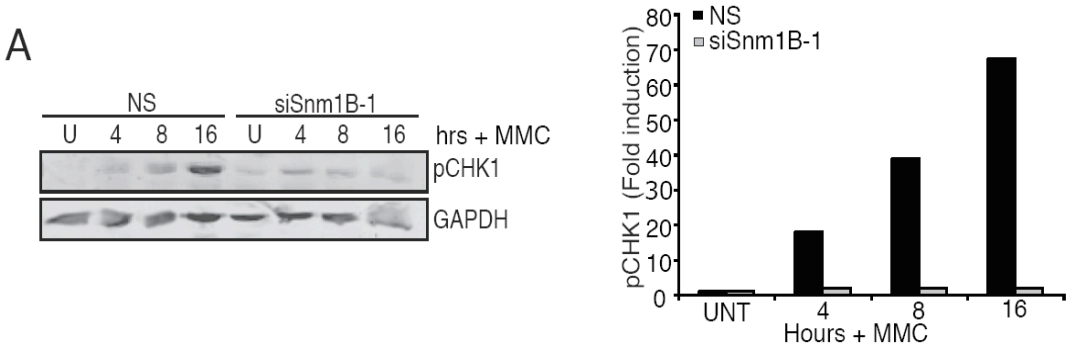
	Levels of Snm1B mRNA (% of control)
HCT116	50-70
HeLa	65-90
Human Fibroblasts	60-90

Supplementary Figure 2.1. Verification of Snm1B depletion. (A) Schematic of siRNAs **(B)** Validation of Snm1B siRNAs. **Top panels:** HCT116 cells stably expressing V5-Snm1B were transfected with siSnm1B-1 and harvested 48 hours post transfection. RT-PCR was performed on total RNA to amplify cDNA containing exons 13 and 14 of Artemis, exons 8 and 9 of Snm1A, and exons 3 and 4 of Snm1B. GAPDH was used as a loading control. **Middle panels:** HeLa cells were transfected with siSnm1B-1 and -2, as indicated, and harvested 48 hours post transfection. RT-PCR was performed on total RNA to amplify the Snm1B cDNA. **Lower panel:** HCT116 cells stably expressing V5-tagged Snm1B was transfected with NS or siSnm1B-1 and harvested 48 hours post transfection. V5-Snm1B was immunoprecipitated from whole cell lysates (200 μg) using the α-V5 antibody and analyzed by Western blotting with α-V5. **(C)** Range of Snm1B knockdown as determined by RT-PCR in three different cell lines. Bands were normalized to RT-PCR of GAPDH.



Supplementary Figure 2.2. S phase checkpoint defect in Snm1B depleted HCT116 cells. **(A)** Cell cycle profiles of Snm1B depleted HCT116 cells in response to MMC exposure. HCT116 cells transfected with NS or siSnm1B-1 were treated with MMC (100 ng/ml) for 1 hour. Drug was removed and cells were collected at indicated times post MMC. Cell cycle profiles were determined using propidium iodide. **(B)** Accumulation of MMC treated HCT116 cells in S phase. Graphical representation of (A). Average of three independent experiments with standard deviation.

Supplementary Figure 2.3. Defective ATR-dependent signaling in Snm1B depleted cells. (A) Levels of CHK1 phosphorylation in siSnm1B-1 transfected HeLa cells. HeLa cells were transfected with NS or siSnm1B-1 and treated with MMC (1μg/ml) for indicated times. Cells were harvested and membranes were probed with α-CHK1ser317 antibodies. GAPDH was used as a loading control. Western blotting was performed at least three times from two independent transfections. Representative blot is shown. Graph represents fold induction relative to the untreated control. **(B)** Levels of CHK1 phosphorylation in siSnm1B-2 transfected HeLa cells. Experiment was performed as described in (A). Western blotting was performed with lysates isolated from three independent transfections. Two representative experiments are shown. **(C)** CHK1 phosphorylation in Snm1B depleted HCT116 cells after MMC exposure. HCT116 were transfected with NS or siSnm1B-1 and were treated with MMC (10 μg/ml) for 1 hour. Cells were collected at indicated times post MMC treatment. Membranes were probed with α-CHK1ser317 antibodies. GAPDH was used as a loading control. Fold induction compared to untreated is indicated below blot. Western blotting was performed at least three times from two independent transfections. Representative blot is shown. **(D)** Defective induction of phospho-RAD17 in Snm1B depleted cells upon interstrand crosslink exposure. HeLa cells transfected with NS or siSnm1B-2 were exposed to MMC (1 μg/ml) for indicated times. Cells were harvested and membranes were probed with α-pRAD17ser645. GAPDH was used as a normalization control. Protein levels were normalized to GAPDH and fold induction was determined relative to untreated control. Representative results are shown.


A
NS
siSnm1B-1
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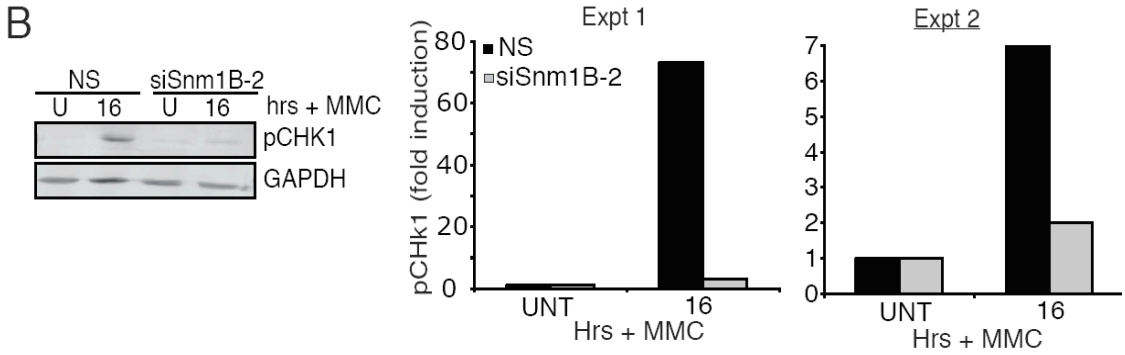
hrs + MMC
pCHK1
GAPDH
NS
siSnm1B-1
pCHK1 (Fold induction)
80
70
60
50
40
30
20
10
0
UNT
4
8
16
Hours + MMC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Expt 1
Expt 2
NS
siSnm1B-2
pCHk1 (fold induction)
80
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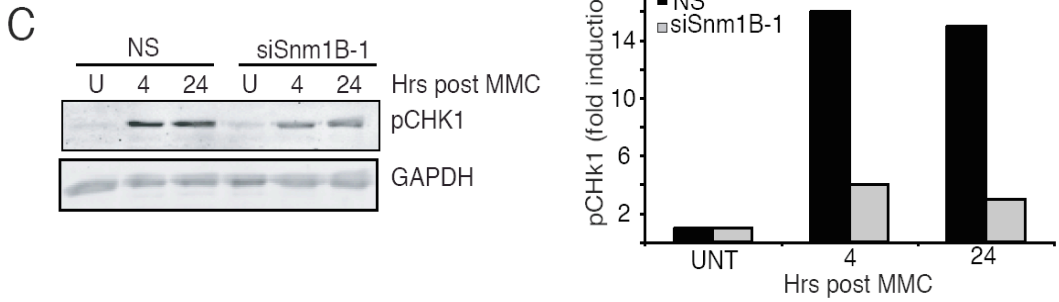
Hrs + MMC


C
NS
siSnm1B-1
U 4 24 U 4 24
Hrs post MMC
pCHK1
GAPDH
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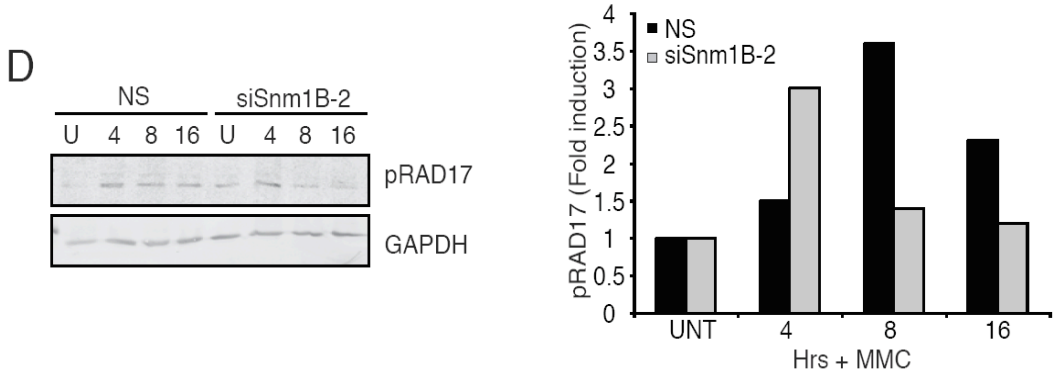
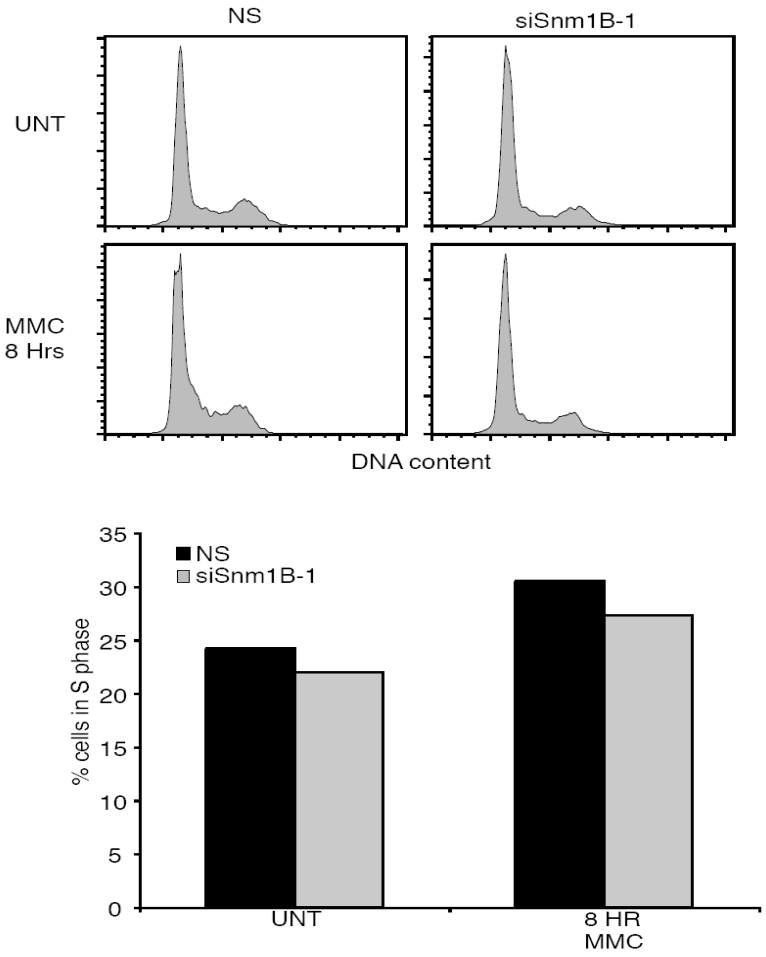
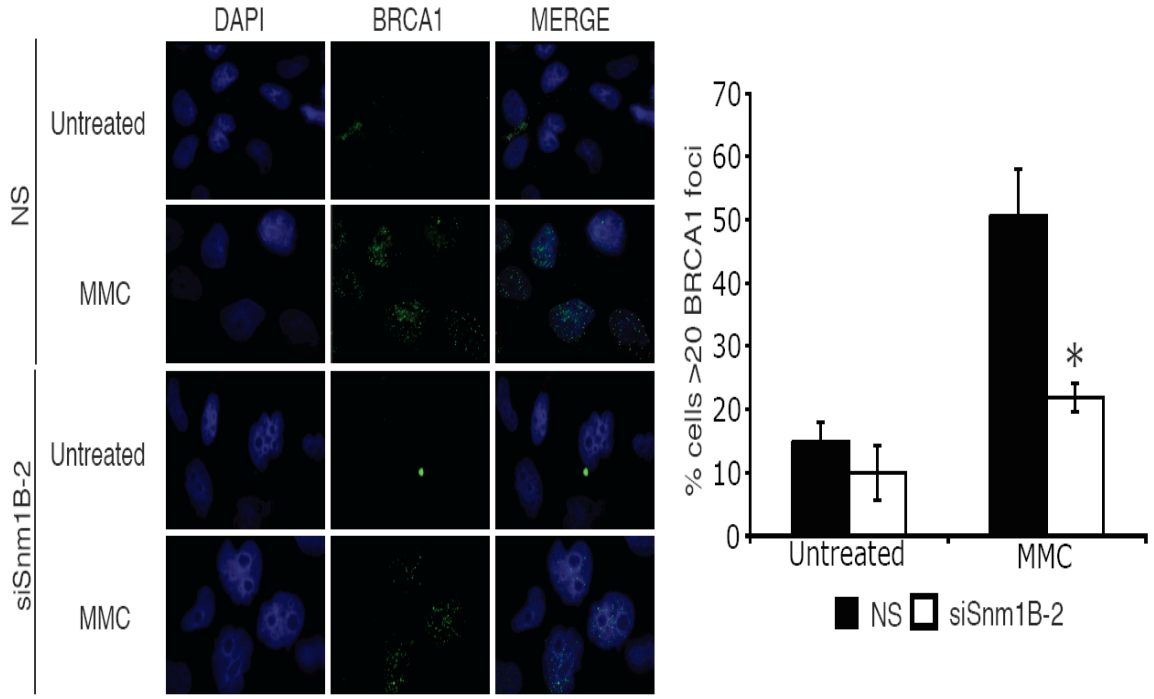
24
Hrs post MMC


D
NS
siSnm1B-2
U 4 8 16 U 4 8 16
pRAD17
GAPDH
NS
siSnm1B-2
pRAD17 (Fold induction)
4
3.5
3
2.5
2
1.5
1
0.5
0
UNT
4
8
16
Hrs + MMC



Supplementary Figure 2.4. Accumulation of S phase cells at 8 hours post MMC treatment. HeLa cells transfected with NS or siSnm1B were treated with MMC (1μg/ml) for 8 hours. Cells were harvested and DNA content was determined using propidium iodide. (top). Graph indicates the percent of cells in S phase as determined by DNA content and is the average of two independent experiments.



Supplementary Figure 2.5. BRCA1 foci formation is impaired in siSnm1B-2 depleted HeLa cells. HeLa cells transfected with siSnm1B-2 and at 48 hours post transfection were treated with MMC (330 ng/ml) for 8 hours. Cells were fixed with p-formaldehyde and stained for α-BRCA1 antibodies (green). Nuclei were stained with DAPI (blue). Graph represents average of at least three independent experiments with standard deviation. *P<0.05, Students two-tailed t-test.

Chapter 3

Snm1B functions within the context of the FA/BRCA1 pathway in response to interstrand crosslinks

Summary

DNA interstrand crosslinks are covalent linkages between the strands of the double helix and represent one of the most cytotoxic forms of DNA damage. Interstrand crosslink repair involves the coordinated activities of multiple proteins that function in distinct repair pathways, including the FA/BRCA protein network. Snm1B/Apollo, a member of metallo-β-lactamase/βCASP family of nucleases, has been demonstrated to function in interstrand crosslink repair; however, its precise roles and functional relationship to the FA/BRCA pathway are not understood. Here we identify a key function for Snm1B in the FA/BRCA network. We establish that Snm1B functions epistatically to the FA factor, FANCD2, in cellular survival after interstrand crosslink damage. Furthermore, we find that Snm1B is required for localization of critical repair factors, including FANCD2 to interstrand crosslink induced subnuclear foci. Our findings establish that Snm1B plays important roles in the context of the FA/BRCA pathway during interstrand crosslink repair.

Introduction

DNA interstrand crosslinks represent one of the most formidable lesions as covalent linkage of the strands of the double helix inhibits essential processes such as transcription and replication. Due to their cytotoxicity, interstrand crosslink inducing agents, such as MMC and cisplatin are widely used as chemotherapeutic agents. However, despite the clinical and biological importance of interstrand crosslinks, the molecular mechanisms underlying the repair of these lesions are poorly understood. In this regard, the sensing and resolution of interstrand crosslinks is a complex process which involves components of multiple DNA repair pathways including nucleotide excision repair, translesion bypass synthesis and homologous recombination (1).

In eukaryotic cells, a double strand break forms as an intermediate during interstrand crosslink repair as a result of replication fork collapse. This results from endonucleolytic cleavage of the stalled fork by MUS81-EME1. Unhooking of the interstrand crosslink is mediated by the endonuclease ERCC1-XPF that incises 5' of the crosslink. Translesion bypass occurs past the unhooked interstrand crosslink and removal is believed to occur by the NER pathway. Once removed, the double strand break is repaired by homologous recombination resulting in replication fork restart.

The FA (FA) factors play critical roles in the repair of interstrand crosslinks in mammalian cells, but the precise mechanism is unknown. FA is an autosomal, recessive chromosomal instability syndrome characterized by developmental abnormalities, aplastic anemia, cancer predisposition and cellular hypersensitivity to interstrand crosslink-inducing agents (2). There are currently 13 identified FA complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M and N), and the protein factors encoded by

the FA genes function within a protein network (1, 2). Central to the FA protein network is the core complex comprised of eight subunits (FANCA, B, C, E, F, G, L and M) that coordinately function to monoubiquitinate FANCD2 and FANCI, a critical event in FA mediated interstrand crosslink repair (3). The monoubiquitination signal targets the ID complex where it colocalizes with the where it colocalizes with proteins required for homologous recombination, including BRCA1, BRCA2 (FANCD1), and RAD51 (4-6).

Previous studies have demonstrated that Snm1B deficiency results in hypersensitivity to interstrand crosslink damage, moderate sensitivity to ionizing radiation, chromosomal instability and increased interstrand crosslink-induced quadriradial chromosome formation (7, 8). These phenotypes are characteristic of cells derived from FA patients, thereby raising the possibility that Snm1B may function within the FA/BRCA pathway. Consistent with this notion, the human *Snm1B* gene has been identified as a candidate gene associated with primary mediastinal B-cell lymphomas as the Snm1B genomic locus was found deleted in 42% of tumors analyzed (23). Cancer predisposition, particularly lymphoid malignancy, is a hallmark phenotype associated with FA (29). However, whether Snm1B functions within the context of the FA/BRCA pathway remains an open question.

In this study, we examine the interplay between Snm1B and the FA/BRCA pathway. Using an siRNA approach in human cell lines, we demonstrate that Snm1B functions epistatically to FANCD2 in cellular interstrand crosslink repair and is required for efficient localization of FANCD2 to repair foci. Furthermore, Snm1B depletion results in a modest reduction in homologous recombination. Together, these findings establish that Snm1B functions in the context of the FA/BRCA network during

interstrand crosslink repair.

Materials and Methods

Knockdown of Snm1B expression by siRNA. The HCT116 colon cancer, HeLa cervical cancer, and FANCD2 mutant and control fibroblast human cell lines were cultured as previously described (9, 21). HCT116 or HeLa cells were plated at a density of $1x10^5$ cells per well of 6 well dish 24 hours prior to siRNA transfection. All siRNAs (25-50 nM) were transfected using Liptofectamine 2000 (Invitrogen) as per manufacturer's instructions. PD20F and control fibroblasts were transfected with 150 nM siRNA as previously described (22). Snm1B mRNA levels were determined via RT-PCR in every experiment to verify the extent of siRNA knockdown.

Semi-quantitative RT-PCR analysis of Snm1B mRNA levels. Total RNA (1 μg) was reverse transcribed using a poly-dT(20) primer and MLV-reverse transcriptase (Invitrogen). cDNA was amplified with Snm1B-specific primers to exons 3 and 4, and GAPDH for normalization of cDNA levels. Bands were quantitated using AlphaImager 2200 (AlphaInnotech).

Western blot analyses. Cells ($1-2x10^6$) were harvested and resuspended in lysis buffer (25 mM HEPES, pH 7.4, 10% glycerol, 200mM KCL, 0.1% NP40, 1mM DTT) containing phosphatase (Roche PhosSTOP) and protease inhibitors (Roche Complete Mini EDTA free). Soluble and chromatin bound FANCD2 fractions were separated as previously described (23). Protein concentration was determined using the Bradford

assay (BioRad). Lysates (50-100 μg) were analyzed by Western blotting with the appropriate primary antibodies and IRdye 800 CW secondary antibodies (Li-Cor). Bands were visualized and quantitated using the Li-Cor scanner and Odyssey 2.1 software. All experiments were performed three times from at least two independent transfections.

Immunofluorescence of subnuclear foci. HeLa cells (4×10^4) were seeded on coverslips in 12 well dishes 24 hours prior to transfection. At 48 hours post-transfection, cells were continuously exposed to MMC (330 ng/ml) for 8 hours, then incubated in cold pre-extraction buffer (20mM HEPES, 50mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% TX-100) for 5 minutes followed by fixation in 3.7% p-formaldehyde, 2% sucrose, 0.5% TX100 for 20 minutes at room temperature and washed 3X with PBS. Cells were incubated with the appropriate primary antibody for 45 minutes then stained with Alexa Fluor 488 or 594 secondary antibodies (Invitrogen Molecular Probes). Coverslips were mounted on slides using ProLong Gold antifade reagent containing DAPI (Invitrogen). Foci were visualized using an Olympus BX61 microscope and FISHview software (Applied Spectral Imaging). All foci experiments were performed blinded. Each experiment was performed at least three independent times.

Mitomycin C survival assay. Cellular survival upon treatment with MMC was determined using a colorimetric assay, as previously described (10). Average percent survival was obtained from at least three independent experiments.

Analysis of homologous recombination frequencies. HeLa cells were transfected with the DR-GFP Puro homologous recombination substrate plasmid (generous gift from Maria Jasin, Sloan-Kettering Institute) (35), and single colonies were expanded after puromycin selection. HeLa cells harboring the stably integrated DR-GFP plasmid were transfected with siSnm1B-1 or -2 or NS RNA (50nM) as above. At 24 hours post transfection, cells were infected with either an adenovirus expressing the I-SceI endonuclease or a negative control adenovirus at 100-200 PFU/cell. Cells were trypsinized at 48 hours post infection, and GFP positive cells were determined by flow cytometry as above. Average was obtained from three independent experiments.

Results

Interplay between Snm1B and the FA/BRCA pathway.

Recently, it has been reported Snm1B and FANCD2 can be co-immunoprecipitated from human cells (7), thereby raising the possibility that Snm1B may function in concert with the FA pathway in interstrand crosslink repair. To assess the genetic interactions between Snm1B and FANCD2 during the repair of MMC induced interstrand crosslink damage, we depleted Snm1B in FANCD2 deficient human fibroblasts (PD20F) and a control cell line complemented with FANCD2 cDNA using siRNAs as described in Chapter 2 (9). Using a colorimetric assay (10, 11), we examined the survival of cells deficient for Snm1B and FANCD2, either alone or in combination, upon MMC treatment. We observed that Snm1B and FANCD2 deficiencies led to increased hypersensitivity to MMC, consistent with previous studies (Figure 3.1A) (3, 7, 8, 10, 12, 13). However, Snm1B depletion in the FANCD2 deficient cell line did not

further increase cellular sensitivity to MMC (Figure 3.1A, Supplementary Figure 3.1A). These results indicate that Snm1B and FANCD2 function epistatically in response to MMC.

We next examined the impact of Snm1B deficiency on FANCD2 chromatin localization and foci formation. In response to interstrand crosslink damage, FANCD2 undergoes monoubiquitination which is dependent on the FA core complex (1). Monoubiquitinated FANCD2 (FANCD2-Ub) is targeted to chromatin and forms DNA repair foci, and proper localization of FANCD2 is required for interstrand crosslink repair (1). We exposed Snm1B depleted and control cells to MMC and examined the cellular localization of FANCD2 by fractionation of the cytosolic and chromatin bound proteins. Monoubiquitination of FANCD2 results in a slower migrating band that can be measured by immunoblotting. Monoubiquitination of FANCD2 is induced upon MMC treatment in control and Snm1B knockdown cells, consistent with previous reports (Figure 3.1B) (7, 8). We observed that the ratios of monoubiquitinated FANCD2 (L) to the unmodified form (S) in the cytosolic and chromatin protein fractions were not significantly different in control and Snm1B-deficient cells (Figure 3.1B). These findings indicate that Snm1B is not required for proper localization of FANCD2-Ub to the chromatin fraction.

The localization of monoubiquitinated FANCD2 to chromatin occurs independently of DNA repair foci formation (14). Therefore, we examined the efficiency of FANCD2 foci formation after MMC treatment in Snm1B depleted cells. As described in Chapter 2, the FANCD2 foci formation was examined after treatment with MMC for 8 hours, which is prior to when a cell cycle defect was observed. After MMC treatment, the percentage of control cells containing FANCD2 foci increased by over 3-fold, thereby

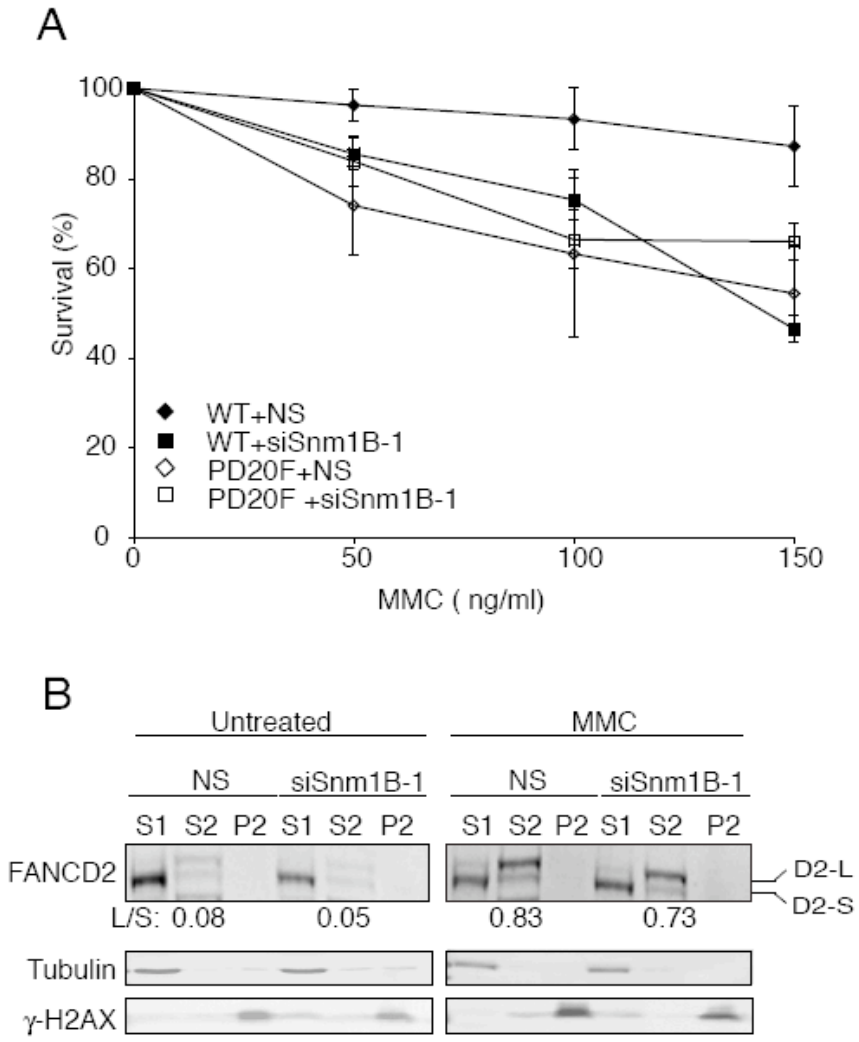


Figure 3.1. Snm1B functions epistatically to the FA gene, FANCD2, in interstrand crosslink repair. (A) FANCD2 deficient PD20F fibroblasts and PD20F cells complemented with FANCD2 cDNA were transfected with NS and siSnm1B-1. At 48 hours post transfection, cells were plated and treated with indicated MMC concentrations for 1 hour. Percent survival compared to untreated sample was determined. Average of at least three independent transfections with standard deviation is shown. **(B)** FANCD2 monoubiquitination in NS and siSnm1B-1 depleted cells. HeLa cells transfected with NS or siSnm1B-1 were treated with MMC (1 μg/ml) for 8 hr at 48 hr post transfection. Cells were collected and the soluble and chromatin associated proteins were fractionated. Membranes were probed with α-FANCD2 antibodies. Tubulin and γ-H2AX were used as controls for S1 and P2 fractions, respectively. The L/S ratio of FANCD2 is shown below blot. S1-Soluble, S2-chromatin bound, P2-chromatin bound (tightly).

indicating robust interstrand crosslink-induced activation of the FA pathway (Figure 3.2). In contrast, Snm1B deficiency markedly impaired induction of FANCD2 foci formation upon MMC exposure (Figure 3.2; Supplementary Figure 3.1B). Taken together, these findings indicate that although Snm1B is dispensable for FANCD2 monoubiquitination and chromatin localization, it is required for interstrand crosslink induced FANCD2 nuclear foci assembly.

Snm1B promotes homology-directed repair of DNA double strand breaks

During the processing of interstrand crosslinks, DNA DSB intermediates are generated and are repaired by the homologous recombination pathway (42). Homologous recombination facilitates replication restart by catalyzing strand invasion of the one-sided DSB between sister chromatids via a mechanism dependent on the activities of BRCA1, BRCA2 and RAD51 (14). Proper resolution of the resulting Holliday junction intermediate allows for progression of the fork beyond the site of interstrand crosslink damage. Factors within the FA network, including FANCA, FANCG, and FANCD2, promote homologous recombination of DSBs (32, 39, 52). Our findings implicating Snm1B in the FA/BRCA pathway led us to examine the impact of Snm1B depletion on homologous recombination levels. To this end, we used the characterized DR-GFP reporter construct that measures levels of homologous recombination of a specific DSB generated by the rare cutting endonuclease, I-SceI (35). Successful homologous recombination of the DR-GFP substrate restores expression of a wildtype GFP cassette, and the levels of homologous recombination can be assessed by flow cytometry.

Snm1B was depleted in a HeLa cell line harboring a chromosomally integrated

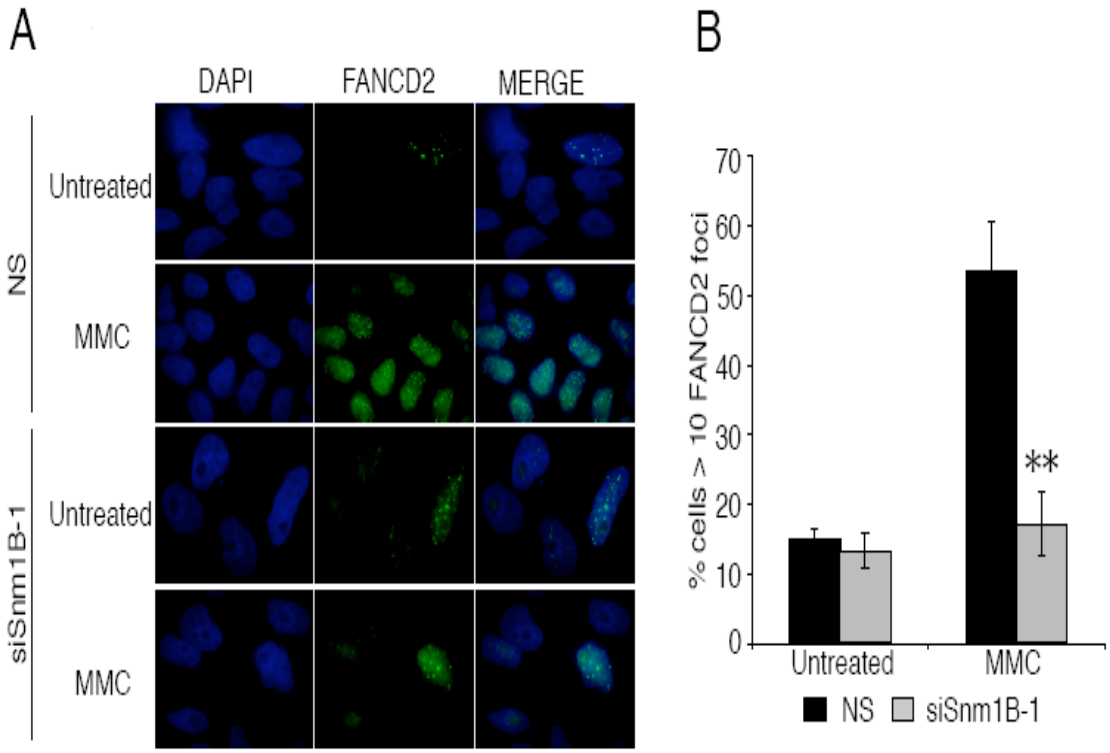


Figure 3.2. Interstrand crosslink-induced FANCD2 foci formation is impaired in Snm1B depleted cells. HeLa cells transfected with NS or siSnm1B-1 were treated with MMC (330 ng/ml) for 8 hr at 48 hr post transfection. Cells were fixed with p-formaldehyde and stained using α-FANCD2 antibodies (green). Nuclei were stained with DAPI (Blue). The percentage of cells with greater than 10 FANCD2 foci was determined. Graph represents average foci positive cells from three independent experiments with standard deviation. **P<0.005, Students two-tailed t-test.

DR-GFP construct (Figure 3.3A). At 24 hours post-transfection, the cells were infected with an adenovirus expressing I-SceI to initiate homologous recombination via the generation of a specific DSB. The levels of the DSB repair via homologous recombination were determined by assessing the percentage of GFP positive cells at 48 hours post-infection (Figure 3.3B). We observed that Snm1B depletion by siSnm1B-1 and -2 consistently resulted in a modest reduction in homologous frequency compared to controls (Figure 3.3C). Thus, these results provide evidence that Snm1B promotes efficient homologous recombination of chromosomal DSBs.

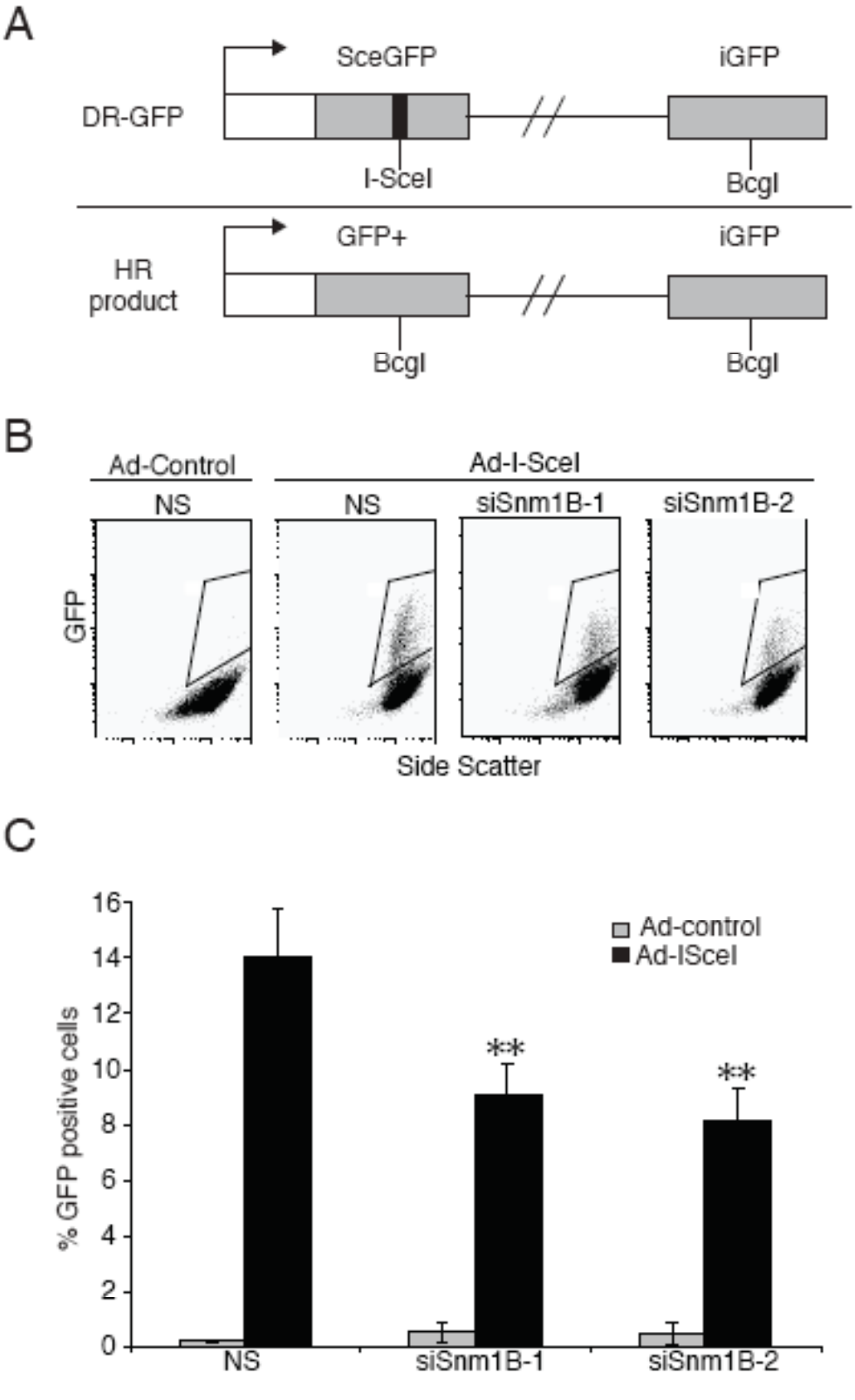
Discussion

Snm1B functions in the context of the FA/BRCA protein network of interstrand crosslink repair.

In this study, we establish that Snm1B functions within the FA/BRCA pathway during repair of interstrand crosslink damage. Snm1B depleted cells exhibit cellular phenoytpes associated with FA including sensitivity to interstrand crosslinks, ionizing radiation, and interstrand crosslink-induced genomic instability (7, 8, 15). Previous studies have indicated that FANCD2 and Snm1B co-immunoprecipitate (7). In this study, we show that Snm1B acts epistatically to FANCD2 in cellular repair of interstrand crosslink damage as Snm1B depletion in FANCD2 mutant cells did not further increase sensitivity to MMC compared to either Snm1B or FANCD2 deficiencies alone.

In response to MMC, Snm1A was found to be non-epistatic to the FA pathway. Snm1A depletion in a FANCA deficient cell line increased MMC-induced chromosomal anomalies when compared to the FANCA deficient cell line alone, indicating that Snm1A

Figure 3.3. Snm1B promotes homology-mediated repair of DNA double strand breaks. (A) DR-GFP reporter construct. The DR-GFP contains a GFP cassette that has been disrupted by the rare cutting endonuclease, I-SceI (top) and an internal GFP fragment downstream (iGFP). Upon I-SceI expression, a double strand break is formed at the I-SceI site. If the resulting double strand break is repair by homologous recombination using the iGFP fragment, the GFP cassette is restored leading to GFP expression (bottom). Adapted from (35). **(B)** Representative plots of HeLa DR-GFP cells infected with Ad-Control or Ad-I-SceI. HeLa cells were transfected with NS, siSnm1B-1, or siSnm1B-2 and infected with the indicated adenovirus. 48 hours post transfection, samples were collected and analyzed for GFP positive cells by flow cytometry. **(C).** Snm1B depletion reduces homologous recombination frequency. Graph indicates the % cells that are GFP positive for each sample and is an average of three independent experiments. Bars are standard deviation. **p-value <0.005 as determine using students t-test.


A
SceGFP
iGFP
DR-GFP
I-SceI
BcgI
GFP+
iGFP
HR
product
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BcgI
B
Ad-Control
Ad-I-SceI
NS
NS
siSnm1B-1
siSnm1B-2
GFP
Side Scatter
C
16
14
12
10
8
6
4
2
0
% GFP positive cells
Ad-control
Ad-ISceI
**
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NS
siSnm1B-1
siSnm1B-2

functions independently of the FA pathway in interstrand crosslink repair (16). Furthermore, deficient DT40 chicken B cells Snm1A and Snm1B were more sensitive to interstrand crosslink inducing agents than Snm1A-deficient or Snm1B-deficient DT40 cells alone, indicating independent for redundant functions (17). Together, this indicates that Snm1B and Snm1A have distinct functions in interstrand crosslink repair.

The role of FANCD2 can be divided into three independent steps: mono-ubiquitination, chromatin localization, and DNA repair foci formation (18). Consistent with previous studies, we found that Snm1B is not required for FANCD2 mono-ubiquitination (7, 8). In this chapter, I demonstrated that Snm1B is also dispensable for localization of Ub-FANCD2 to chromatin. ERCC1- and XPF-deficient cells exhibit WT levels of mono-ubiquitination, but reduced Ub-FANCD2 chromatin localization indicating that unhooking of the interstrand crosslink was critical for efficient localization of FANCD2 to chromatin (19). Previously, Snm1B was shown to interact with MUS81-EME1, which makes an incision 3' of the interstrand crosslink and is thought to promote double strand break formation, thus contributing to interstrand crosslink unhooking (7). Mus81-deficient cells activate FANCD2 consistent with the hypothesis that unhooking is dispensable for FANCD2 monoubiquitination. In this chapter, I show that Snm1B does not affect FANCD2 localization to chromatin (Figure 3.1). However, Mus81-deficient ES cells do not show a defect in FANCD2 chromatin localization indicating the link between interstrand crosslink processing and FANCD2 localization warrants further investigation (19).

In this chapter, I demonstrate that Snm1B is required for efficient MMC-induced FANCD2 foci formation. Deficiency for the ubiquitin protease, USP1, leads to a similar

impairment of assembly of chromatin-associated monoubiquitinated FANCD2 into nuclear foci, thereby suggesting that Snm1B and USP1 may function during the same stage of interstrand crosslink repair (14). It has been suggested that chromatin localization of FANCD2-Ub promotes assembly of DNA repair complexes, and USP1-mediated deubiquitination of FANCD2-Ub induces nuclear foci formation which may facilitate subsequent interstrand crosslink repair events, such as homologous recombination and/or translesion synthesis (14). Indeed, recent evidence in Xenopus cell-free extracts indicates that the ID complex is required for incision of the interstrand crosslink lesion as well as translesion synthesis (20).

Together, these findings support a model in which Snm1B functions in concert with FANCD2 to facilitate the cleavage and/or processing of the interstrand crosslink lesions. In support of this notion, Snm1B has been found to co-immunoprecipitate with FANCD2 via an indirect interaction, thereby suggesting that Snm1B may function coordinately in the context of DNA repair complexes with FANCD2 at sites of interstrand crosslink damage. Snm1B also directly interacts with the Mus81-Eme1 nuclease and is involved in replication fork collapse and formation of DSB intermediates during interstrand crosslink repair (7). The nuclease activities intrinsic to Snm1B are likely involved in a subset of processing events. Thus, it will be of interest to elucidate the full catalytic repertoire of Snm1B nucleolytic activities and to determine their importance in Snm1B cellular functions.

The findings described in this chapter demonstrate that Snm1B functions within the context of the FA/BRCA network in interstrand crosslink repair. As mutations in many of the genes that function within the FA/BRCA pathway are associated with

genome instability disorders, our findings raise the possibility that mutations in Snm1B may lead to similar phenotypic outcomes.

Acknowledgements

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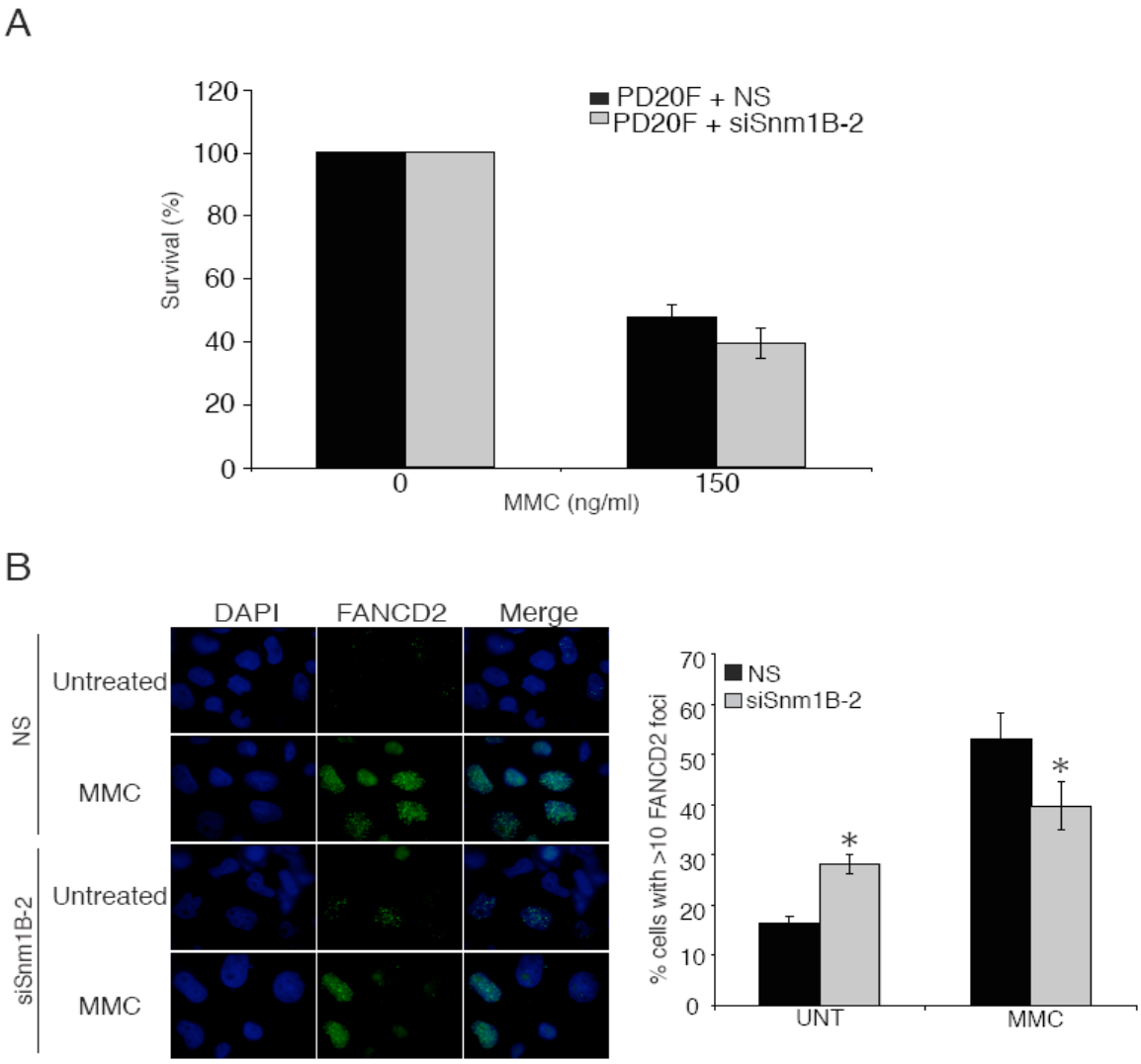


Supplementary Figure 3.1. Snm1B functions within the FA/BRCA pathway. (A) MMC sensitivity of siSnm1B-2 depleted FANCD2 deficiency fibroblasts. PD20F ($FANCD2^{-/-}$) fibroblasts were transfected with NS or siSnm1B-2 and 48 hours later plated at a low density. Cells were treated with MMC (150 ng/ml) for 1 hour. Percent survival compared to untreated samples was determined. Graph represents average of three independent experiments with standard deviation. **(B)** FANCD2 foci formation after MMC. HeLa cells transfected with NS or siSnm1B-2 were treated with MMC (330 ng/ml) 48 hours post transfection for 8 hours. Cells were fixed with p-formaldehyde and stained using α-FANCD2 (green). Nuclei were stained with DAPI (Blue). The percentage of cells with greater than 10 FANCD2 foci was determined. Graph represents average foci positive cells from three independent experiments. Error bars are SD. *P<0.05, Students two-tailed t-test.

Chapter 4

Snm1B functions to maintain genome stability in response to replication stress

Introduction

Snm1B has been implicated in interstrand crosslink repair, double strand break repair and telomere processing. Snm1B depletion results in activation of DNA damage responses even in the absence of exogenous DNA damage as indicated by the formation of 53BP1 and γ-H2AX foci (1, 2) (Chapter 2). Interestingly, DNA damage foci were only observed in S phase indicating a role for Snm1B in repair of spontaneous DNA damage that occurs during replication. Consistent with this notion, Snm1B depleted cells have an increase in chromosomal anomalies in unperturbed cells (3). Together, these data suggest that Snm1B may be involved in repair of lesions that arise during replication.

The Ataxia-telangiectasia and Rad3 related (ATR) kinase is critical for maintaining genomic stability in response to replication perturbation. ATR null mice are early embryonic lethal and mouse embryonic fibroblasts (MEFs) exhibit an increase in chromosomal anomalies even in the absence of exogenous DNA damage (4, 5). An increase in genomic instability is observed when ATR deficient cells are treated with the replication inhibitor aphidicolin indicating that ATR is important for stabilization of replication forks (6, 7). Snm1B has roles in both ATR signaling in response to UV and interstrand crosslinks (8) (chapter 3) and in the FA network (chapter 2). Both of these

pathways have been shown to be critical for the replication stress response. This led us to hypothesize that Snm1B is involved in the cellular response to replication stress.

Common fragile sites are loci in the genome that exhibit gaps and breaks in response to partial replication inhibition by folate deficiency or replication inhibitors such as aphidicolin and hydroxyurea. Although over 80 common fragile sites have been identified, 20 common fragile sites make up over 80% of the lesions that are observed after low dose of aphidicolin(9). Common fragile sites are hot spots for chromosomal rearrangements, deletions, sister chromatid exchanges, and plasmid integration in response to treatment to low doses of aphidicolin, an inhibitor of polymerase α/δ. Furthermore, it has been shown that common fragile sites are sites of rearrangements and deletion in cancer cells, indicating that fragile site stability may contribute to tumorigenesis (9). Indeed, the common fragile sties FRA3B and FRA16D both are located within well-characterized tumor suppressor genes, FHIT and WWOX. Therefore, understanding the mechanism of fragile site stability is not only important for understanding replication, but also for prevention of tumorigenesis.

ATR is critical for maintaining fragile site stability in response to replication perturbation. ATR deficient cells when treated with low doses of aphidicolin exhibit significant increase in gaps/breaks and fragile site expression (6, 7). Interestingly, ATR depletion results in spontaneous fragile site expression indicating that common fragile site stability during unperturbed replication (6). Several ATR targets including CHK1, FANCD2, SMC1, and BRCA1 are important for fragile site stability (10-13). This indicates that S and G2 checkpoints are critical for preventing fragile site expression. Furthermore, proteins involved in double strand break response have been shown to be

involved in repair of double strand breaks that arise at stalled/collapsed replication forks including RAD51, DNA-PKcs, LIG IV, XLF/Cerunnos (9). Together, this supports that fragile site instability is due to stalled or incomplete replication.

In this chapter, I examine the role of Snm1B in the cellular response to replication stress. I found that Snm1B depleted human fibroblasts are hypersensitive to the replication inhibitor, aphidicolin. Furthermore, I show that Snm1B depleted cells exhibit elevated levels of gaps and breaks even in the absence of aphidicolin and this phenotype was further enhanced by addition of aphidicolin. Snm1B depletion results in expression of the common fragile sites FRA3B and FRA16D. Interestingly, we did not observe a defect in CHK1 phosphorylation. This chapter represents the first evidence that Snm1B functions in the cellular response to replication stress to maintain genome stability.

Material and Methods

Knockdown of Snm1B expression by siRNA. The HCT116, p53 -/- HCT116, and control fibroblast human cell lines were cultured as previously described (14, 15). The ATR^{Seckel} human fibroblasts were cultured as previously described (16). HCT116 cells were plated at a density of $1x10^5$ cells per well of 6 well dish 24 hours prior to siRNA transfection. All siRNAs (50 nM) were transfected using Liptofectamine 2000 (Invitrogen) as per manufacturer's instructions. Snm1B mRNA levels were determined via RT-PCR in every experiment to verify the extent of siRNA knockdown.

Aphidicolin sensitivity assay. WT fibroblasts transfected with NS or siSnm1B-1 were plated at low density 48 hours post transfection and incubated with the indicated doses of aphidicolin for 24 hours. Cells were washed with media three times and allowed to

recover 5-7 days. Percent survival was determined using the colorimetric assay for cell survival as previously described (17). The sensitivity curve was performed three independent times.

Chromosome anomalies and FISH. HCT116 cells transfected with NS or siSnm1B-1 were treated with aphidicolin (0.3 or 0.5 uM) for 24 hours. Cells were incubated with colcemid for 1 hr (untreated) or 3 hours (0.3 uM, 0.5uM aphidicolin). Cells were harvested and incubated in 0.075 KCL for 15 minutes at 37oC followed by a series of fixation in Carnoy's fixative (3:1 Methanol: acetic acid). Cells were dropped onto slides and baked prior to Giemsa staining or two- color Fish. Giemsa stained chromosomes were scored for gaps and breaks per metaphases. Average gaps and breaks per metaphase was determined from three independent experiments. For two-color FISH, probes were generated using BACS and YACS that span fragile site regions. YAC 850A6 was used for FRA3B and BAC26L41 was used for FRA216D. Probes were labeled by nick translation synthesis with digioxigenin (Roche) or biotin (Roche). Two-Color FISH was done as previously described (18). Roughly 20 signals have been examined for each sample from one independent experiment. Images were acquired using a Zeiss Axioscope epifluorescence microscope and Olympus DP70 digital camera system.

Western blot analysis. HCT116 cells transfected with NS or siSnm1B-1 were treated with 0.3 uM aphidicolin for the indicated times. Cells were resuspended in protein lysis buffer (25 mM HEPES, pH 7.4, 10% glycerol, 200mM KCL, 0.1% NP40, 1mM DTT) containing phosphatase (Roche PhosSTOP) and protease inhibitors (Roche Complete Mini EDTA free). Protein concentration was determined by Bradford assay. Lysates

(100ug) were analyzed by western blotting using phosho-CHK1ser317 antibodies (Cell signaling) and IRDye 800 CW secondary antibodies (Li-Cor). Bands were visualized and quantitated using Odyssey 2.1 scanner. All experiments were performed at least three independent times.

Results

Snm1B depletion leads to induction of apoptosis in HCT116 cells

HCT116 cells were depleted of Snm1B using siSnm1B-1 as described in Chapter 2. We observed that Snm1B deficiency significantly impaired cellular proliferation in HCT116 cells transfected with either siSnm1B-1 or -2, consistent with previous studies (Figure 4.1A) (1, 2). Flow cytometric analyses of cell cycle profiles revealed that Snm1B depletion led to an accumulation of cells with a sub-G1 content of DNA, which represents cells undergoing apoptosis (Figure 4.1B). The percentage of sub-G1 cells transfected with siSnm1B-1 increased significantly up to 72 hours post transfection to a level that was approximately 3.3-fold higher than the NS controls ($P<0.005$). Thus, the growth defect observed in Snm1B depleted human cell lines is due, at least in part, to the induction of cellular apoptosis. As HeLa cells are infected with HPV, p53 dependent responses are inactivated including apoptosis, therefore we note that a significant subG1 population in Snm1B depleted HeLa cells was not observed (19) (Compare Figure 4.1 and Figure 2.1).

I next assessed the levels of p53 accumulation in Snm1B depleted cells by Western blotting. In response to damaged DNA, p53 undergoes phosphorylation and

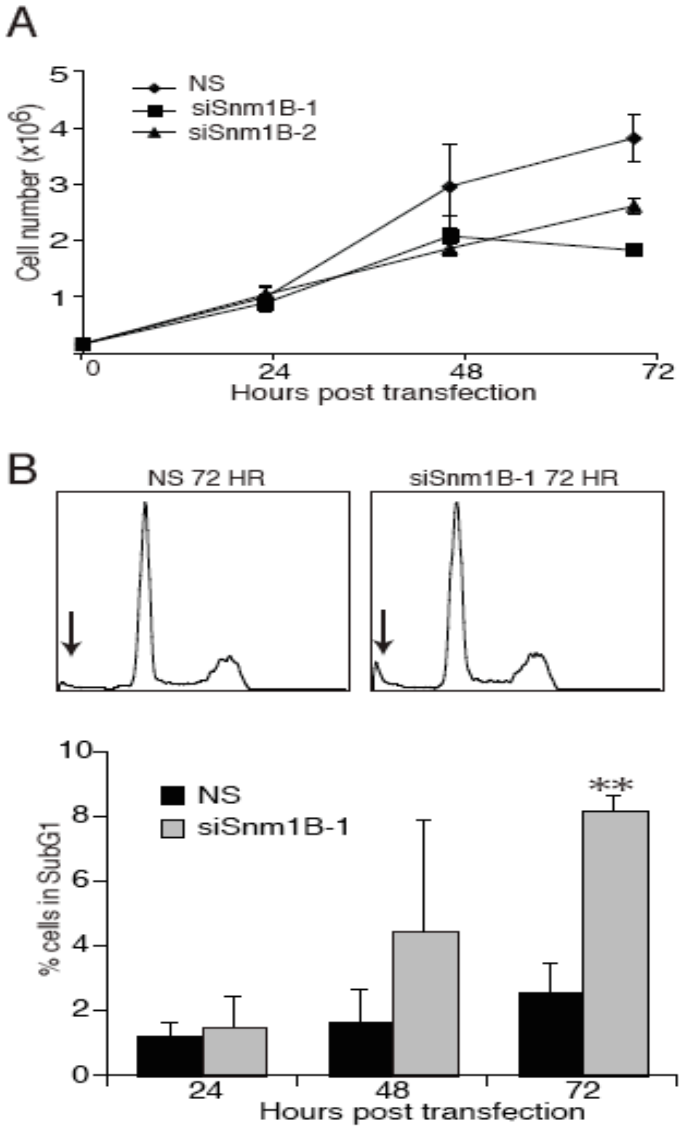


Figure 4.1. Snm1B depletion results in cellular apoptosis in HCT116 cells. (A) Growth curve of Snm1B HCT116 cells depleted with siSnm1B-1 or -2. Cells were harvested and counted at the indicated times post transfection. Snm1B depleted cells exhibit a growth defect. Graph represents three independent experiments. (B) Representative histograms for NS and siSnm1-1 at 72 hours post transfection with SubG1 population indicated by an arrow. Graph depicting the increase in the SubG1 population of Snm1B depleted HCT116 cells at the indicated times post transfection as determined by flow cytometry of propidium iodide stained cells. Graph represents data from at least three independent experiments. Error bars are standard deviation. **p-value < 0.005.

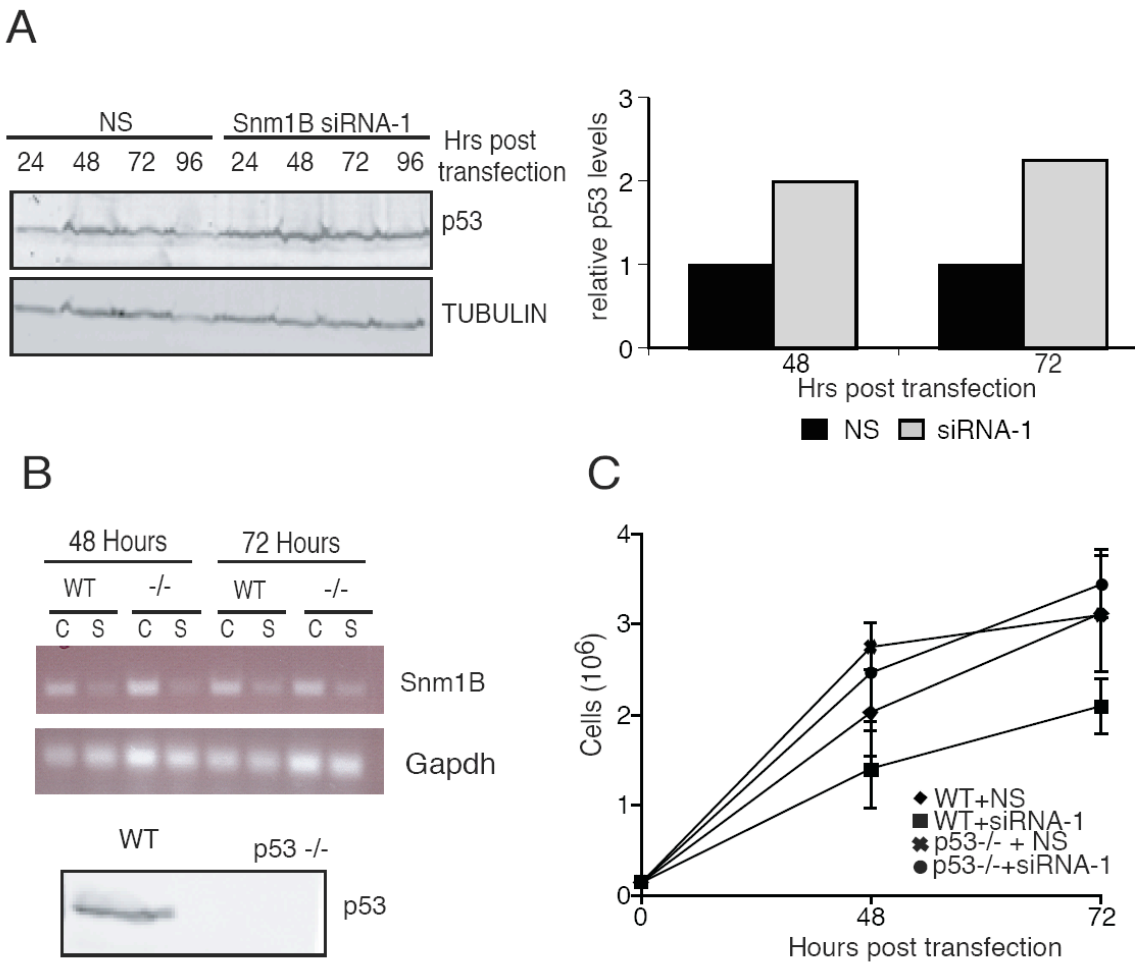


Figure 4.2. Growth defect observed in Snm1B depleted cells is p53 dependent. (A) Elevated p53 levels in Snm1B depleted HCT116 cells. HCT116 transfected with NS or siSnm1B-1 were collected at the indicated time post transfected. P53 levels were determined from whole cell lysates using p53 antibodies. Bands were normalized to Tubulin. Graph represents relative p53 levels when compared to the NS control and is the average of two independent experiments**. (B)** Verification of knockdown in p53 -/- HCT116 cells. Top. RT-PCR of NS (C) and siSnm1B-1 (S) transfected WT and p53 -/- at the indicated times post transfection. Bottom. Western blot showing that p53 is not detectable in the p53 -/- HCT116 line**. (C)** Growth curve of Snm1B depleted cells in the p53-/- HCT116 cell line. WT and p53 -/- were transfected with NS or siSnm1B-1 and counted at the indicated time post transfection. Graph represents average of three independent experiments. Error bars are standard deviation.

accumulates in cells to elicit cell cycle checkpoint activation or apoptosis (20). At 48 and 72 hours post-transfection, I observed that p53 levels were approximately 2-fold greater in HCT116 cells transfected with siRNA-1 or siRNA-2 when compared to the control NS siRNA (Figure 4.2A). These results suggest that unrepaired spontaneous DNA damage in Snm1B knockdown cells induces p53 stabilization which elicits programmed cell death. To test this hypothesis, I examined cellular proliferation in a p53 deficient HCT116 cell line, which harbors inactivating mutations in both p53 alleles (14). Initially, I confirmed that the Snm1B siRNA efficiently knocked down mRNA levels in the p53 null HCT116 cells. We observed that p53 deficiency fully rescued the growth defect caused by Snm1B depletion (Figure 4.2B). Thus, these results indicate that Snm1B deficiency leads to spontaneous DNA damage that induces p53-dependent apoptosis.

Snm1B deficient cells are hypersensitive to aphidicolin

Snm1B depletion results in DNA damage activation in the absence of exogenous damage in S phase. I hypothesized that the spontaneous DNA damage arising due to Snm1B deficiency could be due to stalled/collapsed replication forks. To determine whether Snm1B is critical to the response to replication stress, I determined if Snm1B depleted cells are sensitive to inhibition of replication by the polymerase α/δ inhibitor, aphidicolin (21, 22). WT fibroblasts transfected with NS or siSnm1B-1 were treated with the indicated concentrations of aphidicolin for 24 hours and cellular survival was determined after 5-7 days. Snm1B depleted fibroblasts had significantly reduced survival after aphidicolin treatment when compared to control even at the lowest dose of aphidicolin (Figure 4.3). These findings indicate that Snm1B is important for cellular survival in response to perturbed replication.

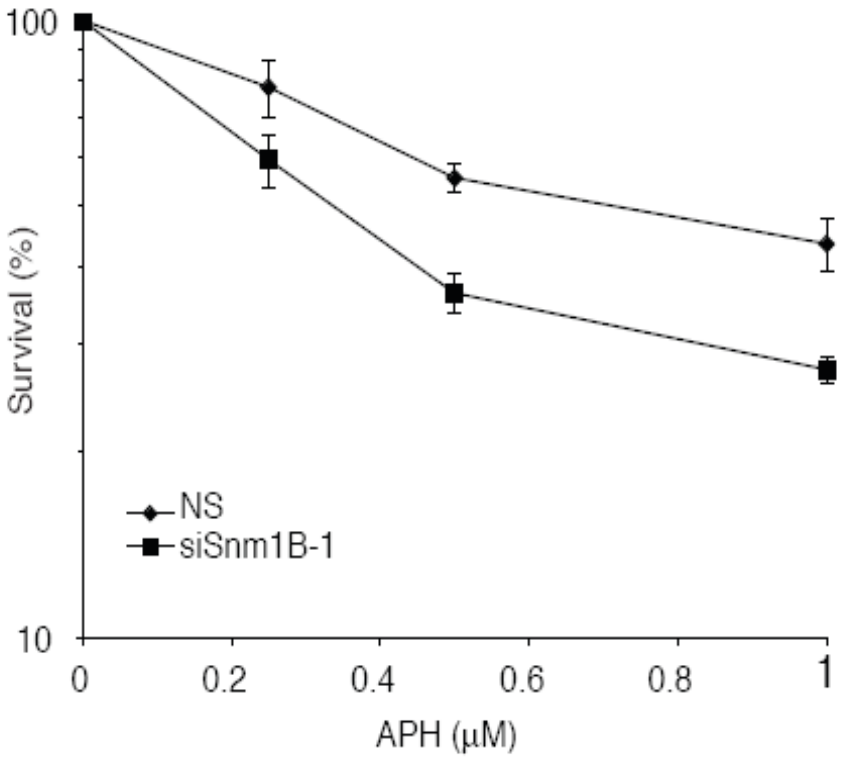


Figure 4.3. Snm1B depleted human fibroblasts are hypersensitive to aphidicolin. NS or siSnm1B-1 depleted WT human fibroblasts were treated with the indicated doses of aphidicolin for 24 hours. Colonies were allowed to from for 5-7 days. Percent survival was determined compared to an untreated control. Graph represents the average of three independent experiments. Bars are standard deviation.

We next examined the impact of Snm1B depletion on chromosome stability in response to replication stress. HCT116 cells depleted of Snm1B were treated with 0.3 μM and 0.5 μM aphidicolin for 24 hours before the cells were harvested and fixed. The number of gaps and breaks were scored using Giemsa band staining of metaphase spreads (Figure 4.4A). Snm1B depletion resulted in slight, but consistent elevation in the levels of gaps and breaks even in the absence of aphidicolin. After 24 hours of aphidicolin treatment, both NS and siSnm1B-1 transfected cells exhibited an increase in chromosomal anomalies. We observed that Snm1B depleted cells exhibited a dose dependent increase in gaps and breaks and at 0.5 μM aphidicolin Snm1B depleted cells showed 2-fold increase in gaps and breaks when compared to NS-transfected HCT116 cells (Figure 4.4B). It is noteworthy that 13-25% of metaphases scored in Snm1B depleted cells contained greater than 20 gaps and breaks per metaphase after aphidicolin treatment and were not included in the average, whereas only 5% of controls metaphases exhibited this level of anomalies. Therefore, the phenotype represented is an underestimate of the extent of replication stress induced chromosomal damage (Figure 4.4C, D). These data indicate a critical role for Snm1B in the maintaining genomic stability in response to replication perturbation.

Mutations in genes required for cellular responses to replication stress, including *Atr* and *Chk1* lead to an increase in instability at common fragile sites (6, 7, 11). My findings that Snm1B depletion leads to increased sensitivity to aphidicolin, led me to hypothesize that Snm1B depletion would likewise result in fragile site instability. To test this hypothesis, I examined expression of two common fragile sites FRA3B and FRA16D (9). NS and Snm1B depleted cells were treated with 0.3 μM aphidicolin for 24 hours prior to

harvesting and fixation. Metaphase spreads of DAPI stained chromosomes were examined for chromosome breaks at FRA3B and FRA16D by two-color fluorescent *in situ* hybridization (FISH) using well-characterized probes that hybridize within these fragile sites (12). In the absence of aphidicolin, 7% of FRA3B signals were localized with a break in siSnm1B-1 depleted HCT116 cells, whereas no breaks at FRA3B were detected in NS transfected HCT116 cells. Aphidicolin treatment of Snm1B depleted cells lead to a substantial increase in fragile site instability. I observed 22% of FRA3B signals localizing with a break, compared to 12% in the NS transfected HCT116 cells (Figure 4.5A). A similar trend was observed at FRA16D. In Snm1B depleted cells, 11% of FRA16D signals were localized within a break and this , which increased to 15% upon aphidicolin treatment compared to 9% in the NS aphidicolin treated controls (Figure 4.5B). Similar to FRA3B, no spontaneous breaks were detected at FRA16D in NS untreated controls. This indicates that Snm1B is important for maintaining fragile site stability in response to replication stress.

Snm1B is not required for CHK1 phosphorylation in response to aphidicolin

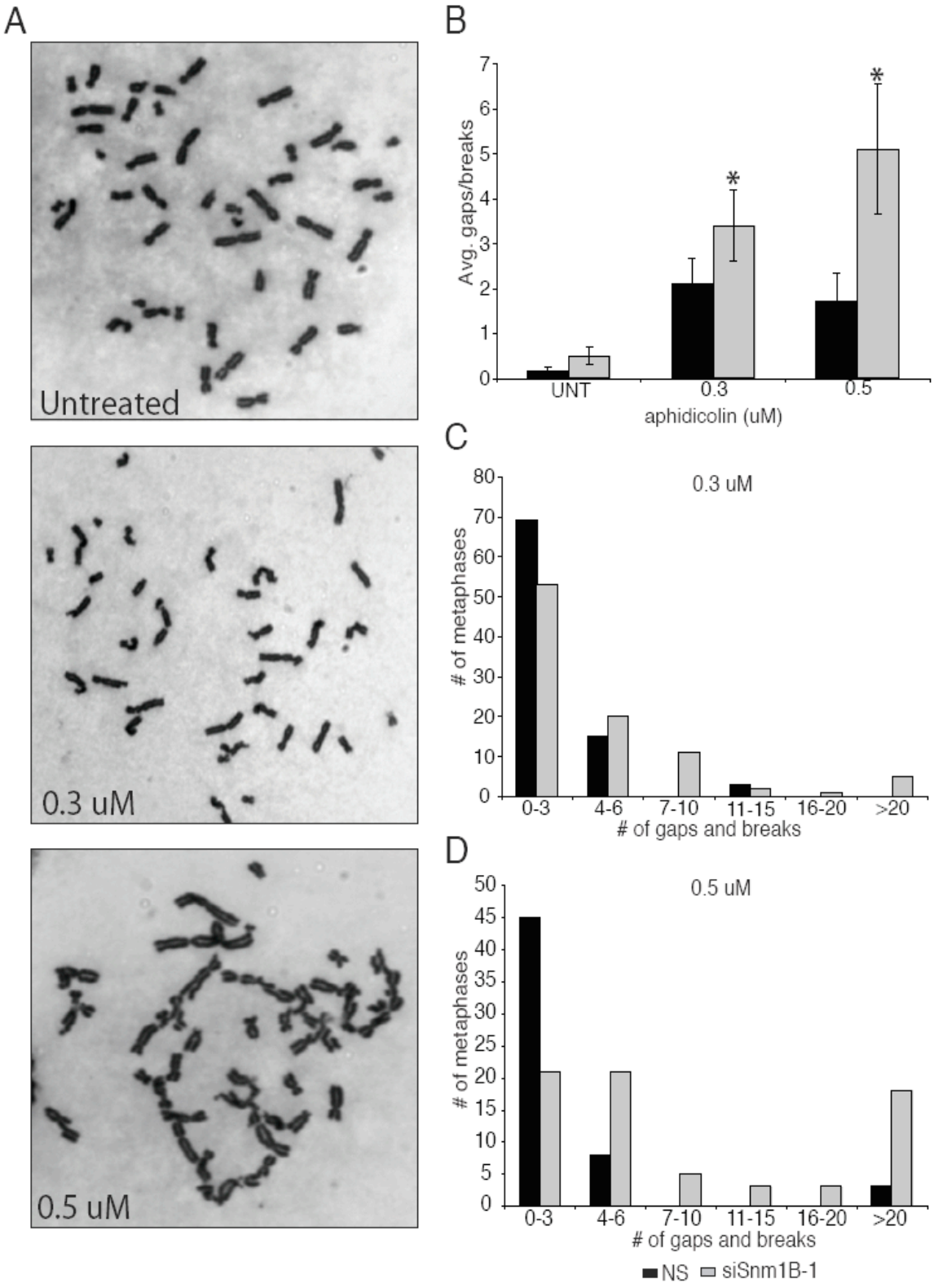
The ATR-CHK1 cell cycle checkpoint is critical for maintaining fragile site stability. CHK1 is phosphorylated on serines 317 and 345 after aphidicolin in an ATR-dependent manner (11). Therefore, we determined whether the increase in fragile site stability that is observed in Snm1B depleted cells is due to a defect in the ATR-CHK1 dependent signaling. To this end, we examined CHK1 phosphorylation on Ser317 in Snm1B depleted HCT116 cells by western blotting. NS or siSnm1B-1 transfected HCT116 cells were treated with 0.3 uM aphidicolin for 6, 12, and 24 hours prior to harvesting. In control cells, we observe a time dependent increase in phosphorylation of

CHK1. We also found that, CHK1 phosphorylation is intact in Snm1B depleted cells (Figure 4.6). In contrast, ATR^{seckel} fibroblasts treated with 0.3 μM aphidicolin for 12 hours exhibited a severe defect in CHK1 phosphorylation. Snm1B was significantly depleted in this experiment as indicated by the elevated pCHK1 levels in siSnm1B-1 untreated sample when compared to the NS untreated sample (Figure 4.6) and the presence of a growth defect in the siSnm1B-1 transfected cells (Figure 4.1, data not shown). Therefore, the absence of a pCHK1 defect after aphidicolin treatment is not due inefficient depletion of Snm1B. This indicates that Snm1B is not required for ATR-dependent CHK1 phosphorylation in response to aphidicolin treatment.

Discussion

In Chapters 2 and 3, I determined the role of Snm1B in interstrand crosslink repair. In this chapter, I provide evidence that Snm1B has important functions in response to the replication inhibitor, aphidicolin. Specifically, Snm1B ensures cellular survival and maintains chromosome integrity, including fragile site stability upon replication stress induction. Even in the absence of aphidicolin, Snm1B depleted cells exhibited expression of the common fragile sites FRA3B and FRA16D which was further increased upon treatment with low doses of aphidicolin. This indicates that the fragile site instability represents a major source of spontaneously arising chromosomal aberrations observed in untreated Snm1B depleted cells. Previous studies have indicated that Snm1B depletion resulted in activation of the DNA damage response in S phase (1). This phenotype is partially due to telomere dysfunction, but only 60% 53BP1 and γH2AX foci localized to telomeres. The evidence presented in this chapter indicates that

Figure 4.4. Snm1B depleted HCT116 exhibit elevated gaps and breaks in response to aphidicolin. (A). Representative Giemsa stained metaphases from Snm1B depleted HCT116 cells that were untreated or were treated with aphidicolin (0.3 uM or 0.5 uM) for 24 hours prior to harvesting. (B). Graph depicting average gaps and breaks per metaphase. Bars are standard deviation. *13-25% of Snm1B depleted metaphases contained >20 gaps/breaks and were not counted so average is an underestimate. Graph is the average of three independent experiments. (C-D) Graphs that depict the number of metaphases containing the indicated number of gaps/breaks at 0.3 uM (C) or 0.5 uM (D) aphidicolin.


A
Untreated
0.3 uM
0.5 uM
B
Avg. gaps/breaks
UNT
0.3
0.5
aphidicolin (uM)
C
0.3 uM
# of metaphases
0-3
4-6
7-10
11-15
16-20
>20
# of gaps and breaks
D
0.5 uM
# of metaphases
# of gaps and breaks
NS
siSnm1B-1

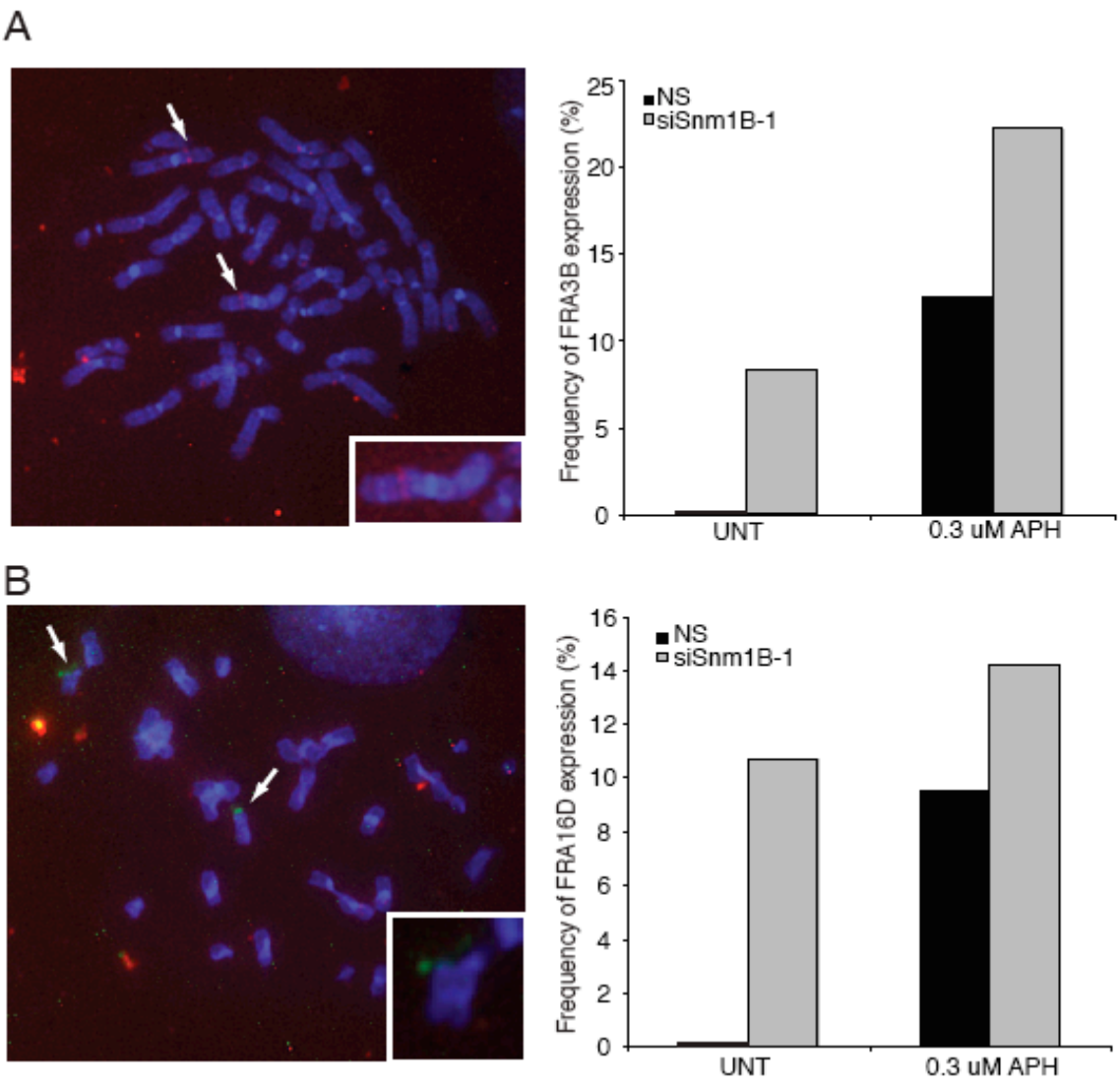


Figure 4.5. Snm1B depleted HCT116 cells have increased fragile site expression. HCT116 cells were transfected with NS or siSnm1B-1 and were treated with 0.3 uM aphidicolin for 24 hours prior to harvest. Untreated samples were used as controls. Two-color FISH was used to determine the frequency of FRA3B (red) and FRA16D (green) expression. (A) Left. Example metaphase from Snm1B depleted HCT116 cells. Arrows indicated FISH signal corresponding to FRA3B. Inset FRA3B signal localizing with a break. Graph indicates the frequency of FRA3B expression. (B) Left. Example metaphase from Snm1B depleted HCT116 cells. Arrows indicate FISH signal corresponding to FRA16D. Inset FRA16D signal localizing with a break. Graph indicates the frequency of FRA16D expression.

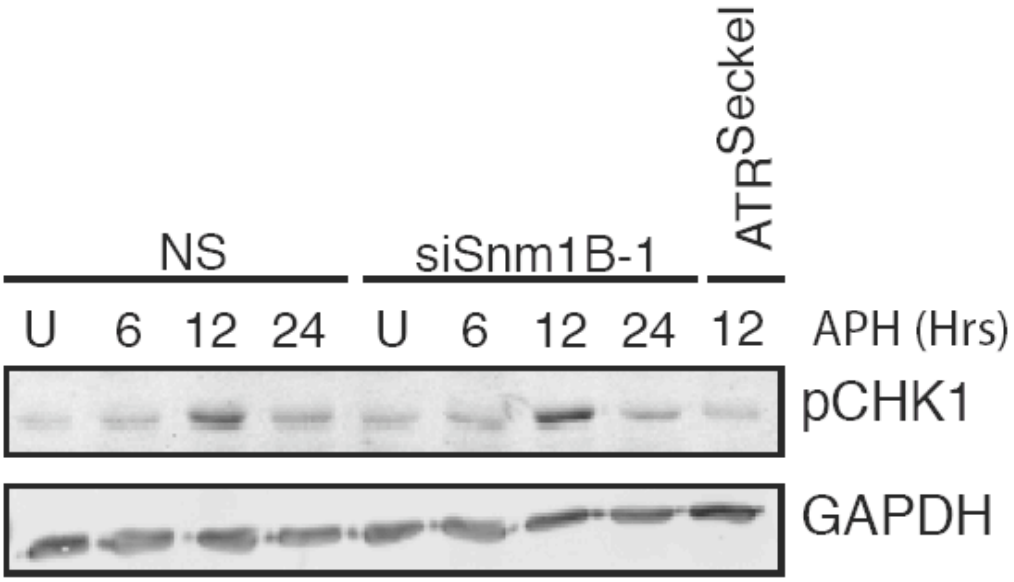


Figure 4.6. Phosphorylation of CHK1 in response to aphidicolin. HCT116 cells transfected with NS or siSnm1B-1 were treated with 0.3 uM aphidicolin for the indicated times. ATR^{Seckel} human fibroblasts were treated with 0.3 uM aphidicolin for 12 hours prior to harvesting. Phosphorylation of CHK1 was determined using pCHK1ser317 antibodies. Representative blot from two independent experiments. GAPDH was used as a loading control.

Snm1B plays a role in the resolution of replication stress and this phenotype likely contributes to the growth defect observed in Snm1B depleted cells.

Previous studies have shown that cell lines derived from ATR^{Seckel} patients exhibit spontaneous fragile site expression that is enhanced with the addition of aphidicolin in a dose dependent manner (7). Studies depleting ATR and CHK1 from HCT116 cells determined that ATR and CHK1 maintain fragile site stability even in the absence of aphidicolin (7, 11). The similarities in the phenotypes between cells with reduced expression of Snm1B and ATR^{Seckel} cells prompted us to investigate whether Snm1B was required for activation of ATR dependent CHK1 phosphorylation. Interestingly, Snm1B depleted cells do not exhibit a defect in CHK1 phosphorylation after aphidicolin. This suggests that Snm1B functions in replication fork stability independent of CHK1 activation. However, it is possible that in response to replication stress, Snm1B is a target of the CHK1 kinase accounting for the similarities in the phenotypic outcomes after aphidicolin treatment.

In addition to the CHK1 dependent pathway, ATR activates a second branch of the S phase checkpoint in response to replication stress that involves the NBS1 and FANCD2 proteins (23). Previous studies have indicated that Snm1B depletion results in defective NBS1 phosphorylation in response to MMC (3). In Chapter 3, I demonstrated that Snm1B is critical for efficient FANCD2 localization in response to interstrand crosslinks. Therefore, I hypothesize that the role of Snm1B in response to replication stress is to mediate the ATR-NBS1-FANCD2 S phase checkpoint, or ensure the efficient localization of DNA repair proteins to the stalled replication fork. It will be of interest to

determine if Snm1B functions within the ATR pathway in response to replication stress and if the function requires Snm1B nuclease activity.

In summary, I have demonstrated that Snm1B functions within the cellular response to replication stress as indicated by sensitivity to the DNA polymerase inhibitor, aphidicolin. Furthermore, Snm1B exhibits a phenotype reminiscent of ATR deficiency in which cells display increased levels of gaps/breaks as well as fragile site expression, but interestingly, CHK1 phosphorylation in Snm1B depleted cells is unaffected. Further investigation into activation of ATR targets and into FA/BRCA protein interaction is warranted to determine the precise role of Snm1B in maintaining fragile site stability. Together, this data indicates that Snm1B is critical in maintaining genome stability during replication.

Acknowledgments

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Chapter 5

Conclusions

Accurate and efficient repair of DNA damage is critical for maintaining genome stability. If mis-repaired, DNA damage leads to an accumulation of mutations and/or chromosomal anomalies. Inherited mutations in DNA repair genes result in human disorders characterized by chromosomal instability, developmental defects, and a predisposition to cancer. Understanding the cellular responses to DNA damaging agents is important to understanding of how genome integrity is maintained. Snm1B, a DNA nuclease, functions within double strand break repair, interstrand crosslink repair, and in prevention of interstrand crosslink-induced chromosomal anomalies, but the functions of Snm1B within these processes is largely unknown. This thesis establishes important roles for Snm1B in the repair of spontaneous and induced DNA damage. I have identified previously unknown functions for Snm1B in the repair of interstrand crosslinks and in the cellular response to replication stress. In the preceding chapters, I demonstrate that Snm1B regulates the activation ATR-dependent signaling and functions within the FA pathway indicating that Snm1B is required during the early cellular responses to interstrand crosslinks. In addition, I determine that Snm1B plays an important role in the cellular response to stalled replication forks and maintenance of genome stability even in the absence of exogenous DNA damage. In this chapter, I will discuss the novel findings of this dissertation and their implications with regard to our understanding of DNA

repair. Furthermore, future directions that would lead to a better understanding of Snm1B within DNA repair processes will be proposed.

Role of Snm1B in ATR-dependent signaling in response to interstrand crosslinks

In Chapter 2, I demonstrate that Snm1B is required for S phase checkpoint activation in response to interstrand crosslinks. Depletion of Snm1B resulted in a reduction in CHK1 and RAD17 phosphorylation indicating that Snm1B functions within the ATR-dependent S phase checkpoint. Two parallel ATR-dependent pathways have been described, ATR-CHK1 and ATR-NBS1-FANCD2 (1). The defect in CHK1 phosphorylation indicates that Snm1B is required for the ATR-CHK1 dependent pathway.

How does Snm1B depletion affect ATR-dependent signaling? Because CHK1 and RAD17 phosphorylation is S phase dependent, a significant difference in the S phase population between Snm1B depleted cells and controls could account for the defect in ATR signaling. I demonstrated that a defect in CHK1 and RAD17 phosphorylation in Snm1B depleted cells was observed as early at 4 and 8 hours after MMC addition (Figure 2.2), which is prior to when a significant reduction in the S phase population was observed (Figure 2.1, Supplemental Figure 2.4). Therefore, the reduction in ATR-dependent phosphorylation is not due to the defect in S phase accumulation.

The defect in the S phase checkpoint can also be due to a defect in activation of the ATR in response to interstrand crosslinks, thereby affecting ATR-dependent phosphorylation of downstream targets. Consistent with this notion, Snm1B depleted HeLa cells exhibited a defect in RPA foci formation after MMC. ATR does not

recognize the DNA lesion itself, but long stretches of ssDNA coated by RPA (2, 3). ATR is recruited to RPA-ssDNA intermediates along with its binding partner, ATRIP and is subsequently activated (2, 3). Therefore, one possibility is that Snm1B mediates formation of ssDNA-RPA intermediates that are critical for recruitment and activation of ATR. RPA-ssDNA intermediates form at the stalled replication fork and resected double strand breaks indicating that the defect in RPA foci formation could also be due to the proposed role of Snm1B in promoting replication fork collapse leading to the formation of the double strand break intermediate (4).

Role of Snm1B in the S phase checkpoint: ATR- or ATM-dependent?

Previously, a study suggested that the S phase checkpoint defect observed in Snm1B depleted cells is due to a defect in ATM-mediated signaling as a result of impaired formation of the double strand break intermediate (4). A defect in CHK1 phoshorylation was not observed in this study. In Chapter 2, I determined that Snm1B results in a defect in the ATR-dependent S phase checkpoint. There are several lines of evidence that support my conclusions.

Cell lines derived from patients lacking ATM or ATM depleted cells are not sensitive to MMC, indicating ATM does not play a major role in interstrand crosslink repair (5, 6). Furthermore, the S phase checkpoint in response to interstrand crosslinks has been shown to be ATM-independent in regards to both S phase progression (7) and inhibition of DNA synthesis (1). Therefore, this indicates that the defect in the S phase checkpoint cannot be solely attributed to a defect in ATM-dependent signaling.

Why was a defect in CHK1 phosphorylation not observed in the previous study? I observed that CHK1 phosphorylation is slightly elevated in Snm1B depleted cells when compared to controls even in the absence of exogenous agents (Figure 2.2, Supplemental Figure 2.3, Figure 4.6). Bae *et al*. (2008) used only one shRNA stable HeLa cell line to examine the role of Snm1B in ATM- and ATR-dependent signaling. This cell line contained high levels of CHK1 phosphorylation even prior to MMC treatment (4). Therefore, it is plausible that the high level of spontaneous CHK1 phosphorylation in Snm1B depleted cells has masked the defect in CHK1 phosphorylation after interstrand crosslink treatment. Consistent with an ATR-dependent defect, Bae *et al.* (2008) observed a significant reduction in NBS1 phosphorylation in Snm1B depleted cells upon MMC treatment, which in response to MMC, has been established to be ATR-dependent (1).

In contrast to the previous study, I demonstrated that Snm1B depletion results in a defect of CHK1 phosphorylation in two different cell lines, with two different siRNAs, at several different MMC concentrations (Chapter 2, data not shown). Therefore, the S phase defect observed in Snm1B depleted cells likely has a major impact on ATR signaling. To further provide evidence that the CHK1 defect is due to Snm1B deficiency, complementation with Snm1B cDNA should be performed using a V5-tagged Snm1B construct that contains silent mutations making V5-tagged Snm1B resistant to siSnm1B-1 mediated knockdown. Complementation of the pCHK1 defect by over expression of WT Snm1B will provide strong evidence that the phenotype we observe is not due to off target effects of the siRNAs. Furthermore, examination of the phosphorylation status of other direct targets of ATR including DNA repair proteins BRCA1, RPA, and NBS1,

would provide additional supporting evidence that Snm1B is important for ATR activation(8).

Snm1B functions within the context of the FA/BRCA network of proteins in response to interstrand crosslinks

Snm1B depleted cells are hypersensitive to interstrand crosslinks, exhibit moderate sensitivity to ionizing radiation, and exhibit an increase in chromosomal anomalies upon exposure to interstrand crosslinking drugs. These phenotypes are characteristic of the genomic instability disorder, FA suggesting that Snm1B may function within the FA pathway in interstrand crosslink repair. In Chapter 3, I determined that Snm1B functions epistatically to the FA factor, FANCD2. Furthermore, Snm1B is not required for activation of the FA pathway because monoubiquitination of FANCD2 by the FANC core complex is unaffected by Snm1B depletion (Chapter 3) (4, 9). Furthermore, Snm1B does not affect the ability of modified FANCD2 to bind to chromatin after MMC. Interestingly, Snm1B had a profound effect on the ability of FANCD2 to form DNA repair foci after the treatment of cells with MMC. Therefore, Snm1B functions downstream of FANCD2 activation, but upstream of FANCD2 foci formation.

Once localized to chromatin, FANCD2 colocalizes with other DNA repair factors including BRCA1, RAD51 and BRCA2 (10). Therefore, it is possible that Snm1B is required for FANCD2 to form higher order DNA repair complexes. Consistent with this, Snm1B and FANCD2 co-immunoprecipitate, but do not directly interact suggesting they are both factors of the same protein complex (4). Snm1B may be recruited the sites of

DNA damage prior to FANCD2 and Snm1B depletion results in disruption of the FANC containing protein complexes at the site of DNA damage. A second possibility is that Snm1B is required for nucleolytic processing of the interstrand crosslink allowing FANCD2 to bind. To test this possibility, the ability of FANCD2 foci to form in a Snm1B nuclease dead cell line needs to be performed. Finally, the ability of FANCD2 foci to form may be a result of the defect in ATR signaling. I determined that Snm1B results in a reduction of BRCA1 foci formation (Figure 2.5, Supplemental Figure 2.5). BRCA1 has been shown to mediate FANCD2 foci formation, but have no effect on FANCD2 monoubiquitination (11, 12). BRCA1 is phosphorylated by ATR in response to stalled replication forks, perhaps leading to its relocalization to DNA damage sites (13). Therefore, a defect in ATR-dependent BRCA1 phosphorylation could lead to the reduction in FANCD2 foci. It would be interesting to determine the status of BRCA1 phosphorylation in Snm1B depleted cells after MMC treatment.

Evidence for ATR-independent activation of FANCD2

Both FANCD2 and FANCI undergo phosphorylation by ATR in response to interstrand crosslinks (1, 14, 15). Although the function of FANCD2 phosphorylation is unclear, phosphorylation of FANCI by ATR has been shown to be critical FANCD2 monoubiquitination (15). In Chapter 2, I have uncovered a role for Snm1B in ATR mediated signaling, however, FANCD2 monoubiquitination is unaffected in Snm1B depleted cells (4, 9) (Chapter 3.1) suggesting that Snm1B only affects certain ATR-mediated events in response to interstrand crosslinks. Recently, replication-independent activation of FANCD2 was reported. Shen and colleagues introduced an interstrand crosslink into a plasmid that contains an Epstein-Barr virus (EBV) replication origin, so

replication of the plasmid only occurs in cell lines expressing Epstein-Barr nuclear antigen (EBNA). Using this assay, it was demonstrated that the presence of an interstrand crosslink was sufficient to induce FANCD2 activation (16) even in the absence of replication. Because ATR activation is dependent upon replication stalling at the site of the interstrand crosslink, this suggests that mechanisms of FANCD2 activation independent of ATR-mediated phosphorylation may exist in mammalian cells. Consistent with this notion, expression of kinase dead ATR in cells eliminates FANCD2 phosphorylation in response to interstrand crosslinks, but does not affect FANCD2 foci formation (1). Therefore, the monoubiquitination observed in Snm1B depleted cells may occur independent of ATR phosphorylation, but further investigation into this mechanism is warranted by examining phosphorylation of FANCD2 after MMC in Snm1B depleted cells.

Snm1B functions in the repair of spontaneous DNA damage

Depletion of Snm1B results in activation of a spontaneous DNA damage response. Previous studies have indicated that this phenotype is partially due to a role of Snm1B in telomere processing during S phase (17, 18). I have uncovered a role for Snm1B in the cellular response to replication stress (Chapter 4). Together this indicates that Snm1B is important for maintaining genome stability during replication. The potential roles for Snm1B in replication at telomeres and in the general response to replication stress are discussed below.

Snm1B functions to protect telomeres during S phase

Snm1B interacts with the shelterin component, TRF2, and has critical functions in maintaining genome stability at telomeres (17-19). The importance of the role of Snm1B at telomeres is highlighted by the identification of a patient with Hoyeraal-Hreidarsson (HH) syndrome, a rare and severe form of dyskeratosis congenita, harboring a splice variant in Snm1B (20). HH is characterized by growth defects, immunodeficiency, and bone marrow failure. The variant is the result of a frameshift deletion of 293 base pairs resulting in a premature stop codon (20), deleting the TRF-homology domain of Snm1B, which is critical for the interaction between Snm1B and TRF2 (21). Interestingly, this mutation only affects the role of Snm1B at telomeres and not in global DNA repair, because cells expressing the mutant version of Snm1B are not sensitive to DNA damaging agents such as MMC and ionizing radiation (20). This indicates that structure/function analysis of Snm1B either by the introduction of mutations in conserved domains or by the generation of deletion mutants may be able to identify separation of function mutants that can be utilized to further define the role of Snm1B in DNA repair processes versus a role at telomeres.

Previous studies indicate that Snm1B has important roles in telomere processing during S phase (18). Depletion of Snm1B by shRNA from human cells results in the activation of the DNA damage response at telomeres, as indicated by the formation of 53BP1 and γ-H2AX foci in cells undergoing replication (17, 18). Snm1B depleted cells also show an increase in chromosomal anomalies at telomeres including chromatids that contain multiple telomere signals, and telomere fusions (17, 18). Recently, telomere replication was examined in human cells over-expressing the HH variant of Snm1B using

chromosome-orientation FISH (CO-FISH) (20). This procedure distinguishes between leading and lagging strand DNA synthesis (22). Using this method, Snm1B depletion was observed to reduce the G-rich signal intensity (20). These findings indicate that Snm1B dysfunction results in either a defect in lagging strand DNA synthesis or promotes degradation of the lagging strand at telomeres. However, the precise role of Snm1B in telomere replication is currently unknown.

One possibility is that Snm1B is involved in processing of secondary structures that form only in the G-rich sequences of the lagging strand at telomeres, such as G quadruplexes. G quadruplexes are highly stable, unusual DNA structures consisting of four DNA strands held together by "G quartets" (23). The WRN helicase has been implicated in resolving G-rich secondary structures at telomeres and has similar phenotypes as Snm1B depleted cells, including activation of ATM-mediated DNA damage response at telomeres and loss of the lagging strand specific telomeres (17, 18, 20, 24). Therefore, it would be of interest to determine whether WRN and Snm1B and interact and co-localize at telomeres during replication.

A second possibility is that Snm1B is required for efficient replication through telomeres. Recently, telomeres were found to behave as common fragile sites and deletion of the telomere binding protein, TRF1, led to an increase in fragile telomeres, as indicated by activation of the DNA damage response specifically as telomeres, and the the presence of chromatids with multiple telomere signals (25). The phenotypes observed in TRF1 deficiency are strikingly similar to those observed in Snm1B depleted cells, suggesting that Snm1B may have similar functions in telomere replication (17, 18, 25). In this study, replication of telomeric sequences was monitored by visualization of single

DNA molecules that had been labeled with different halogenated nucleotides (IdU and CidU) to mark replicating DNA. Using this method, it was determined that TRF1 deletion results in replication fork stalling at telomeric DNA (25). It would be of interest to use similar approaches to determine is Snm1B depletion results in fragile telomeres and is important for replication at telomeres.

Snm1B functions in the cellular response to replication stress independent of ATR mediated activation of CHK1

Although Snm1B has critical roles at telomeres during S phase, only 60% of the DNA repair foci observed in Snm1B depleted cells localized at telomeres (18), indicating Snm1B may be involved in the repair of spontaneous DNA damage outside of telomeres. In Chapter 4, I have demonstrated that Snm1B is involved in the cellular response to replication stress. Snm1B depleted cells accumulated gaps and breaks and this was further enhanced upon aphidicolin treatment. Consistent with a role in the response to replication fork stalling, Snm1B depleted cells exhibit spontaneous expression of the common fragile sites, FRA3B and FRA16D. This indicates that instability at fragile sites significantly contributes to the increased levels of gaps and breaks observed in Snm1B depleted cells. It will be of great interest to determine the extent of the role of Snm1B in fragile site stability by investigating expression of other common fragile sites in Snm1B depleted cells by G-banding of metaphase chromosomes after aphidicolin treatment.

The phenotype of Snm1B depletion is similar to cell lines derived from patients with Seckel syndrome resulting from a hypomorphic mutation in ATR in response to replication stress. ATR^{Seckel} cell lines exhibited elevated levels of gaps and breaks that

was further enhanced by aphidicolin treatment in a dose-dependent manner and increased fragile site expression (26). A similar phenotype is observed when cells are depleted of the ATR target, CHK1 (27). This suggests that Snm1B functions within the ATR pathway in response to replication stress. Surprisingly, I did not observe a defect in CHK1 phosphorylation after aphidicolin treatment, indicating that in response to replication stress Snm1B is not required for ATR mediated phosphorylation of CHK1 (Figure 4.6). Further investigation into activation of the ATR pathway is warranted to determine if Snm1B is required for checkpoint activation in response to aphidicolin.

One possibility is that Snm1B functions downstream of ATR, perhaps either as a direct target of ATR and/or in the parallel branch of the ATR-mediated S phase checkpoint involving NBS1 and FANCD2 (1). Snm1B contains ATR/ATM phosphorylation consensus sites and is phosphorylated after ionizing radiation (28). However, it is currently whether Snm1B is phoshorylated after aphidcolin, or if the ATR/ATM consensus sites in Snm1B are critical for the function of Snm1B within DNA repair. It would be of interest to generate Snm1B constructs with mutated ATR/ATM phosphorylation sites to perform complementation studies with the Snm1B mutants. If the mutants are unable to complement the genome instability observed after aphidcolin in Snm1B depleted cells, this would provide evidence that the phoshorylation sites in Snm1B are functionally relevant. Alternatively, imminoprecipitation of V5-tagged Snm1B followed by immunoblotting with antibodies that recognize phosphorylated serines and threonines will determine if Snm1B undergoes phosphorylation after aphidicolin treatment (28).

To determine if Snm1B functions with the ATR-NBS1-FANCD2 S phase checkpoint, phosphorylation of NBS1 and FANCD2 after aphidicolin treatment should be examined to determine if Snm1B functions within the context of the ATR pathway in response to replication stress. It would be of interest to examine FANCD2 monoubiquitination and localization after aphidicolin treatment to determine if Snm1B functions in context of the FA pathway in response to replication stress. Together, this will determine if Snm1B is functioning either within the ATR mediated checkpoint response or within the FA pathway in response to replication stress.

Proposed functions of Snm1B at stalled replication forks

In Chapter 2, I demonstrated that Snm1B depleted HeLa cells have a severe defect in inducing the phosphorylation of in response to interstrand crosslinks. However, in Chapter 4, I demonstrate that Snm1B is not required for CHK1 phosphorylation in response to replication stress. These findings provide insight into the multiple roles of Snm1B in DNA repair processes. First, Snm1B may function at several steps of DNA repair at stalled replication forks. The difference in signaling defects observed in CHK1 phosphorylation after MMC and aphidicolin indicates that Snm1B may function both upstream and downstream of the ATR kinase. A similar scenario is observed with Mre11-RAD50-NBS1 (MRN) which is required for ATM activation in response to double strand breaks, but is also a target of ATM (29, 30).

Secondly, Snm1B may be involved in ssDNA formation at stalled replication forks in response to interstrand crosslinks. Formation of ssDNA coated by the protein RPA, and is important for recruitment of ATR and its subsequent activation (2, 31). The

mechanism by which ssDNA is formed differs between MMC and aphidicolin. Aphidicolin is an inhibitor of polymerases α/δ leading to uncoupling of the helicase and polymerase (8). Therefore, the helicase continues to unwind the DNA leading to the formation of long streteches of ssDNA. In contrast, interstrand crosslinks are a physical barrier that prevent strand separation, therefore both the helicase and the polymerase are stalled at the interstrand crosslink (8). The ssDNA at the replication fork is thought to occur by nucleolytic resection of the fork. Using a plasmid based system, it was recently shown that during interstrand crosslink repair 5' resection of the lagging strand occurs at the site of the interstrand crosslink and it was proposed that this is the mechanism of ssDNA formation leading to ATR activation (32). The nuclease responsible for the resection of DNA at the stalled replication fork is unknown. My findings establish that Snm1B impairs Snm1B CHK1 phosphorylation in response to interstrand crosslinks, but not after aphidicolin. Together these results indicate that Snm1B may be involved in nucleolytic processing of the interstrand crosslink, thereby generating regions of ssDNA that serves as a substrate for RPA binding.

Therefore, I propose the following mechanisms for Snm1B function at stalled replication forks (Figure 5.1). Upon encounter of an interstrand crosslink, Snm1B is recruited to the stalled replication fork where it promotes nucleolytic 5' resection of the lagging strand. Snm1B is a 5' to 3' exonuclease and possesses endonuclease activity (17) (Karumbati and Sekiguchi personal communation). An equally plausible possibility is Snm1B is required for recruitment of the nuclease responsible for resection to the stalled replication fork. Consistent with this notion, Snm1B has been shown to directly interact with Mre11 (4). Mre11 is a member of the DNA damage sensor complex Mre11-

RAD50-NBS1 and previous studies have indicated that Mre11 promotes end resection of double strand breaks (33). Once the lagging strand is resected, RPA can bind and ATR subsequently activated. In contrast, in response to replication stress, ssDNA is generated by uncoupling and RPA-ssDNA leads to ATR activation (8). In this scenario, Snm1B may function downstream of ATR either as a direct target of ATR or within the ATR dependent NBS1-FANCD2 S phase checkpoint.

To test this hypothesis, I propose several lines of investigation. The experiments and the predictions based on the model will be discussed in detail below. The proposed experiments will determine if Snm1B is required for ssDNA formation after interstrand crosslink repair, and will provide valuable insights into the role of Snm1B nucleolytic activity in DNA repair processes.

If Snm1B is required for the resection of the lagging strand at the stalled replication fork after MMC, Snm1B depleted cells should have a defect in ssDNA formation. It would be of interest to examine ssDNA formation at replication forks in BrdU-pulsed cells after Snm1B depletion. Under non-denaturing conditions, ssDNA is visible by microscopy as punctate foci. Recognition of BrdU by antibodies requires denaturing the DNA to single stranded molecules. Under non-denaturing conditions, BrdU is detectable in areas that contain long stretches of ssDNA (*i.e.,* resected DNA ends). This method has been used previously to examine ssDNA formation after interstrand crosslink-inducing drugs such as MMC. To distinguish between ssDNA at the fork and ssDNA that forms after resection of the double strand break, BrdU

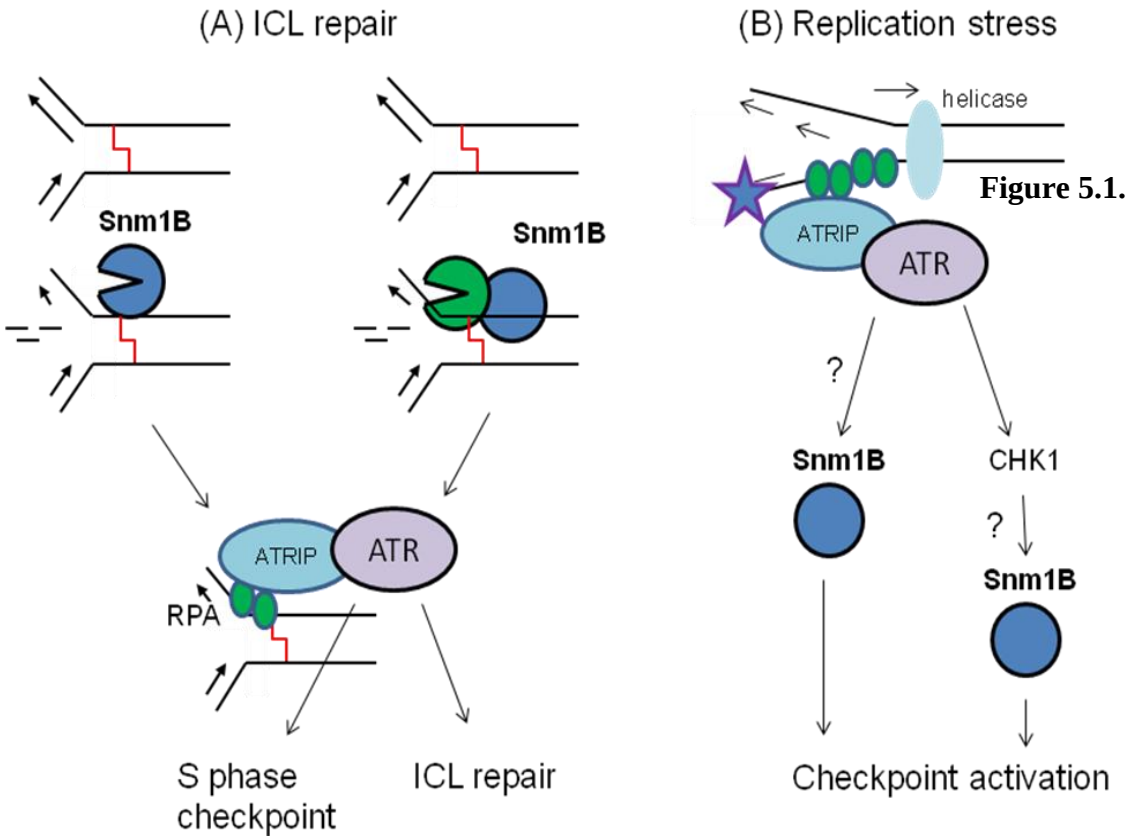

Figure 5.1.

Figure 5.1. Proposed functions of Snm1B in DNA repair processes. (A) Interstrand crosslink repair. Snm1B promotes ssDNA formation at the site of the interstrand crosslink by nucleolytic processing of the lagging strand (left) or by recruiting a DNA nuclease that results in 5' resection of the lagging strand. The resulting ssDNA is coated by RPA leading to recruitment of ATR and its subsequent activation leading to activation of the S phase checkpoint and interstrand crosslink repair proteins. (B) Replication stress. Inhibition the DNA polymerase (star) results in uncoupling from the helicase (light blue circle) resulting in formation of ssDNA. ATR is recruited and phosphorylates CHK1. In this scenario, Snm1B functions downstream of ATR activation in an unknown pathway, perhaps as either a target of ATR or downstream of CHK1.

foci can be localized with PCNA, to identify replication forks, and 53BP1, a marker of double strand breaks (34, 35).

Another prediction based on the mode. is that Snm1B would be required for the localization of ATR, ATRIP, and RPA after MMC treatment, but not after treatment of cells with aphidcolin. In Chapter 2, I demonstrated that Snm1B is critical for RPA foci formation after MMC. Therefore, it would be interesting to examine RPA foci formation after aphidicolin and localization of ATR and ATRIP after MMC and aphidicolin using antibodies that recognize endogenous proteins or over expression of tagged-ATR in Snm1B depleted cells.

To test the hypothesis that Snm1B recruits a DNA nuclease responsible for resection at interstrand crosslink induced fork stalling, it would be of interest to determine if Mre11 is recruited to the site of stalled replication forks. If Mre11 were recruited to the site of DNA damage, it would be important to determine if Mre11 recruitment is dependent upon Snm1B and the nucleolytic activity of Snm1B by examining localization of Mre11 in Snm1B depleted cells. Together, these experiments will provide insight into whether Snm1B and/or Mre11 are important for the early processing events at the stalled replication fork.

Perhaps the most important future area of study is to examine the role of the nucleolytic activity of Snm1B in both interstrand crosslink repair and in the response to replication stress. Although Snm1B has been implicated in several DNA repair processes, a role for Snm1B in DNA processing has not been identified. Complementation studies by over expression of tagged-Snm1B that is resistant to

siSnm1B-1 will provide valuable insight into the functions of Snm1B as a nuclease in DNA repair. Using site directed mutagenesis, conserved residues within the nuclease domain can be mutated to generate a nuclease dead allele(36, 37). If over expression of nuclease dead Snm1B were able to complement the phenotypes observed after MMC and aphidicolin, this would indicate that the nuclease activity of Snm1B is dispensable for these functions. However, if the mutants are unable to complement, this would indicate that this particular function was dependent on the nuclease activity of Snm1B. This study would provide valuable insight into the roles of Snm1B in signaling and the recruitment of DNA repair proteins versus a role in nucleolytic processing. Indeed, Mre11 has roles that are independent of its nuclease activity such as the activation of the ATM kinase (29).

Relationship between Snm1B and the βCASP paralogs

Snm1B is a member of the βCASP family of nucleases able to catalyze DNA. Along with Snm1A and Artemis, emerging evidence indicates that all three family members have critical roles in DNA repair (38). However, one question that has remained is if redundancy exists between the Snm1 family members. In this thesis, I have uncovered roles for Snm1B within the interstrand crosslink repair pathway. Artemis is not sensitive to interstrand crosslinking agents indicating it is not required for this pathway of DNA repair (39). In contrast, Snm1A cells are hypersensitive to MMC, but not cisplatin (40). In DT40 chicken B cells, it was found that Snm1A and Snm1B deficiency are additive in their effect to sensitivity to MMC, which indicated that Snm1A and Snm1B either have redundant functions, or function within separate pathways of interstrand crosslink repair (41). The mild phenotypes of both Snm1A deficient cells and

Snm1B knockdown lead to the hypothesis that perhaps A and B are able to compensate for each other in interstrand crosslink repair.

In Chapter 3, I presented data indicating that Snm1B functions epistatically with the FA pathway. Previous studies have indicated that Snm1A is non-epistatic with the FA pathway because Snm1A depletion in a FANCA deficient cell line increased the interstrand crosslink-induced chromosomal anomalies when compared to FANCA deficiency alone indicating Snm1A functions independently of FANCD2 in interstrand crosslink repair (39). This indicates that Snm1A and Snm1B function within different branches of interstrand crosslink repair. Together, this indicates that the βCASP family members may have distinct functions in DNA repair. Because the conservation of the βCASP nucleases is restricted to the nucleolytic domains, this indicates that the non-conserved regions of the protein contain motifs may contain regulatory domains that mediate their functions. Further investigation into the roles of the non-conserved C terminus of Snm1B is warranted as well as the relationship between the βCASP family members.

Implications for human disease

The DNA damage response is essential for the prevention of tumorigenesis. Defects in DNA damage signaling is thought to be one of the earliest events in cancer progression. Indeed, many factors involved in interstrand crosslink repair are linked to tumorigenesis both as inherited genomic instability syndromes and inhibition in sporadic cancers. Defects in ATR signaling result in Seckel syndrome, a rare inherited human disorder in which patients exhibit similar phenotypes as FA patients, including growth

retardation, microcephaly, increased genome instability and cellular hypersensitivity to interstrand crosslink agents (42-44). The findings in this thesis indicate that Snm1B plays key roles in activation of ATR-dependent signaling as well as in the context of the FA/BRCA protein network of interstrand crosslink repair, suggesting that mutations in *Snm1B* may lead to similar phenotypic outcomes. In this regard, deletion of the *Snm1B* chromosomal locus has been found in nearly half of primary mediastinal B cell lymphomas analyzed in a recent study (45), thereby suggesting that Snm1B could function as a tumor suppressor.

In conclusion, I have uncovered key roles for Snm1B in the repair of spontaneous and induced DNA damage indicating that Snm1B is important for the maintenance of genome stability. The data presented here suggests that Snm1B promotes resection of the stalled replication fork at the site of interstrand crosslink to generate ssDNA that is critical for ATR activation, and provides valuable insight into initial events of interstrand crosslink repair. Furthermore, in response to replication stress, Snm1B likely functions downstream of ATR to maintain fragile site stability. Therefore, Snm1B likely has multiple functions in response to stalled replication forks. Snm1B is critical for ATR-mediated signaling and the FA/BRCA pathway, which are both mutated in genome instability disorders suggesting that mutations in Snm1B may lead to diseases such as FA.

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