Roles of the SNM1B DNA Nuclease in Resolution of Replication Stress and Maintenance of Genome Stability

by

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Dedication

I would like to dedicate this thesis to
my loving and supportive parents,
Himadri and Kakuli Das

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List of Abbreviations

ATM	Ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interaction protein
β-CASP	Beta-Csp1, Artemis, Snm1, Pso2
BER	Base excision repair
BLM	Bloom helicase
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
CDK	Cyclin-dependent kinase
CFS	Common fragile site
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CMG	Cdc45-MCM2-7-GINS
CNV	Copy number variants
CtIP	carboxy-terminal binding protein interaction protein
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DNA2	Human nuclease/helicase 2
DSB	Double-strand break
DUB	Deubiquitylating enzymes
EME1	Essential meiotic endonuclease 1
EXO1	Exonuclease 1
FA	Fanconi anemia
FAAP20	Fanconi anemia-associated protein, 20 kDa
FAAP24	Fanconi anemia-associated protein, 24 kDa
FAN1	FANCD2/FANCI-associated nuclease 1
FANCA	Fanconi anemia complementation group A
FANCB	Fanconi anemia complementation group B
FANCD2	Fanconi anemia complementation group D2
FANCF	Fanconi anemia complementation group F
FANCI	Fanconi anemia complementation group I
FANCJ	Fanconi anemia complementation group J
FANCL	Fanconi anemia complementation group L
FANCM	Fanconi anemia complementation group M
FANCP	Fanconi anemia complementation group P
γH2AX	Phosphorylated histone variant 2AX
HLTF	Human helicase-like transcription factor
HR	Homologous recombination

HU	Hydroxyurea
ICL	Interstrand crosslink
ID	FANCI and FANCD2 complex
IR	Ionizing radiation
MBL	Metallo-β-lactamase
MCM	Mini-chromosome maintenance
MMC	Mitomycin C
MRN	Mre11/Nbs1/Rad50 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
ORC	origin recognition complex
PALB2	Partner and localizer of BRCA2
PARP	Poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PIKK	Phosphoinositide-3-kinase-like protein kinase
PTM	Post-translational modification
RPA	Replication protein A
SMARCAL1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1
SNM1	Sensitivity to nitrogen mustard 1
ssDNA	Single-stranded DNA
SUMO	Small ubiquitin like modifier
TLS	Translesion synthesis
TOPBP1	Topoisomerase 2 binding protein 1
TRF2	Telomeric repeat binding factor 2
UBD	Ubiquitin binding domain
UPS	Ubiquitin proteasome system
UV	Ultraviolet light
WRN	Werners syndrome
XPF	Xeodermun pigmentosum complementation group

Abstract

Genomic DNA is damaged through exposure to exogenous and endogenous agents as well as during cellular processes such as DNA replication. Defects in cellular responses to DNA damage can lead to an accumulation of unrepaired or misrepaired lesions and ultimately, increased genome instability. DNA replication ensures the accurate transmission of the information encoded in the genome to daughter cells. Progression of DNA replication can be impaired or blocked, which leads to replication fork stalling. If stalled forks are not properly restarted, they can collapse, resulting in chromosomal breaks, deletions, and translocations. Therefore, replication-associated DNA damage has been hypothesized as one important source of genome instability associated with cancer initiation and progression. Furthermore, mutations in DNA repair genes result in inherited genome instability disorders characterized by developmental defects and cancer predisposition. Uncovering the cellular mechanisms that repair DNA damage is critical for understanding how cells maintain genome stability and thereby prevent deleterious human diseases.

DNA nucleases play a key role in resolving stalled and collapsed replication forks, but the molecular events involved in these processes are not fully defined. This dissertation addresses how the DNA nuclease SNM1B plays critical roles in preventing replication-associated DNA damage. I demonstrate that SNM1B is not required for the initial detection of a stalled replication fork or for initiating early signaling events. I show that SNM1B is instrumental in stabilizing stalled replication forks by nucleolytically processing aberrant DNA structures at stalled and collapsed forks which allows for the recruitment of key DNA repair factors, FANCD2, BRCA1, and Rad51. Furthermore, I found that SNM1B plays a key role in preventing stalled and collapsed replication forks in unperturbed cells suggesting it is critical in responding to replication-associated DNA damage that occurs spontaneously during cellular proliferation. I also identified a

residue within SNM1B that is critical for its localization to sites of stalled forks and established that SNM1B protein levels are modulated in the cell. Altogether, these findings illustrate that SNM1B has critical functions during the repair of replication-associated DNA damage, thereby ensuring successful replication of the genome and preventing potentially deleterious chromosomal aberrations.

Chapter 1: Introduction

Summary

The genetic information contained within our DNA is essential for the functionality of all cellular processes. The DNA comprising our genome is constantly under assault from endogenous and exogenous sources that cause many different types of damage. If unrepaired, DNA lesions can accumulate, thereby leading to aberrant chromosomal rearrangements, deletions, insertions, substitutions, and translocations. Detrimental genomic alterations may result in cell death, proliferative arrest, or cellular transformation. Cells have evolved a multitude of DNA repair mechanisms to repair different types of DNA damage to maintain genome stability. Mutations in DNA repair genes can cause numerous inherited genome instability disorders characterized by phenotypes including developmental defects, immune system defects, and cancer predisposition. Therefore, understanding the cellular mechanisms that repair DNA damage is critical for understanding how defects in DNA repair mechanisms can lead to human disease.

DNA replication is critical for the survival of proliferating cells

The genetic information contained within our DNA is essential for the functionality of all cellular processes. Complete replication of the genome ensures the accurate transmission of this genetic information from parental cells to daughter cells during every cell division. To prepare for cell division, a cell progresses through the following stages of the cell cycle: G1 (Gap1), S (Synthesis), G2 (Gap2), and M (Mitosis). In the G1 phase, proteins are expressed that are needed for DNA synthesis, which occurs in S-phase. Once the genome is replicated, the cell cycles to G2 where it prepares to enter into mitosis where cellular division occurs. During DNA synthesis, 3 billion base pairs of human DNA have to be replicated accurately and efficiently, so a series of very tightly controlled processes, discussed below, are employed by cells to ensure accurate genomic duplication [1-3].

Before the cell enters S-phase, origins of replication, sites on the chromosome where DNA replication is initiated, are recognized by the origin recognition complex (ORC). Replication initiation proteins load the MCM2-7 (mini-chromosome maintenance) helicase onto the origin thereby assembling the pre-RC (pre-replication complex) and 'licensing' these areas of the genome as origins [1,4]. Additional factors are loaded onto this complex to form an active replication fork including GINS and Cdc45 which together with MCM form the CMG helicase complex [5]. The MCM helicase is then activated and the replisome, which contains the DNA polymerases, is loaded on, and the cell is ready for DNA synthesis in S-phase. These steps are controlled by CDKs (cyclin-dependent kinases) which are activated at specific times during the phases of the cell cycle [6,7].

Eukaryotic cells have thousands of origins of replication which initiate replication bi-directionally once the cell enters S-phase [6]. The two strands of the DNA double helix are separated by the MCM replicative helicase which allows each strand to be copied during the replication process [8]. DNA polymerases are responsible for adding complementary nucleotides to the parental strand. Nucleotides can only be added to an available 3'-OH terminus. Therefore, once parental DNA strands are separated, different mechanisms are utilized to synthesize the nascent DNA strands [9]. One DNA strand that is synthesized is the leading strand which is formed by DNA polymerase ε in

a single, continuous segment [10-12]. The other DNA strand is termed the lagging strand which is synthesized by DNA polymerases δ and α in short segments called Okasaki fragments in a discontinuous process [13-15]. These high processivity and high fidelity polymerases have intrinsic 3' to 5' exonuclease activity, which provides them proofreading functions allowing the chance for more accurate copying of the parental DNA strand [9,16]. Consequently, this results in approximately one error per 10^9 – 10^{10} nucleotides [17]. Once the genome is completely replicated, the cell continues through the cell cycle and enters mitosis. Now that the cell contains a replicated genome, the copied chromosomes are separated during mitosis to allow for cell division, and the daughter cells each now have an intact, complete genome [2].

Sources of replication stress

'Replication stress' can result from endogenous and exogenous sources, but how 'replication stress' is defined is not concrete and is continually changing. This thesis focuses on replication stress that results in the stalling or slowing of replication fork progression thereby resulting in slowed replication elongation. There are various endogenous sources of replication stress that occur spontaneously and cause stalling of replication forks during cellular proliferation (Figure 1.1). The replication fork can encounter unrepaired single-strand lesions in the parental DNA strand which need to be repaired before replication can progress [18]. The replication fork can also encounter secondary structures in the DNA, like a hairpin loop structure, that are difficult to replicate and need to be unwound before replication can continue [19]. Certain areas of the genome that are GC-rich can form secondary structures called G-quadruplexes that have to be unwound by specific helicases [20]. Proteins tightly bound to DNA can also inhibit progression of the replication fork [21].

Another endogenous source of replication fork impediment is the collision between DNA replication and transcription machinery, since both these critical processes occur simultaneously [22,23]. One structure that can form is a R-loop which is a RNA:DNA hybrid between the DNA template strand and a nascent RNA transcript. The other DNA strand is then single-stranded, exposed, and unstable, which could result in DNA breaks [24,25]. Therefore, removal of R-loops by the enzyme RNaseH1 results in hydrolysis of the RNA from the RNA:DNA hybrid and inhibits replication stress [26-29].

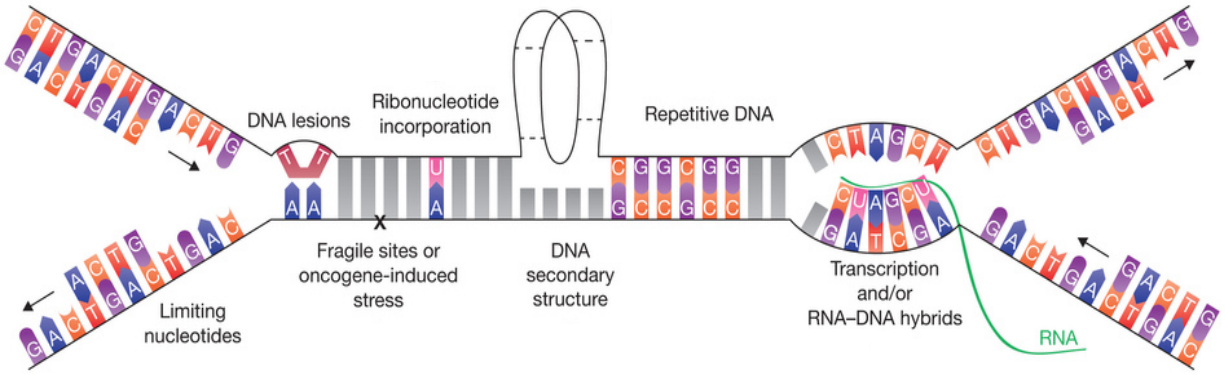

Figure 1.1 Sources of replication stress.

Progression of the replication fork can be blocked or stalled through various ways. This diagram shows some of these sources of replication stress including DNA lesions, secondary structures, collision with transcription machinery and RNA:DNA hybrids, oncogene-induced stress, common fragile sites, and nucleotide depletion. Adapted from Zeman, et al. [30]

Another instigator of endogenous replication stress is oncogene-induced, which results in cells being abnormally driven into S-phase, although the mechanism of this type of replication stress is still being addressed [31-33]. Overexpression of certain oncogenes, including c-myc, results in the dysregulation of cell cycle checkpoints, which are activated upon DNA damage. If the cells do not arrest at the G1 cell cycle checkpoint, too many cells enter S-phase [34]. Hyper-proliferation of cells results in DNA hyper-replication which can consequently result in DNA damage [35-37].

Certain regions of the genome called common fragile sites (CFSs) are prone to breakage under certain conditions of replication perturbation resulting in gaps and breaks in metaphase chromosomes [38,39]. Multiple explanations of what makes these sites fragile have been proposed and are still being investigated. It has been suggested these sites are late replicating regions [40,41]. Furthermore, it has been shown that CFSs are located in regions of very large genes that often take more than one cell cycle to transcribe, thereby implying that transcription and replication machinery collide at CFSs [28,42,43]. It has also been suggested that the number of origins in CFSs is lower compared to other regions of the genome; therefore, CFSs cannot rely on dormant origins to ensure complete replication of the region [44]. CFSs have also been suggested to be intrinsically difficult to replicate regions [45,46].

There are also many exogenous sources of replication stress. Depletion of the nucleotide pool through hydroxyurea (HU) treatment results in stalling of replication fork progression. While stalled replication forks can be restarted, DNA lesions that block progression of the replication fork have to be removed. For example, interstrand crosslinks (ICLs), which covalently link the two strands of the DNA double helix and block replication, can occur spontaneously through metabolic intermediates and oxidized lipids and also through chemotherapeutic agents like cisplatin and mitomycin C (MMC) [47,48]. ICLs need to be removed and then the replication fork can restart progression. Ultraviolet (UV) light induces pyrimidine dimers which can result in helix distorting lesions, and therefore the dimers need to be removed before replication can continue [49]. Furthermore, Topoisomerase I is an enzyme that relieves torsional stress during DNA replication, and an inhibitor of Topoisomerase I, camptothecin, results in the formation of a protein adduct which inhibits replication progression [50]. This thesis

focuses on replication stress caused by aphidicolin-induced stalled replication forks. Aphidicolin inhibits the replicative DNA polymerases α, δ, and ε by blocking the binding of cytosine leading to arrest specifically at template guanine residues [51]. DNA polymerase inhibition results in the uncoupling of helicase and polymerase activities. The helicase continues unwinding the DNA double helix while the polymerase is unable to add nucleotides to the parental strand resulting in long stretches of ssDNA [8,52-54]. As described above there are many diverse causes of replication stress (Figure 1.1). Therefore, how the cell responds to and repairs different types of replication-associated DNA damage through different mechanisms is still not understood completely.

DNA damage response

The DNA damage response (DDR) is comprised of all the cellular responses that become activated upon DNA damage (Figure 1.2). The DDR is firstly responsible for detecting any type of DNA damage including DNA mismatches, single-strand breaks, double-strand breaks (DSBs), ICLs, and replication-associated DNA damage [49]. Sensor proteins recognize the DNA damage and then activate transducer and effector proteins, which activate cell cycle checkpoints so that cells stop progression through the cell cycle allowing time for the cell to fix the damaged DNA. Additionally, initiation of transcription of certain genes needed for the repair process and recruitment of DNA repair proteins occur. If the damage is too severe and cannot be repaired, some cells may be targeted for apoptosis. Defects in the DDR resulting from mutations in the genes involved in this cellular response can lead to decreased cellular survival [55].

There are multiple DNA repair pathways within the cell that are activated in response to all the different types of DNA damage. Single-strand breaks are repaired through base excision repair (BER) [56]. Single-strand lesions that result in more bulky adducts, like UV light induced dimers, are repaired through nucleotide excision repair (NER) [57]. Any base mismatches that occur during DNA replication are repaired through the mismatch repair pathway. DSBs are repaired either through non-homologous end joining (NHEJ), which can occur throughout the cell cycle, or homologous recombination (HR), which acts primarily in S and G2 phases of the cell cycle once a homologous template is available on the sister chromatid [58-61]. ICLs are repaired by the Fanconi

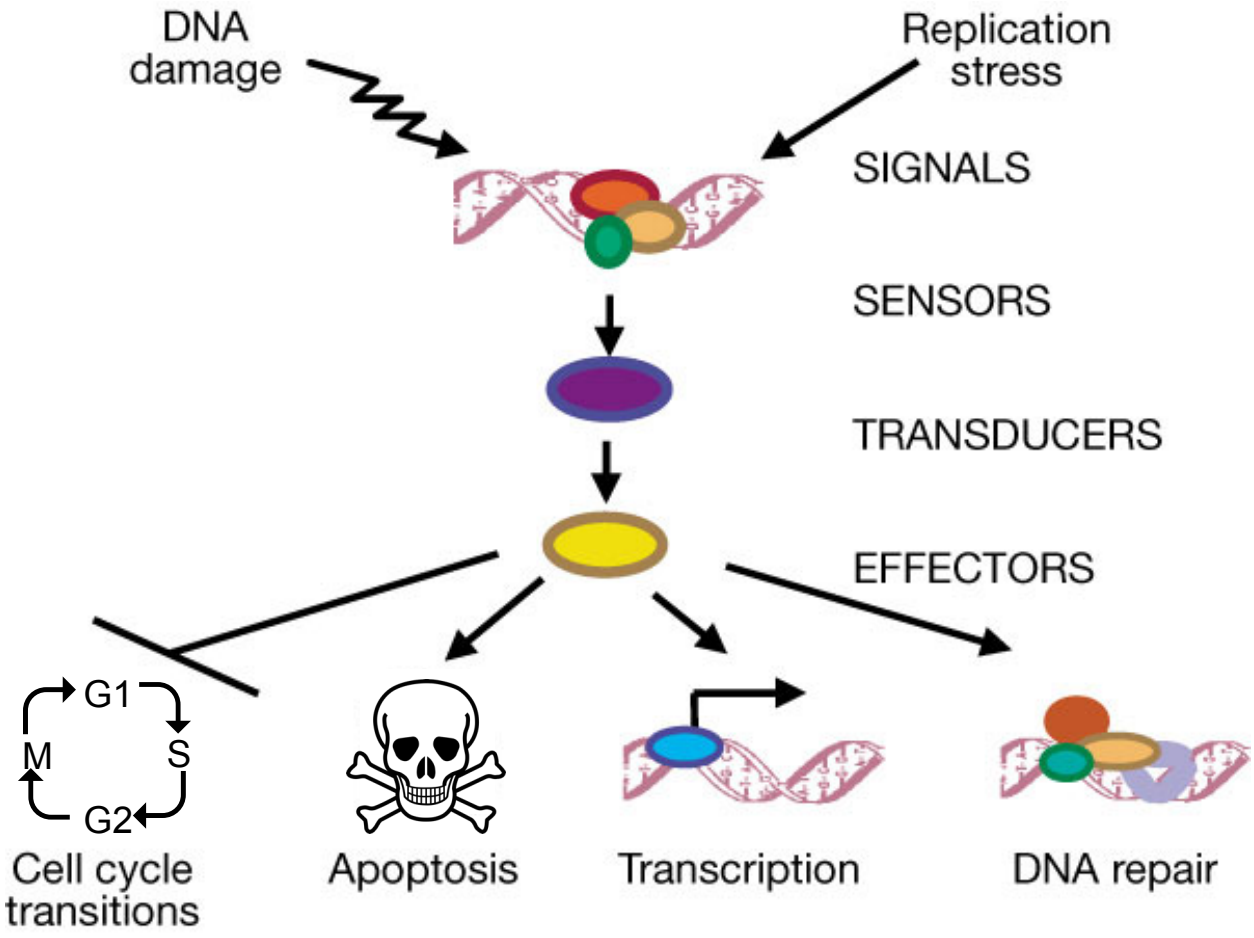

Figure 1.2 DNA Damage Response.
Upon genomic damage, the DNA Damage Response (DDR) is activated. This cellular response recognizes the damage and initiates a signaling pathway which activates transducer and effector proteins that turn on cell cycle checkpoints, promote apoptosis, initiate transcription, and recruit DNA repair proteins. Adapted from Zhou et al. [62]

anemia (FA) pathway with the help of low-fidelity translesion synthesis DNA polymerases [63].

The FA pathway is comprised of 15 known complementation groups (A,B,C,D1,D2,E,F,G,I,J,L,M,N,O,P), which are genes that have been found mutated in FA patients. The FA core complex, comprised of A,B,C,E,F,G,L,M and accessory proteins FAAP20 and FAAP24, is activated in response to ICLs. FANCL which possesses ubiquitin ligase activity, monoubiquitinates FANCD2 and FANCI (ID complex) which localizes to the site of damage and coordinates downstream repair proteins [64-66]. FA patient cells are not only hypersensitive to ICL agents but also ionizing radiation (IR), UV, HU, and aphidicolin [63,67,68]. Therefore, the FA pathway clearly has additional roles beyond ICL repair in the cell. The role of the FA pathway in stalled replication fork repair is not as clear, but it is needed for replication fork stability. Specifically the FA-BRCA network including FANCJ (BRIP1), BRCA2 (FANCD1), and SLX4 (FANCP) functions after the ID monoubiquitination step [69-72]. These proteins have critical roles in protecting stalled forks and repairing collapsed replication forks which are discussed in the next section.

Repair of Stalled Replication Forks

A stalled replication fork can be characterized by having long stretches of ssDNA which can result from the uncoupling of the replicative helicase from the DNA polymerase. The helicase continues unwinding the parental DNA double helix while the polymerase is inhibited forming long stretches of ssDNA [8,52,54]. The heterotrimeric Replication Protein A (RPA) binds to this exposed ssDNA and recruits the ATR kinase (ataxia telangiectasia and Rad3 related protein) with its binding partner ATRIP (ATR-interacting protein). A separate checkpoint clamp complex made up of Rad9-Rad1-Hus1 (also called the (9-1-1 complex) is recruited to RPA-ssDNA [73]. Subsequently, TOPBP1 (topoisomerase-binding protein 1) is recruited and activates ATR which phosphorylates downstream substrates [73-78]. These steps initiate the S-phase checkpoint by recruiting downstream DNA repair proteins including the effector protein kinase CHK1 and activation of the FA pathway [21,79-83].

When the replication fork encounters an unrepaired or misrepaired lesion in the template DNA, the cell employs the DNA damage tolerance (DDT) pathway to bypass the damaged DNA and continue replication. One component of DDT is translesion synthesis, which recruits low-fidelity DNA polymerases that replicate past the damaged DNA. Template switching can also be activated which results in using the damaged sister chromatid as a template for replication. The ubiquitination of PCNA, the replicative sliding clamp, aids in determining if translesion synthesis or template switching will be activated. It has been shown that monoubiquitination of PCNA promotes recruitment of translesion synthesis polymerases, while it has been suggested that polyubiquitination of PCNA is important in facilitating template switching [84].

One mechanism through which template switching can occur at a stalled fork to help in restarting DNA replication is fork regression/reversal, where the separated DNA strands are rewound leading to annealing of the complementary nascent DNA strands forming a 'chicken-foot' structure (Figure 1.3) [85]. Various proteins have been implicated in initiating this regression process including PARP (poly(ADP-ribose) polymerase), BLM (Bloom helicase), FANCM (Fanconi anemia complementation group M), HLTF (human helicase-like transcription factor), and SMARCAL1 DNA translocase [86-90]. Until recently there was not much evidence that fork regression occurs in mammalian cells. Therefore, the mechanism through which regression occurs is still not well understood. One model is that HLTF, which has DNA translocase activity, unwinds the leading and lagging strands of the replication fork which is followed by annealing of these nascent strands and rewinding the parental strands to form the 'chicken-foot' structure [91,92].

Once fork regression occurs, this intermediate 'chicken-foot' structure has to be resolved, and this occurs through various ways (Figure 1.3). One process is through digestion of the nascent annealed strands. The nucleases Mre11 (a component of the MRN (Mre11/Rad50/Nbs1) complex), FAN1 (FANCD2/FANCI-associated nuclease1), and DNA2 (human nuclease/helicase 2) have been implicated in this step [93-98]. Another way to resolve this structure is through template switching as previously mentioned, by extension of these strands using the complementary nascent strand as a template. A DNA polymerase extends the 3'OH end of the leading nascent strand by copying from the nascent lagging strand [30].

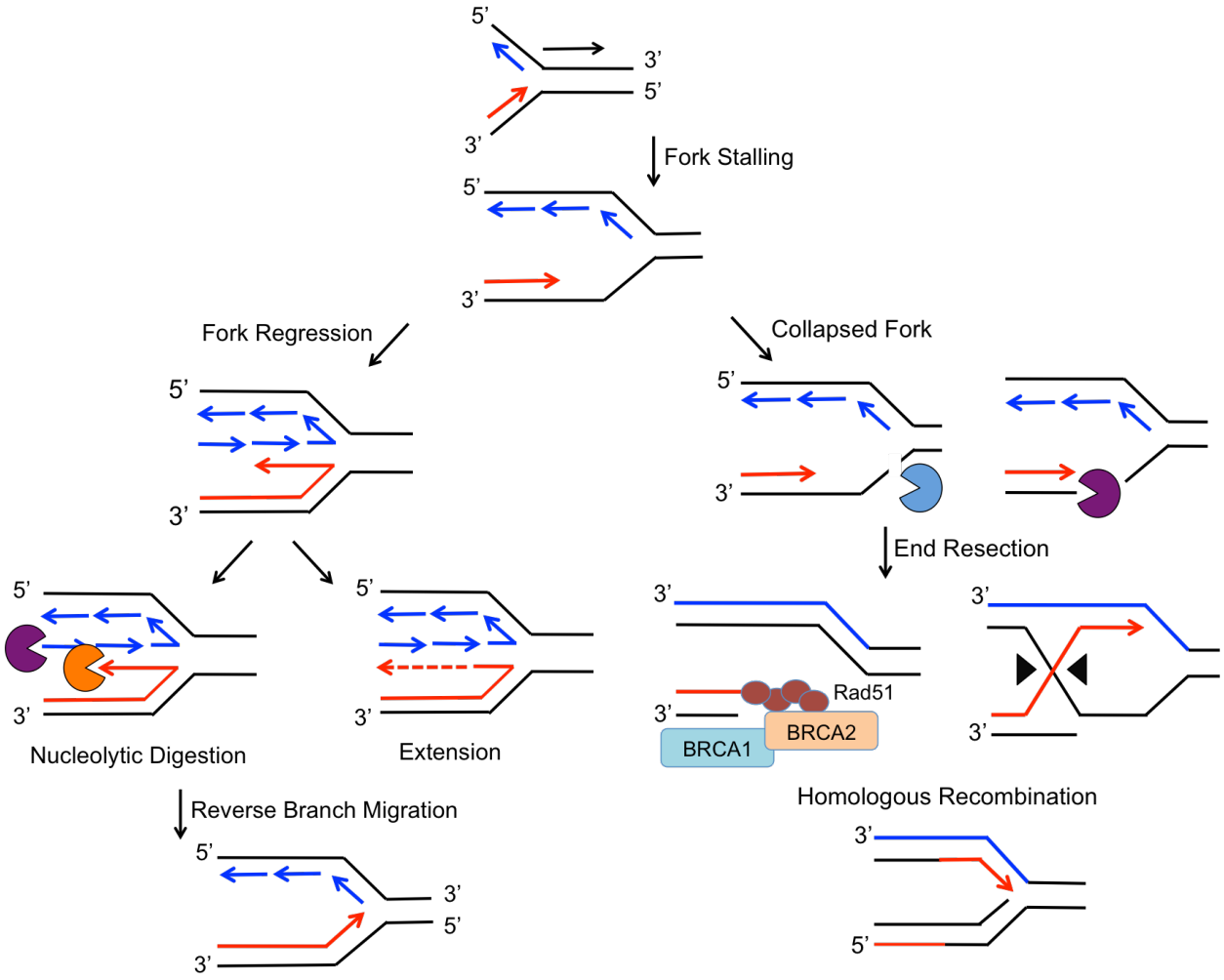

Figure 1.3: Resolution of Stalled Replication Forks
Once the helicase and polymerase uncouples, the helicase continues unwinding the DNA double helix resulting in long stretches of ssDNA. One mechanism through which stalled forks are restarted is fork regression, which forms a 'chicken-foot' structure. This aberrant DNA intermediate either undergoes nuclease digestion or extension followed by reverse branch migration to restart replication. If a stalled fork cannot be restarted, it can collapse resulting in a double-strand break. This break is then repaired through homologous recombination.

The regressed fork then needs to be restructured back to a normal replication fork in a process called reverse branch migration, and BLM helicase has been shown to be important in restoring replication forks. BLM is a RECQ-like 3' to 5' DNA helicase known to unwind bubble structures, forked duplexes, G-quadruplex structures, and resolve Holliday junctions. BLM localizes to aberrant fork structures and works to inhibit formation of breaks at the stalled fork. It has been shown that FANCD2 plays a role in recruiting BLM to chromatin where it helps to restart stalled replication forks [99]. FANCD2 has also been shown to recruit FAN1 and SLX4/FANCP, a scaffold protein that will be discussed in a later section [100-103].

If a stalled fork cannot be restarted efficiently it can collapse resulting in a DSB at the fork (Figure 1.3). A break in the ssDNA region at a stalled fork, and further digestion by a nuclease results in a one-ended DSB. This is accompanied by dissolution of components of the replisome [104]. This DSB is then sensed by DDR proteins (including the MRN complex) which recruit and activate the ATM (ataxia telangiectasia mutated) kinase, which then activates further downstream repair proteins. The DSB is processed so that a 3' overhang is generated. RPA binds to this exposed ssDNA and is then displaced by Rad51 which eventually forms filaments on this overhang [105]. This results in strand invasion to the homologous template to initiate HR-mediated repair. Components of the FA/BRCA network including BRCA2, PALB2, and FANCJ have functions during this HR step [106]. It is known that BRCA2 has a critical role in this HR step of repairing a collapsed fork, but it has also been shown that BRCA2 works with FANCD2 in protecting stalled forks from extensive degradation by Mre11, which processes regressed forks. Therefore, BRCA2 is important beyond its role in the DSB response [95,96,98]. It has also been demonstrated that FANCD2 may play a role in Rad51 filament stabilization during this process [96].

BRCA2 and Mre11 are clear examples of how DNA repair proteins can have multiple roles at different stages during the DDR. This also highlights that there are still many unanswered questions as to the precise steps that occur in repairing stalled forks, what pathways and proteins are involved, how they collaborate, and how these processes are all regulated. Understanding how cells respond to and restart stalled replication forks and repair collapsed forks is important in determining how cells

maintain genome stability.

Consequences of incomplete replication

DNA damage that results from incomplete replication and inefficient repair of stalled replication forks results in an overall increase in genome instability. The DSB at a collapsed fork can engage in deleterious rearrangements resulting in copy number variants (CNVs) in the genome or chromosomal anomalies like harmful translocations that may lead to cancer. Faulty template switching events during repair processes at stalled forks or inefficient DSB repair at these collapsed forks can also cause CNVs [107]. Many neurodevelopmental diseases including autism and schizophrenia have been linked to CNVs [107,108]. CNVs are also seen at a high frequency in cancer cells [109,110]. These genomic alterations can be detrimental by resulting in cell death, proliferative arrest, or cellular transformation, which can manifest as disease phenotypes, including developmental delay, immune system defects, and cancer predisposition [26,41,111,112].

CFSs, as previously mentioned, are regions of the genome prone to breakage especially upon aphidicolin treatment [39]. Two of the most broken CFSs (FRA3B and FRA16D) are located within tumor suppressor genes (FHIT, WWOX, respectively). Therefore, a break at this loci can inactivate expression of the tumor suppressor gene, which can ultimately lead to tumor formation [113]. CFSs are hot spots for chromosomal rearrangements, deletions, gaps, and breaks [38,114,115].

Mutations in genes involved in the cellular response to replication stress and repairing stalled replication forks can ultimately manifest as inherited genome instability disorders in humans. For example, mutations in the ATR kinase leads to Seckel syndrome characterized by growth retardation and microcephaly. Werner syndrome and Bloom syndrome result from mutations in those respective helicases (WRN, BLM) and have phenotypes including premature ageing, growth retardation, and cancer predisposition [63]. FA which is caused by mutations in any one of the 15 known FA genes results in developmental defects, abnormal skeletal features, bone marrow failure, and cancer predisposition [68]. By further studying the cellular mechanisms of repairing stalled replication forks we can gain a better understanding of how defects in these processes lead to human disease.

Role of nucleases in repairing stalled replication forks

DNA nucleases clearly have a significant role in the repair processes involved in restarting and repairing stalled replication forks as described above. Nucleases are needed in multiple stages during this cellular response, and the activity of the nuclease also needs to be tightly regulated so that it does not act inappropriately through hyper-degradation of the DNA which can lead to fork instability.

DNA nucleases are needed at regressed forks to digest the annealed nascent strands resulting in a formation of a normal fork (Figure 1.3). The nuclease Mre11 may be involved in this digestion process through its 3'-5' exonuclease activity, although this activity needs to be tightly regulated to prevent hyper-degradation of the DNA [95,96,98]. FAN1, in a complex with FANCD2 and BLM, has also been shown to digest nascent DNA strands, but again this digestion seems to be tightly regulated by FANCD2 and Mre11 [94,99]. Furthermore, DNA2, which possesses 5'-3' exonuclease activity, has been shown to be important in degrading reversed forks with the aid of the WRN helicase [97].

If a stalled fork cannot be efficiently restarted, the fork is persistently stalled, and the fork may collapse (Figure 1.3). To form a collapsed fork, an endonuclease is first needed to generate the break on the ssDNA, and Mus81-Eme1 has been shown to be important in this step [116,117]. An exonuclease can then digest the DNA to form a one-ended DSB. To initiate HR this DSB then needs to be processed through end resection where Mre11 and CtIP may act. DNA2 and EXO1 (exonuclease 1) have also been implicated in this resection step, but the nucleases involved are not well-characterized [118,119]. After a 3' overhang is generated, RPA binds to this ssDNA, is displaced, and Rad51 forms filaments and recruits HR factors as described above to initiate strand invasion into the homologous template. Moreover, during HR, structure-specific endonucleases (Mus81-Eme1, SLX1) bound to SLX4 engage in Holliday junction resolution. SLX4 is a scaffold protein for these endonucleases (Mus81, SLX1, XPF).

As discussed above, many nucleases are known to be involved in resolving stalled replication forks, but how they collaborate, what other nucleases are needed, and how their functions are regulated is not well characterized. This thesis seeks to determine how the DNA nuclease SNM1B/Apollo (described below) plays a role in resolving stalled replication forks and maintaining genome integrity.

Identification of the *SNM1B* gene

The SNM1 (sensitivity to nitrogen mustard) gene family was first found in *Saccharomyces cerevisiae* as the gene *SNM1/Pso2* which when mutated led to hypersensitivity to two agents that induce ICLs: nitrogen mustard and psolaren [120-122]. Mammalian homologs of *SNM1/Pso2* were then identified and named *SNM1A*, *SNM1B/Apollo*, *SNM1C/Artemis*, *CPSF73*, and *ELAC2* [123-125]. SNM1A, SNM1B, and Artemis are involved in DNA processing while CPSF73 and ELAC2 are involved in RNA processing [126-128]. These proteins are part of the metallo-β-lactamase (MBL)/βCASP (CPSF-Artemis-SNM1-PSO2) superfamily of proteins, and these two domains possess nucleolytic function [129]. The MBL fold has four conserved motifs where metal zinc ions can bind [130]. One motif (HxHxDH) is the nuclease active site [129,131]. The βCASP domain is unique to the family and has a nucleic acid binding domain. There are three motifs with conserved amino acids predicted to be located near the catalytic center of the MBL fold [129]. These two domains are conserved between the three members but the rest of the sequence is distinct (Figure 1.4) [132,133]. It has been shown that SNM1A is involved in ICL repair [125]. Artemis is important in processing intermediates that form during V(D)J recombination and in repairing DSBs within the NHEJ pathway [134,135]. SNM1B has roles in ICL repair, DSB repair, and telomere maintenance as discussed below.

SNM1B possesses 5' to 3' exonuclease activity on both double-stranded and single-stranded DNA [136]. Moreover, our lab has unpublished data that shows SNM1B also has endonuclease activity and it can specifically digest a flap-like structure and hairpin structure. SNM1B depleted human and chicken DT40 cells have been shown to be sensitive to ICL agents including MMC and cisplatin [137-140]. There is conflicting data about if SNM1B depleted cells are sensitive to IR [137,138]. Two studies used siRNA depletion of SNM1B in either U2OS cells or HeLa cells and found hypersensitivity to IR [138,141]. On the contrary, another study found shRNA stable knockdown of SNM1B in 293T cells had no difference in cellular survival upon IR [137]. However, in this experiment the control cells decreased in survival substantially with 4Gy of IR suggesting these cells may not be the best controls. Therefore, the results are hard to interpret and the conclusion the authors come to is not concrete. This same study also found that SNM1B depletion does not render cells sensitive to UV damage [137]. These studies

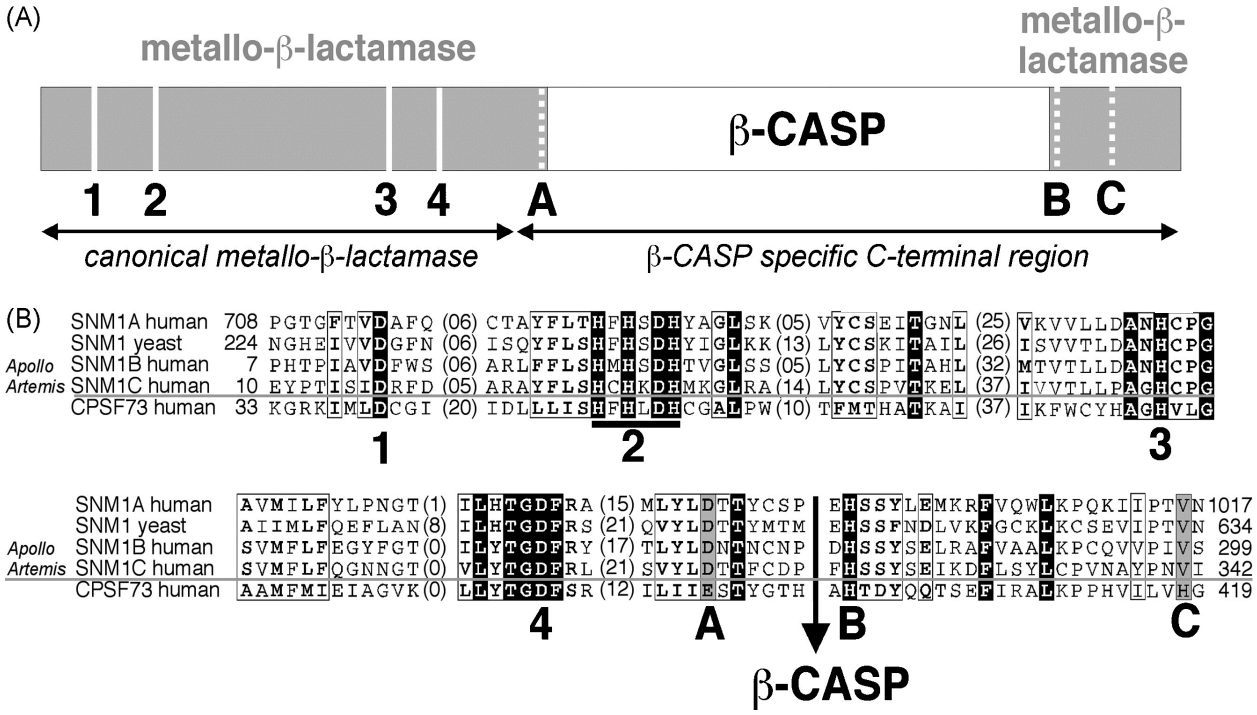

Figure 1.4: SNM1B is a DNA nuclease that is comprised of a highly conserved catalytic domain.

A. Schematic showing the location of the metallo-β-lactamase (MBL) fold and the β-CASP domain in SNM1B.

B. Alignment of the SNM1 proteins. The four conserved motifs of the MBL fold are numbered (1-4) with emphasis on motif 2, which contains the HxHxDH nuclease active site. The three conserved motifs in the β-CASP domain are labeled A-C. Black: conserved amino acids; Boxed: similar amino acids. Adapted from de Villartay et al. [142]

implicate SNM1B in having important roles in the DDR and cellular survival in response to damage, which will be further discussed in a later section.

In studies where SNM1B knockout mice were generated, it was found that homozygous SNM1B null mice are perinatal lethal. Double null embryos were analyzed and were found to be smaller and have hypocellularity in multiple organs (brains, lungs, thymus) compared to wildtype or heterozygous embryos. All these embryos died at postnatal stage PO [143]. Primary mouse embryonic fibroblasts (MEFs) from E13.5 were generated and found to exhibit increased apoptosis, proliferation defects, and aberrant metaphase chromosomes, including telomere fusions [144]. A double knockout mice with Ku70, a factor involved in NHEJ, rescued the embryonic lethality phenotype, showing that the telomere fusions resulted from unprotected telomeres activating the DDR and being 'repaired' by NHEJ. This study also found that double knockout mice of SNM1B and p53 or SNM1B and ATM did not rescue the perinatal lethality phenotype, suggesting that these phenotypes are not a result of activation of p53-dependent apoptosis or ATM activated DNA repair pathways [143,144].

The role of SNM1B in protecting telomeres

Telomeres, the ends of chromosomes, are bound by the shelterin complex which protects telomeres from being recognized as broken DNA and activating the DDR. G-rich 3' overhangs are present at telomeres and is necessary for loading of the shelterin complex [145,146]. One component of this complex is TRF2 [147]. Through mass spectrometry, SNM1B was found to associate with TRF2 and the shelterin complex [148]. Co-immunoprecipitation experiments found that TRF2 interacts with the C-terminus of SNM1B [136,148-150]. Specifically, it was found that SNM1B 5' to 3' exonuclease activity is involved in the resection of leading strand telomeres forming the 3' overhangs [144,151,152]. Knockdown of SNM1B therefore results in unprotected telomeres which are therefore recognized by the DDR as a DNA break that needs to be repaired resulting in telomere fusions [136,153]. By associating with the shelterin complex and protecting telomeres, SNM1B prevents these NHEJ mediated chromosome end-to-end telomere fusions.

The clear lethality of SNM1B knockout mice shows the importance of SNM1B on survival. Interestingly, a mutant allele of *SNM1B* was identified in a patient with

Hoyeraal-Hreidarsson syndrome, a severe form of dyskeratosis congenita, who exhibited phenotypes including aplastic anemia, pre-mature aging, microcephaly, bone marrow failure, and immunodeficiency; features similar to many genome instability disorders, including FA. This patient possessed a splice variant of SNM1B that led to a truncation mutant which disrupted the interaction of SNM1B with TRF2 in the C-terminus. This mutant however was found to not be hypersensitive to ICL agents suggesting that SNM1B may have distinct and separate roles in telomere maintenance and the DNA damage response [154].

SNM1B protein interactions

In understanding how SNM1B functions in DNA repair it is imperative to look at what proteins it interacts with to determine what pathways it may be involved in. SNM1B was found to interact with the nuclease Mre11, which is a component of the MRN complex that is involved in the recruitment of the ATM kinase to DSBs to initiate checkpoint signaling and repair through HR [137,155,156]. Mre11 has also been implicated in alternative-NHEJ and replication stress, and it may be acting at different stages of the repair process as described earlier [95,96,157]. SNM1B was also found to interact with the FA protein FANCD2 which is involved in repairing stalled forks as described above [137]. The specific role of FANCD2 in the response to replication stress may be different than its role in ICL repair, which is more clearly defined. It was also found that SNM1B interacts with Mus81, a nuclease that also has roles in ICL repair which are distinct from its roles in repairing stalled forks [137]. Mus81 has specifically been implicated in generating the break that results in a DSB which forms a collapsed fork as explained previously [116,117].

SLX4, another FA protein, was previously mentioned as a scaffold protein that binds to Mus81, SLX1, and XPF, and it was also found to interact with SNM1B [141]. No studies were performed that showed direct interaction, so more investigation into the functionality of this interaction needs to be done. Interestingly, SNM1B was also found to interact with PSF2 in a yeast two hybrid screen [158]. PSF2 is part of the CMG helicase complex [159]. It is known that PSF2, as part of the GINS complex, accumulates on chromatin and forms a complex with Cdc45 and MCM2-7. Interestingly, FA proteins

FANCA, FANCB, and FANCF also interact with PSF2 and have all been shown to associate with the replication fork independent of the DDR [160]. The function of this specific interaction of SNM1B is still unknown although it suggests SNM1B may be at the replication fork during both normal and aberrant replication.

Roles of SNM1B in DNA repair

As mentioned above SNM1B has a role in ICL repair. We have previously shown that SNM1B functions epistatically to FANCD2 and FANCI in ICL repair. Specifically depletion of both SNM1B and FANCD2 did not increase hypersensitivity to MMC when compared to cells depleted of either SNM1B or FANCD2. Similar results were found when analyzing MMC induced chromosomal anomalies. SNM1B is also important for the efficient recruitment of HR factors BRCA1 and Rad51 to sites of MMC induced ICLs [140]. These data suggest that SNM1B functions within the FA pathway to repair ICLs. During the repair of ICLs, the ICL is unhooked by endonucleases which generates a DSB at the fork that is then repaired through HR [161,162].

Multiple studies have shown evidence that SNM1B has a role in DSB repair. One study looked at IR induced breaks and using laser micro-irradiation immunofluorescence experiments, found that endogenous SNM1B localizes to the laser stripe 10 minutes post irradiation. Using live cell imaging it was also shown that SNM1B localizes to regions of induced DNA breaks by 10 seconds and that this SNM1B localization is ATM independent. From these results they concluded that SNM1B is important in the early response to DSBs including autophosphorylation of ATM and downstream ATM targets [149]. When analyzing DSBs that result from ICL inducing agents, one group found that SNM1B depleted cells treated with MMC are defective in the DSB response, but IR cells are normal, which conflicts with the previous study [137]. In the previous study (Demuth et al.) the authors used up to 20Gy of IR where the most drastic differences were noticed, while in this study 2Gy was used. The amount of breaks produced by 20 Gy versus 2 Gy is higher, and therefore, it is difficult to directly compare the two experiments since the cellular response may be different in the two conditions. SNM1B may have a role in promoting ATM signaling when there are higher numbers of DNA breaks.

Several studies have also found additional roles of SNM1B in maintaining genome integrity but the mechanisms have not been elucidated yet. One study found that SNM1B binds to Astrin and is important in responding to spindle stress by activating the prophase checkpoint which occurs in early mitosis [163]. Another study found that SNM1B interacts with HSP70 (heat shock proteins 70) which have roles in the DDR but the functionality of this interaction has not been characterized [164].

Since the FA pathway is involved in ICL repair and in resolving replication stress, this thesis sought to determine if SNM1B is involved within the FA pathway in replication restart at stalled replication forks. As described above, nucleases are critical in these repair processes, so specifically the nuclease properties of SNM1B may have critical functions. Investigation into how SNM1B is involved in repairing collapsed replication forks that results from stalled forks has not been elucidated. SNM1B may play several roles at multiple stages of repairing replication-associated damage. It is also emerging that the *SNM1B* locus is genomically altered or mutated in a variety of cancer types and has been mapped at a chromosomal breakpoint associated with Wilms tumor [165,166]. Furthermore, SNM1B was found mutated in half of primary mediastinal B cell lymphomas [167]. Thus, SNM1B could potentially function as a tumor suppressor and may have important roles in preventing oncogenic chromosomal events in association with human disease.

Regulation of DNA repair processes

The DDR and subsequent DNA repair processes are tightly monitored and regulated events. Specifically, some DNA repair proteins are involved in multiple pathways to repair different types of DNA damage. Therefore, the timely activation and recruitment of these proteins need to be tightly controlled to ensure maintenance of genome integrity. The functions of DDR and DNA repair proteins are regulated at multiple levels including post-translational modifications (PTMs), cell cycle regulation, protein stability, and proteasomal degradation.

Post-translational modifications (PTMs)

PTMs have a critical role in the DDR as they impact protein functions, interactions, enzymatic activity, and localization. Some PTMs that are commonly

observed during DNA repair processes are phosphorylation, ubiquitination, SUMOylation, acetylation, methylation, and PARylation. The critical importance of kinases for phosphorylation events during the DDR is exemplified by the three protein kinases central in DNA repair: ATM, ATR, and DNA-PK (DNA-dependent protein kinase) which are members of the PIKK (phosphoinositide-3-kinase-like protein kinase) family [168-170]. One key phosphorylation event that all three kinases perform is phosphorylation of the histone variant H2AX [171-175]. Once H2AX is phosphorylated (denoted as γH2AX) further downstream events are initiated including the recruitment of repair proteins [173,176]. These kinases also have the ability to autophosphorylate which affects their own activities and functions [177-180]. The DDR is comprised of a complex signaling cascade. Therefore, ATM phosphorylates a multitude of downstream proteins including the effector kinase CHK2 while ATR phosphorylates another effector kinase CHK1. These events ultimately result in cell cycle checkpoint activation to allow time for the cell to repair the damage and regulation of p53-induced apoptosis pathways if the damage is too severe to repair [83,181-183].

Ubiquitination is another PTM that is commonly utilized to regulate DNA repair proteins. Ubiquitin is a 76 amino acid polypeptide that is covalently attached to lysine residues on proteins through E1 (activating), E2 (conjugating), and E3 (ligase) enzymes, and proteins are either monoubiquitinated or polyubiquitinated [184-186]. Ubiquitin itself has seven lysine residues and polyubiquitin chains can form [187]. While polyubiquitination primarily targets proteins for proteasomal degradation, discussed later, K63 linked polyubiquitin chains have roles in DNA repair [188,189].

Monoubiquitination of a protein can promote its localization to a specific site. For example, two FA proteins, FANCD2 and FANCI (ID complex), are monoubiquitinated by the FA core complex which possesses E3 ubiquitin ligase function. This monoubiquitination event results in localization of the ID complex to sites of blocked or stalled replication forks [190]. Additionally, monoubiquitination can promote protein:protein interactions through ubiquitin-binding domains (UBD) which can potentially recruit a DNA repair protein complex to the site of damaged DNA [191,192]. For example, the monoubiquitination of the ID complex binds to the UBD of the nuclease FAN1 resulting in the recruitment of FAN1 to the replication fork [100,101,193,194]. Monoubiquitination also

plays a role in regulation of functions by activating specific repair pathways. Monoubiquitination of the replication sliding clamp PCNA (proliferating cell nuclear antigen protein) is a prominent response to blocked replication forks. This modification induces translesion synthesis (TLS) mediated repair by displacing high-fidelity replicative polymerases with low-fidelity TLS polymerases which can bypass DNA lesions [18,195-197].

Another example of a PTM is SUMOylation which adds SUMO (small ubiquitin-like modifier) to the protein [198]. For example, SUMOylation of RPA results in facilitating the interaction between RPA and Rad51, thereby recruiting Rad51 to DSBs to initiate repair through HR [199]. PARylation, characterized by the addition of ADP-ribose polymers to proteins through PARP, is another PTM [200]. PARP is known to bind to DNA breaks and has been shown to PARylate the catalytic subunit of DNA-PK (DNA-PKcs) and stimulate its kinase activity *in vitro* [201]. Furthermore, PARylation of targets recruits DDR proteins to these breaks. Histone methylation and acetylation regulates opening up of the chromatin allowing room for localization of repair proteins [202]. An example of PTMs affecting protein stability and turnover is the WRN helicase which is acetylated upon DNA damage and this stabilizes the protein and prevents protein degradation [203].

Cross-talk between these different types of PTMs can be involved in regulating one protein, especially if this protein has several functions in multiple repair pathways. One example of this is CtIP (carboxy-terminal binding protein interacting protein) which plays a key role in DSB repair. CtIP is phosphorylated by CDK2 resulting in BRCA1 and MRN binding which promotes end resection at a DSB and initiates HR-mediated repair [204-207]. CDK2 is present mainly in S and G2 phases and since its phosphorylation of CtIP occurs during these phases, CtIP plays an important role in regulating which DSB repair process HR or NHEJ should be used [208]. CtIP is also ubiquitinated by BRCA1 and both these PTMs are critical for the localization of CtIP to sites of breaks [209].

Removal of PTMs is just as important in the regulation of proteins. For example, DUBs (deubiquitylating enzymes) promote disassembly of protein complexes, inactivation of protein functions, and disruption of interactions that are not required anymore once the damage is fixed [210,211]. If proteins are not properly modified, their functions could be impaired resulting in the persistence of damaged DNA and an overall

increase in genome instability.

Mechanisms to stabilize and regulate protein levels

The ubiquitin-proteasome system (UPS) regulates protein levels and stabilization of proteins which is a key component of the DDR. While monoubiquitination is involved in localization, protein:protein interactions, and repair functions as described above, polyubiquitin chains (K48 linked) target proteins to the proteasome for degradation [212,213]. Specifically, many helicases have been shown to be regulated through this UPS. BLM helicase is ubiquitinated by RNF8/RNF168 which affects its localization to stalled replication forks. When BLM is ubiquitinated by other E3 ligases like CUL3, it is targeted for degradation [214,215]. These two types of ubiquitination work together to monitor the activity of BLM during the restart of stalled replication forks. Another helicase FANCM is known to process DNA structures found during the repair of stalled replication forks and also to initiate fork regression [216]. Once FANCM is no longer required at the replication fork it is targeted for degradation by the proteasome [217].

Having higher levels of certain proteins in normal proliferating cells may result in deleterious phenotypes, so the cell rapidly degrades these proteins in normal conditions. Upon cellular stress, which may require the protein to fix the damage, it is not targeted for proteasomal degradation. Furthermore, protein levels of some DNA repair proteins are regulated in response to DNA damage. p53 is an example of this form of regulation of stabilization and activation in response to cellular stress including DNA damage [218]. ATM, ATR, and DNA-PK have all been shown to phosphorylate p53 in response to DNA damage [219-223]. This phosphorylation of p53 interrupts its interaction with Mdm2, which normally is bound to p53 and targets it for degradation [224,225]. Once activated, p53, which is a transcription factor, initiates transcription of genes needed for the DDR and activation of cell cycle checkpoints, or if the damage is too severe, to initiate apoptosis [226-230].

As the cell progresses through the cell cycle, DNA repair protein functions are coordinated depending on what phase of the cell cycle the damage has occurred and also what specific type of lesion is present [231]. Protein levels of DNA repair factors may be regulated by the cell cycle; for example, CtIP. CtIP protein levels are low in G1 and as the cell progresses though S phase the protein levels increase (with consistent

transcription) [232]. It has also been demonstrated that an interaction of Mre11 with CDK2-CyclinA which occurs when this complex is available (S-phase) results in the regulation of CtIP protein levels, and these events occur in unperturbed proliferating cells [233]. These data provide a potential way through which CtIP plays a role in deciding between HR and NHEJ by promoting HR-mediated repair.

The importance of PTM mediated protein:protein interactions was discussed above as being critical for the assembly of repair complexes to sites of DNA damage for efficient repair. The stability of a protein can also be regulated through its interaction with other proteins. For example, the binding of two helicases important in the stabilization and restart of stalled forks, FANCJ and BLM, stabilizes the BLM protein while this interaction does not affect protein levels of FANCJ [234-236]. The MRN complex is another example of a protein complex where removal of one component results in destabilization of the complex and decreased protein levels of the other components [237-239].

Regulation of nucleases in DNA repair

DNA nucleases have critical functions in repairing all types of DNA damage. Specifically, nucleases are absolutely necessary in repairing stalled replication forks. A number of nucleases including Mre11, Mus81, FAN1, DNA2, Exo1, and SNM1B have been implicated in this process, but how they all work together and are coordinated and regulated is unknown. Furthermore, nuclease activities have to be tightly controlled during the repair process as unregulated or uncontrolled nuclease activity can degrade the DNA, resulting in even more damage and increased genome instability. Mre11 is controlled by the FA/BRCA network during its role in processing regressed forks to ensure these structures are not highly degraded [95].

The 5’ to 3’ exonuclease activity of SNM1B generates 3’ overhangs at telomeres which is necessary for telomere protection. TRF2, a component of the shelterin complex, recruits SNM1B to telomeres through this direct interaction. It has been shown that at telomeres another member of the shelterin complex, POT1b, inhibits hyper-resection of leading-end telomeres by SNM1B. However, the specific mechanism through which POT1b regulates SNM1B functions at telomeres is not defined [151]. One study found that the interaction of SNM1B with TRF2 stabilized SNM1B protein levels. It

was shown that upon TRF2 binding to SNM1B, polyubiquitination of SNM1B was inhibited therefore preventing degradation of SNM1B via the proteasome [150,240]. In addition to TRF2, SNM1B has also been shown to interact with DNA repair proteins including Mre11, FANCD2, Mus81, SLX4 [137,141]. However, the functional significance of these interactions and how they are mediated is not known.

It is important to recognize that the functions of proteins can be regulated at multiple levels, and the interplay between these different levels of regulation including PTMs, cell cycle regulation, protein stability, and proteasomal degradation could all be important in the recruitment, localization, and enzymatic functions of a protein. Furthermore, the specific mechanism through which a protein is regulated could vary depending on the type of DNA damage.

Summary

Accurate DNA replication is critical for the cellular survival of proliferating cells. The DNA replication machinery may encounter various endogenous and exogenous sources of damage many of which are still being identified. A functioning DDR resolves stalled replication forks; however, if stalled forks cannot be restarted, collapsed forks with DSBs may result. These breaks are then repaired, but if the repair mechanisms are defective, this could lead to deleterious chromosomal anomalies. This increase in genome instability can lead to diseases associated with defects in the cellular response to replication stress including cancer. Understanding the cellular mechanisms that repair DNA damage is critical for understanding how defects in DNA repair mechanisms can lead to human disease. There are multiple processes to resolve replication-associated genomic damage and what proteins are involved, how they function together, and how their functions are regulated is still ambiguous. DNA nucleases play critical roles in the restart and repair of stalled or collapsed replication forks but the specifics are not well characterized. In this thesis, I sought to elucidate the role of the DNA nuclease SNM1B in responding to stalled replication forks and maintaining genome stability.

In Chapter 2, I show that SNM1B is critical for cellular survival upon replication fork stalling caused by DNA polymerase inhibition. I also provide evidence that SNM1B is important for the localization of key repair factors FANCD2 and BRCA1 to sites of

stalled replication forks and in preventing chromosomal aberrations specifically at CFSs. In Chapter 3, I demonstrate that SNM1B is important in preventing the accumulation of ssDNA and localization of Rad51 to stalled forks. I also show that in unperturbed conditions SNM1B prevents the accumulation of stalled and collapsed replication forks. In Chapter 4, I have identified a residue that is important for efficient SNM1B localization to stalled replication forks. I have also found that SNM1B protein levels are regulated in response to DNA damage and during normal cellular proliferation. Finally, implications of my findings and future directions are discussed in Chapter 5.

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Chapter 2: SNM1B has functions in the resolution of replication stress and maintenance of common fragile site stability

Summary

SNM1B/Apollo is a DNA nuclease that has important functions in telomere maintenance and repair of DNA interstrand crosslinks (ICLs) within the Fanconi anemia (FA) pathway. SNM1B is required for efficient localization of key repair proteins, such as the FA protein, FANCD2, to sites of ICL damage and functions epistatically to FANCD2 in cellular survival to ICLs and homology-directed repair. The FA pathway is also activated in response to replication fork stalling. Here we sought to determine the importance of SNM1B in cellular responses to stalled forks in the absence of a blocking lesion, such as ICLs. We found that depletion of SNM1B results in hypersensitivity to aphidicolin, a DNA polymerase inhibitor that causes replication stress. We observed that the SNM1B nuclease is required for efficient localization of the DNA repair proteins, FANCD2 and BRCA1, to subnuclear foci upon aphidicolin treatment; thereby indicating SNM1B facilitates direct repair of stalled forks. Consistent with a role for SNM1B subsequent to recognition of the lesion, we found that SNM1B is dispensable for upstream events, including activation of ATR-dependent signaling and localization of RPA, γH2AX, and the MRE11/RAD50/NBS1 complex to aphidicolin induced foci. We determined that a major consequence of SNM1B depletion is a marked increase in spontaneous and aphidicolin-induced chromosomal gaps and breaks, including breakage at common fragile sites. Thus, this study provides evidence that SNM1B functions in resolving replication stress and preventing accumulation of genomic damage.

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Ishita Das contributed to Figures 2.1B, 2.2A,B,C, 2.3B, 2.4, 2.5, 2.6, 2.8; Suppl. Fig 2.1, 2.2, 2.3, 2.4, 2.5
Jennifer Mason contributed to Figures 2.1A,B, 2.2A, 2.3A,B, 2.7, 2.8; Suppl. Fig 2.4, 2.6

Introduction

Replication of the genome is essential for faithful transmission of genetic information to daughter cells and for maintenance of genomic integrity. The DNA replication machinery is highly processive and accurate; however, progression of the replication fork can be impeded by secondary DNA structures or physical blocks, such as DNA interstrand crosslinks (ICLs). Blocked or stalled forks can be stabilized and restarted upon arrest; however, they may also collapse, leading to genomic damage in the form of DNA double strand breaks (DSBs). Replication associated DSBs have potential to engage in mutagenic events such as chromosomal deletions or aberrant rearrangements [1,2]. Replication stress represents a constant threat to the genome; thus, it is of importance to elucidate the cellular mechanisms that ensure efficient resolution of blocked or stalled replication forks.

SNM1B/Apollo is a DNA nuclease that is comprised of a highly conserved, catalytic metallo-β-lactamase/β-CASP N-terminal domain and a unique C-terminus [3]. Previous studies have demonstrated critical functions for its intrinsic 5' to 3' exonuclease activity in the processing of leading strand telomeres to protect them from end-to-end joining [4-6]. SNM1B also plays important roles in the repair of DNA damage. In this regard, depletion of SNM1B in mammalian cells results in hypersensitivity to ICL inducing agents such as mitomycin C (MMC) and a moderate sensitivity to ionizing radiation (IR) [7-10]. We demonstrated that SNM1B is required for efficient localization of key repair proteins, including the Fanconi anemia (FA) protein, FANCD2, and the homologous recombination proteins, BRCA1 and RAD51, to sites of ICL induced damage [10].

FA is an inherited genome instability disorder characterized by bone marrow failure, skeletal defects, cancer predisposition, and cellular hypersensitivity to ICLs [11,12]. There are currently fifteen known FA complementation groups (FANCA, B, C, D1/BRCA2, D2, E, F, G, I, J, L, M, N, O, P). A "core" complex comprised of eight FA proteins (FANCA, B, C, E, F, G, L and M) possesses ubiquitin ligase activity and is activated by the presence of ICLs. The core complex monoubiquitinates FANCD2 and FANCI, which localize to chromatin and form subnuclear foci at the sites of damage [11,12]. The FA pathway plays central roles in ICL repair, and FA patient cell lines are hypersensitive to ICLs and exhibit spontaneous and replication stress induced chromosomal aberrations. SNM1B depletion also results in increased levels of spontaneous and ICL induced chromosomal anomalies, including gaps, breaks, and radial structures. The cellular phenotypes of SNM1B depleted cells parallel those observed in FA cells, and indeed, the functions of SNM1B in ICL repair and maintaining chromosomal stability are epistatic to FANCD2 and FANCI [10].

The FA pathway is also activated in response to replication fork slowing or stalling [13]. The mechanisms involved in resolving replication stress are distinct from those required for removal or bypass of ICLs and are not fully defined. One outstanding question is the identification of DNA nucleases involved in processing the nascent DNA strands to facilitate replication restart or repair upon fork collapse [14]. SNM1B is one of several candidate nucleases that may participate in nucleolytic processing of replication intermediates. It has been demonstrated to form complexes with proteins that localize to and function in repair of stalled forks. SNM1B interacts directly with MRE11 [7], an endo/exonuclease that facilitates replication fork restart [15,16] and localizes to stalled replication forks [17-19]. MRE11 also catalyzes enhanced resection of unprotected stalled replication forks [20-22]. In addition, SNM1B interacts with the FA proteins, FANCD2 [7] and FANCP/SLX4 [23]. Monoubiquitinated FANCD2 localizes to sites of stalled forks [13] and functions in stabilizing and protecting stalled forks from extensive nucleolytic degradation [21]. FANCP/SLX4 is a scaffold protein that interacts with several structure-specific nucleases and regulates their activities in response to different types of DNA damage, including replication stress [24-28]. Like FANCD2, FANCP/SLX4 functions epistatically to SNM1B in repair of ICLs [23].

Given the evidence that SNM1B functions within the FA pathway in the repair of ICLs and interacts with proteins involved in resolving replication stress, we have examined the importance of the SNM1B nuclease in the repair of stalled forks. We find that depletion of SNM1B from human cell lines results in hypersensitivity to the DNA polymerase inhibitor, aphidicolin, which causes replication fork slowing and stalling. We demonstrate that SNM1B depleted cells are not defective for sensing or signaling aphidicolin-induced DNA damage. However, the SNM1B nuclease is required for efficient localization of key repair proteins, FANCD2 and BRCA1, to stalled replication forks. We also observe that SNM1B depleted cells exhibit elevated levels of spontaneous and aphidicolin induced gaps and breaks, including increased instability at the common fragile sites, FRA3B and FRA16D. These findings provide evidence that the SNM1B nuclease plays critical roles in the resolution of stalled replication forks to maintain genome stability.

Materials/Methods

Knockdown of SNM1B expression by siRNA.

The HCT116 colon cancer and WT fibroblast human cell lines were cultured as previously described [29]. HCT116 cells were plated at a density of $1x10^5$ cells per well of a 6 well dish in McCoy's media (10% FBS, 1% Pen/Strep) 24 hours prior to siRNA transfection. HeLa cells were plated at the same density in DMEM (10% FBS, 1% Pen/Strep). All siRNAs (50 nM) were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. SNM1B mRNA levels were determined via semi-quantitative RT-PCR in every experiment to verify the extent of siRNA knockdown as previously described [10].

Aphidicolin sensitivity assay.

WT fibroblasts transfected with non-specific (NS) or siSnm1B-1 (SNM1B specific) siRNAs 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 [30]. The sensitivity curve was

performed three independent times. HeLa cells transfected with NS, siSnm1B-1, or siSnm1B-2 siRNAs and HCT116 cells transfected with NS or siSnm1B-1 siRNAs were plated and analyzed as above. The aphidicolin sensitivity curve was performed two independent times.

Chromosome anomalies and FISH.

HCT116 cells transfected with NS or siSnm1B-1 were treated with aphidicolin (0.3 or 0.5 μM) for 24 hours. Cells were incubated with colcemid for 1 hr (untreated) or 3 hours (0.3 or 0.5μM aphidicolin). Cells were harvested and incubated in 0.075M KCl for 15 minutes at 37^{o}C followed by a series of fixations in Carnoy's fixative (3:1 methanol:acetic acid). Fixed cells were dropped onto slides and baked prior to Giemsa staining or two-color fluorescence *in situ* hybridization (FISH). Giemsa stained chromosomes were scored for gaps and breaks. The average gaps and breaks per metaphase was calculated from three independent experiments. For two-color FISH, probes were generated using BAC and YAC constructs containing human genomic inserts that span the fragile site regions. YAC 850A6 was used for FRA3B and BAC26L41 was used for FRA16D [31]. Probes were labeled by nick translation synthesis with digoxigenin or biotin (Roche). Two-color FISH was done as previously described [31]. Approximately 30 signals were examined for each sample from at least three independent experiments. The gaps/breaks and common fragile site analyses were performed in a blinded manner. Images were acquired using a Zeiss Axioscope epifluorescence microscope and Olympus DP70 digital camera system.

Western blot analyses.

HCT116 cells transfected with NS or siSnm1B-1 were treated with 0.3μM aphidicolin for 24 hours. Cells were harvested and 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). For the FANCD2-Ub experiments, soluble and chromatin-bound FANCD2 fractions were separated as previously described [32]. Protein concentration was determined by Bradford assay. Lysates (100 mg) were analyzed by western blotting

using the appropriate primary antibodies and IRDye 800 CW secondary antibodies (Li-Cor). Bands were visualized using Odyssey 2.1 software. All experiments were performed at least three independent times.

HCT116 cell lines expressing wildtype and mutant SNM1B were harvested and resuspended in protein lysis buffer (10mM PIPES, pH 6.8, 100mM NaCl, 300mM sucrose, 1mM $MgCl_2$, 0.1% Triton-X 100) containing phosphatase (Roche PhosSTOP) and protease inhibitors (Roche Complete Mini EDTA free), and Benzonase (Purity >99% Novagen). Expression of SNM1B protein was analyzed by western blotting using V5 antibody (Invitrogen).

Generation of HCT116 cell lines expressing siRNA-1 resistant wildtype and mutant SNM1B.

An siSnm1B-1 resistant cDNA containing three silent point mutations within the siRNA-1 core sequence was used for the complementation experiments and to generate the site specific mutants [10]. The wildtype and mutant siRNA resistant cDNAs were subcloned into the pLL IRES GFP lentiviral vector (UM vector core). Lentiviruses expressing the SNM1B-IRES-GFP cassettes were generated as previously described [10]. HCT116 cells ($2.0x10^5$) were incubated with 1 mL of virus containing media with 4 mg/mL polybrene, 1 mL DMEM, and 10% FBS for 24 hours. Cells were harvested 24 hours later, and expression of the SNM1B-IRES-GFP expression cassette was determined by flow cytometry to determine the percentage of GFP positive cells.

GFP positive cells were sorted (University of Michigan Flow Cytometry Core), cultured, and resorted. SNM1B expression levels were assessed by semi-quantitative RT-PCR using primers specific for the siRNA resistant cDNAs. Early passage sorted cell lines with comparable levels of SNM1B expression were used for complementation experiments (Fig. 2.4B, 2.5B, Suppl. Fig. 2.4). HCT116 cells infected with the pLL empty vector control were used for all experiments. For complementation experiments, HCT116 cells expressing wildtype or mutant SNM1B-IRES-GFP constructs were transfected with the siRNAs and FANCD2 and BRCA1 foci were quantitated in a blinded manner. Images were acquired at 100 X magnification at the same fluorescence

intensity. Data represent three or more independent experiments. Error bars indicate standard error of the mean (SEM).

Immunofluorescence of subnuclear foci.

HCT116 cells ($4x10^4$) were plated on coverslips in 12 well dishes 24 hrs prior to siRNA transfection. Cells were treated with aphidicolin (0.3 μM) 48 hours post transfection for either 6 or 24 hours. HeLa cells were plated the same way for NBS1, FANCD2, and BRCA1 foci experiments. Empty vector-IRES-GFP and WT-SNM1B-IRES-GFP HCT116 cells were plated on coverslips in 12 well dishes and then treated with aphidicolin (0.3 μM) for 24 hours. For RPA, BRCA1, MRE11, NBS1, and FANCD2 foci experiments, cells were incubated in cold extraction buffer (20mM HEPES, 50mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 0.5% TX-100) for 5 min followed by fixation in 3.7% p-formaldehyde, 2% sucrose, 0.5% TX-100 for 20 min and then washed 3x with PBS. For γH2AX foci, cells were fixed in 3.7% p-formaldehyde, 2% sucrose for 20 min followed by incubation in cold extraction buffer for 5 min and then washed 3x with PBS. For V5-SNM1B foci, cells were fixed with ice cold 70% methanol, 30% acetone at -20°C for 20 min and then air dried at room temperature. Cells were then stained with primary antibody for 45 min and then Alexa Fluor 488 or 594 (Invitrogen Molecular Probes) secondary antibodies for 45 min. Prolong Gold antifade reagent with DAPI (Invitrogen) was used to mount coverslips on slides. Images were acquired using Olympus BX61 microscope and FISHview Software (Applied Spectral Imaging). At least three independent experiments were conducted.

Cell cycle analysis.

HCT116 cells were plated in a 6 well dish (1×10^5 in each well) and then transfected with siRNA 24 hours later. Cells were treated with 0.3μM aphidicolin 48 hours post transfection. Cells were then fixed with cold 70% ethanol, stored at -20°C overnight, and stained with propidium iodide (PI) for 30 min at room temperature. FACS analysis was performed using an Accuri C6 flow cytometer and cell cycle profiles were analyzed using FlowJo (TreeStar) software.

Antibodies.

α-pRPA32 Ser4/8 was from Bethyl (cat# A300-245A). α-pCHK1 Ser317 was from R&D Systems (cat# AF2054). α-PCNA was from SantaCruz (PC10). α-Topoisomerase I was from BD Biosciences (cat# 556597). α-RPA and α-BRCA1 were from Calbiochem (cat# NA19L, OP92). α-γH2AX was from Millipore (cat# 05-636). α-FANCD2, α-MRE11, and α-NBS1 were from Novus Biologicals (cat# 100-182, 100-142, 110-57272). α-V5 was from Invitrogen (cat# R960-25). α-Ku70 was from Abcam (cat# 10878).

Results

Impact of SNM1B depletion on cellular survival and signaling in response to aphidicolin treatment.

We examined the impact of SNM1B depletion on cellular survival upon exposure to the DNA polymerase inhibitor, aphidicolin. Wildtype human fibroblasts were transfected with a previously characterized siRNA specific for SNM1B (siSnm1B-1) or a nonspecific (NS) siRNA control [10]. At 48 hours post-transfection, cultures were treated with 0, 0.25, 0.5, and 1 μM aphidicolin for 24 hours, and the number of surviving cells was quantitated. We observed that SNM1B depleted fibroblasts exhibited significantly reduced survival after aphidicolin treatment when compared to controls (Figure 2.1A). To confirm these results, I examined aphidicolin sensitivity in HeLa cells transfected with siSnm1B-1 and a distinct siRNA, siSnm1B-2, that binds to a downstream sequence within the mRNA [10]. I observed that depletion of SNM1B with either siRNA significantly reduced survival of HeLa cells upon exposure to aphidicolin (Suppl. Fig. 2.1A). I further examined aphidicolin sensitivity in HCT116 cells transfected with siSnm1B-1 and observed a similar reduced survival of HCT116 cells depleted of SNM1B in response to aphidicolin treatment (Suppl. Fig. 2.1B).

The presence of long stretches of ssDNA generated upon replication fork stalling activates signaling pathways, and defects in DNA damage sensing or signaling can manifest as decreased cellular survival. Thus, we examined the cellular responses to inhibition of DNA polymerase activity in SNM1B depleted cells. The ATR protein kinase plays a central role in initiating the cellular responses to replication stress. The canonical signaling pathway is initiated upon binding of the RPA heterotrimeric complex

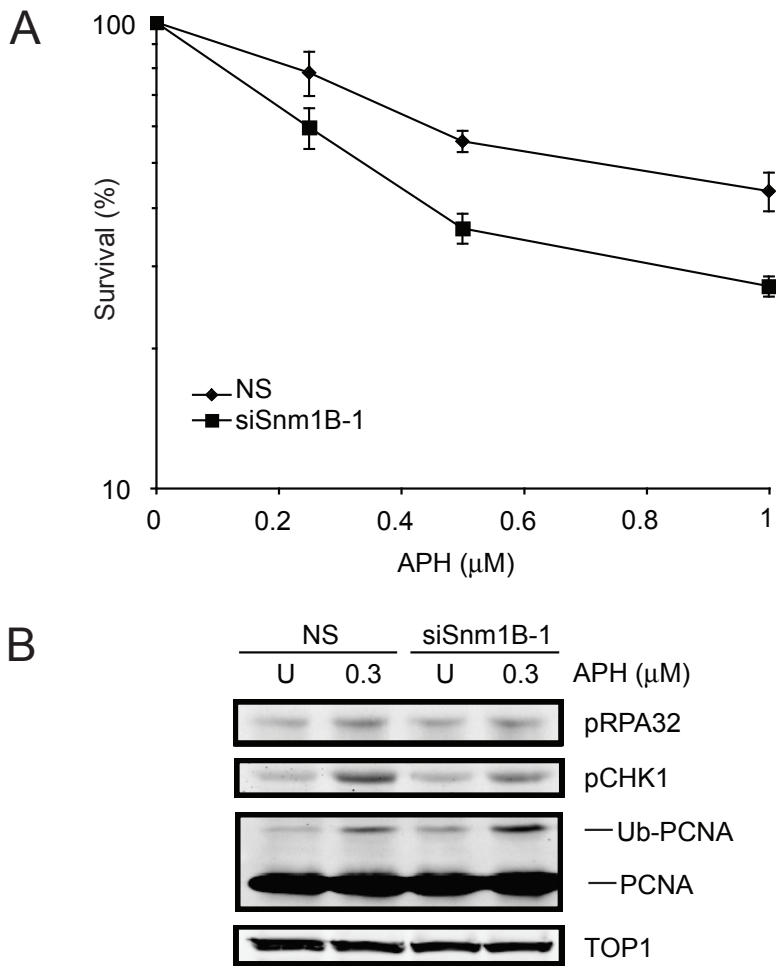

Figure 2.1. Cellular responses to replication stress upon SNM1B depletion.
A. Cellular survival in response to aphidicolin treatment. Wildtype human fibroblasts transfected with NS or siSnm1B-1 were treated with the indicated doses of aphidicolin for 24 hr at 48 hr post transfection. Cells were allowed to proliferate for 5-7 days. Percent survival was determined compared to an untreated control. Graph represents the average of three independent experiments. Error bars; standard deviation.
B. ATR-dependent signaling in SNM1B depleted cells. NS or siSnm1B-1 transfected HCT116 cells were treated with aphidicolin (0.3 μM) for 24 hr, and whole cell lysates were analyzed by western blotting. Phosphorylation of RPA (pRPA32) and CHK1 (pCHK1) and monoubiquitination of PCNA (Ub-PCNA) were examined. Representative blots from at least six independent experiments are shown. Topoisomerase I (TOP1), loading control; U, untreated controls.

to ssDNA at stalled forks and recruitment of ATR to ssDNA-RPA via its binding partner, ATRIP. Subsequent activation of ATR-ATRIP results in phosphorylation of downstream substrates to potentiate damage induced signaling [33].

One key signaling event indicative of ATR activation in response to replication stress is phosphorylation of the effector protein kinase, CHK1 [34]. To assess the importance of SNM1B in ATR dependent signaling, we depleted SNM1B in the HCT116 human colon cancer cell line and assessed pCHK1 S317 levels upon exposure to aphidicolin (0.3 μM) by western blotting. We observed that aphidicolin treatment induced phosphorylation of CHK1 in both siSnm1B-1 and NS transfected cells, and the levels of CHK1 p-S317 were approximately equivalent (Figure 2.1B). ATR-dependent phosphorylation of the RPA32 subunit is another well-characterized event in response to replication stress. Thus, we next examined levels of RPA32 phosphorylation and observed that aphidicolin induced similar levels of p-RPA in the SNM1B depleted cells compared to controls (Figure 2.1B).

PCNA is the homotrimeric sliding clamp that tethers DNA polymerases to replication forks. It becomes monoubiquitinated in a CHK1-dependent, ATR-independent manner and facilitates translesion DNA synthesis upon replication fork stalling [35,36]. We found that SNM1B depletion did not have a significant impact on Ub-PCNA levels compared to NS transfected controls (Figure 2.1B). These results indicate that SNM1B does not play a critical role in the cellular signaling response to replication stress, including PCNA ubiquitination and ATR-dependent phosphorylation of CHK1 and RPA32. Consistent with these findings, we observed that SNM1B depleted and control cells exhibit similar proportions of cells accumulating in S phase in response to low dose aphidicolin treatment (Suppl. Fig. 2.2). However, higher percentages of sub-G1 cells were observed in SNM1B depleted cells, consistent with the reduced cell survival observed in Fig. 1A (Suppl. Fig. 2.2).

SNM1B is not required for localization of RPA, γH2AX, and the MRN complex to aphidicolin induced subnuclear foci.

RPA localization to regions of ssDNA at replication forks can be visualized as punctate, subnuclear foci by immunofluorescence microscopy (Figure 2.2A). We

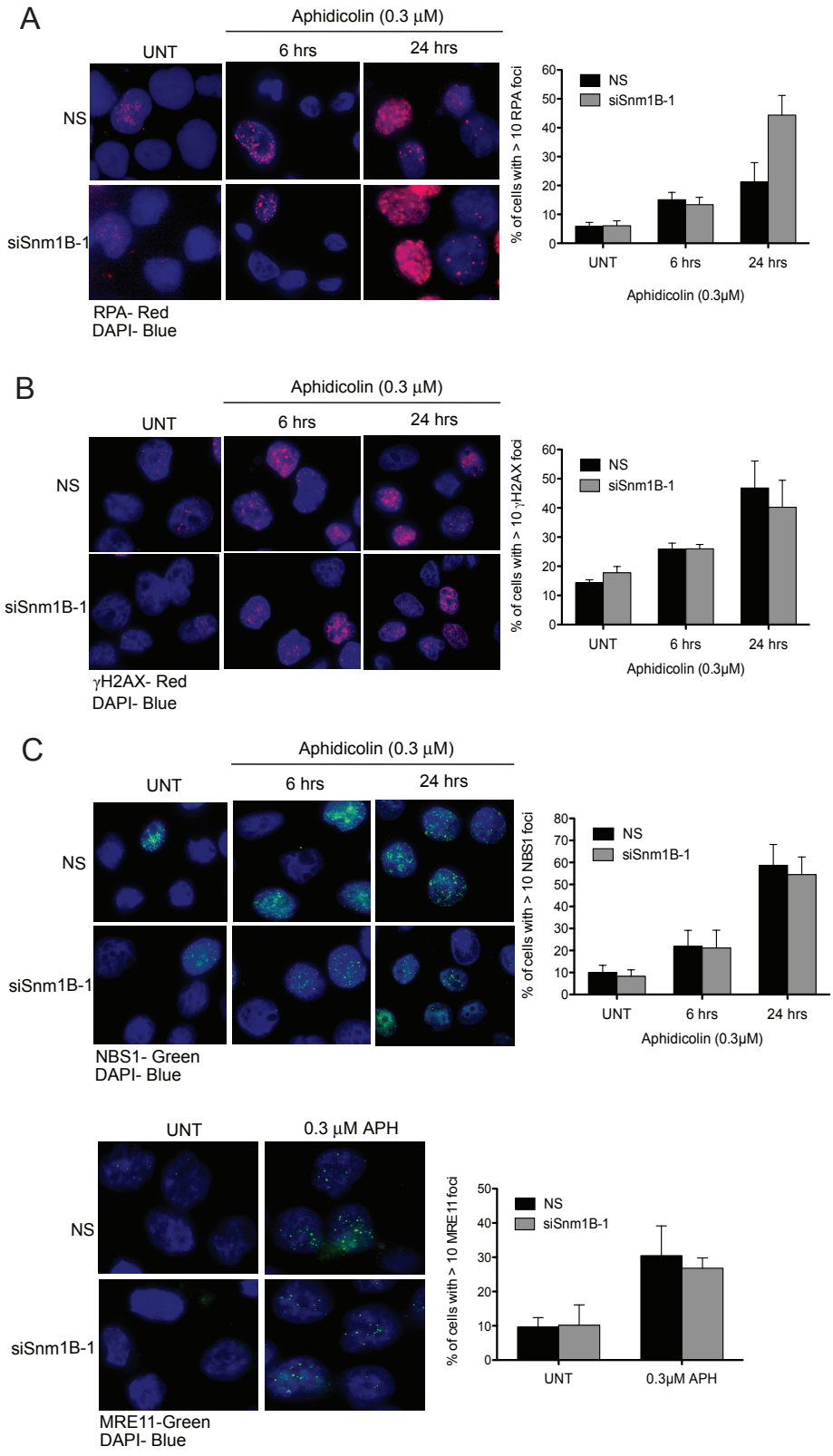
A
Aphidicolin (0.3 μM)
UNT
6 hrs
24 hrs
NS
siSnm1B-1
RPA- Red
DAPI- Blue
% of cells with > 10 RPA foci
NS
siSnm1B-1
UNT
6 hrs
24 hrs
Aphidicolin (0.3μM)
B
Aphidicolin (0.3 μM)
UNT
6 hrs
24 hrs
NS
siSnm1B-1
γH2AX- Red
DAPI- Blue
% of cells with > 10 γH2AX foci
NS
siSnm1B-1
UNT
6 hrs
24 hrs
Aphidicolin (0.3μM)
C
Aphidicolin (0.3 μM)
UNT
6 hrs
24 hrs
NS
siSnm1B-1
NBS1- Green
DAPI- Blue
% of cells with > 10 NBS1 foci
NS
siSnm1B-1
UNT
6 hrs
24 hrs
Aphidicolin (0.3μM)
UNT
0.3 μM APH
NS
siSnm1B-1
MRE11-Green
DAPI- Blue
% of cells with > 10 MRE11 foci
NS
siSnm1B-1
UNT
0.3μM APH

Figure 2.2. SNM1B is not required for localization of RPA, γH2AX, and the MRN complex to aphidicolin-induced subnuclear foci.

A. RPA foci formation in SNM1B depleted cells. RPA foci (red fluorescence) were quantitated in SNM1B depleted and control HCT116 cells exposed to aphidicolin (0.3 μM) for 6 or 24 hr. The average percentage of cells containing >10 RPA foci is plotted. The results represent data from at least three independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (blue). Error bars, SEM; UNT, untreated controls.

B. γH2AX foci formation in SNM1B depleted cells. γH2AX foci (red) were quantitated in SNM1B depleted and control HCT116 cells treated with aphidicolin for 6 or 24 hr, as described in A.

C. NBS1 and MRE11 foci formation in SNM1B depleted cells. NBS1 foci (green) were quantitated in SNM1B depleted and control HeLa cells treated with aphidicolin for 6 or 24 hr, as described in A. MRE11 foci (green) were quantitated in SNM1B depleted and control HCT116 cells treated with aphidicolin for 24 hours, as described in A.

examined the impact of SNM1B depletion on RPA foci formation in HCT116 cells exposed to 0.3μM aphidicolin at early (6 hrs) and later (24 hrs) time points. Upon aphidicolin treatment, we observed an approximately 2-fold increase in RPA foci positive cells in both siSnm1B-1 and NS transfected cells at 6 hours and an even further increase at 24 hours (Figure 2.2A). We noted that the percentages of aphidicolin induced RPA foci positive cells in SNM1B depleted cells were consistently higher at 24 hours compared to controls. These findings demonstrate that SNM1B is not required for RPA localization and suggest that SNM1B depletion results in increased ssDNA formation upon replication fork stalling.

The histone variant, H2AX, is phosphorylated by ATR in response to stalled or blocked replication forks. Recent studies have demonstrated that phosphorylated H2AX (γH2AX) is localized at stalled forks prior to the detection of DNA breaks and is required for efficient recruitment of other repair proteins, including FANCD2 and BRCA1 [37-39]. I observed that aphidicolin treatment of siSnm1B-1 and NS transfected cells induced γH2AX foci formation (Fig. 2B). However, SNM1B depletion did not impact the percentage of γH2AX foci positive cells at either 6 or 24 hours post aphidicolin treatment compared to controls.

MRE11 is a DNA nuclease that functions within the context of the heterotrimeric MRN protein complex. MRN plays central roles in the repair of DNA DSBs, and it also has functions during DNA replication. MRE11 and NBS1 have been demonstrated to co-localize with RPA, γH2AX, and FANCD2 at stalled replication forks [17-19], and both MRE11 and NBS1 physically interact with RPA [40]. Previous studies identified physical interactions between SNM1B and the MRE11 and RAD50 components of MRN [7]. Therefore, I next examined the impact of SNM1B depletion on replication stress induced MRE11 and NBS1 foci formation. I found that, similar to RPA and γH2AX foci, MRE11 and NBS1 localization to sites of stalled forks was not dependent on SNM1B (Figure 2.2C).

Impact of SNM1B depletion on FANCD2 monoubiquitination, chromatin localization, and foci formation.

The FA protein, FANCD2, plays a central role in cellular responses to stalled forks. It forms a stable complex with FANCI, and both proteins undergo monoubiquitination by the FA core complex in response to genotoxic stress. Monoubiquitinated FANCD2-FANCI becomes associated with nuclear chromatin and subsequently assembles into foci. FANCD2 then recruits additional repair factors required for resolution of stalled replication forks [41,42]. We examined the levels of aphidicolin induced FANCD2 ubiquitination (FANCD2-Ub) and chromatin localization of FANCD2-Ub in SNM1B depleted cells. The cytosolic and chromatin bound proteins were fractionated from siSnm1B-1 and NS transfected cells, and the levels of FANCD2 (S) and FANCD2-Ub (L) in each fraction were determined by western blotting. We observed that SNM1B depletion did not significantly impact the extent of monoubiquitination of FANCD2 (FANCD2-L) upon aphidicolin treatment nor did it affect accumulation of FANCD2-Ub in the chromatin fraction (P1) (Figure 2.3A).

We next examined replication stress induced FANCD2 foci in siSnm1B-1 and NS transfected cells exposed to 0.3 μM aphidicolin. We observed that the percentage of control cells containing FANCD2 foci increased approximately 3.5- and 13-fold at 6 and 24 hrs of aphidicolin treatment, respectively (Figure 2.3B). In contrast, SNM1B depletion markedly reduced the percentage of aphidicolin induced FANCD2 foci to approximately 50% of controls at both 6 and 24 hrs ($p < 0.01$). I observed a similar decrease in FANCD2 formation in HCT116 cells transfected with siSnm1B-2 upon aphidicolin treatment (Suppl. Fig. 2.3A). Furthermore, I examined replication stress induced FANCD2 foci in HeLa cells and confirmed that depletion of SNM1B significantly impairs recruitment to sites of stalled forks. I observed a decrease in the percentage of HeLa cells with FANCD2 foci to approximately 50% and 35% of controls upon transfection with siSnm1B-1 and siSnm1B-2, respectively (Suppl. Fig. 2.3B). Thus, these results clearly establish that SNM1B is required for efficient assembly of FANCD2 into DNA repair foci; however, it is dispensable for activation and chromatin localization of FANCD2.

SNM1B possesses intrinsic 5' to 3' DNA exonuclease activity on single and double strand substrates [4,5,43,44]. This nuclease activity is required for DNA end resection of telomeres to generate 3' single strand overhangs for protection against

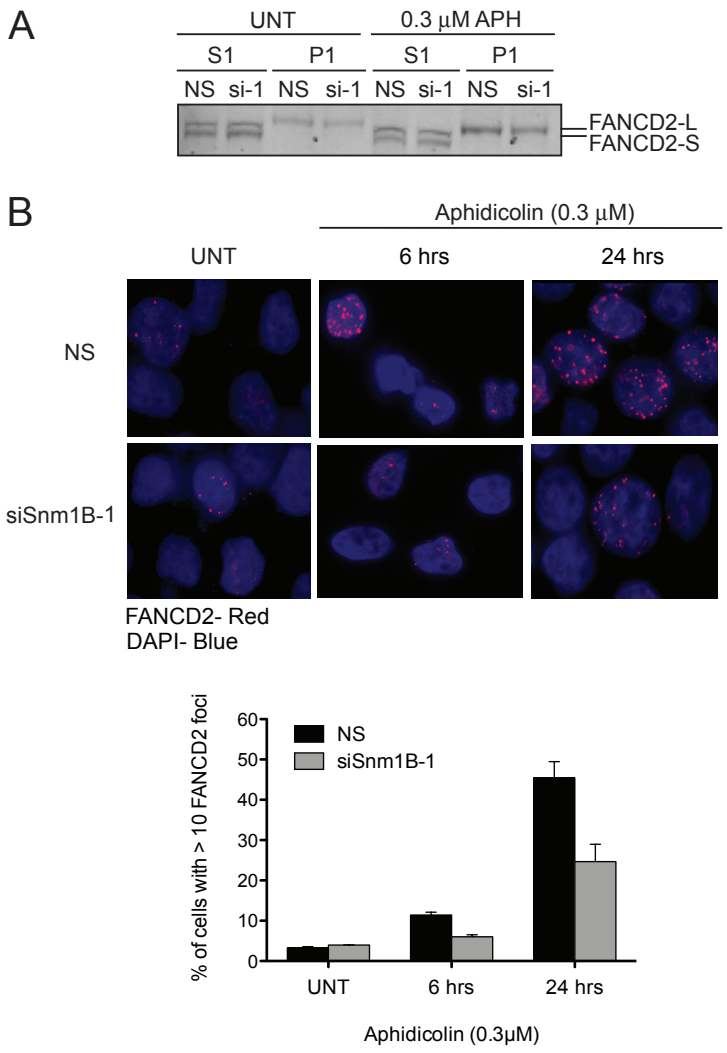

Figure 2.3. Replication stress induced FANCD2 foci formation is impaired in SNM1B depleted cells.

A. FANCD2 monoubiquitination in SNM1B depleted cells. HCT116 cells transfected with NS or siSnm1B-1 (si-1) were treated with aphidicolin (0.3 μM) for 24 hr. Cells were harvested, and the soluble (S1) and chromatin associated (P1) proteins were fractionated. Western blotting to detect the unmodified (FANCD2-S) and monoubiquitinated (FANCD2-L) forms of FANCD2 was performed using α-FANCD2 antibodies. UNT, untreated controls.

B. FANCD2 foci formation. HCT116 cells transfected with NS or siSnm1B-1 were treated with aphidicolin (0.3 μM) for 6 or 24 hr, as indicated. The average percentage of cells containing >10 FANCD2 foci (red) is plotted. The results represent data from three independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (Blue). Error bars, SEM. UNT, untreated controls.

inappropriate end-to-end joining of chromosomes [4,5,43]. We assessed the importance of SNM1B nuclease activity on localization of FANCD2 to DNA repair foci in response to replication stress. To this end, we generated an siRNA-resistant lentiviral SNM1B cDNA with a C-terminal V5 epitope tag and mutated a residue within the highly conserved metallo-β-lactamase domain required for nucleolytic activity, D14N (Figure 2.4A, Suppl. Fig. 2.4A,B). This residue has been previously demonstrated to be essential for SNM1B 5' to 3' exonuclease activity *in vitro* [4,45] and for the functions of SNM1B in telomere processing *in vivo* [4,45]. The SNM1B-D14N mutant and wildtype siRNA resistant cDNAs were expressed from a construct harboring an IRES-GFP cassette, and cells expressing GFP were sorted and cultured. We confirmed expression of the siRNA resistant cDNAs by immunoblotting using α-V5 antibodies and observed both the wildtype and SNM1B-D14N proteins in NS and siSnm1B-1 transfected cells (Suppl. Fig. 2.4C).

I observed that the wildtype SNM1B expressing cells fully complemented the defect in aphidicolin induced FANCD2 foci formation in siSnm1B-transfected cells (Figure 2.4B). In contrast, the nuclease deficient SNM1B-D14N construct did not complement this defect and exhibited a significantly lower percentage of FANCD2 foci containing cells (45% vs. 22%, $p < 0.05$; wildtype vs. SNM1B-D14N, respectively). These findings indicate that the nucleolytic activity intrinsic to SNM1B facilitates the localization of FANCD2-Ub to stalled replication forks.

Impact of SNM1B depletion on BRCA1 localization to DNA repair foci.

FANCD2 co-localizes with the breast cancer suppressor protein, BRCA1, in response to UV-induced stalled replication forks, and its localization is dependent on BRCA1 [37]. Recently, BRCA1 has also been demonstrated to play central roles in stabilizing replication forks prior to collapse [21]. Given our observations that SNM1B is required for FANCD2 foci formation in response to replication stress, I also examined the impact of SNM1B depletion on BRCA1 localization. Upon exposure to aphidicolin, I observed a significant, approximately 5-fold, increase in the percentage of control cells containing BRCA1 foci ($p < 0.008$) (Figure 2.5A). However, BRCA1 foci formation was markedly impaired in siSnm1B-1 transfected HCT116 cells. I observed a minimal

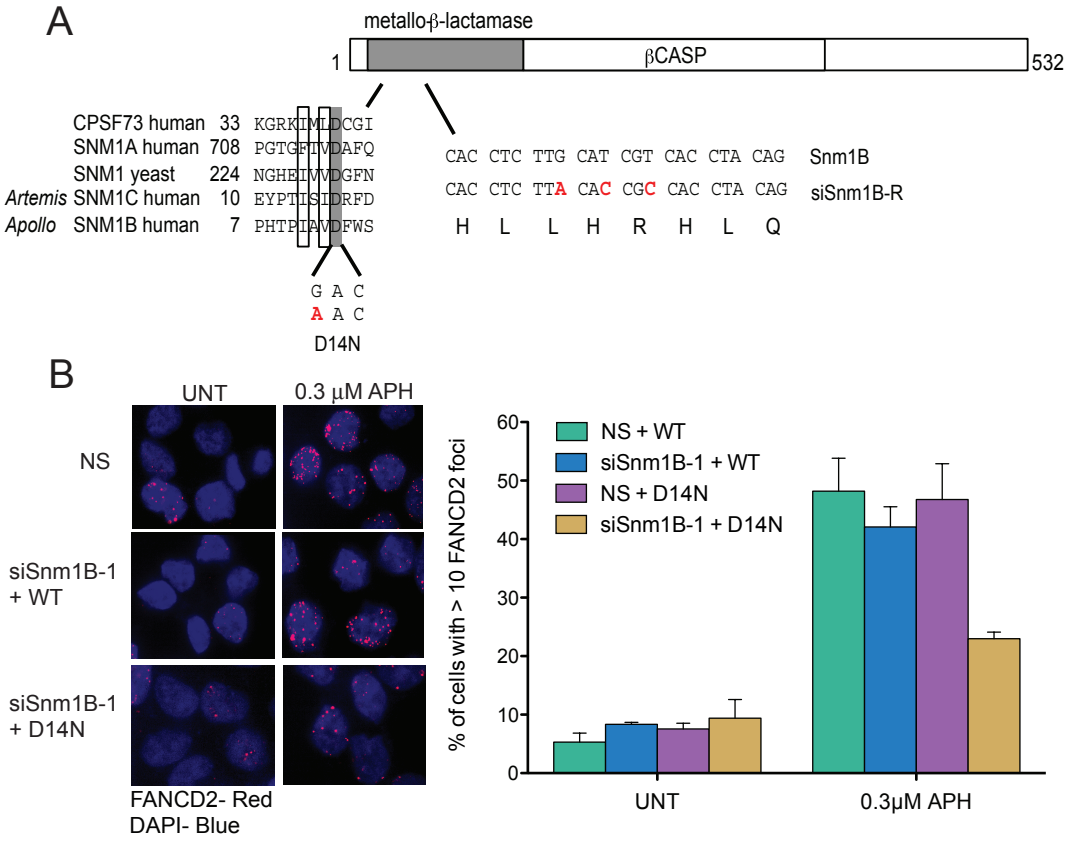

Figure 2.4. SNM1B nuclease activity is required for efficient FANCD2 foci formation.

A. Nuclease deficient SNM1B cDNA. Diagram of SNM1B cDNA encoding the conserved metallo-β-lactamase/βCASP domain. Alignment between the *S. cerevisiae* and *H. sapiens* orthologous amino acid sequences of βCASP family members adjacent to the inactivating D14N mutation is shown. Identical residues, grey box; conserved residues, open boxes. Base changes within the target region of the SNM1B siRNA are indicated below wildtype cDNA sequence as siSnm1B-R (resistant).

B. FANCD2 foci formation with nuclease deficient SNM1B. HCT116 cells transduced with WT-SNM1B-IRES-GFP (WT) or D14N-SNM1B-IRES-GFP (D14N) retroviruses were sorted, then transfected with NS or siSnm1B-1 and treated with aphidicolin (0.3 μM) for 24 hr. FANCD2 foci (red) were visualized by immunofluorescence (left panels). The percentage of cells with >10 FANCD2 foci were quantitated (right panel). Graph represents the results from at least three independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (Blue). Error bars, SEM. UNT, untreated controls.

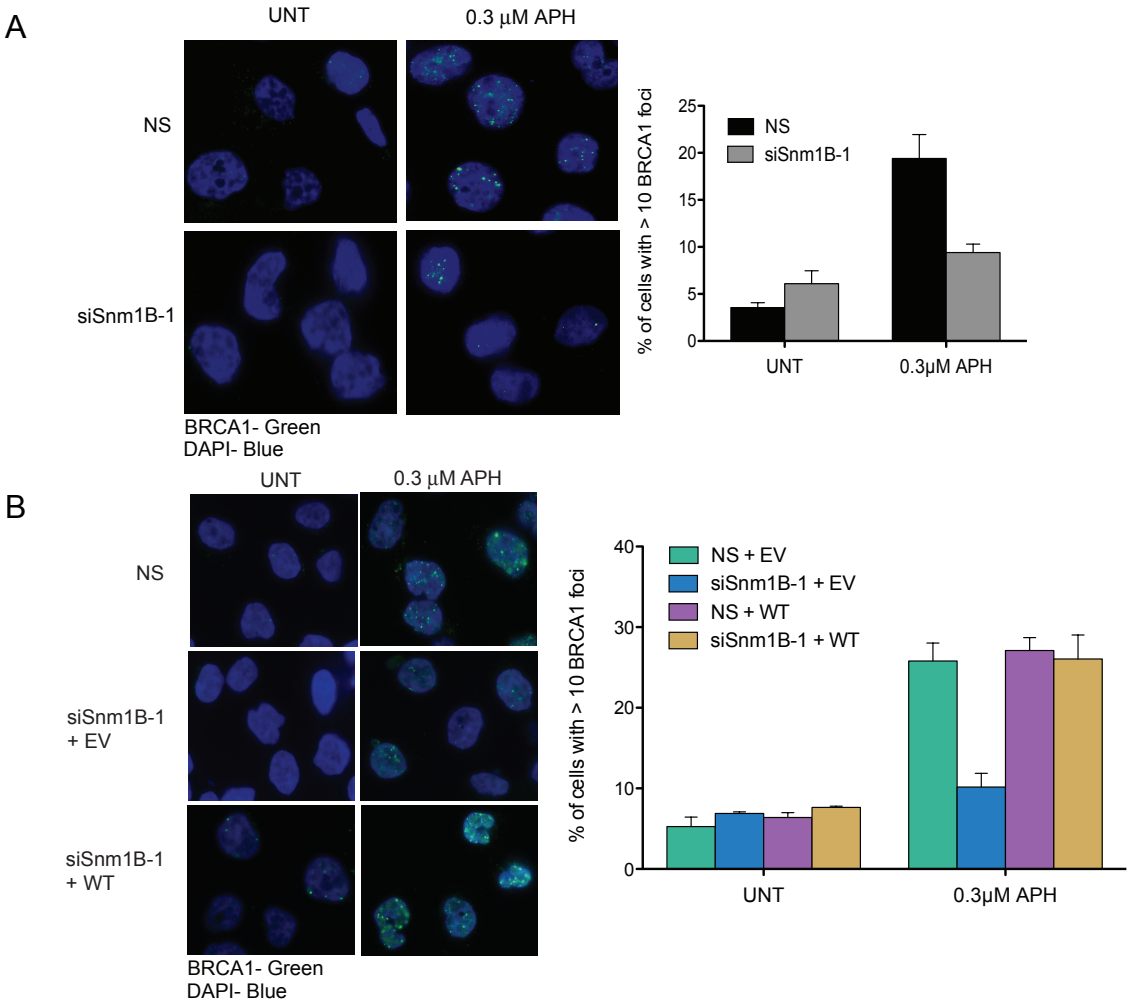

Figure 2.5. Replication stress induced BRCA1 foci formation is impaired in SNM1B deficient cells.

A. BRCA1 foci formation. HCT116 cells transfected with NS or siSnm1B-1 were treated with aphidicolin (0.3 μM) for 24 hr. The percentage of cells containing > 10 BRCA1 foci (green) was determined. Graph represents average of four independent experiments; at least 100 cells were scored from each experiment.

B. Complementation of defective BRCA1 foci formation. HCT116 cells transduced with pLL-IRES-GFP empty vector (EV) or WT-SNM1B-IRES-GFP (WT) retroviruses were sorted, then transfected with NS or siSnm1B-1 and treated with aphidicolin (0.3 μM) for 24 hr. BRCA1 foci (green) were visualized by immunofluorescence (left panels). The percentage of cells with >10 BRCA1 foci were quantitated (right panel). Graph represents the results from two independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (Blue). Error bars, SEM.

increase in replication stress induced localization (approximately 1.5-fold), and the percentage of aphidicolin induced BRCA1 foci containing cells was significantly lower compared to controls ($p < 0.05$). I observed a similar defect in BRCA1 foci formation in HeLa cells transfected with siSnm1B-1, which reduced the percentage of cells with aphidicolin-induced BRCA1 foci to 50% of controls (Suppl. Fig. 2.5). This impairment in localization of BRCA1 to sites of stalled forks was fully complemented upon expression of the siRNA resistant SNM1B cDNA (Figure 2.5B). Together, these findings demonstrate that SNM1B is required for efficient recruitment of the key repair proteins, BRCA1 and FANCD2, to sites of stalled replication forks.

SNM1B localizes to subnuclear foci upon aphidicolin treatment.

Based on our observations that depletion of SNM1B impairs localization of FANCD2 and BRCA1 to aphidicolin induced subnuclear foci, we hypothesized that SNM1B may be recruited to sites of stalled forks to facilitate repair. Thus, I treated cells expressing V5-tagged SNM1B with 0.3 μM aphidicolin and examined SNM1B foci formation. I observed a marked 8-fold increase in cells containing SNM1B foci upon exposure to aphidicolin (Figure 2.6). These findings indicate that, similar to other proteins required for resolution of replication stress, SNM1B localizes to stalled forks to facilitate repair.

SNM1B prevents accumulation of spontaneous and replication stress induced chromosome damage.

Defects in the resolution of stalled replication can result in fork collapse and the generation of DNA DSB intermediates that undergo repair via RAD51-mediated homologous recombination. Inefficient repair of collapsed forks results in accumulation of gaps and breaks, and these DNA lesions accumulate in cells defective in responses to replication stress, including ATR, CHK1, and FANCD2 deficiencies [13,31,46,47]. Therefore, we examined the impact of SNM1B depletion on spontaneous and aphidicolin induced gaps and breaks.

We observed that SNM1B depletion in HCT116 cells resulted in significantly elevated levels of spontaneous gaps and breaks compared to controls, as previously

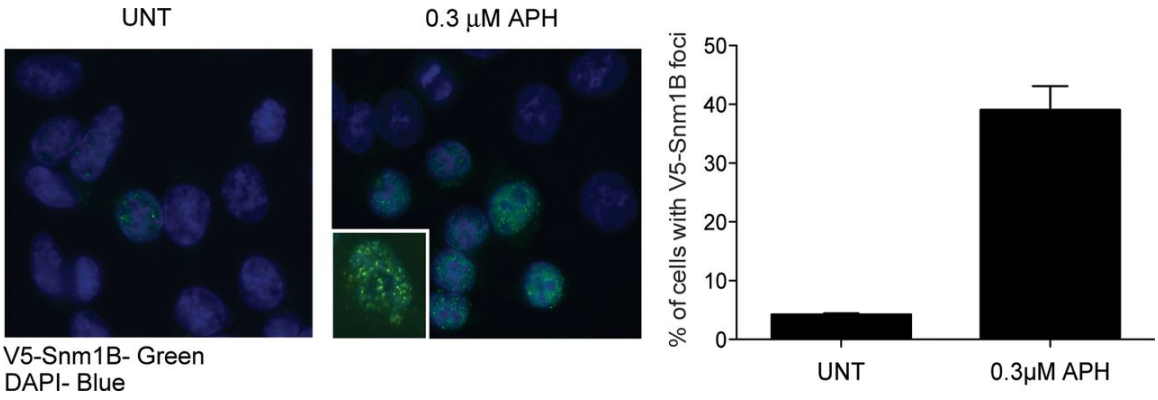

Figure 2.6. SNM1B forms subnuclear foci in response to aphidicolin.
HCT116 cells transduced with WT-SNM1B-IRES-GFP (WT) retrovirus were sorted and treated with aphidicolin (0.3 μM) for 24 hr. V5-SNM1B foci (green) were visualized by immunofluorescence (left panels). The average percentage of cells with V5-SNM1B foci was quantitated, and the percentage of cells containing >5 foci is plotted (right panel). Graph represents the results from four independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (Blue). Error bars, SEM. UNT, untreated controls.

reported [7,9,10,48] (Figure 2.7A). Untreated NS transfected cells harbored an average of 0.17 gaps and breaks per metaphase, whereas siSnm1B-1 transfected cells contained approximately 3-fold more anomalies (0.5 gaps/breaks per metaphase) (Figure 2.7B). SNM1B depleted cells exhibited a dose dependent increase in gaps/breaks, and at 0.5 μM aphidicolin, siSnm1B-1 transfected cells harbored a substantially higher average number of anomalies per metaphase compared to NS transfected controls (5 vs. 1.7 gaps/breaks per metaphase, respectively; Figure 2.7B). A Poisson distribution analysis of the number of spontaneous and aphidicolin induced gaps/breaks revealed that the differences in mean rate of anomalies in SNM1B depleted versus control cells were significant in all cases ($p < 0.001$; Fig. 7C).

We note that approximately 26% of metaphases scored in SNM1B depleted cells treated with 0.5 μM aphidicolin contained greater than 20 gaps and breaks per metaphase, and in some cases, the number of anomalies was too numerous to quantitate. In contrast, only 5% of control metaphases exhibited greater than 20 gaps/breaks at this aphidicolin dose (Suppl. Fig. 2.6). These metaphases were not included in the quantitative analyses; therefore, the phenotypes graphically shown underestimate the extent of replication stress induced chromosomal damage in SNM1B depleted cells (Figure 2.7B,C). Together, these findings demonstrate a critical role for SNM1B in preventing chromosomal gaps/breaks in response to replication perturbation.

SNM1B is required to suppress spontaneous and replication stress induced common fragile site expression.

Common fragile sites are genomic loci that recurrently exhibit gaps and breaks on metaphase chromosomes in response to partial replication inhibition [49]. Thus, we hypothesized that SNM1B depletion would result in increased common fragile site instability. Two of the most frequently expressed common fragile sites in the human genome are FRA3B and FRA16D, located at 3p14.2 and 16q23, respectively [49]. We examined the impact of SNM1B depletion on the frequency of spontaneous and aphidicolin induced instability at both FRA3B and FRA16D [49] using a fluorescence *in situ* hybridization (FISH) approach with YAC/BAC probes located at the fragile sites [13].

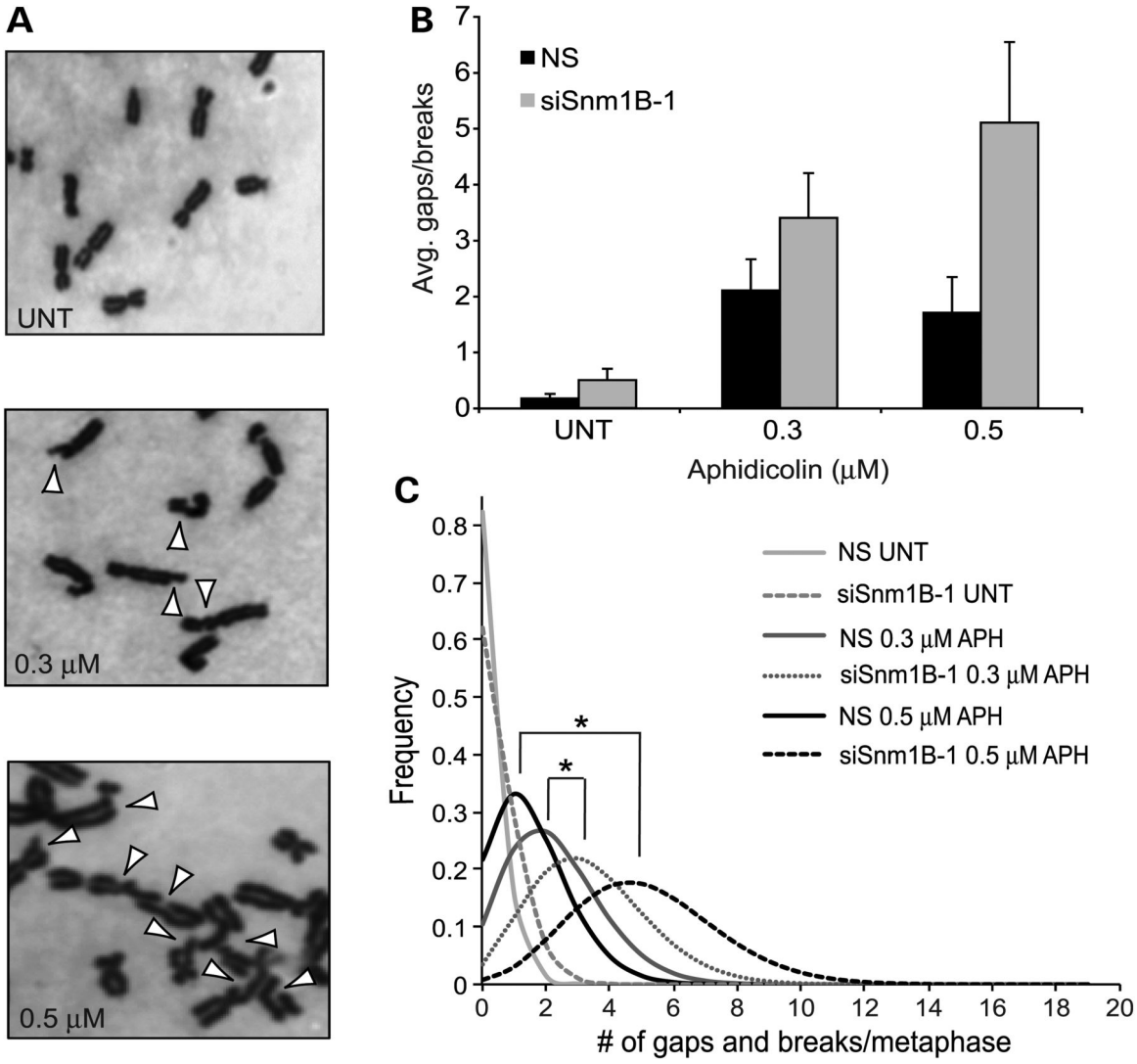

Figure 2.7. SNM1B depletion results in elevated gaps and breaks in response to aphidicolin.

A. Chromosomal anomalies in SNM1B depleted cells. Representative Giemsa stained metaphases from SNM1B depleted HCT116 cells that were untreated (UNT) or were treated with aphidicolin (0.3 or 0.5 μM) for 24 hr prior to harvesting. Arrows indicate chromosomes with gaps or breaks.

B. Quantitation of gaps and breaks in SNM1B depleted cells. Graphical representation of the average number of gaps/breaks in metaphase chromosomes that were untreated or treated with aphidicolin. Graph represents data from three independent experiments with SEM.

C. Poisson distributions of gaps and breaks. Poisson distributions illustrating the frequencies of gaps and breaks per metaphase observed in untreated (light gray), 0.3 μM (dark gray), and 0.5 μM (black) aphidicolin treated NS (solid) and siSnm1B-1 (dotted) transfected cells ($p < 0.001$).

We observed low or undetectable levels of chromosomal breaks within either the FRA3B (1.4% of FRA3B signals with breaks) or FRA16D (0% of signals with breaks) loci in metaphases from NS transfected, untreated cells (Figure 2.8A,B). In contrast, SNM1B depletion significantly increased the frequency of spontaneous breaks at both FRA3B and FRA16D (to 6.9% and 4.6%, respectively). Aphidicolin treatment of SNM1B depleted cells lead to a further increase in fragile site instability, and the percentages of FRA3B (22%) and FRA16D (10%) signals localized to a break were consistently higher in comparison to NS transfected controls (approximately 7% for both FRA3B and FRA16D) (Figure 2.8A,B). These findings indicate that SNM1B is important for maintaining fragile site stability not only in response to partial inhibition of DNA polymerase, but also in the context of unperturbed DNA replication.

Discussion

In this study, we provide evidence that the SNM1B/Apollo DNA nuclease has critical functions in the resolution of DNA replication stress. We demonstrate that SNM1B is required for cellular survival in response to replication fork stalling. Depletion of SNM1B does not significantly affect ATR dependent signaling events or localization of proteins involved in the early response to stalled replication forks prior to DSB formation, i.e., RPA, γH2AX, and the MRN complex [20,38]. In contrast, SNM1B depletion markedly impairs localization of the critical repair proteins, FANCD2-Ub and BRCA1, to replication stress induced foci. We also found that SNM1B protects the genome from accumulation of spontaneous and aphidicolin induced gaps and breaks, including those at common fragile sites. Thus, our findings demonstrate that SNM1B is dispensable for recognition of the lesion and activation of the DNA damage response, but is required during the downstream events to facilitate fork stabilization and repair.

During DNA synthesis, the replication fork frequently encounters barriers that impede progression and cause stalling. These barriers can be in the form of blocking DNA lesions or intrinsic, natural impediments to fork progression, such as secondary DNA structures, highly transcribed regions, or tightly bound proteins [50-52]. Evidence indicates that the replication machinery remains stably associated with the stalled fork and is poised for replication restart. Prolonged stalling or defects in maintaining fork

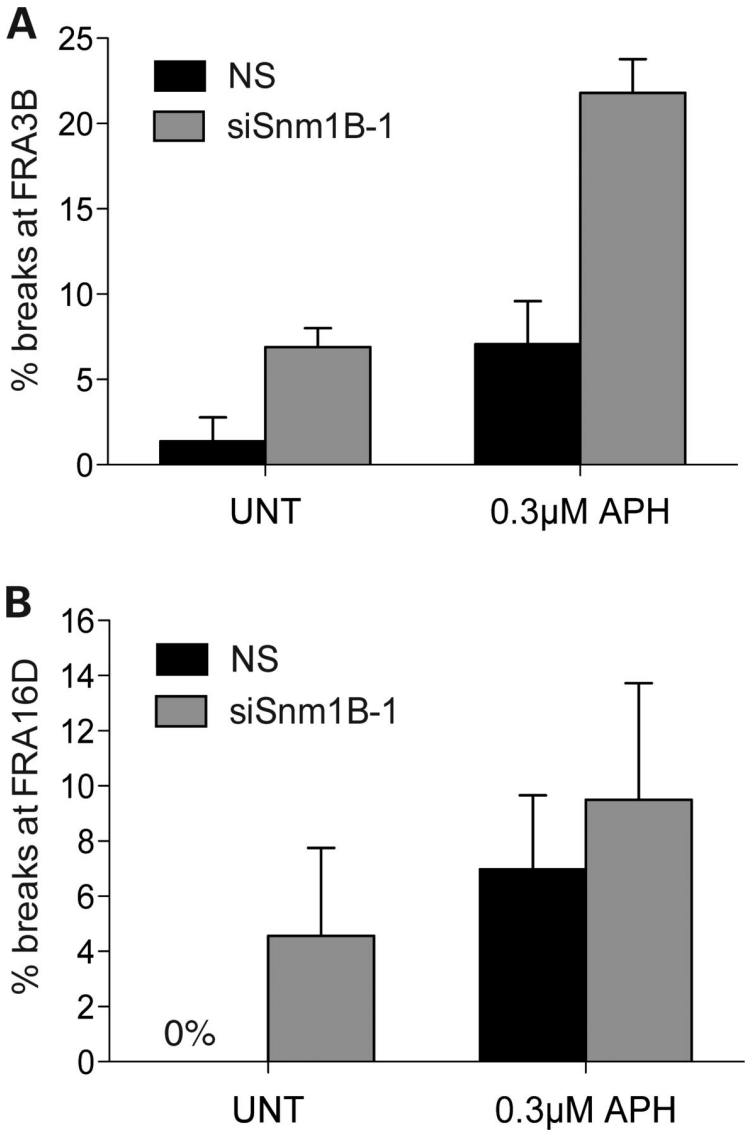

Figure 2.8. SNM1B depleted cells exhibit increased fragile site expression. HCT116 cells were transfected with NS or siSnm1B-1 and treated with aphidicolin (0.3 μM) for 24 hr. Untreated samples were used as controls. FISH analyses using YAC/BAC probes were used to determine the frequency of FRA3B and FRA16D expression.

A. Expression of FRA3B in SNM1B depleted cells. Quantitation of FRA3B expression in NS and siSnm1B-1 transfected cells treated with 0 (UNT) or 0.3 μM aphidicolin. Bar graph represents average percentage of FRA3B signals localized at breaks from at least 3 independent experiments; Error bars, SEM.

B. Expression of FRA16D in SNM1B depleted cells. Quantitation of FRA16D expression in NS and siSnm1B-1 transfected cells treated with 0 (UNT) or 0.3 μM aphidicolin. Bar graph represents average percentage of FRA16D signals localized at breaks from at least 3 independent experiments; Error bars, SEM.

stability can lead to fork collapse and increased genome instability, including chromosomal deletions, duplications, or more complex rearrangements. Previous studies have provided evidence that FANCD2, BRCA1, BRCA2, and RAD51 act in concert to stabilize stalled replication forks by protecting nascent DNA present from excessive MRE11 dependent resection [20-22,53]. We demonstrated impaired recruitment of FANCD2 and BRCA1 to sites of aphidicolin-induced stalled forks in SNM1B depleted cells (Figure 2.3B, 2.5). This defect in FANCD2/BRCA1 localization could result in excessive resection, which would manifest as long stretches of ssDNA and an increased percentage of RPA positive cells, as observed in our study (Figure 2.2A). Thus, our findings suggest that SNM1B facilitates the stabilization and repair of stalled replication forks.

We find that the intrinsic SNM1B nuclease activity plays an important role during resolution of stalled replication forks, as the D14N mutant protein is unable to restore aphidicolin-induced FANCD2 foci formation. While the precise functions of SNM1B nuclease activity during replication have yet to be uncovered, the roles of SNM1B in telomere processing provide some insights. At telomeres, the 5' to 3' exonuclease activity of SNM1B has been demonstrated to be involved in the generation of the 3' overhang at leading strand telomeres [4-6]. SNM1B nuclease activity could also relieve topological strain induced during replication of telomeres [45]. Both SNM1B catalyzed end resection and/or regulation of DNA topology may have relevance at replication forks. In this regard, unregulated unwinding of DNA ahead of a stalled fork, caused by uncoupling of polymerase and helicase activities, can result in positive supercoiling that promotes fork regression [54]. SNM1B may play a role in relieving this torsional strain, similar to its proposed roles in unwinding superhelical strain at telomeres [45]. Alternatively, SNM1B-mediated 5' to 3' exonucleolytic processing of nascent lagging strand DNA could generate ssDNA regions necessary for loading of fork stabilizing proteins, such as FANCD2, BRCA1, and RAD51 [20,21], or degrade reversed forks to facilitate replication restart [2]. It is likely that SNM1B collaborates with other DNA nucleases at stalled forks, as it physically interacts with MRE11 [7] and MUS81 [23] and the nuclease scaffold protein, SLX4, each of which have been implicated in the repair of blocked or stalled forks [26,55,56]. Detailed molecular analyses of nascent DNA strand

degradation and synthesis will provide significant insights into the roles of SNM1B and functional interactions with other DNA nucleases during resolution of replication stress.

Our studies demonstrated that SNM1B is required for preventing chromosomal damage, including common fragile site instability, not only in response to aphidicolin induced replication inhibition, but also during unperturbed DNA replication. Common fragile sites are difficult to replicate loci in the genome and are hot spots for chromosomal rearrangements, deletions, sister chromatid exchanges, and plasmid integration in response to treatment with low doses of aphidicolin [49]. Rearrangements and deletions at common fragile sites are observed in cancer cells, indicating that fragile site instability may contribute to tumorigenesis [57]. Indeed, the common fragile sites FRA3B and FRA16D both are located within well-characterized tumor suppressor genes, *FHIT* and *WWOX* [49,58]. In addition, replication stress induced by aphidicolin or hydroxyurea, produces copy number alterations, which arise frequently in cancer cells [59]. Thus, it will be of interest to further define the functions of the SNM1B nuclease activity in DNA processing at stalled replication forks and examine its roles in suppressing genomic instability, including potentially oncogenic chromosomal rearrangements.

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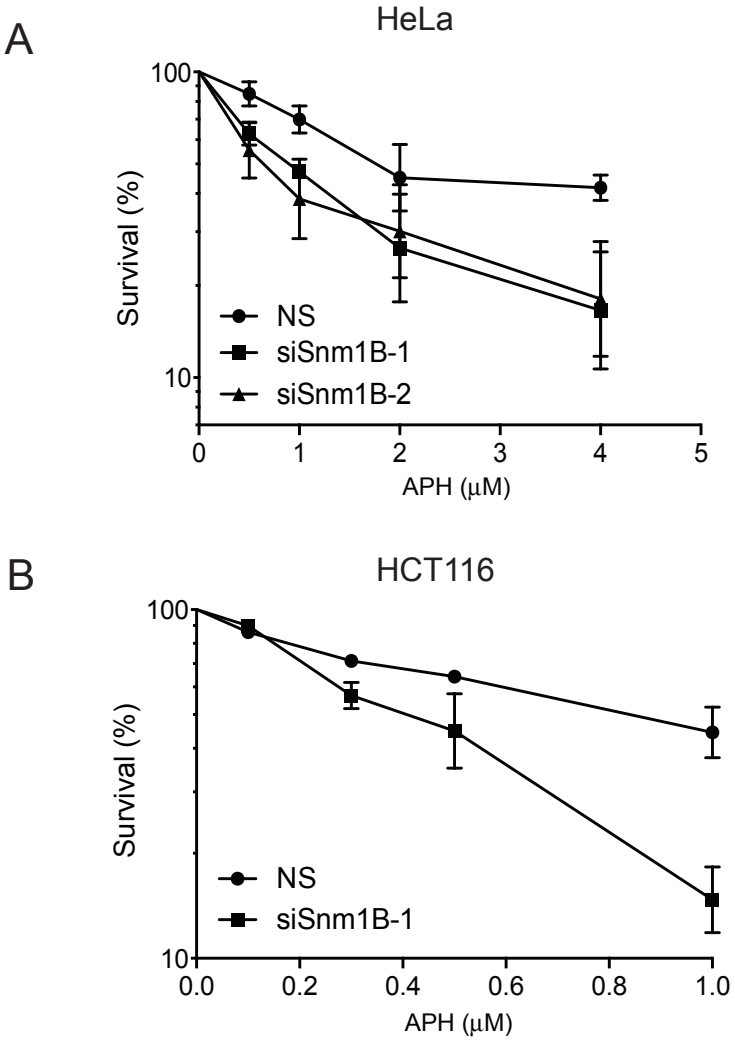
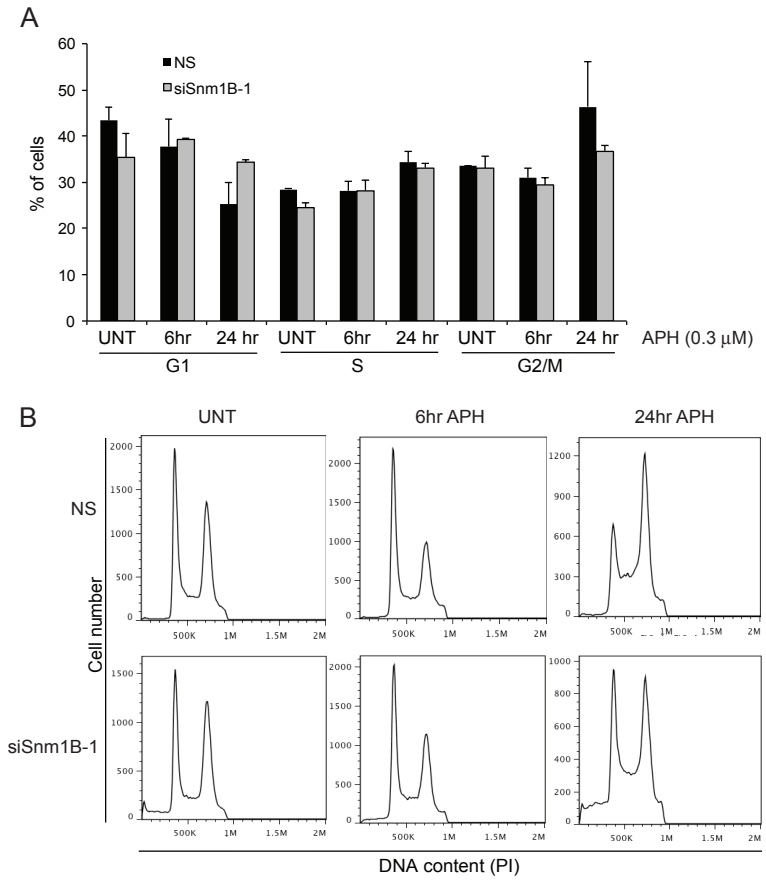
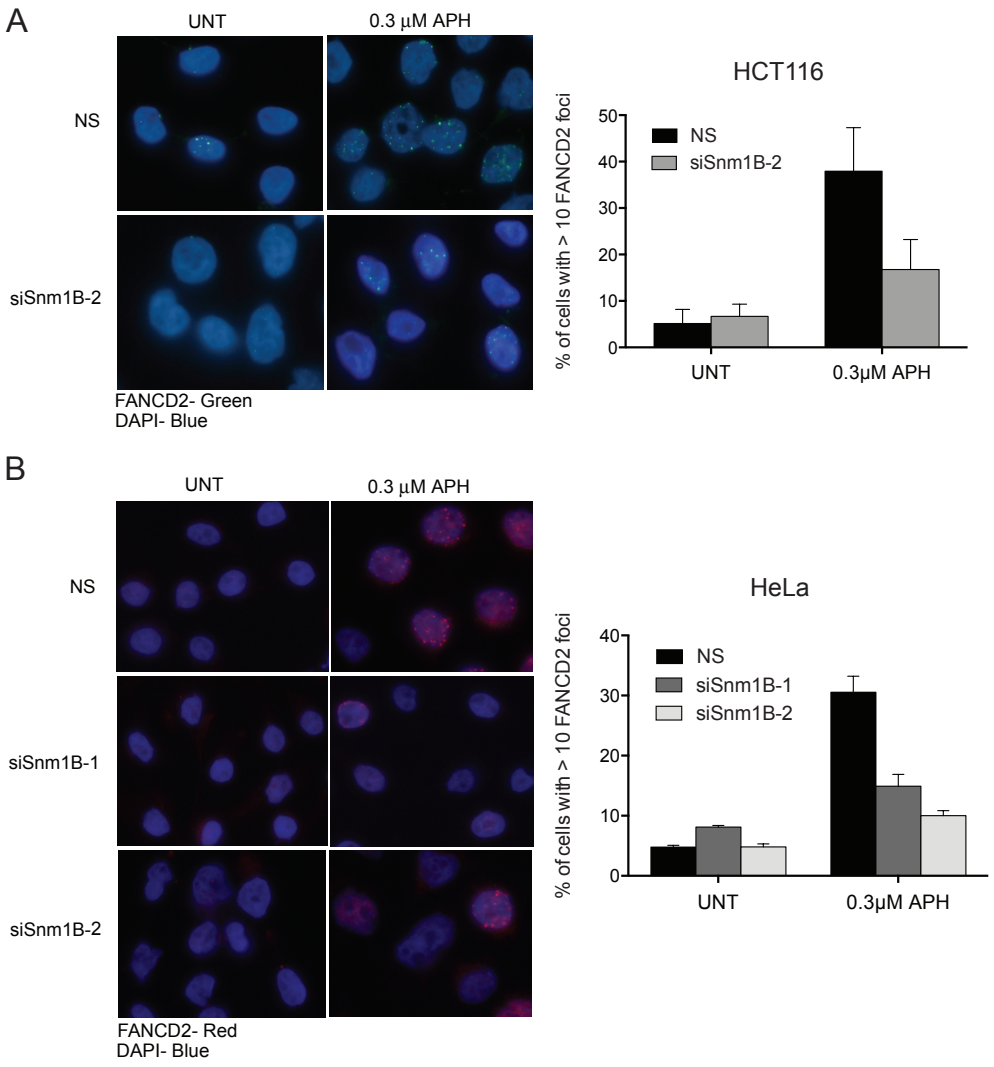


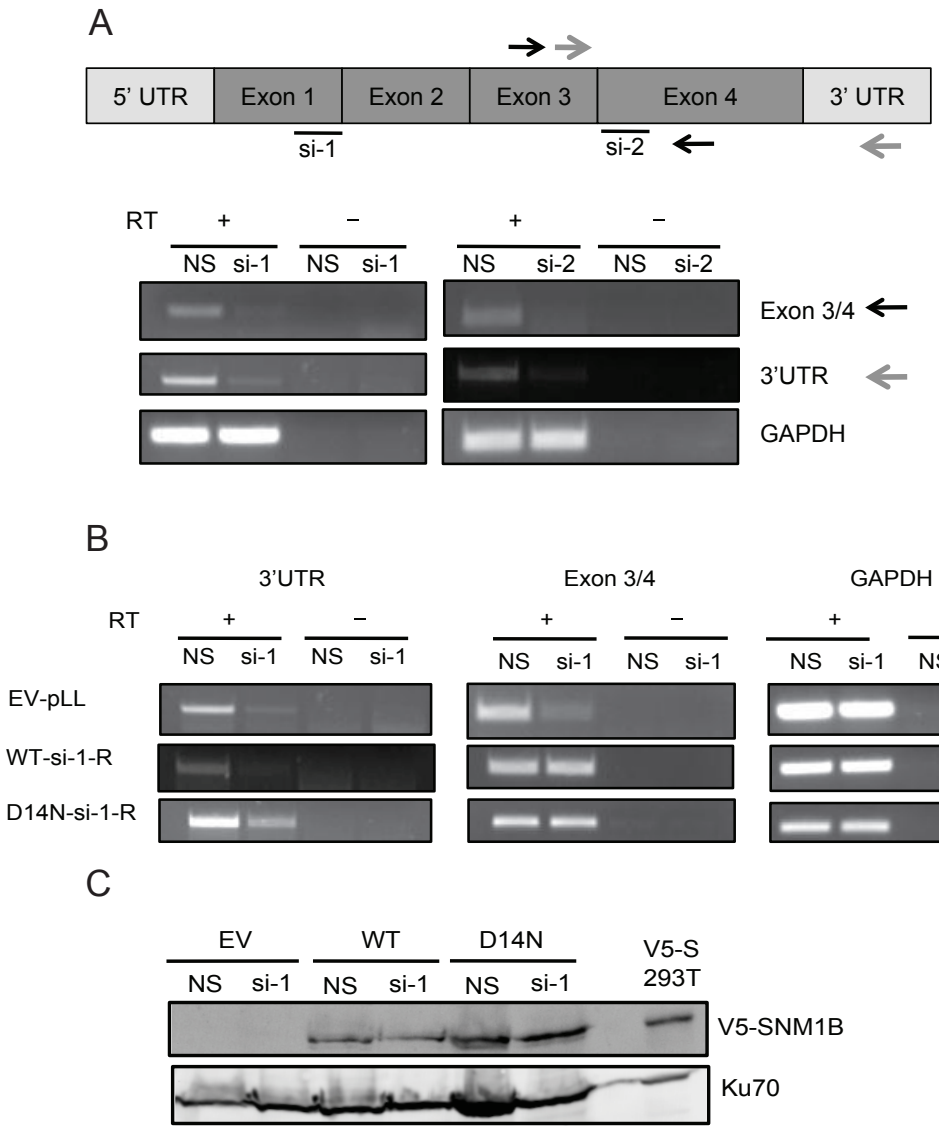
Supplemental Figure 2.1. SNM1B depletion in HeLa and HCT116 cells results in hypersensitivity to aphidicolin treatment.
A. HeLa cells transfected with NS, siSnm1B-1, or siSnm1B-2 were treated for 24 hr with the indicated doses of aphidicolin at 48 hr post transfection. The concentrations of aphidicolin used in the survival curve were optimized for the HeLa cell line and were higher than those used for HCT116 cells and fibroblasts. Cells were allowed to proliferate for 5-7 days. The survival fraction was determined compared to untreated controls.
B. HCT116 cells transfected with NS or siSnm1B-1 were plated and analyzed as above. Graphs represent the average of two independent experiments. Error bars, SEM.



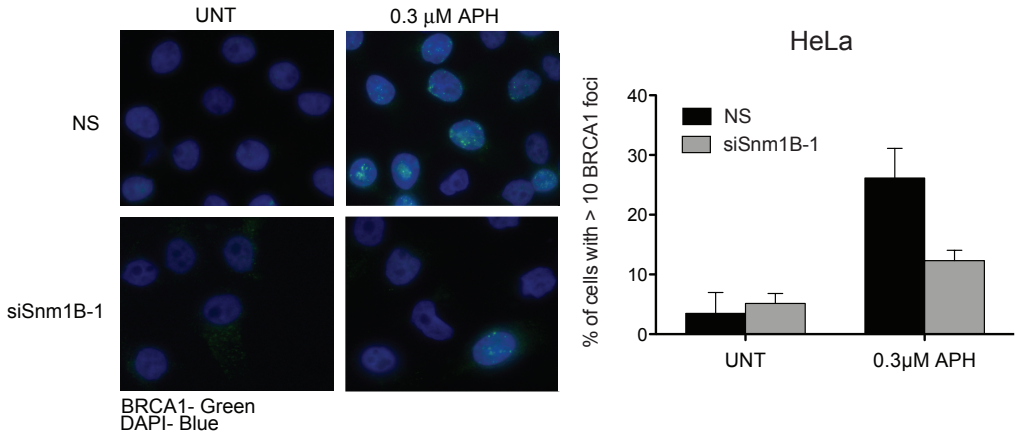
Supplemental Figure 2.2. Cell cycle analysis of SNM1B depleted cells. HCT116 cells were untreated (UNT) or treated with 0.3μM aphidicolin 48 hours post siRNA transfection. After 6 and 24 hrs of aphidicolin treatment, DNA content was assessed by fixing then staining cells with propidium iodide and analyzing DNA content by FACS. **A. Similar percentages of NS and siSnm1B-1 transfected cells in S phase.** Quantitation of cells in the G1, S, and G2/M phases of the cell cycle using the Watson algorithm (FlowJo, TreeStar). The percentage of live cells in each cell cycle phase is plotted as a function of fluorescence intensity as an indicator of DNA content (PI, propidium iodide). No significant differences in the percentages of S phase cells upon SNM1B depletion compared to controls were observed in either untreated or aphidicolin treated cultures. Exclusion of the sub-G1 populations yielded similar results (data not shown). We observed an increase in the sub-G1 population of cells in the siSnm1B-1 transfected untreated and aphidicolin treated cells, consistent with the notion that unrepaired DNA damage associated with replication stress in SNM1B depleted cells induces apoptosis (Average % of cells in sub-G1: UNT, 8.5%; 0.3 μM aphidicolin 6h, 7.9%; 0.3 M aphidicolin at 24h, 8.3%). In comparison, the average percentages of sub-G1 cells in NS transfected cells were significantly lower (UNT, 0%; 0.3 μM aphidicolin 6h, 0.7%; 0.3 μM aphidicolin at 24h, 0.6%). Data represent an average of 2 independent experiments.
B. Flow cytometry analyses of propidium iodide stained cells. Representative cell cycle profiles of untreated and 0.3μM aphidicolin treated NS and siSnm1B-1 transfected cells at 6 and 24 hrs post treatment.



Supplemental Figure 2.3. FANCD2 foci formation is significantly decreased in SNM1B depleted HCT116 and HeLa cells.
A. Transfection of a distinct siRNA, siSnm1B-2, impairs recruitment of FANCD2 in HCT116 cells. HCT116 cells transfected with NS or siSnm1B-2 were treated with aphidicolin (0.3 μM) for 24 hr. The percentage of cells containing > 10 FANCD2 foci (green) was determined.
B. FANCD2 foci formation in HeLa cells. HeLa cells transfected with NS, siSnm1B-1, or siSnm1B-2 were treated with aphidicolin (0.3 μM) for 24 hr. The percentage of cells containing > 10 FANCD2 foci (red) was determined. Graphs represent average of two independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (Blue). Error bars, SEM. UNT, untreated controls.

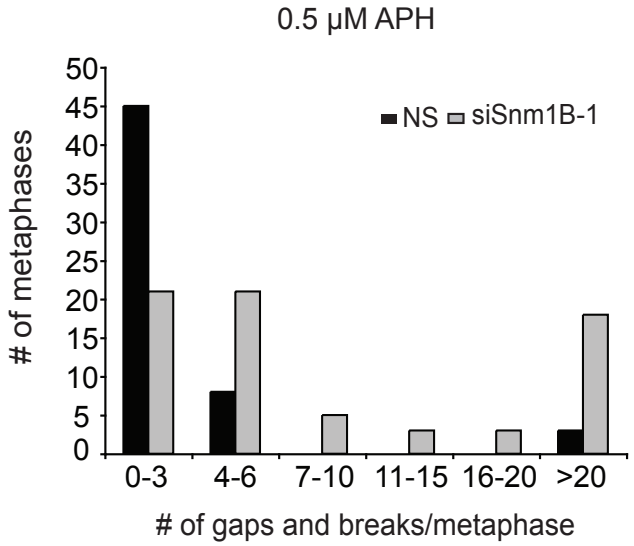

A
5' UTR
Exon 1
Exon 2
Exon 3
Exon 4
3' UTR
si-1
si-2
RT
+
–
NS
si-1
si-2
Exon 3/4
3'UTR
GAPDH
B
3'UTR
Exon 3/4
GAPDH
EV-pLL
WT-si-1-R
D14N-si-1-R
C
EV
WT
D14N
V5-S
293T
V5-SNM1B
Ku70

Supplemental Figure 2.4. Semi-quantitative RT-PCR for SNM1B expression.
A. RT-PCR strategy to detect endogenous and siRNA-resistant, lentiviral cDNA expression of SNM1B. Upper panel. Diagram of SNM1B cDNA exonic structure with positions of siSnm1B-1 (si-1) and siSnm1B-2 (si-2) target sites and RT-PCR primers. Lower panel. Total RNA (1μg) was reverse transcribed, and the cDNA was PCR amplified with SNM1B-specific primers to exons 3/4 (black arrows) for detection of both endogenous and siRNA-resistant cDNA transcripts, and primers that amplified a region containing the 3'UTR (grey arrows), which only amplify endogenous SNM1B mRNAs. A representative gel of RT-PCR products from HCT116 cells transfected with NS, si-1, or si-2 siRNAs at 48h post-transfection is shown. RT-PCR of GAPDH was used for normalization of cDNA levels. Bands were quantitated using AlphaImager 2200 (Alpha Innoten) to verify efficient knockdown of SNM1B expression to between 20-30% of NS transfected controls.
B. Expression levels of endogenous and siRNA-resistant SNM1B transcripts. HCT116 cell lines harboring pLL IRES GFP lentiviral expression constructs were generated, and GFP expressing cells were sorted. RT-PCR was performed on total RNA isolated from sorted lines expressing GFP alone (EV-pLL), siRNA-resistant wildtype V5-tagged SNM1B (WT-si-1-R), and siRNA-resistant nuclease dead V5-tagged SNM1B-D14N (D14N-si-1-R) at 48 hr post-transfection of NS or siSnm1B-1 siRNAs. The negative control EV-pLL cell line showed a marked decrease of SNM1B RT-PCR products upon siSnm1B-1 knockdown with primers amplifying both the 3'UTR and Exon 3/4, thereby indicating efficient knockdown. In contrast, the siSnm1B-1 transfected cells expressing siRNA-resistant WT and D14N mutant SNM1B constructs exhibit knockdown of endogenous SNM1B expression using the 3'UTR primers; however, expression of the exon 3/4 region, which is common to both endogenous and cDNA transcripts, is not decreased.
C. Western blot analysis of siRNA resistant SNM1B protein levels. HCT116 cells transduced with pLL-IRES-GFP empty vector (EV), siRNA-resistant wildtype V5-tagged SNM1B (WT), and siRNA-resistant nuclease dead V5-tagged SNM1B-D14N (D14N) retrovirus were sorted and harvested 48 hr post-transfection with NS or siSnm1B-1 siRNAs, as indicated. Whole cell lysates were then analyzed by immunoblotting with V5 antibody. HEK293T cells transiently transfected with V5-SNM1B PEF6 vector was used as a positive control (V5-S 293T). Representative blots from at least three independent experiments are shown. Ku70, loading control.



Supplemental Figure 2.5. BRCA1 foci formation is significantly impaired in SNM1B depleted HeLa cells.

HeLa cells transfected with NS or siSnm1B-1 were treated with aphidicolin (0.3 μM) for 24 hr. The percentage of cells containing > 10 BRCA1 foci (green) was determined. Graph represents average of two independent experiments; at least 100 cells were scored from each experiment. Nuclei, DAPI stained (Blue). Error bars, SEM. UNT, untreated controls.



Supplemental Figure 2.6. SNM1B depletion results in a high proportion of metaphases harboring excessive chromosomal damage.
The total number of metaphases harboring the indicated number of gaps/breaks in NS or siSnm1B-1 transfected HCT116 cells treated with 0.5 μM aphidicolin is plotted. Some metaphases in aphidicolin treated siSnm1B-1 transfected cells contained gaps/breaks that were too numerous to quantitate and were categorized as >20 gaps/breaks per metaphase. A subset of metaphases from SNM1B depleted cells treated with 0.3 μM aphidicolin also exhibited greater than 20 gaps and breaks (average of 5% in SNM1B depleted cells, compared to 0% in controls at 0.3 μM aphidicolin; data not shown). Metaphases with excessive chromosomal damage were rarely observed in NS transfected control cells treated with either 0.3 or 0.5 μM aphidicolin. Thus, the quantitative results shown in Figure 2.7 underestimate the levels of replication stress induced chromosomal anomalies in SNM1B depleted cells.

Chapter 3: The nuclease activity of SNM1B is important in the stabilization and repair of stalled replication forks

Summary

The SNM1B DNA nuclease is a member of the metallo-β-lactamase/β-CASP superfamily of proteins which has functions in telomere processing, interstrand crosslink repair, double-strand break repair, and resolving replication stress. SNM1B functions within the FA/BRCA network to respond to stalled replication forks, but the precise mechanism of the nuclease activity of SNM1B in this process is not defined. In this study, I found that SNM1B localizes to aphidicolin-induced stalled replication forks after early response proteins (Mre11 and RPA) but prior to FANCD2 and is therefore not needed to sense the stalled fork. Additionally, I demonstrate that SNM1B prevents the accumulation of long stretches of unstable single-stranded DNA and promotes Rad51 filament formation at aberrant DNA intermediate structures or collapsed forks after aphidicolin treatment. I have also identified that in normal proliferating cells SNM1B depletion results in increased stalled and collapsed replication forks suggesting SNM1B has critical roles in resolving spontaneous replication stress that occurs during every cell division cycle. Thus, this study provides evidence that the nuclease activity of SNM1B is important in the stabilization and restart/repair of stalled replication forks, thereby preventing genomic instability.

Introduction

Complete replication of the genome is of fundamental importance to ensure the accurate transmission of genetic information during every cell division. Due to the essential nature of DNA replication for all cellular processes, it is a tightly monitored and controlled process. During DNA synthesis, the replication machinery can encounter obstacles including secondary DNA structures, unrepaired lesions in the DNA template, highly transcribed regions, or protein-DNA complexes that impair progression of the replication fork resulting in slow or stalled replication [1]. Multiple mechanisms are employed by cells to sense and resolve stalled replication forks. Incomplete or faulty DNA replication can lead to deleterious consequences including accumulation of mutations, deletions, insertions, translocations, and cell growth defects. Mutations in DNA repair genes can ultimately cause genome instability disorders in humans, some of which are characterized by developmental defects, immunodeficiency, neurodegeneration, and cancer predisposition [1-3].

DNA polymerase inhibition, through treatment with aphidicolin, leads to the uncoupling of DNA polymerase and helicase activities, resulting in the helicase continuing to unwind the DNA double helix [4]. This generates long stretches of single-stranded DNA (ssDNA) which become bound by the heterotrimeric complex RPA (replication protein A) [5]. The ATR kinase (ataxia telangiectasia and Rad3 related protein) is then recruited and activated which triggers signaling of downstream effector proteins that initiate cellular responses including the recruitment of DNA repair proteins to the site of damage [5-7].

One mechanism through which stalled forks are restarted is a process called fork regression, which leads to the annealing of the nascent complementary DNA strands, thereby forming an intermediate 'chicken foot' structure at the fork (Figure 1.3) [8-11]. A number of DNA repair proteins have been implicated in stimulating fork regression including PARP (poly(ADP-ribose) polymerase), BLM helicase, FANCM (Fanconi anemia complementation group M), HLTF (human helicase-like transcription factor), and SMARCAL1 DNA translocase [12-17].

DNA nucleases are involved in resolving the 'chicken-foot' structure by digesting the annealed nascent leading and lagging strands thereby resetting the fork so that

replication can continue. It has been suggested that the nuclease Mre11, which is a component of the MRN (Mre11/Rad50/Nbs1) complex, may be involved in this digestion process, although this nuclease activity needs to be tightly regulated to prevent hyper-degradation of the DNA [18,19]. FAN1 (FANCD2/FANCI-associated nuclease1) and DNA2 (human nuclease/helicase 2) are both 5' to 3' exonucleases that have also been implicated in processing regressed forks [20,21]. Furthermore, extension of strands at regressed forks using the respective complementary strand followed by reverse branch migration, which resets the replication fork, is another mechanism to restart stalled forks (Figure 1.3) [22,23]. The DNA translocase SMARCAL1 has roles in promoting efficient reverse branch migration [14]. The roles of DNA repair proteins in resolving regressed forks and initiating replication restart are not well understood. Specifically, the functions of nucleases in resolving intermediate DNA structures that form at stalled replication forks is not well characterized.

SNM1B/Apollo is a DNA nuclease that plays critical roles in preventing genome instability through telomere maintenance and DNA repair. SNM1B is comprised of a highly conserved, catalytic metallo-β-lactamase/β-CASP N-terminal domain, responsible for its 5' to 3' exonuclease activity, and a unique C-terminus [24-27]. Previous work from our lab has demonstrated that SNM1B has important functions in the repair of ICLs and stalled replication forks [28,29]. We established that the intrinsic 5' to 3' single and double-strand exonuclease activity of SNM1B is required for its repair functions at stalled replication forks, thereby indicating that it may be involved in processing DNA intermediates [28].

Ineffective repair or restart of stalled forks can lead to replication fork collapse, which results in the dissociation of components of the replisome and generation of a DNA double-strand break (DSB) at the fork [30-34]. These DSBs can then be repaired through homologous recombination (HR) by using the available homologous template [9,35-37]. The repair of collapsed forks through HR also requires the enzymatic activity of both endonucleases and exonucleases. Firstly, an endonuclease makes an initial break in the ssDNA region at the stalled fork to generate a one-ended DSB, and Mus81-Eme1 is a nuclease that has been implicated in this role [35,38]. Upon formation of this one-ended DSB, proteins involved in the DSB response including the MRN complex sense

this break and recruit and activate the ATM kinase (Ataxia-telangiectasia mutated), which goes on to phosphorylate downstream targets [39-41]. In order to initiate HR, the DSB has to be acted on by a 5' to 3' exonuclease which resects one strand of the DSB resulting in a 3' overhang. An HR factor Rad51 binds to this overhang and forms filaments which initiates events that lead to strand invasion to the homologous template to begin repair of the break [2]. During HR, intermediate structures called Holliday junctions form which are processed by structure-specific endonucleases (Mus81-Eme1, SLX1) that are bound to the scaffold protein SLX4/FANCP [42]. Once HR-mediated repair takes place, the collapsed fork is repaired, the replisome is reloaded and intact, and replication restarts and continues to progress. If the collapsed fork is not repaired, these DSBs persist, resulting in chromosomal aberrations, such as deletions, insertions, and deleterious translocations [1]. Therefore, DNA nucleases play multiple roles in generating and resolving intermediate structures arising at stalled or collapsed replication forks.

It has been shown that SNM1B interacts with the nucleases Mre11 and Mus81, which have critical roles in resolving stalled and collapsed replication forks as stated above [43]. SNM1B was also found to interact with SLX4 which has important roles in stabilizing stalled forks and facilitating Holliday junction resolution [44]. Interestingly, SNM1B was also found to interact with PSF2, a component of the GINS complex, which along with Cdc45 and MCM2-7 helicase, forms the CMG helicase complex that is important for DNA replication initiation and progression [45,46].

These interactions of SNM1B with key DNA repair and replication factors suggest that SNM1B has multiple functions during normal DNA replication and in stabilizing and resolving stalled or collapsed replication forks through its collaborations with other DNA repair proteins. However, the precise roles of SNM1B in these processes have not been elucidated.

In this study, I found that localization of SNM1B to stalled replication forks does not occur immediately upon replication fork stalling but after the initial DNA damage response proteins have sensed the stalled fork. SNM1B was also found to be important in preventing the accumulation of ssDNA regions. Furthermore, I demonstrate that SNM1B is important in maintenance of replication fork stability and the prevention of collapsed replication forks. SNM1B may also have roles in repairing collapsed

replication forks by promoting Rad51 filament formation at the DSB. These results also suggest that SNM1B has important functions during normal DNA replication by responding to spontaneous replication stress. Overall, these findings present evidence that SNM1B plays a key role in maintaining replication fork stability, facilitating replication recovery, and potentially in HR-mediated repair of replication-associated genomic damage.

Materials/Methods

Cell systems and knockdown of SNM1B expression by siRNA

The HCT116 colon cancer cell line was cultured in McCoy's media (10% FBS, 1% Pen/Strep) as previously described [28]. All siRNAs (50 nM) were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. SNM1B mRNA levels were determined via semi-quantitative RT-PCR in every experiment to verify the extent of siRNA knockdown as previously described [29].

Generation of HCT116 cell lines expressing siRNA-1-resistant wild-type and mutant SNM1B

A siSnm1B-1 resistant cDNA containing three silent point mutations within the siRNA-1 core sequence was used for the complementation experiments and to generate the site specific mutant [29]. The wildtype and mutant siRNA resistant cDNAs were subcloned into the pLL IRES GFP lentiviral vector (UM vector core). Lentiviruses expressing the SNM1B-IRES-GFP cassettes were generated as previously described [29]. HCT116 cells ($2.0x10^5$) were incubated with 1 mL of virus containing media with 4 mg/mL polybrene, 1 mL DMEM, and 10% FBS for 24 hours. Cells were harvested 24 hours later, and expression of the SNM1B-IRES-GFP expression cassette was determined by flow cytometry by analyzing the percentage of GFP positive cells.

GFP positive cells were sorted (University of Michigan Flow Cytometry Core), cultured, and resorted. SNM1B expression levels were assessed by semi-quantitative RT-PCR using primers specific for the siRNA resistant cDNAs. Early passage sorted cell lines with comparable levels of SNM1B expression were used for complementation

experiments. HCT116 cells infected with the pLL empty vector (EV) control were used for all experiments.

Immunofluorescence of subnuclear foci

WT-SNM1B-IRES-GFP HCT116 cells (6 x 10^4) were plated on coverslips in 12 well dishes. Cells were treated with 0.3μM aphidicolin for either 1, 2, 4, 6, or 24 hours. For RPA, Mre11, and FANCD2 foci experiments, cells were incubated in cold extraction buffer (20mM HEPES, 50mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 0.5% TX-100) for 5 min followed by fixation in 3% paraformaldehyde/2% sucrose for 20 min. For V5-SNM1B foci, cells were fixed with ice cold 70% methanol/30% acetone at -20°C for 20 min and then air dried at room temperature. Cells were blocked in 0.5% BSA, 0.05% Tween-20, 1X PBS for 1 hr. Cells were then stained with primary antibodies for 45 min and then Alexa Fluor secondary antibodies for 45 min. Prolong Gold antifade reagent with DAPI was used to mount coverslips on slides.

HCT116 cells (4 x 10^4) were plated on coverslips in 12 well dishes 24 hours prior to siRNA transfection. Forty-eight hours post transfection, cells were treated with 2μM aphidicolin for 1, 6, and 24 hours. For Rad51 foci, cells were fixed in 3% paraformaldehyde/2% sucrose with 0.5% TX-100 for 20 minutes. Cells were blocked in 5% FBS, 1% goat serum, 0.05% Tween-20, 1X PBS for 1 hour and stained with primary antibody overnight at 4°C. Cells were then stained with Alexa Fluor secondary antibody for 45 minutes. Prolong Gold antifade reagent with DAPI was used to mount coverslips on slides. Images were acquired using Olympus BX61 microscope and FISHview Software (Applied Spectral Imaging). At least three independent experiments analyzing approximately 100 cells per sample were conducted.

ssDNA immunofluorescent assay

HCT116 cells and EV, WT-SNM1B, D14N-SNM1B-IRES-GFP HCT116 cells (4 x 10^4) were plated on coverslips in 12 well dishes. Cells were depleted of SNM1B using siSnm1B-1 or a nonspecific control (NS) for 48 hours. Cells were then labeled for 24 hours with 20μM BrdU and then treated with 1μM aphidicolin for either 6 or 24 hours. Without denaturing the DNA, cells were incubated in cold extraction buffer (20mM

HEPES, 50mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 0.5% TX-100) for 5 minutes and then fixed with 3% paraformaldehyde/2% sucrose solution for 20 minutes. For experiments performed to ensure the specificity of BrdU detection as being indicative of ssDNA, cells were treated with hydrochloric acid (HCl) for 30 mins before the fixation step.

Cells were then incubated with α-BrdU antibody followed by Alexafluor secondary antibody to visualize BrdU staining using immunofluorescence microscopy. Images were acquired with an Olympus BX61 microscope and the FISHview Software (Applied Spectral Imaging). Using the contours setting on this software, the fluorescence intensity for every nucleus was measured along with the area, shape, and perimeter measurements. This fluorescence intensity was normalized to background levels for each experiment. At least three independent experiments analyzing approximately 100 cells per sample were performed.

DNA fiber assay

HCT116 cells were depleted of SNM1B using siSnm1B-1 or a nonspecific control (NS). For untreated experiments, cells were labeled with 50μM IdU for 20 min and then labeled with 100μM CldU for 20 min. Cells were harvested and re-suspended in ice-cold PBS with 200 cells/μL. Two microliters of cell suspension was deposited on a silanized slide (Sigma), and 15μL of lysis buffer (200mM Tris pH 7.4, 0.5% SDS, 50mM EDTA) was added to the cells on the slide for 10 minutes. The slides were tilted to 15° to stretch the DNA fibers and allowed to dry for 6-8 hours. Slides were then fixed in methanol:acetic acid (3:1) for 2 minutes and then allowed to dry overnight in the dark. Slides were placed at -20°C for 24 hours.

The DNA was then denatured with 2.5M HCl for 30 minutes and stained with mouse α-IdU and rat α-CldU and then Alexa Fluors 594 and 488 secondary antibodies to visualize the fibers using immunofluorescence microscopy. Fibers were imaged with an Olympus BX61 microscope and the FISHview software (Applied Spectral Imaging). At least three independent experiments were conducted with at least 200 fibers scored for each sample for every experiment. When scoring images, replication tracts with a red to green transition were labeled as ongoing, only red tracts were labeled as stalled

forks, and only green tracts were newly originated forks. The average percentage of stalled forks was calculated by dividing the number of stalled forks by the total number of ongoing and stalled forks. The average percentage of new forks was calculated as the number of new forks divided by the total number of ongoing, stalled, and new forks.

Pulse Field Gel Electrophoresis

HCT116 cells were transfected with NS or siSnm1B-1 for 48 hours and then 2μM aphidicolin was added for 24 hours. Cells were harvested and washed in PBS and 10^6 cells were used to make low melting point agarose (lmt) plugs (Lonza) using BiorRAD 50-well plug molds (#170-3713). To prepare these plugs, 10^6 cells were re-suspended in 50μL of washing buffer (10mM Tris, 100mM EDTA; TE_{100}) and mixed with 50μL of 1% agarose in water. Then 80μL of this solution was added to each plug mold, and the agarose plugs were allowed to polymerize at 4°C for 30 minutes. The plugs were then lysed at 37°C in buffer (10mM Tris, 100mM EDTA, 1% sodium lauryl sarcosine, 0.2% sodium deoxycholate, and 1 mg/ml proteinase K) for 48 hours. The plugs were then washed 3 times for 30 minutes each in TE_{100}.

The plugs were then loaded onto a 1% agarose gel in 0.5X TBE. In order to seal the plugs in the gel, 1% lmt agarose in 0.5X TBE was added to each well and allowed to polymerize. The gel was run for 24 hr at 14°C in the BioRAD CHEF III system using the following parameters: initial switch time 60s, final switch time 240s, 4 Volts/cm, 120° angle. Gels were then stained with 1.25μg/mL ethidium bromide in 0.5X TBE for 30 min and visualized using Alpha Imager 2200. The Spot Denso analysis tool, which measures the Integrated Density Value (IDV) was used to quantitate bands, including broken DNA versus intact chromosomes in the wells. The percent of broken DNA was calculated as the intensity of DNA in the migrated segment over the total intensity of DNA in the well and in the lane. This was then normalized to NS untreated samples for each experiment. At least 3 independent experiments were performed.

Cell cycle analysis

HCT116 cells were plated in a 6 well dish (1 x 10^5 in each well) and then transfected with siRNA 24 hours later. Cells were treated with 1μM aphidicolin 48 hours

post transfection. Cells were then fixed with cold 70% ethanol, stored at -20°C overnight, and stained with propidium iodide (PI) for 30 min at room temperature. FACS analysis was performed using an Accuri C6 flow cytometer and cell cycle profiles were analyzed using FlowJo (TreeStar) software.

Antibodies

α-IdU was from Becton Dickinson (347580). α-CldU was from AbD Serotec (OBT0030G). α-BrdU was from BD Pharmingen (555627). α-RPA was from Calbiochem (NA19L). α-FANCD2 and α-Mre11 was from Novus Biologicals (100-182, 100-142). α-V5 was from Invitrogen (R960-25). α-Rad51 was from GeneTex (GTX70230).

Results

Determining SNM1B localization to repair foci relative to DNA repair proteins that are critical for the cellular response to stalled replication forks.

We previously demonstrated that SNM1B is not required for efficient ATR signaling in response to aphidicolin-induced stalled replication forks. Furthermore, we have shown that SNM1B is not required for the localization of DNA repair proteins that are critical in the initial response to stalled forks, including RPA and Mre11. However, SNM1B is required for the efficient localization of FANCD2 (the Fanconi anemia complementation group D2) [28]. FANCD2 functions with the rest of the FA (Fanconi anemia) pathway in stabilizing stalled forks and is recruited to the stalled fork after the initial recognition step [19,47].

To further examine SNM1B localization to stalled forks, the timing of SNM1B localization to sites of aphidicolin-induced stalled forks relative to other DNA repair proteins was assessed. To address this question, HCT116 cells stably expressing V5-tagged SNM1B were exposed to 0.3μM aphidicolin, and localization of SNM1B was determined at 1, 2, 4, 6, and 24 hours of aphidicolin treatment. The localization of SNM1B to sub-nuclear foci was examined by immunofluorescence microscopy using α-V5 antibody. Foci formation of RPA, Mre11, and FANCD2 was also determined at the same time points (Figure 3.1). I observed that RPA and Mre11 formed foci as early as 1 hour of aphidicolin treatment and increased to approximately 40% foci positive cells at

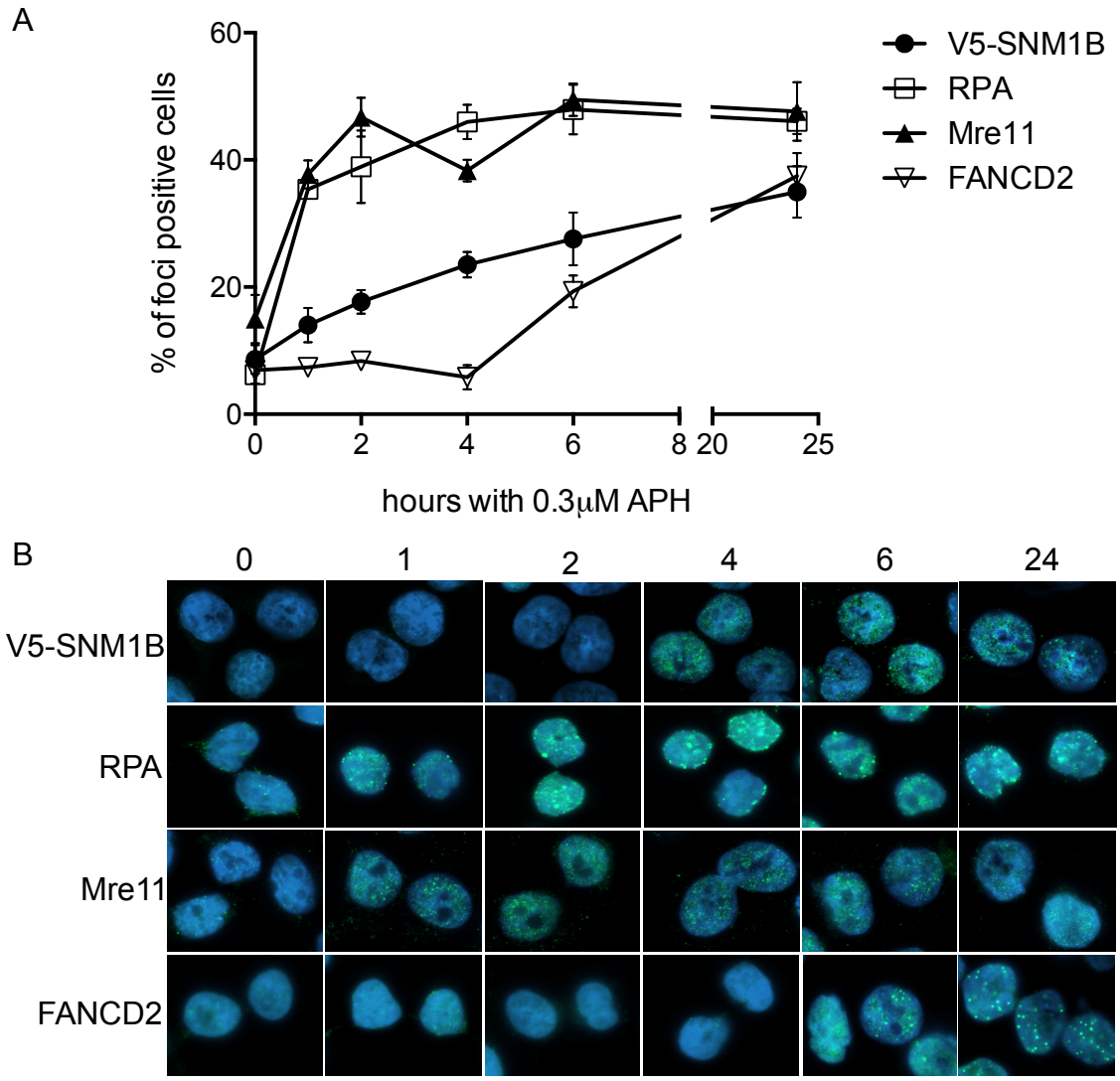


Figure 3.1 SNM1B foci formation to stalled forks occurs after RPA and Mre11 but before FANCD2.

A. WT-SNM1B-IRES-GFP HCT116 cells were treated with 0.3μM aphidicolin for various times (1, 2, 4, 6, 24 hr). Plotted here are the average percentages of foci positive cells for V5-SNM1B, RPA, Mre11, and FANCD2 foci. The results represent data from at least three independent experiments; at least 100 cells were analyzed in each experiment. Error bars, SEM.

B. Representative images of V5-SNM1B, RPA, Mre11, and FANCD3 foci (green) after 0, 1, 2, 4, 6, and 24 hours of 0.3μM aphidicolin treatment. Nuclei, DAPI stained (blue).

2 hours, which persisted through 24 hours of aphidicolin treatment. An induction of FANCD2 foci beginning at 6 hours of aphidicolin treatment further increased to approximately 40% at 24 hours. I observed a two-fold induction of SNM1B foci after 4 hours of aphidicolin treatment compared to untreated samples, which further increased with 6 and 24 hours of aphidicolin treatment. All proteins reached an approximately similar percentage of foci positive cells (40%) by 24 hours of aphidicolin treatment (Figure 3.1A). These results indicate that RPA and Mre11 localize to stalled forks initially. SNM1B is subsequently recruited, and FANCD2 localizes at a later time point suggesting a requirement for nucleolytic processing for efficient FANCD2 foci formation.

SNM1B is important in preventing the accumulation of single-stranded DNA during spontaneous and induced stalled replication forks.

Our previous studies demonstrated that knockdown of SNM1B by siRNA consistently results in increased numbers and intensity of RPA foci after aphidicolin treatment [28]. This finding suggests that SNM1B depletion may lead to increased availability of ssDNA resulting in increased RPA loading. Therefore, I examined how SNM1B depletion in cells would affect formation of regions of ssDNA.

To directly examine regions of ssDNA upon DNA polymerase inhibition, SNM1B depleted and control HCT116 cells were exposed to the thymidine analog, bromodeoxyuridine (BrdU) for 24 hours, which allowed for incorporation into both parental and nascent DNA strands. Cells were then treated with 1μM aphidicolin for either 6 or 24 hours. This higher dose of aphidicolin (compared to 0.3μM) was used to ensure that the majority of progressing replication forks would be stalled to allow detection of regions of ssDNA. Without denaturing the DNA, cells were fixed and stained with α-BrdU antibody, which recognized exposed and labeled ssDNA. The regions of ssDNA were then detected using immunofluorescence microscopy (Figure 3.2A). BrdU staining within the nucleus was quantitated and the dot plot shows the BrdU fluorescence intensity of every nucleus analyzed (Figure 3.2B). The average fluorescence intensity of SNM1B knockdown cells with and without 24 hours of aphidicolin treatment was significantly higher compared to NS controls while there was no difference after 6 hours of aphidicolin treatment. To further analyze a subset of cells

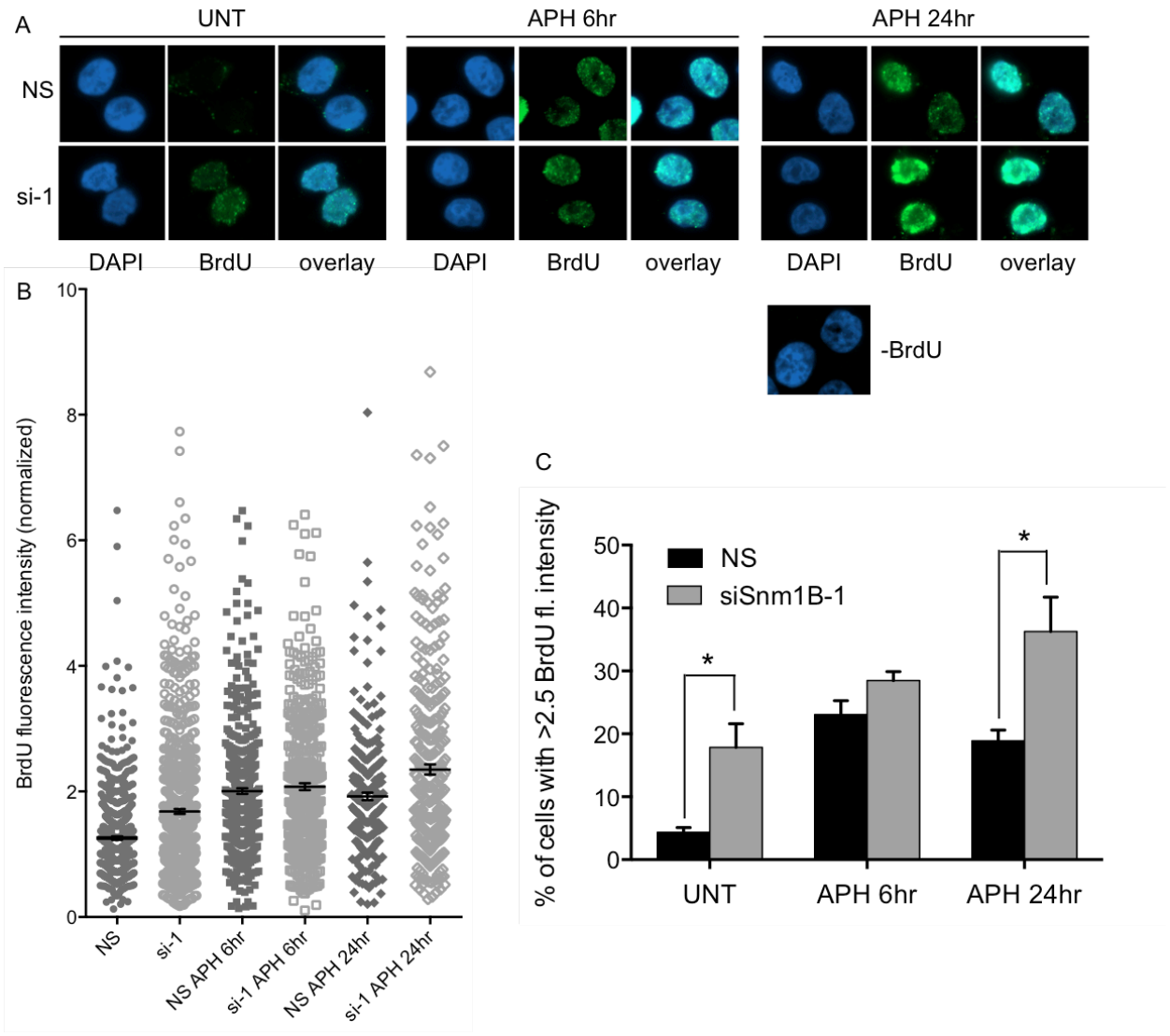


Figure 3.2 SNM1B is important for preventing the accumulation of single-stranded DNA during spontaneous and induced replication stress.
A. SNM1B depleted and control HCT116 cells were allowed to incorporate BrdU for 24 hr, which labeled both parental and nascent DNA strands, and then were treated with 1μM aphidicolin for 6 and 24 hr. Cells were fixed and stained with α-BrdU antibody without denaturing the DNA. Representative images are shown. An image of cells with no BrdU incorporation is shown as a negative control. BrdU (green). Nuclei, DAPI stained (blue).
B. BrdU staining within the nucleus was analyzed and quantitated using Applied Spectral Imaging software, which measures fluorescence intensity. This normalized BrdU fluorescence intensity per nucleus is shown on the dot plot. Error bars, SEM.
C. The average percentage of cells with high intensity BrdU staining (>2.5 fluorescence intensity) is plotted on the bar graph. The results represent data from at least three independent experiments; at least 100 cells were analyzed in each experiment. Error bars, SEM. * $p < 0.05$.

with large amounts of detectable ssDNA, the average percentage of cells with high intensity BrdU staining (>2.5 fluorescence intensity) was calculated (Figure 3.2C). In control cells treated with hydrochloric acid to completely denature the DNA and then stained with α-BrdU antibody, I observed that every nucleus contained bright BrdU fluorescence staining. This indicates that the staining detected in the non-denaturing experiments was ssDNA regions (Supplemental Figure 3.1A).

SNM1B depletion in untreated cells resulted in a 4-fold higher percentage of cells with high intensity BrdU fluorescence compared to controls, indicating an increase in regions of ssDNA (Figure 3.2C). Since these cells were not synchronized, the number of cells in S-phase, and therefore the number of cells incorporating BrdU, could vary in the SNM1B knockdown cells compared to the controls, which could affect the results. Therefore, the cell cycle profiles were analyzed and the percentage of cells in S-phase in the control (35%) and siSnm1B-1 (28%) cells was found to be relatively similar (Supplemental Figure 3.1B). Therefore, the increase in BrdU fluorescence observed in SNM1B depleted cells is not a result of more cells in S-phase. These findings suggest that SNM1B is important for preventing the accumulation of ssDNA during spontaneous replication stress.

When comparing the untreated and aphidicolin treated NS control cells, I observed a 5-fold increase in high BrdU fluorescence intensity after 6 hours of aphidicolin treatment. However, there was no significant difference in the percent of cells with ssDNA in the SNM1B knockdown cells compared to NS after 6 hours of aphidicolin treatment (Figure 3.2C). This result provides evidence that SNM1B is not needed for the generation of ssDNA through the initial uncoupling and resection events at the stalled fork. On the contrary, after 24 hours of aphidicolin treatment when many stalled forks have collapsed, the amount of high BrdU fluorescence intensity in SNM1B depleted cells was significantly higher compared to controls (Figure 3.2C). Therefore, these findings indicate that with longer aphidicolin treatment, SNM1B functions to prevent extensive generation of ssDNA.

To test the importance of the intrinsic nuclease activity of the SNM1B protein in preventing the accumulation of ssDNA during spontaneous replication stress, a conserved residue within the SNM1B catalytic domain that has been previously

identified to be essential for exonuclease activity was mutated to impair exonucleolytic activities (D14N) [24,27,48]. The BrdU immunofluorescence assay with HCT116 cells stably expressing siSnm1B-1 resistant WT-SNM1B or the nuclease mutant D14N-SNM1B was performed. In unperturbed cells, an increase in BrdU staining in cells expressing the SNM1B nuclease mutant compared to WT-SNM1B was observed, while the WT-SNM1B cells complemented the SNM1B knockdown phenotype (Figure 3.3). Altogether, these data suggest that the nuclease activity of SNM1B prevents the accumulation of ssDNA during the repair of spontaneous stalled forks. These results also suggest that SNM1B may have a distinct function in resolving spontaneous stalled forks versus aphidicolin-induced stalled forks since we did not observe any phenotypes in SNM1B depleted cells after 6 hours of aphidicolin treatment. However, longer inhibition of DNA polymerase (24 hours) did result in increased ssDNA after SNM1B depletion (Figure 3.2C).

SNM1B is important in stabilizing stalled replication forks and efficiently restarting replication.

In order to further define SNM1B functions at stalled replication forks, specifically its roles in replication fork stability, restart of stalled replication forks, and new origin firing, the established DNA fiber assay was utilized. This assay uses two different thymidine analogs, iodo-deoxyuridine (IdU) and chloro-deoxyuridine (CldU), which are incorporated into the DNA. DNA fibers are stained with different fluorescent antibodies that recognize IdU and CIdU and are visualized using immunofluorescence microscopy (Figure 3.4A). DNA fiber tracts that incorporated both IdU and CldU (red to green transition) are indicative of ongoing replication forks. DNA fibers that only incorporate IdU (only red DNA tract) indicate forks that initially were progressing but then stalled or stopped during the second labeling period. Finally, DNA fibers that only incorporated CldU (only green DNA tract) were scored as newly originated replication forks (Figure 3.4B).

I observed that in unperturbed cells, knockdown of SNM1B resulted in a significant increase in the percent of stalled forks, while the percent of new forks remained unchanged when compared to control cells (Figure 3.4C,D). These findings

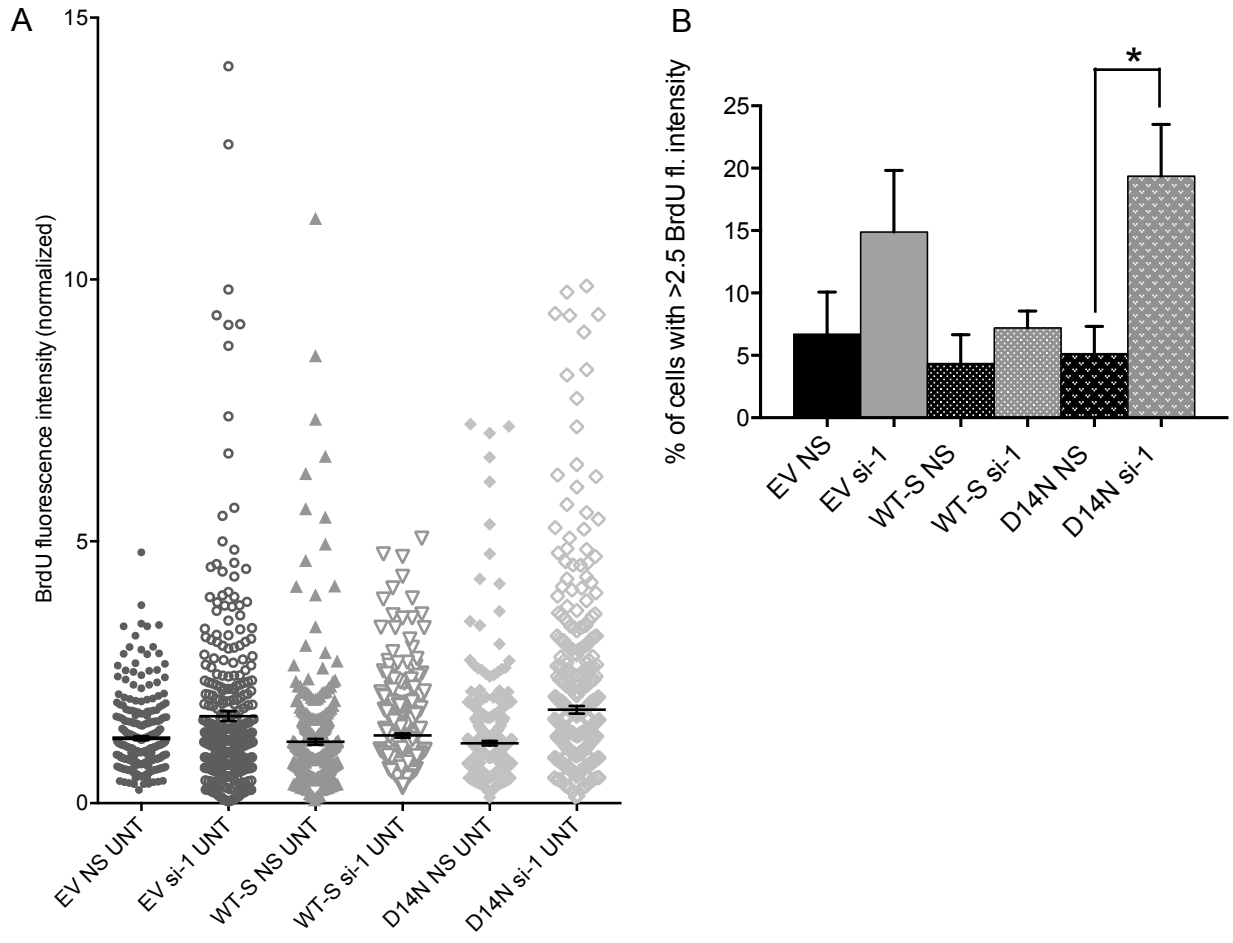


Figure 3.3 The nuclease activity of SNM1B prevents the accumulation of single-stranded DNA during spontaneous replication stress.
A. HCT116 cells stably expressing siSnm1B-1 resistant WT-SNM1B or the nuclease mutant D14N-SNM1B were transfected with NS or siSnm1B-1. Cells were allowed to incorporate BrdU. The dot plot shows the normalized BrdU fluorescence intensity of every nucleus analyzed in unperturbed cells. Error bar, SEM.
B. The average percentage of cells with >2.5 fluorescence intensity is plotted in the bar graph. The results represent data from at least three independent experiments; at least 100 cells were analyzed in each experiment. Error bars, SEM. * $p < 0.05$.

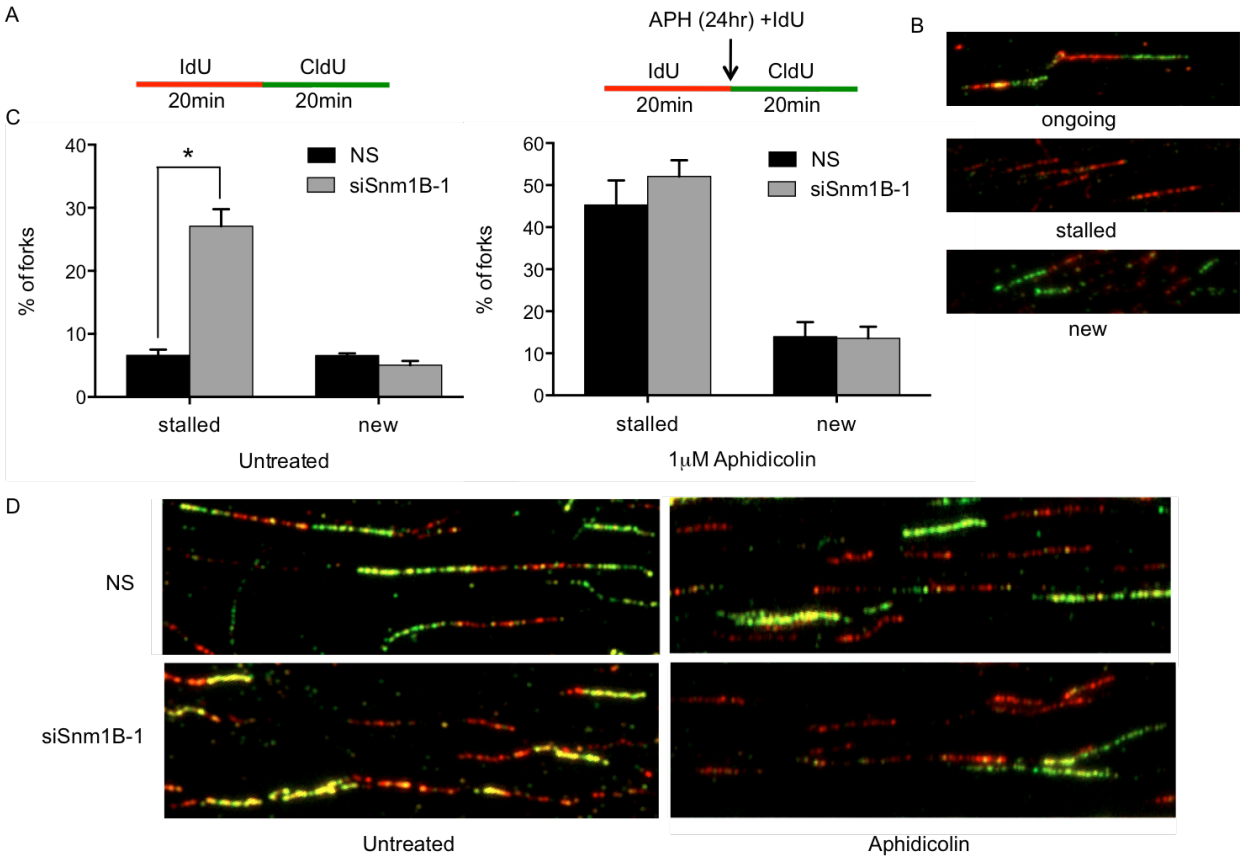


Figure 3.4: SNM1B is important in stabilizing stalled replication forks.
A. HCT116 cells were transfected with NS or siSnm1B-1. For untreated experiments, log-phase cells were labeled with IdU for 20min and then CldU for 20min. For aphidicolin treated experiments, cells were labeled with IdU for 20min, 1μM aphidicolin for 24 hr with additional IdU, and then CldU for 20min as shown by the schematics.
B. Representative DNA fiber images of ongoing forks, stalled forks, and new forks are shown. IdU (red), CldU (green).
C. The average percent of stalled forks was calculated by dividing the number of stalled forks by the total number of ongoing and stalled forks. The average percent of new forks was calculated as the number of new forks divided by the total number of ongoing, stalled, and new forks. The graphs represent the average of at least three independent experiments; at least 200 fibers were analyzed in each experiment. Error bars, SEM. *$p < 0.01$.
D. Representative DNA fiber images of NS and siSnm1B-1 untreated and 1μM aphidicolin treated cells.

indicate that SNM1B is important in stabilizing spontaneous stalled replication forks and allowing for efficient replication recovery.

For cells treated with aphidicolin for 24 hours, the percent of stalled forks was similar in SNM1B depleted cells compared to controls (Figure 3.4C,D). These results indicate that after 24 hours of aphidicolin treatment with 20 minutes of recovery time, SNM1B depletion does not affect the stability of stalled forks compared to controls. After aphidicolin treatment the percent of ongoing forks decreased (Supplemental Figure 3.2) while the percent of new forks increased, which may be a result of stalled replication forks not being processed and repaired. Studies have shown that during replication stress, the cell may begin to fire dormant origins to ensure the whole genome is duplicated [49-51]. Therefore, the cell may be compensating for this replication defect caused by aphidicolin treatment by firing more origins to aid in completing the replication process. In this experiment, the cells were allowed a 20 minute recovery after aphidicolin treatment which may not be enough time for the efficient restart of stalled forks even in the NS samples. Thus, the increased ssDNA we observed in SNM1B depleted cells (Figure 3.2C) may not necessarily be due to increased stalled forks.

SNM1B is important in preventing the accumulation of chromosomal breaks during DNA replication.

The above results of increased regions of ssDNA and increased numbers of stalled forks in unperturbed SNM1B knockdown cells suggest that the pathways that are in place to efficiently resolve stalled forks are defective upon SNM1B depletion. These persistent stalled forks are then more vulnerable to nuclease digestion which would generate breaks at the stalled fork resulting in collapsed replication forks. Therefore, in order to analyze the appearance of collapsed replication forks upon SNM1B depletion, Pulse Field Gel Electrophoresis (PFGE) was performed which allows the detection of broken DNA from intact chromosomes. HCT116 cells depleted of SNM1B were treated with 2μM aphidicolin for 24 hours to ensure the formation of collapsed replication forks. Using PFGE, broken DNA was then separated from intact chromosomes, and the percentage of faster migrating DNA was determined as a measure of the extent of

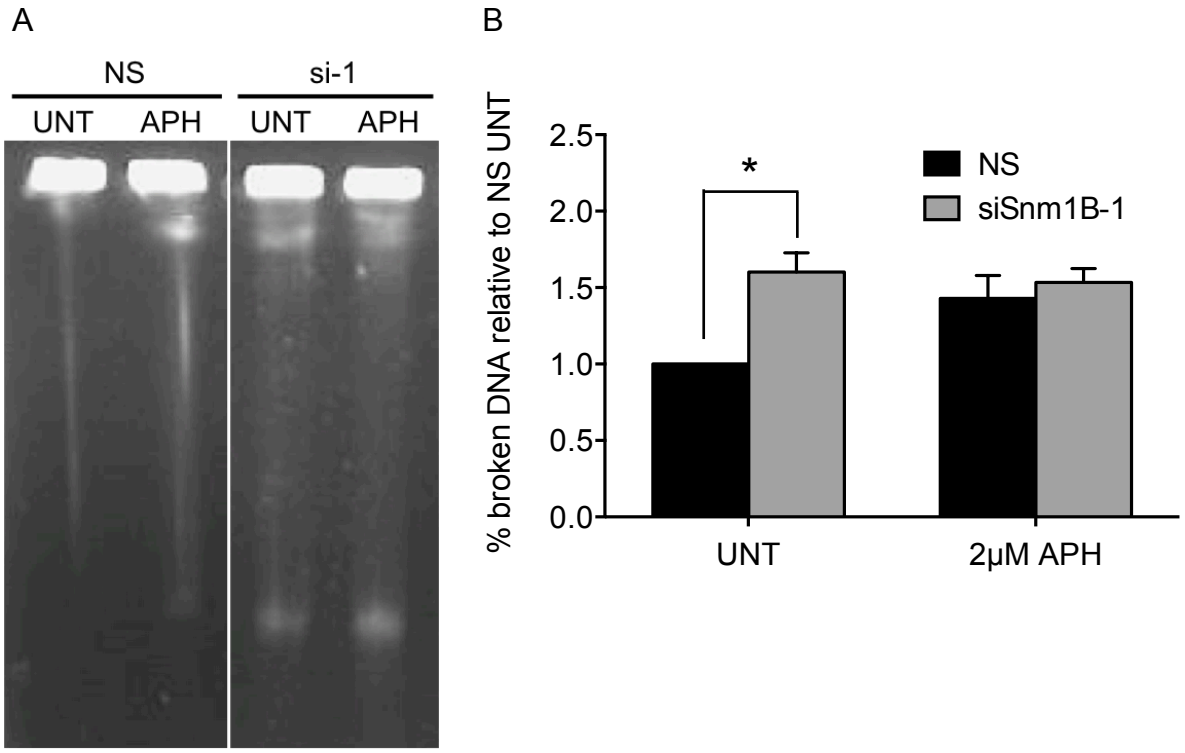


Figure 3.5 SNM1B prevents the accumulation of double-strand breaks during DNA replication.
A. PFGE image of HCT116 cells transfected with NS or siSnm1B-1 and untreated or 2μM aphidicolin treated (24 hours). Intact chromosomes are in the wells while broken DNA has migrated into the lanes.
B. The percent of broken DNA in each sample relative to NS untreated samples (set at 1) is plotted. The graph represents the average of at least three independent experiments. Error bars, SEM. * $p < 0.02$.

chromosomal breakage (Figure 3.5A).

In the untreated samples, SNM1B depletion consistently resulted in a significant increase in broken DNA. The percent of broken DNA was plotted relative to NS controls (which was set to 1). Therefore, I observed an average 1.6 fold increase in breaks upon SNM1B depletion compared to controls. In cells treated with aphidicolin however, there was no significant difference in the amount of broken DNA in SNM1B depleted cells compared to NS cells. Aphidicolin treatment did result in an increase in broken DNA when compared to untreated in NS control cells (Figure 3.5B). These data demonstrate that DSBs accumulate in SNM1B depleted cells during normal DNA replication; however, aphidicolin does not further increase the levels of DSBs in SNM1B depleted cells compared to controls.

SNM1B plays a role in promoting efficient Rad51 filament formation

Since SNM1B depletion results in an increase in broken DNA upon spontaneous replication stress, SNM1B may play a role in DSB repair at a collapsed fork. SNM1B is known to be involved in the 5' to 3' resection of telomeres, resulting in a 3' overhang which allows for binding of the shelterin complex that works to protect telomeres [24,27]. Therefore, the exonuclease activity of SNM1B may have a role in the resection step that occurs at DSBs at collapsed forks to initiate HR-mediated repair. Upon formation of the 3' overhang at collapsed replication forks, Rad51 binds to the ssDNA forming Rad51 filaments to which HR factors BRCA2, PALB2, and BRCA1 are recruited, and this initiates strand invasion to the homologous template resulting in HR [2]. Furthermore, it has also been demonstrated that Rad51 is important in stabilizing stalled replication forks by binding to ssDNA regions either at the stalled fork or that are present at a regressed fork [9,31]. Therefore, Rad51 has multiple roles at different stages during the stabilization and resolution of stalled replication forks and also in HR-mediated repair of collapsed forks.

Rad51 foci formation was analyzed in SNM1B depleted cells after 2μM aphidicolin treatment for 1, 6, and 24 hours to determine if SNM1B, and specifically its nuclease activity, is involved in the resection step at the DSB at collapsed replication forks (Figure 3.6A). If SNM1B is involved in this step, I would expect a decrease in

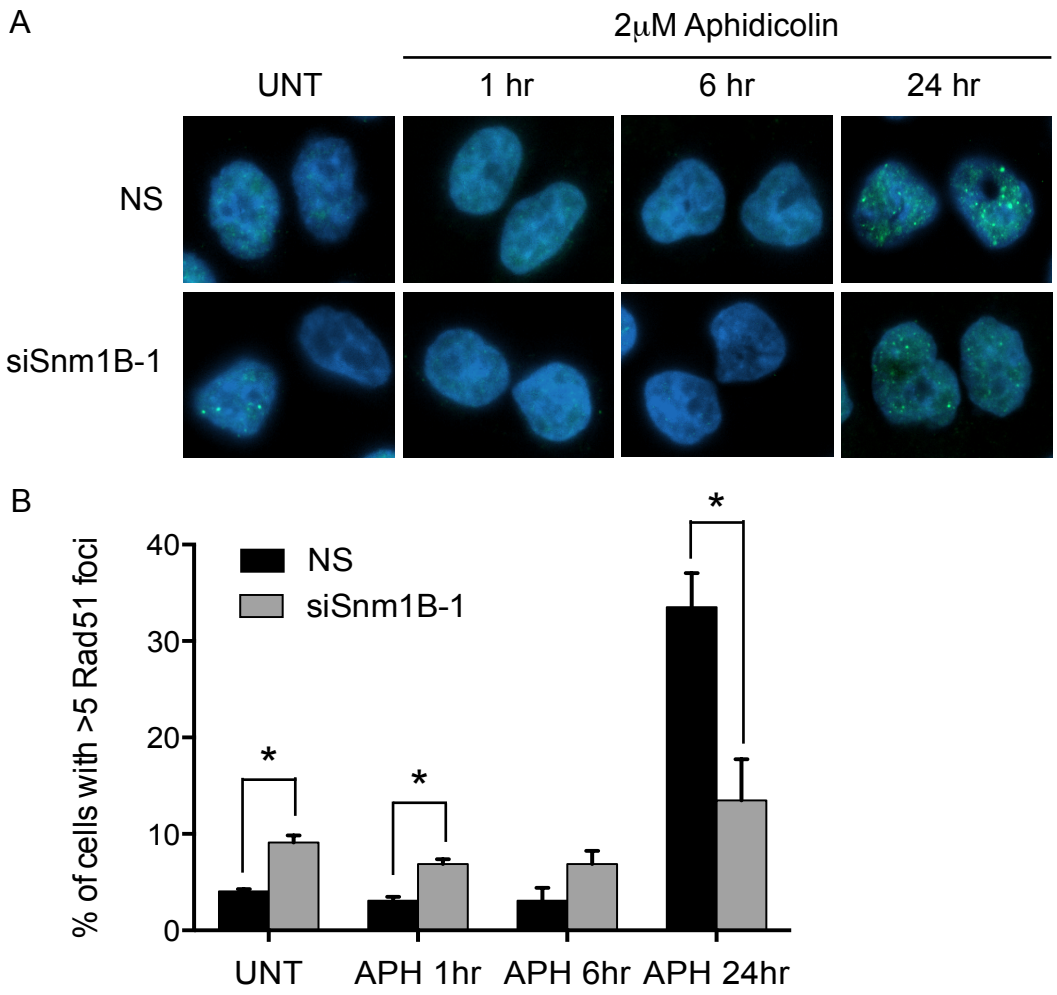


Figure 3.6 SNM1B is important for efficient Rad51 filament formation.
A. SNM1B depleted and control HCT116 cells were treated with 2μM aphidicolin for 1, 6, and 24 hrs. Cells were fixed and stained with Rad51 antibody. Representative images are shown. Rad51 (green). Nuclei, DAPI stained (blue).
B. The average percentage of cells with >5 Rad51 foci is plotted on the bar graph. The results represent data from at least three independent experiments; at least 100 cells were analyzed in each experiment. Error bars, SEM. * $p < 0.03$

Rad51 foci after SNM1B depletion upon 24 hours of aphidicolin treatment. After 24 hours of aphidicolin treatment, SNM1B knockdown cells exhibited a significantly lower percent of Rad51 foci positive cells compared to controls (Figure 3.6B). In untreated cells and cells treated with aphidicolin for 1 hour, knockdown of SNM1B resulted in a significant two-fold increase in Rad51 foci compared to NS samples; although still at a relatively low percentage (10%). Interestingly, Rad51 foci formation did not increase upon 1 or 6 hours of aphidicolin treatment in NS cells suggesting that using this method we are not able to detect Rad51 filaments at forks that have been stalled for short time periods [52]. Therefore, the Rad51 foci I detect with 24 hours of aphidicolin are Rad51 filaments at DSBs or extensively long stretches of ssDNA. These data suggest that during the repair of aphidicolin-induced collapsed forks, SNM1B is important for efficient Rad51 localization. Since at the 24 hour timepoint of aphidicolin treatment I observed similar levels of DSBs in SNM1B depleted and NS cells as measured by PFGE, this suggests that Rad51 is able to form filaments and initiate HR in controls but not in SNM1B depleted cells.

Discussion

In this study, I provide evidence to demonstrate that the DNA nuclease SNM1B plays critical roles in the stabilization of stalled replication forks and in repairing collapsed replication forks. In elucidating the precise roles of SNM1B, I have analyzed its role at early and later events in the resolution of stalled forks. Furthermore, I have also uncovered that SNM1B is important during spontaneous replication stress, which may be distinct to its roles at aphidicolin-induced stalled forks.

Upon aphidicolin-induced replication fork stalling, the uncoupling of the helicase and polymerase activities results in long stretches of ssDNA. RPA binds to the long stretches of ssDNA at stalled forks and Mre11 has been shown to have functions in this initial response to stalled forks. For example, the MRN complex has been shown to colocalize with RPA upon replication fork stalling [53,54]. I have shown that within 1 hour of aphidicolin treatment both RPA and Mre11 form foci.

I observed a significant increase in SNM1B foci formation after 4 hours of aphidicolin treatment (Figure 3.1). Furthermore, no difference was detected between

SNM1B knockdown and NS samples when analyzing regions of ssDNA at 6 hours of aphidicolin treatment, suggesting the ssDNA needed to activate signaling events has already been generated (Figure 3.2). These data along with previous data that SNM1B is not required for efficient RPA and Mre11 foci formation, suggest that SNM1B is not needed for sensing stalled forks or activating early events during the repair process [28]. At this timepoint of aphidicolin treatment, many stalled forks have begun the process of fork regression in an attempt to initiate fork restart [35,36]. As mentioned previously, Mre11 is involved in degrading nascent DNA at a regressed fork, and BRCA2, FANCD2, and Rad51 regulate the 3' to 5' exonuclease activity of Mre11 to prevent unregulated degradation of nascent DNA [18,19]. Once Mre11 digests the nascent leading strand, a 5' to 3' exonuclease, like SNM1B, could digest the nascent lagging strand. The timing of localization through foci formation suggests sequential actions of Mre11 and SNM1B.

I found that FANCD2 foci formation induction is detectable 6 hours post aphidicolin treatment, once SNM1B has already begun to localize to the stalled fork (Figure 3.1). Previous results also show that SNM1B is required for efficient FANCD2 foci formation [28]. Therefore, once SNM1B localizes to the area of damage, it can nucleolytically process intermediate DNA structures and allow room or generate binding sites for further downstream proteins like FANCD2, BRCA1, and Rad51 to localize to the stalled fork and function in restarting replication. Additionally, SNM1B may collaborate with other nucleases that are also responsible for degrading regressed forks.

SNM1B may also have roles at later events during the repair process. When analyzing regions of ssDNA using the BrdU immunofluorescence assay after 24 hours of aphidicolin treatment, I observed a significant increase in ssDNA in SNM1B depleted cells compared to controls (Figure 3.2). More ssDNA could either indicate the presence of more stalled forks and/or longer stretches of ssDNA resulting from the helicase continuing to unwind the DNA while the stalled forks are being repaired. Increased ssDNA could also result from uncontrolled resection at stalled forks. If these stalled forks are not resolved, many of them by this timepoint of aphidicolin treatment may have collapsed. To further examine how SNM1B depletion affects replication fork progression, the DNA fiber analysis was performed. I did not observe any difference in

the percent of stalled forks when comparing SNM1B depleted cells compared to controls (Figure 3.4). These results show that with a 20 minute restart and recovery time after aphidicolin treatment SNM1B depletion does not affect the percent of stalled forks but there is an increase in unstable ssDNA (Figure 3.2). Allowing for longer recovery may give more insight into prolonged stalled or collapsed forks upon SNM1B depletion.

To analyze collapsed replication forks, I performed PFGE and found no difference in the percent of DSBs that separated from intact chromosomes in SNM1B depleted cells compared to controls after 24 hours of aphidicolin treatment (Figure 3.5). These data suggest that SNM1B is not needed to generate the breaks at collapsed replication forks, a function that Mus81 has been implicated in [35,55,56]. Instead, SNM1B may be involved in helping to sense the DSB. Furthermore, the exonuclease activity of SNM1B may be important in the initial resection step at DSBs at collapsed forks, which forms a 3' overhang that initiates Rad51 filament formation and HR-mediated repair. To determine if SNM1B has a role in this resection step, Rad51 filament formation was analyzed after aphidicolin treatment, and there was a significant decrease in Rad51 foci formation in SNM1B depleted cells compared to control cells (Figure 3.6). This suggests that upon aphidicolin treatment, SNM1B depletion results in collapsed forks at similar levels to control cells, but Rad51 is unable to form filaments to initiate HR. SNM1B could play a role in promoting Rad51 loading onto ssDNA thereby preventing extensive resection of aberrant DNA structures at stalled and collapsed forks by other nucleases. If SNM1B is absent and Rad51 filament formation is inhibited, these other nucleases can act on these DNA structures in an uncontrolled manner possibly resulting in increased unstable ssDNA (Figure 3.2C).

There are several pieces of evidence from our lab and others that suggest SNM1B is important in DSB repair. We have previously shown that BRCA1 foci formation is impaired after aphidicolin treatment in SNM1B depleted cells lending evidence that decreased BRCA1 localization to DSBs at collapsed forks could be the reason there is defective Rad51 filament formation [28]. As previously mentioned, SNM1B exonuclease activity is important in resecting telomeres which allows for telomere protection [24,27,57]. We have shown, using the established DR-GFP assay, that SNM1B is needed for efficient repair of I-SceI induced breaks [29]. Other studies have found that

SNM1B localizes to IR (ionizing radiation) induced breaks independent of ATM and is important in the DSB response signaling events [58]. All these studies exemplify how SNM1B may play different roles at DSBs depending on the source of DNA damage. Our data in this study show that SNM1B localizes to stalled forks and plays important roles at the later events that occur during the stabilization and restart of stalled replication forks or in the repair of collapsed replication forks.

It is important to note the differences between aphidicolin-induced stalled forks versus spontaneous replication stress. Spontaneous fork stalling can result from a multitude of sources as previously described including secondary structures, protein:DNA adducts, or encounters with the transcription machinery. Uncoupling of polymerase and helicase activities may not necessarily occur in the response to all these spontaneous sources [1]. We have found some of the most striking and interesting phenotypes in untreated SNM1B depleted cells, which are dealing with spontaneous replication stress that occurs during every S phase of the cell cycle.

When SNM1B is absent or nuclease deficient, I observed a significant increase in regions of ssDNA compared to NS suggesting that the stalled forks are not repaired efficiently (Figures 3.2,3.3). SNM1B was also found to be important in preventing the occurrence of stalled replication forks in untreated cells, during spontaneous replication stress using DNA fiber analysis (Figure 3.4). These results indicate that there is clearly a role for SNM1B in preventing stalled replication upon spontaneous replication stress. If SNM1B nuclease activity is involved in digesting or processing regressed forks, during its absence or nuclease deficiency, these structures remain, resulting in persistent stalled forks. SNM1B nucleolytic activity may digest the nascent DNA strands at stalled forks to provide binding sites for recruitment of repair proteins, as previously discussed.

In normal proliferating cells when SNM1B is absent, I found a significant increase in the percent of broken DNA from intact chromosomes using PFGE, indicating an increase in genome instability (Figure 3.5). In conclusion, I have demonstrated there are increased regions of ssDNA, numbers of stalled forks, and broken DNA in unperturbed SNM1B depleted cells. These results exemplify that SNM1B is critical in stabilizing stalled replication forks, preventing aberrant accumulation of ssDNA, and also preventing collapsed replication forks in normal proliferating cells.

One responsibility of SNM1B in normal proliferating cells could be to relieve torsional stress, especially since it has a role in unwinding supercoiled DNA structures at telomeres with its association with Topoisomerase 2α [48]. During spontaneous replication stress, SNM1B may also process topological barriers to avoid fork regression and maintain replication fork progression. Future studies determining the specific roles of SNM1B in responding to spontaneous and induced stalled forks would address the interplay of SNM1B and other nucleases and DNA repair factors involved in these processes.

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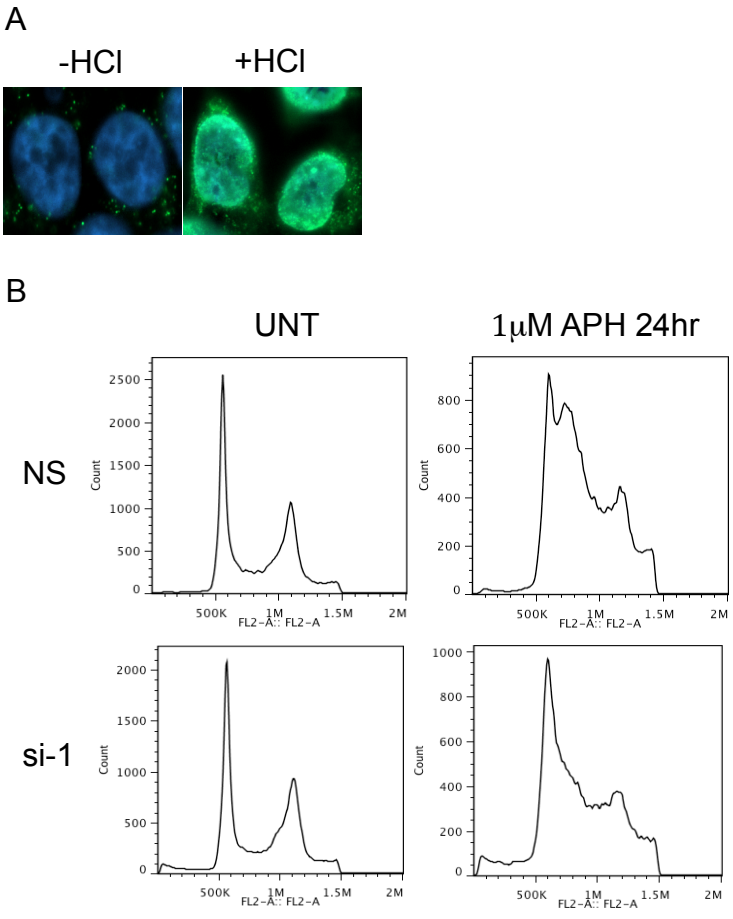
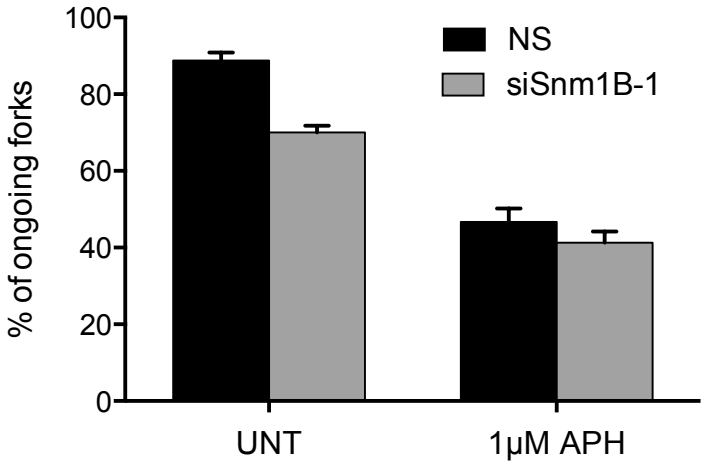


Supplemental Figure 3.1 Control experiments for BrdU immunofluorescence assay.

A. HCT116 cells were allowed to incorporate BrdU and then treated with hydrochloric acid (HCl) to completely denature the DNA. Cells were then fixed and stained with α-BrdU antibody. BrdU (green). Nuclei, DAPI stained (blue).

B. SNM1B depleted and control HCT116 cells were untreated or treated with 1μM aphidicolin for 24 hours. DNA content was assessed by fixing then staining cells with propidium iodide and analyzing DNA content by FACS. Cell cycle profiles are shown, and quantitation of the percent of cells in the G1, S, and G2/M phases of the cell cycle were assessed using the Watson algorithm (FlowJo, TreeStar).



Supplemental Figure 3.2 Aphidicolin treated cells result in a decrease in ongoing replication forks.
HCT116 cells were transfected with NS or siSnm1B-1. For untreated experiments, log-phase cells were labeled with IdU for 20min and then CldU for 20min. For aphidicolin treated experiments, cells were labeled with IdU for 20min, 1μM aphidicolin for 24 hr with additional IdU, and then CldU for 20min. The average percent of ongoing forks was calculated by dividing the number of ongoing forks divided by the total number of ongoing, stalled, and new forks. The graphs represent the average of at least three independent experiments; at least 200 fibers were analyzed in each experiment. Error bars, SEM.

Chapter 4: Regulation of SNM1B functions during the repair of stalled replication forks.

Summary

SNM1B/Apollo is a DNA nuclease involved in telomere maintenance, double-strand break repair, interstrand crosslink repair, and resolution of replication stress. It has been shown that SNM1B functions within the FA/BRCA network to repair interstrand crosslinks and stalled replication forks; however, how SNM1B functions are regulated is not well defined. In this chapter, I sought to determine how SNM1B localization, functions, and protein stabilization is regulated in response to aphidicolin-induced stalled replication forks. We identified a residue within a conserved monoubiquitination motif in SNM1B as being critical for SNM1B and FANCD2 localization to subnuclear foci. I also determined that SNM1B protein levels are elevated upon aphidicolin treatment suggesting SNM1B protein stabilization may be induced by DNA damage or regulated by the cell cycle. I further found that SNM1B protein levels are regulated through proteasomal degradation. In conclusion, this study provides evidence that multiple mechanisms are employed by cells to regulate SNM1B functions by controlling SNM1B localization to stalled replication forks and modulating SNM1B protein stability.

Introduction

The DNA comprising our genome is constantly under assault from endogenous and exogenous sources that cause many different types of damage. If unrepaired, DNA lesions can accumulate, thereby leading to insertions, deletions, and aberrant chromosomal rearrangements [1]. The DNA Damage Response (DDR) is a signaling network which senses the damage, initiates cell cycle checkpoints, induces apoptosis if the damage is too severe, and activates signaling pathways that recruit DNA repair proteins which fix the damaged DNA through different repair mechanisms [2]. All these cellular events that occur during the DDR are tightly monitored and regulated. The recruitment and timely activation of repair proteins is critical for the maintenance of genome integrity. The functions of DNA repair proteins are regulated at multiple levels including post-translational modifications (PTMs), cell cycle regulation, protein stability, and proteasomal degradation.

PTMs affect protein functions, interactions, enzymatic activity, and localization, and therefore are a critical part of the DDR and repairing DNA damage. Some PTMs that commonly occur during DNA repair processes are phosphorylation, ubiquitination, SUMOylation, acetylation, methylation, and PARylation [3,4].

There are three central protein kinases responsible for key phosphorylation events during the DDR: ATM (Ataxia telangiectasia mutated), ATR (Ataxia telangiectasia and Rad3 related), and DNA-PK (DNA-dependent protein kinase) which are members of the PIKK (phosphoinositide-3-kinase-like protein kinase) family [5-7]. All of these kinases phosphorylate the histone variant H2AX, which is a key phosphorylation event that signals that DNA damage has occurred [8-12]. Phosphorylation of H2AX (denoted as γH2AX) initiates downstream events including the recruitment of repair proteins [10,13]. ATM and ATR phosphorylate the effector kinases CHK2 and CHK1, respectively which ultimately results in cell cycle arrest to allow time for the cell to repair the damage [14-17].

Another PTM that is utilized to regulate DNA repair proteins is ubiquitination. Ubiquitin is a 76 amino acid polypeptide that is covalently attached to lysine residues on proteins through E1 (activating), E2 (conjugating), and E3 (ligase) enzymes [18-20]. Ubiquitin itself has seven lysine residues and therefore, polyubiquitin chains can form

and this primarily targets the protein for proteasomal degradation [21-23]. Monoubiquitination of a protein can aid in its localization to a specific site and promote protein:protein interactions through ubiquitin-binding domains (UBD) [24,25]. FANCD2 and FANCI (ID complex), two Fanconi anemia (FA) proteins, are monoubiquitinated by the FA core complex which possesses E3 ubiquitin ligase functions, which results in localization of the ID complex to sites of blocked or stalled replication forks [26]. The monoubiquitinated ID complex binds to the UBD of the nuclease FAN1 (Fanconi-associated nuclease 1) resulting in the recruitment of FAN1 to the replication fork [27-30].

SUMOylation, which covalently links SUMO (small ubiquitin-like modifier) to a protein, is another example of a PTM [31]. SUMOylation of RPA (replication protein A) facilitates the interaction between RPA and Rad51, which recruits Rad51 to DSBs to initiate homologous recombination (HR) [32]. PARylation, characterized by the addition of ADP-ribose polymers to proteins through PARP (poly(ADP-ribose) polymerase) enzymes is another important PTM because PARylation of targets recruits DDR proteins to DNA breaks. [33]. Histone methylation and acetylation regulates opening up of the chromatin allowing room for localization of repair proteins [34]. Cross-talk between these different types of PTMs can be involved in regulating one protein, especially if this protein has multiple functions in several different repair pathways. One example of this is CtIP (carboxy-terminal binding protein interacting protein) which plays a key role in regulating if a DSB is repaired through HR or non-homologous end joining (NHEJ) [35].

Removal of PTMs is just as critical in regulating protein functions. For example, DUBs (deubiquitylating enzymes) promote inactivation of protein functions, disassembly of protein complexes, and disruption of interactions that are no longer required at the site of DNA damage [36,37]. If proteins are not modified in a timely manner, their functions can be impaired resulting in unrepaired DNA damage and genome instability.

The ubiquitin-proteasome system (UPS) is involved in protein stability which regulates protein levels. While monoubiquitination is involved in localization, protein interactions, and repair functions as described above, K48 linked polyubiquitin chains target proteins to the proteasome for degradation [38,39]. The BLM helicase is monoubiquitinated which promotes its localization to stalled replication forks and binding to proteins with UBDs. BLM is also polyubiquitinated and targeted for degradation [40,41].

These two types of ubiquitination work together to monitor the activity of BLM during the restart of stalled replication forks.

Having higher levels of certain proteins in normal proliferating cells may result in deleterious phenotypes, so the cell rapidly degrades these proteins in normal conditions. Upon DNA damage, which may require the protein to fix the damage, it is not targeted for proteasomal degradation. p53 is an example of this form of regulation as it is stabilized and activated in response to DNA damage. [42-44]. Protein levels of DNA repair factors may also be regulated by the cell cycle. CtIP functions are not only regulated by PTMs but also by the cell cycle. CtIP protein levels are low in G1 and as the cell progresses though S phase the protein levels increase (with consistent transcription) suggesting a mechanism through which CtIP plays a role in deciding between HR and NHEJ by promoting HR-mediated repair [45]. Interactions between proteins is another mechanism through which a protein can be stabilized. For example, the binding of two helicases important in the stabilization and restart of stalled forks, FANCJ and BLM, stabilizes the BLM protein while this interaction does not affect protein levels of FANCJ [46-48].

DNA nucleases have critical functions in repairing all types of DNA damage, including restarting stalled replication forks. A number of nucleases including Mre11, Mus81, FAN1, DNA2, Exo1, and SNM1B have been implicated in this process, but how their functions are regulated and how they collaborate is unknown. The nuclease activities of these proteins have to be tightly controlled because unmonitored nuclease activity can degrade the DNA, resulting in even more damage and increased genome instability. It has been shown that Mre11-mediated nucleolytic processing of regressed forks is controlled by the FA/BRCA network thereby ensuring these structures are not highly degraded [49]. Therefore, we sought to determine how SNM1B nuclease activity, functions, and localization during the repair of stalled replication forks are regulated.

Through its 5’ to 3’ exonuclease activity, SNM1B forms 3’ overhangs at telomeres which is necessary for telomere protection. A member of the shelterin complex, POT1b, inhibits hyper-resection of leading-end telomeres by SNM1B. However, the specific mechanism through which POT1b regulates SNM1B functions at telomeres is not known [50]. Furthermore, SNM1B interacts with TRF2, another

component of the shelterin complex, and this interaction promotes recruitment of SNM1B to telomeres and also stabilizes SNM1B protein levels by inhibiting polyubiquitination of SNM1B, thereby preventing proteasomal degradation [50-52]. In addition to TRF2, SNM1B has also been shown to interact with DNA repair proteins Mre11, FANCD2, Mus81, and SLX4 [53,54]. However, how these interactions are mediated and their functional significance is unknown.

SNM1B functions may be regulated at multiple levels including PTMs, cell cycle regulation, protein stability, and proteasomal degradation, and the interplay between these different types of regulation can mediate its recruitment, localization, and enzymatic functions. Furthermore, since SNM1B has been implicated in repairing ICLs, DSBs, stalled replication forks, and spontaneous replication stress, the specific mechanisms through which it is regulated could vary depending on the type of DNA damage.

In this chapter, a residue on the C terminus of SNM1B was found to be critical for SNM1B localization to aphidicolin-induced stalled forks. This residue was also shown to be important for efficient FANCD2 foci formation upon aphidicolin treatment. Furthermore, I found that SNM1B protein levels increase upon aphidicolin treatment and are regulated by the proteasome, suggesting that SNM1B functions are regulated at multiple levels of protein stability and turnover along with localization to stalled replication forks.

Materials/Methods

Cell systems and knockdown of SNM1B expression by siRNA

The HCT116 colon cancer cell line was cultured in McCoy's media (10% FBS, 1% Pen/Strep) as previously described [55]. All siRNAs (50 nM) were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. SNM1B mRNA levels were determined via semi-quantitative RT-PCR in every experiment to verify the extent of siRNA knockdown as previously described [55,56].

Generation of HCT116 cell lines expressing siRNA-1-resistant wild-type and mutant SNM1B

A siSnm1B-1 resistant cDNA containing three silent point mutations within the siRNA-1 core sequence was used for the complementation experiments and to generate the site specific mutants [56]. The wildtype and mutant siRNA resistant cDNAs were subcloned into the pLL IRES GFP lentiviral vector (UM vector core). Lentiviruses expressing the SNM1B-IRES-GFP cassettes were generated as previously described [56]. HCT116 cells ($2.0x10^5$) were incubated with 1 mL of virus containing media with 4 mg/mL polybrene, 1 mL DMEM, and 10% FBS for 24 hours. Cells were harvested 24 hours later, and expression of the SNM1B-IRES-GFP expression cassette was determined by flow cytometry to determine the percentage of GFP positive cells.

GFP positive cells were sorted (University of Michigan Flow Cytometry Core), cultured, and re-sorted. SNM1B expression levels were assessed by semi-quantitative RT-PCR using primers specific for the siRNA resistant cDNAs. Early passage sorted cell lines with comparable levels of SNM1B expression were used for complementation experiments. HCT116 cells infected with the pLL empty vector (EV) control were used for all experiments.

Immunofluorescence of subnuclear foci

Empty vector (EV), WT-SNM1B and K398R-IRES-GFP HCT116 cells were plated on coverslips in 12 well dishes. For some experiments, cells were then transfected with NS or siSnm1B-1. Cells were treated with aphidicolin (either 0.3, 0.5, or 1μM) 48 hours post transfection for 24 hr. For FANCD2 foci, cells were incubated in cold extraction buffer (20mM HEPES, 50mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 0.5% TX-100) for 5 min followed by fixation in 3% paraformaldehyde/2% sucrose for 20 min. For V5-SNM1B foci, cells were fixed with ice cold 70% methanol, 30% acetone at -20°C for 20 min and then air dried at room temperature. For experiments co-staining for GFP and V5-SNM1B, cells were incubated in 3% p-formaldehyde/2% sucrose for 20 min followed by incubation in cold extraction buffer (20mM HEPES, 50mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 0.5% TX-100) for 5 min. Cells were blocked in 0.5% BSA, 0.05% Tween-20, 1X PBS for 1 hr. Cells were then stained with primary antibody for 45 min and then Alexa Fluor 488 or 594 (Invitrogen Molecular Probes) secondary antibodies for 45 min. Prolong Gold antifade reagent with DAPI (Invitrogen) was used to mount

coverslips on slides. Images were acquired using Olympus BX61 microscope and FISHview Software (Applied Spectral Imaging). At least three independent experiments analyzing approximately 100 cells per sample were conducted.

Western blot analyses

WT-SNM1B and K398R-SNM1B HCT116 cells were treated with either 1μM aphidicolin for 24 hr, 10μM MG-132 for 8 hr, or 1μM MMC for 8 hr. Cells were harvested and re-suspended in protein lysis buffer (10mM PIPES pH 6.8, 100mM NaCl, 300mM sucrose, 1mM $MgCl_2$, 0.1% Triton-X 100) containing phosphatase (Roche PhosSTOP) and protease inhibitors (Roche Complete Mini EDTA free), and Benzonase (Purity >99% Novagen). Expression of SNM1B protein was analyzed by western blotting using α-V5 antibody and IRDye 800 CW secondary antibodies (Li-Cor). Bands were visualized and quantitated using the Odyssey 2.1 software. All experiments were performed at least three independent times.

WT-SNM1B and K398R HCT116 cells were treated with 1μM aphidicolin for 24 hr and then cells were harvested for cellular fractionation experiments. Cells were first lysed with buffer 1 (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 0.2% NP-40, containing phosphatase and protease inhibitors) for 5 min on ice. After centrifugation (1000g, 5min) the supernatant was labeled as the cytoplasmic fraction. The pellet was then lysed with buffer 2 (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40, containing phosphatase and protease inhibitors) for 40 min on ice. After centrifugation (16000g, 15min) the supernatant was labeled as the nuclear fraction. The final pellet was then re-suspended in RIPA buffer (50mM Tris-Cl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150mM NaCl) containing the chromatin-associated fraction. GAPDH and H2AX were used as loading controls for cytoplasmic and chromatin fractions, respectively. Expression of SNM1B protein was analyzed by western blotting using α-V5 antibody and IRDye 800 CW secondary antibodies (Li-Cor). FANCD2 monoubiquitination was also analyzed using α-FANCD2 antibody. Bands were visualized and quantitated using the Odyssey 2.1 software. All experiments were performed at least three independent times.

Cell Cycle Analysis

WT-SNM1B HCT116 cells were plated in a 6 well dish (1 x 10^5 in each well) and treated with 1μM aphidicolin for 24 hours and 1μM MMC for 8 hours. Cells were then fixed with cold 70% ethanol, stored at -20°C overnight, and stained with propidium iodide (PI) for 30 min at room temperature. FACS analysis was performed using an Accuri C6 flow cytometer and cell cycle profiles were analyzed using FlowJo (TreeStar) software.

Antibodies

α-FANCD2 was from Novus Biologicals (100-182). α-V5 was from Invitrogen (R960-25). α-GAPDH was from Santa Cruz (32233). α-H2AX was from Millipore (07-627). α-GFP was from Abcam (ab5450). α-Topoisomerase I was from BD Biosciences (556597).

Results

Identification of a residue within SNM1B that is important for FANCD2 localization to stalled replication forks

One key PTM of the FA pathway in responding to ICLs and stalled replication forks is the monoubiquitination of the ID complex as mentioned above. Monoubiquitination results in subnuclear localization of the ID complex to sites of damage [26]. Interestingly, SNM1B possesses a lysine residue within a motif (K**K**QL) similar to the monoubiquitination consensus sequence in FANCD2 (R**K**QL) and FANCI (R**K**AM) [57]. Shown in the diagram is the conserved sequence of SNM1B in humans and mice and of FANCD2 and FANCI in humans, mice, and amoeba (Figure 4.1A).

To determine if the localization and function of SNM1B in the response to replication stress could possibly be regulated through this potential monoubiquitination motif, the lysine residue of SNM1B was mutated to arginine (K398R). Lysine and arginine are both polar, positively charged amino acids, but ubiquitin cannot be added to arginine; therefore, this conservative amino acid substitution is commonly used to mutate ubiquitination sites. HCT116 cell lines which stably express the K398R-SNM1B mutant protein were generated using a pLL-IRES-GFP lentiviral vector. Since we have previously found that both depletion of SNM1B and expression of a nuclease deficient

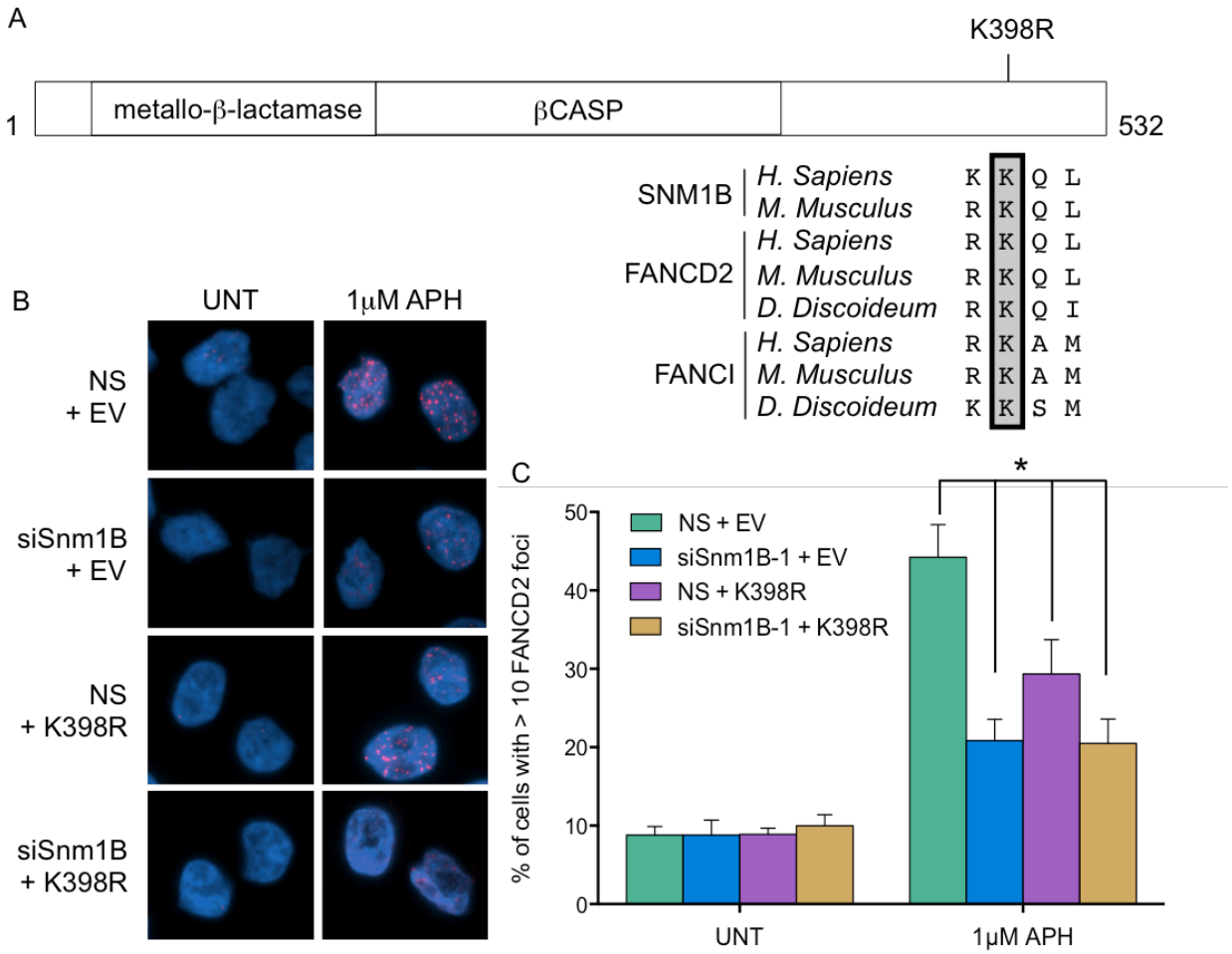


Figure 4.1: Identification of a residue within SNM1B that is important for FANCD2 foci formation

A. Schematic of SNM1B showing the lysine residue (K398) that is located within the monoubiquitination consensus sequence found in FANCD2 and FANCI. Protein sequence in human and mice are shown for all three plus amoeba for FANCD2 and FANCI. This potential mono-ubiquitination lysine residue of SNM1B was mutated to arginine (K398R).

B. HCT116 cells transduced with pLL-IRES-GFP empty vector (EV) or K398R-SNM1B retroviruses were sorted, transfected with NS or siSnm1B-1, and treated with 1μM aphidicolin treatment for 24 hr. FANCD2 foci formation was visualized by immunofluorescence microscopy. Representative images are shown. FANCD2 (red). Nuclei, DAPI stained (Blue).

C. The average percentage of cells with >10 FANCD2 foci was quantitated. The graph represents the results from at least three independent experiments; at least 100 cells were scored in each experiment. Error bars, SEM. *p<0.01

SNM1B mutant (D14N) results in defective FANCD2 foci formation in response to aphidicolin-induced stalled replication forks, I examined FANCD2 foci formation in the K398R-SNM1B cells (Figure 4.1B) [55]. In EV cells, knockdown of SNM1B resulted in decreased aphidicolin-induced FANCD2 foci as previously reported [55]. I also found that the K398R-SNM1B mutant cells exhibited a significant decrease in the percent of cells with >10 FANCD2 foci after aphidicolin treatment, even in cells containing endogenous SNM1B (Figure 4.1B,C). These results suggest that mutation of this residue may have a dominant negative effect on SNM1B functions and is important for efficient FANCD2 foci formation upon aphidicolin-induced stalled forks.

Identification of a residue within SNM1B that is important for SNM1B localization to aphidicolin-induced stalled replication forks.

I have also previously found that SNM1B localization to aphidicolin-induced repair foci occurs before FANCD2 (Figure 3.1). Therefore, I next determined if the K398R-SNM1B mutant can localize to aphidicolin-induced sites of stalled replication forks. WT-SNM1B and K398R-SNM1B cells were treated with increasing doses of aphidicolin (0.3, 0.5, and 1μM) for 24 hours. Cells were then co-stained with α-GFP and α-V5 antibodies and V5-SNM1B foci were visualized by immunofluorescence microscopy. Only the GFP positive cells were analyzed since these cells contain the pLL IRES GFP vector and express the V5 tagged SNM1B protein (Figure 4.2A). While WT-SNM1B cells showed increased percentages of V5-SNM1B foci positive cells with increasing doses of aphidicolin compared to untreated controls, the K398R-SNM1B mutant was severely defective in localization to aphidicolin-induced sites of stalled forks (Figure 4.2B). V5-SNM1B foci formation was also determined by analyzing the whole population of cells and similar results were found (Figure 4.2C). These results suggest that the K398 residue is important for efficient localization of SNM1B to sites of stalled replication forks.

I also assessed the ability of a nuclease deficient SNM1B (D14N) to localize to aphidicolin-induced stalled replication forks. D14N-SNM1B exhibited a moderate defect in forming foci (~25% foci positive cells) when compared to WT-SNM1B (~40%), but there was not as severe a defect in foci formation like what I observed with the K398R-

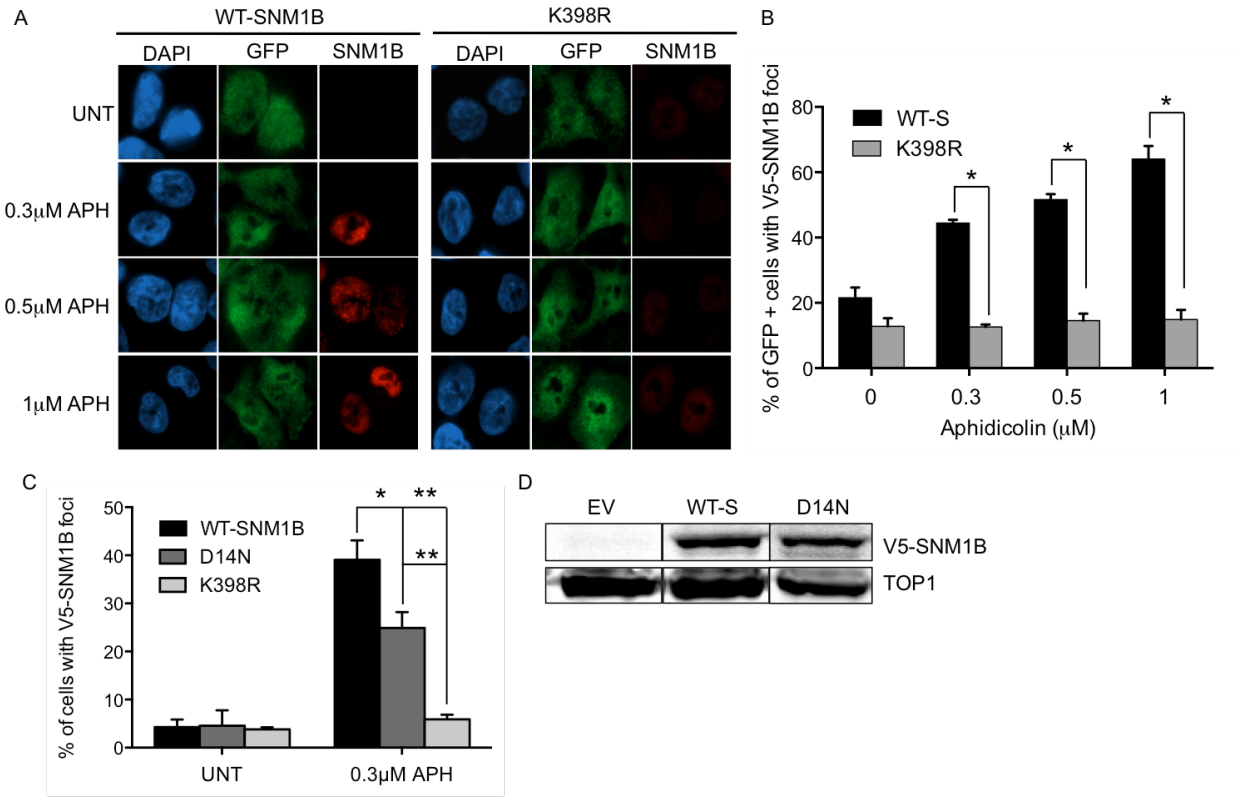


Figure 4.2 Identification of the K398 residue as being important for SNM1B localization to aphidicolin-induced stalled replication forks.

A. WT-SNM1B and K398R-SNM1B HCT116 cells were treated with increasing doses of aphidicolin (0.3, 0.5, and 1μM) for 24 hr. Cells were then co-stained with α-GFP and α-V5 antibodies, and V5-SNM1B foci were visualized by immunofluorescence microscopy. Representative images are shown. GFP (green). V5-SNM1B (red). Nuclei, DAPI stained (Blue).

B. The average percentage of GFP positive cells (green) that also contained V5-SNM1B foci staining (red) was quantitated. The graph represents the results from three independent experiments; at least 100 cells were scored in each experiment. Error bars, SEM. *p<0.02

C. WT-SNM1B, D14N, and K398R HCT116 cells were treated with 0.3μM aphidicolin for 24 hours and then fixed and stained with α-V5 antibody to detect V5-SNM1B foci formation. The average percent of cells with V5-SNM1B foci is plotted. Error bars, SEM. *p<0.05, **p<0.005

D. Whole cell lysates of EV, WT-SNM1B, and D14N-SNM1B pLL-IRES-GFP HCT116 cells were analyzed for V5-SNM1B protein levels. TOP1, loading control.

SNM1B mutant (~6%) (Figure 4.2C). Protein levels of the D14N SNM1B mutant was expressed at similar levels to WT-SNM1B in our stably expressing cell lines, as shown by the western blot (Figure 4.2D). Therefore, a nuclease deficient SNM1B can localize to stalled forks but not as effectively as WT-SNM1B, and the K398 residue is clearly important for ensuring SNM1B subnuclear localization.

SNM1B protein levels increase upon aphidicolin treatment and are regulated by proteasomal degradation.

Since the K398R-SNM1B mutant results in decreased FANCD2 foci formation and exhibits a marked impairment in forming foci upon aphidicolin treatment, protein levels of this mutant SNM1B were compared to WT-SNM1B to assess if this mutation results in an unstable SNM1B protein. WT-SNM1B and K398R-SNM1B HCT116 cells were treated with 1μM aphidicolin for 24 hours. Interestingly, an increase in SNM1B protein levels after aphidicolin treatment was consistently observed (a two-fold increase) in both WT-SNM1B and K398R-SNM1B cells (Figure 4.3A,B). As transcription of SNM1B is driven by a constitutive CMV promoter in the WT-SNM1B and K398R-SNM1B stably expressing cell lines, these results suggest that the SNM1B protein may be stabilized in response to DNA damage.

To assess if the stabilized SNM1B protein is chromatin associated, cellular fractionation experiments were performed. An increase in SNM1B protein levels after aphidicolin treatment in all fractions (cytoplasmic, nuclear, and chromatin-associated) for both WT-SNM1B and K398R-SNM1B (at least two-fold increase) was observed. There was also more SNM1B protein in the chromatin-associated fraction for untreated and aphidicolin treated cells in both WT-SNM1B and K398R cells (at least three-fold increase) (Figure 4.3C). These results indicate that SNM1B protein levels increase overall after aphidicolin treatment and that there is more chromatin-associated SNM1B protein in unperturbed proliferating cells. FANCD2 monoubiquitination was also analyzed as a control since the modified form of FANCD2 has previously been shown to be enriched in the chromatin fraction upon replication stress, which is what I observed [58].

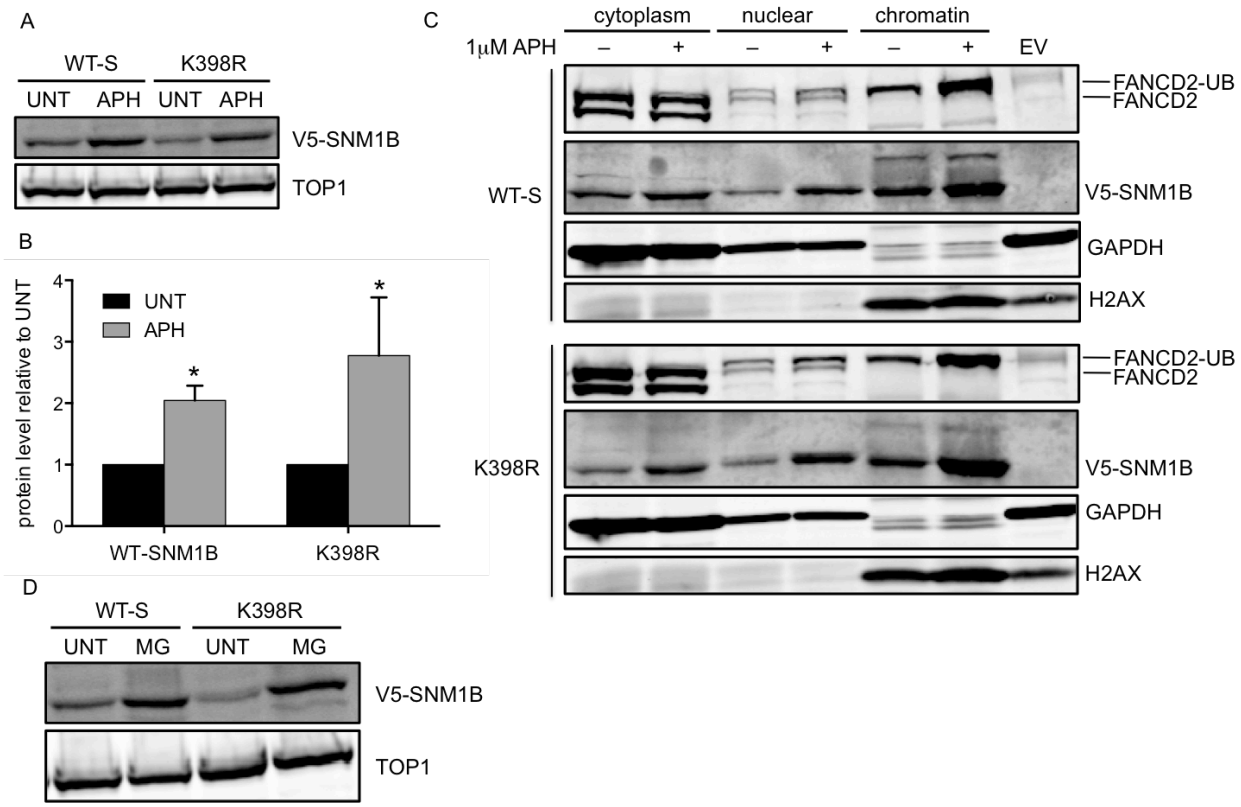


Figure 4.3 SNM1B protein levels increase upon aphidicolin treatment and proteasome inhibition

A. WT-SNM1B and K398R-SNM1B HCT116 cells were treated with 1μM aphidicolin for 24 hr, and whole cell lysates were analyzed by western blotting. V5-SNM1B protein levels were examined. Representative blots from at least three independent experiments are shown. TOP1, loading control.

B. Quantitation of western blots is shown. The protein levels were calculated relative to the untreated samples for each cell line. Error bars, SEM. *$p<0.05$

C. WT-SNM1B and K398R-SNM1B HCT116 cells were treated with 1μM aphidicolin for 24 hr. Cells were harvested and separated into cytoplasmic, nuclear, and chromatin-associated fractions. Western blotting to detect the unmodified and monoubiquitinated forms of FANCD2 was performed as a control. V5-SNM1B protein levels in the different fractions were assessed. GAPDH was a control for the cytoplasmic fraction while H2AX was a control from the chromatin-associated fraction. Representative blots from at least 2 independent experiments are shown.

D. WT-SNM1B and K398R-SNM1B HCT116 cells were treated with 10μM MG-132 (proteasome inhibitor) for 8hr and whole cell lysates were analyzed by western blotting. V5-SNM1B protein levels were examined. Representative blots from two independent experiments are shown. TOP1, loading control.

Since aphidicolin treatment results in an accumulation of cells in S-phase, the increase in SNM1B protein levels that I observe could be a result of SNM1B being under cell cycle control. SNM1B protein levels could potentially increase during the S-phase of normal proliferating cells. If SNM1B protein levels oscillate as a cell progresses through the cell cycle, inhibition of the proteasome should result in increased SNM1B protein levels since the protein is not degraded. Therefore, WT-SNM1B and K398R cells were treated with the proteasome inhibitor MG-132 and an increase (3 fold) in SNM1B protein levels in WT-SNM1B and K398R cell lines was found (Figure 4.3D). These results demonstrate that SNM1B protein levels are regulated through proteasomal degradation.

I have shown that protein levels of both WT-SNM1B and the K398R mutant increase after aphidicolin treatment, associate with the chromatin, and are under proteasome regulation (Figure 4.3). However, unlike WT-SNM1B, the K398R-SNM1B mutant is defective in aphidicolin-induced foci formation and FANCD2 foci formation (Figure 4.1, 4.2). Furthermore, since K398R-SNM1B could associate with the chromatin but was not detected to form foci, this residue could be important in localization of SNM1B to the specific site of a stalled replication fork. These results suggest that SNM1B protein levels, localization, and functions are regulated at multiple levels through different mechanisms. Moreover, there is the possibility that SNM1B has different roles repairing spontaneous replication stress versus induced stalled replication forks and consequently, how its functions are controlled are distinct for each type of damage.

Discussion

SNM1B localizes to aphidicolin induced stalled replication forks before FANCD2, and we have previously shown that depletion of SNM1B results in defective FANCD2 foci formation (Figure 3.1) [55]. Additionally, I now have found that mutation of a residue, K398, located within the conserved monoubiquitination motif in SNM1B, results in a significant defect in FANCD2 and SNM1B foci formation (Figure 4.1, 4.2). Taken together, these data demonstrate that this amino acid is required for SNM1B cellular functions. Furthermore, I showed that a nuclease deficient SNM1B is able to form foci

more efficiently that the K398R mutant but not to levels of WT-SNM1B (Figure 4.2). These data suggest that SNM1B is able to localize to the stalled fork, but since it cannot nucleolytically process any DNA structures that are present, perhaps it is removed. Because the orthologous residue in FANCD2 and FANCI undergoes monoubiquitination and plays critical roles for these proteins in proper localization, I hypothesize that SNM1B could also be modified on this residue to initiate its localization to stalled replication forks. Consequently, SNM1B nucleolytically processes intermediate DNA structures allowing room or generating binding sites for downstream repair proteins like FANCD2 to localize and initiate replication restart or repair.

Not only is SNM1B localization regulated by this lysine residue, but I also found evidence to suggest SNM1B protein levels are modulated. Increased SNM1B protein levels after aphidicolin treatment could be caused by cell cycle regulation of SNM1B protein (Figure 4.3A,B). Since aphidicolin induces arrest of cells in S phase, SNM1B protein levels may be elevated during the S phase of every cell cycle. Additionally, we observed an increase in SNM1B protein levels after aphidicolin treatment in the whole cell (cytoplasm, nucleus, and chromatin-associated fraction) (Figure 4.3C). If SNM1B plays a role during normal replication or responding to spontaneous replication stress, it would make sense that it is up-regulated during every S phase within the cell cycle similar to CtIP [45]. Furthermore, proteasome inhibition also resulted in a significant increase in SNM1B protein levels similar to aphidicolin treatment (Figure 4.3). These results show that SNM1B is proteasomally regulated and provide some evidence that it could be cell cycle regulated which may explain the aphidicolin-induced protein levels as well. In previous chapters I have shown that depletion of SNM1B in unperturbed cells results in increased chromosomal gaps and breaks, breaks at CFS, regions of unstable ssDNA, stalled forks, and collapsed replication forks, indicating SNM1B is critical during normal cellular proliferation [55,56].

The increase in SNM1B protein levels after aphidicolin treatment could also be damage induced. We did not observe a similar increase in SNM1B protein levels when cells were treated with mitomycin C (MMC) suggesting that the up-regulation of SNM1B protein levels may be damage specific since MMC induces cell cycle arrest in S phase similar to aphidicolin (Suppl. Fig. 4.1). If SNM1B protein is stabilized in response to

stalled replication forks, there would be an accumulation of the protein specifically in S phase. Therefore, one line of investigation would be to examine SNM1B protein levels in G1, S, and G2 phases of the cell cycle. Further studies analyzing cell cycle progression and investigating other damaging agents could define the mechanisms through which SNM1B protein levels are regulated.

Interestingly, when I analyzed protein levels of the K398R-SNM1B mutant, which is defective in foci formation, I found that its levels were similar to WT-SNM1B. K398R protein levels increased with both aphidicolin treatment and proteasome inhibition. K398R-SNM1B also localized to the chromatin fraction and the increase in SNM1B protein after aphidicolin was across all fractions, although more pronounced in the chromatin fraction (Figure 4.3). It has been shown that FANCD2 chromatin localization can be independent of foci formation as well [59]. Together, this suggests that SNM1B is regulated at multiple levels. The stability of SNM1B protein and its target for degradation is one way through which it is regulated while its localization is somehow regulated through the K398 residue. This lysine residue may be monoubiquitinated and future studies will address this hypothesis. Alternatively, this residue could be important in promoting SNM1B interactions with other proteins which are important for its recruitment or localization. Furthermore, SNM1B binding partners like the scaffold protein SLX4 or other nucleases like Mre11 and Mus81 could potentially have protein stabilizing effects that suppress degradation of SNM1B when it is needed for cellular functions.

The importance of nucleases in resolving replication stress is becoming increasingly evident. Therefore, understanding how the localization, enzymatic activities, and functions of these nucleases, including SNM1B, are regulated is a critical question to address. Faulty or absent regulation of DNA nucleases during replication and the DDR could have adverse affects resulting in genome instability. Determining how SNM1B functions are controlled may uncover mechanisms through which other nucleases or DNA repair proteins, including SNM1B binding partners, are regulated. Additionally, spontaneous replication stress occurs during every cell cycle in normal proliferating cells, and nucleases are critical in ensuring that the resolution of replication stress occurs correctly.

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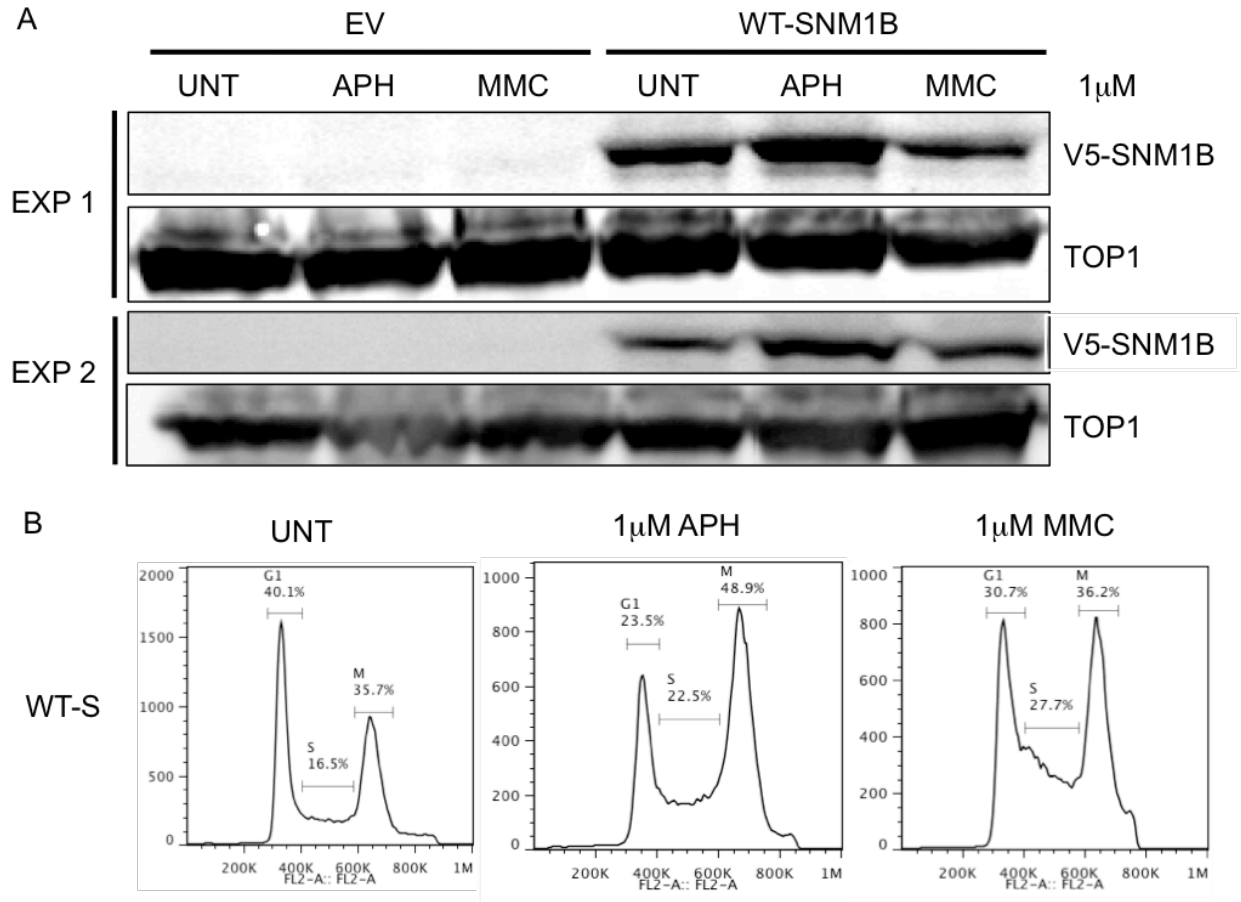

Supplemental Figure 4.1 SNM1B protein levels do not increase upon MMC treatment.

A. EV and WT-SNM1B HCT116 cells were treated with 1μM aphidicolin for 24 hours or 1μM MMC for 8 hours. Whole cell lysates were analyzed by western blotting. V5-SNM1B protein levels were examined. Blots from two independent experiments are shown. TOP1, loading control.

B. WT-SNM1B cells treated as above were stained with PI to analyze the cell cycle profile. FACS analysis was performed and cell cycle profiles were analyzed using FlowJo (TreeStar) software.

Chapter 5: Conclusions

Summary

Genomic DNA is constantly damaged through both exogenous and endogenous sources. Remarkably, one source of DNA damage is the fundamental process of DNA replication. This occurs during every cell cycle in proliferating cells. Progression of the replication fork can be blocked or disrupted through various ways leading to stalled replication. If these stalled replication forks are not restarted, they can collapse resulting in a double-stranded break (DSB). If not repaired, this DSB can engage in deleterious chromosomal rearrangements including deletions, insertions, and translocations. The DNA damage response (DDR) senses stalled replication forks and activates DNA repair pathways to resolve this genomic damage. Mutations in DNA repair proteins can result in genome instability disorders with phenotypes including developmental defects, immunodeficiency, and cancer predisposition. Therefore, understanding the mechanisms involved in the cellular response to replication-associated genomic damage is critical in uncovering how genome integrity is maintained.

The DNA nuclease SNM1B has functions in telomere protection, DSB repair, and repair of interstrand crosslinks (ICLs). In this thesis, I have established that SNM1B also has important roles in repairing stalled replication forks that result from DNA polymerase inhibition and during normal cellular proliferation. We have found that SNM1B is important for cellular survival upon aphidicolin-induced replication fork stalling. We showed that SNM1B is not required for sensing stalled replication forks, the activation of ATR-dependent signaling, or localization of DNA repair proteins that function during early events in the DDR. We demonstrated that SNM1B is important for efficient localization of key repair proteins during the later steps of these repair processes. We found that SNM1B plays a key role in preventing replication- associated DNA damage. I also have begun to uncover how SNM1B protein stability, localization, and functions are regulated during the repair of stalled replication forks. These findings indicate that

SNM1B is important for restarting stalled replication forks and repairing collapsed forks and thereby critical for preventing genome instability.

In this chapter, I will discuss the implications of these novel functions of the SNM1B DNA nuclease. Future directions will also be proposed to further understand the precise mechanisms through which SNM1B prevents replication-associated genomic damage and maintains overall genome stability.

SNM1B is not required for the detection of stalled replication forks or the activation of early events in the DDR.

My studies have provided evidence that SNM1B has functions in restarting stalled replication forks after the early signaling events of the DDR have been activated. In order to activate the DDR at a stalled fork, long stretches of single-stranded (ssDNA) are needed for RPA (Replication Protein A) binding which initiates ATR (ataxia telangiectasia and Rad3 related) kinase signaling. The MRN (Mre11/Rad50/Nbs1) complex has been shown to colocalize with RPA upon replication fork stalling [1,2]. I found that both RPA and Mre11 localize quickly (within 1 hour of aphidicolin treatment) to foci (Figure 3.1). SNM1B depletion did not affect localization of RPA and the MRN complex or ATR-dependent signaling, which are all early events that occur in the cellular response to stalled forks (Figures 2.1B, 2.2).

Interestingly, we have previously found that SNM1B is needed for ATR-dependent signaling in response to MMC-induced ICLs [3]. However, we have now shown that SNM1B is dispensable for these signaling events after aphidicolin-induced replication stress [4]. This phenotypic difference suggests that SNM1B has different roles in responding to blocked versus stalled forks. At a blocked fork, ssDNA needs to be generated while upon DNA polymerase inhibition, the polymerase and helicase uncouple, resulting in the helicase unwinding the DNA which forms long stretches of ssDNA [5-7]. Therefore, SNM1B may be responsible for generating ssDNA at an ICL to activate ATR-dependent signaling while at an aphidicolin-induced stalled fork, SNM1B functions after ATR-depending signaling has already been initiated.

Once a stalled fork has been recognized and the DDR is activated, the cell initiates processes that will restart the stalled fork. One mechanism is through fork

regression as previously explained in Chapter 1 (Figure 1.3). Mre11 has been implicated in nucleolytically digesting the nascent leading strand at a 'chicken-foot' structure with its 3' to 5' exonuclease activity. At a regressed fork, once a 3' to 5' exonuclease, like Mre11, begins to digest the nascent leading strand, a 5' to 3' exonuclease needs to resect the nascent lagging strand. I found that SNM1B forms foci upon aphidicolin treatment, providing evidence that it localizes to sites of stalled replication forks (Figure 2.6). Furthermore, I demonstrated that SNM1B localizes to these stalled forks after both RPA and Mre11 localization, suggesting SNM1B functions after Mre11 perhaps by digesting the nascent lagging strand at a regressed fork (Figure 3.1). It has been shown that degradation of a regressed fork by Mre11 is regulated by the FA/BRCA network [8-10].

SNM1B functions within the FA/BRCA network in the repair of stalled replication forks by recruiting key DNA repair proteins

The Fanconi anemia (FA) pathway has roles in ICL repair and responding to replication stress. We have previously shown that SNM1B functions within the FA pathway in repair of MMC induced ICLs [3]. Furthermore, SNM1B depleted cells have similar phenotypes to FA patient cells including hypersensitivity to ICL inducing agents and ionizing irradiation (IR) and exhibition of chromosomal anomalies [11-13]. I found that SNM1B localizes to stalled forks before FANCD2 and that SNM1B is needed for efficient FANCD2 foci formation to aphidicolin-induced stalled replication forks (Figures 3.1, 2.5). Importantly, FANCD2 monoubiquitination, which is important for FANCD2 foci formation, is not affected by SNM1B depletion (Figure 2.3). Interestingly, the nuclease activity of SNM1B was found to be important for FANCD2 foci formation (Figure 2.4). These data provide additional evidence that SNM1B may act in processing a regressed fork, after Mre11 has begun digesting the nascent leading strand, by nucleolytically digesting the nascent lagging strand, and thereby generates binding sites for FANCD2 and other DNA repair complexes which work to stabilize the fork and promote restart. I also found that SNM1B depletion results in deficient BRCA1 foci formation upon aphidicolin treatment (Figure 2.5). BRCA1 depletion results in defects in FANCD2 foci formation but does not affect monoubiquitination, similar to what is observed upon

SNM1B knockdown [14,15]. Therefore, SNM1B could generate a binding site for BRCA1 which then recruits FANCD2, forming a complex with classic HR proteins, BRCA1, BRCA2, and Rad51, which have interestingly been shown to be important prior to the formation of DSBs at collapsed forks by stabilizing stalled forks and facilitating restart of replication [16-19]. This FA/BRCA complex then prevents uncontrolled degradation of regressed forks by Mre11 [9,20].

In this model (Figure 5.1), SNM1B acts on regressed forks after Mre11 has begun nucleolytic digestion of the annealed nascent strands. Once SNM1B nucleolytically processes the regressed fork and generates binding sites, the FA/BRCA complex is recruited to the stalled fork and works to stabilize the fork, one mechanism of which is to prevent extensive degradation of the nascent strands by Mre11.

SNM1B prevents accumulation of ssDNA and chromosomal aberrations during the repair of stalled replication forks by processing aberrant DNA intermediates.

If SNM1B functions within the FA/BRCA network, when SNM1B is absent we would expect replication fork destabilization. For example, if SNM1B is not present, the nascent lagging strand remains exposed and single-stranded while Mre11 continues to degrade the nascent leading strand with no regulation. When I analyzed regions of ssDNA, using the BrdU immunofluorescence assay explained in Chapter 3, SNM1B depleted cells exhibited an increase in ssDNA after 24 hours of aphidicolin treatment but not after 6 hours (Figure 3.2). Similar results were found when analyzing RPA foci formation which binds to ssDNA (Figure 2.2). These results show that SNM1B is important in preventing the accumulation of ssDNA once the DNA polymerase has been inhibited for a longer time (24hr). However, upon SNM1B depletion, I did not observe a significant difference in the percent of stalled forks after aphidicolin treatment (24h) compared to treated controls using the DNA fiber assay (Figure 3.4). Furthermore, in order to analyze collapsed replication forks, I used PFGE to assess the amount of broken DNA from intact chromosomes and found that SNM1B depletion did not result in a significant increase in collapsed replication fork formation after aphidicolin treatment

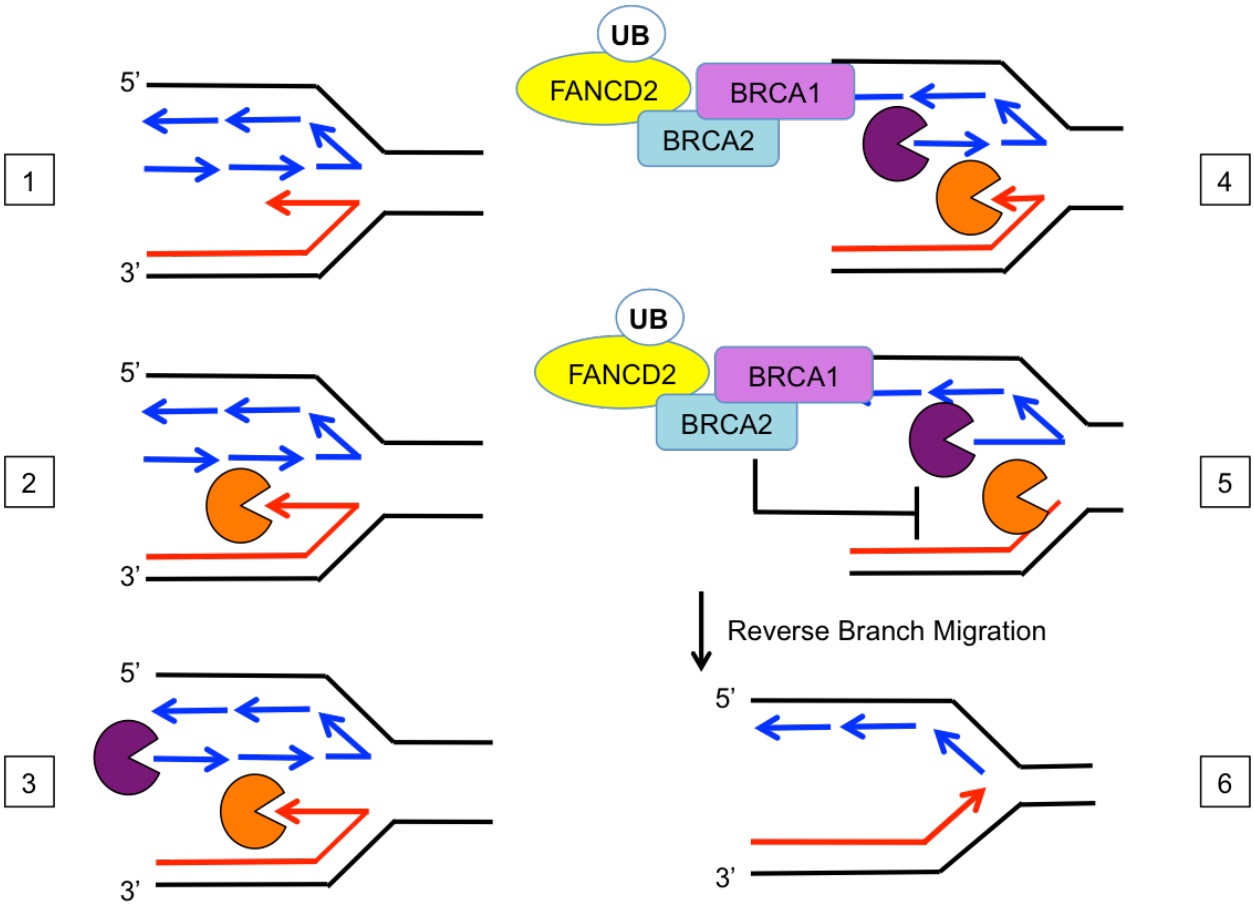

Figure 5.1: Potential role of SNM1B in processing a regressed fork

One mechanism through which a stalled fork is restarted is (1) fork regression, which results in a 'chicken-foot' structure where the nascent leading (red) and lagging (blue) strands have annealed together. (2) Nuclease digestion to process the regressed fork can involve Mre11 (orange) mediated 3' to 5' exonuclease activity of the nascent leading strand. (3) One working model is that SNM1B (purple) could then process the nascent lagging strand with its 5' to 3' exonuclease activity. (4) This processing results in formation of binding sites for downstream repair factors like members of the FA/BRCA network. (5) Once localized to the fork, these factors work to regulate Mre11 digestion to prevent hyper-resection of the fork. (6) Once nuclease digestion of the regressed fork is complete, reverse branch migration resets the replication fork.

compared to controls (Figure 3.5). Therefore, the unstable regions of ssDNA seem to result from excessive resection at the regressed fork forming longer stretches of ssDNA potentially from uncontrolled Mre11 digestion in the absence of SNM1B (Figure 5.1).

There can also be another nuclease that uncontrollably digests the DNA at the regressed fork suggesting SNM1B could be involved in regulating the functions of other nucleases. For example, the nuclease FAN1 (FANCD2/FANCI-associated nuclease 1) has been implicated in digesting nascent DNA strands at regressed forks. FAN1 is in a complex with FANCD2 and BLM helicase, and its functions seem to be regulated by both FANCD2 and Mre11 [21,22]. Therefore, SNM1B is likely collaborating with other nucleases to ensure proper restart of stalled replication forks. Studies determining if nucleases like Mre11, FAN1, or DNA2 (human nuclease/helicase 2) regulate SNM1B localization or functions, or vice versa, would provide insights into how nucleases function in concert to respond to stalled replication forks. Moreover, SNM1B is known to associate with Mre11, Mus81, and SLX4; therefore, it would be interesting to examine the functional importance of these interactions in the response to stalled replication forks [23-27]. Furthermore, DNA combing experiments will allow us to analyze the dynamics of replication fork progression besides replication fork restart; for example, replication tract lengths and replication rates. These experiments will provide a way to examine if SNM1B depletion results in hyper-resection at a stalled replication fork.

The persistence of longer stretches of ssDNA upon SNM1B depletion suggests that this region of ssDNA is not unstable because it is available for nuclease digestion and therefore has potential to break. Even though we did not find an increase in collapsed forks in SNM1B depleted cells, we did observe an increase in breaks at common fragile sites (CFSs) after aphidicolin treatment (Figures 3.5, 2.8). CFSs are regions that are more ‘fragile,’ so more unstable ssDNA might be present and therefore these regions are more prone to break. To analyze replication fork dynamics at CFSs, DNA combing experiments combined with FISH using probes that recognize CFSs can be performed. We also observed an overall increase in gaps and breaks in metaphase chromosomes from SNM1B deleted cells after aphidicolin treatment, which provides evidence that SNM1B is important for preventing replication-associated genomic damage.

Although after aphidicolin treatment in SNM1B depleted cells, we did not observe increased stalled or collapsed forks, in unperturbed cells we saw a significant increase in both. Spontaneous replication fork stalling, if not repaired, can lead to collapsed forks; therefore, SNM1B may also play a role in repairing collapsed forks. There may be specific regions of the genome or DNA structures that are more susceptible to breakage when SNM1B is depleted.

SNM1B facilitates the recruitment of HR proteins to collapsed replication forks

Previous evidence has shown that SNM1B has a role in general DSB repair, as explained in Chapter 1. For example, SNM1B is known to generate 3' overhangs at leading end telomeres through its 5' to 3' exonuclease activity. Once this resection results in enough room for a shelterin complex component, POT1b, to bind to the overhang, POT1b limits the nuclease activity of SNM1B. This results in the exonuclease, Exo1, to generate a longer 3' overhang which recruits further downstream factors important in telomere maintenance [28,29]. Therefore at telomeres, SNM1B works with another nuclease to maintain telomere length, and similar mechanisms could be employed at collapsed forks.

In the previous section I described a model in which SNM1B plays a role prior to fork collapse by processing a regressed fork and generating binding sites for the recruitment of repair proteins. Another model is that SNM1B plays a role after fork collapse once the break has already been generated. In this model, SNM1B may have functions in facilitating events that occur to repair a collapsed replication fork through HR (Figure 5.2). I have shown that SNM1B is not needed for the generation of the break that forms a collapsed fork, which has been partly attributed to Mus81 [30,31]. The 5' to 3' exonuclease activity of SNM1B could be responsible for generating a 3' overhang at the one-ended DSB at a collapsed fork, which results in Rad51 filament formation along with assembly of HR proteins and strand invasion for HR. I found that SNM1B depletion in cells treated with aphidicolin for 24 hours results in a defect in Rad51 foci formation, which could lead to defects in FA/BRCA complex formation, as we have shown (Figure 3.6, 2.3, 2.5). Another possibility is that SNM1B may play a role in regulating RPA

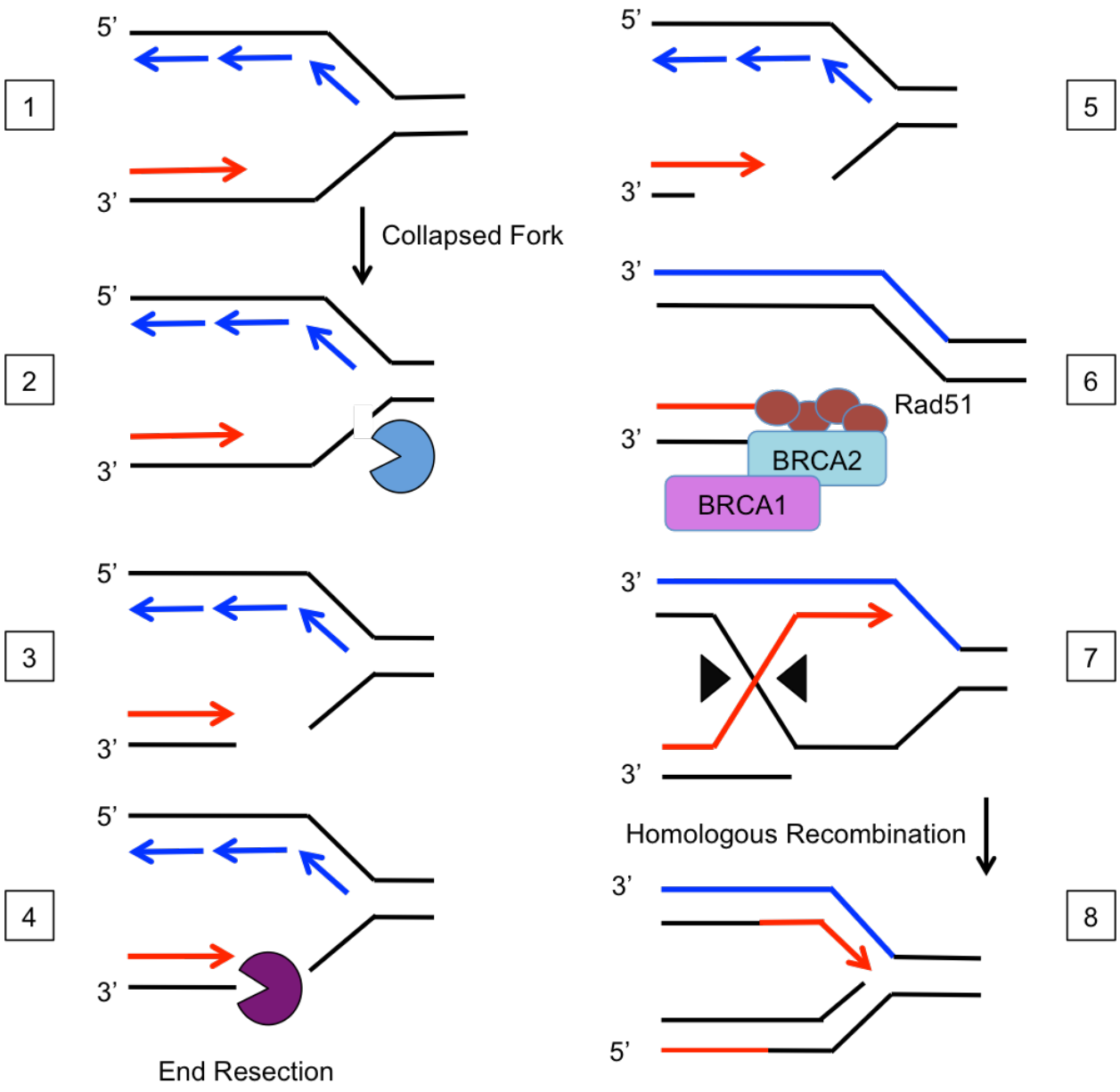

Figure 5.2: Potential role of SNM1B in repairing collapsed replication forks

If a stalled replication fork cannot be restarted, (1) it can collapse. (2) An endonuclease like Mus81 (blue) generates a break in the unstable ssDNA region at a stalled replication fork. (3) This results in a one-ended DSB, which activates the DSB response. (4) SNM1B (purple) through its 5' to 3' exonuclease activity could be involved in end resection of the DSB, (5) resulting in a 3' overhang. (6) This leads to Rad51 filament formation and recruitment of HR proteins, (7) which initiate HR (8) to repair the break, and the replication fork continues to progress.

displacement from the ssDNA to allow for Rad51 filament formation. Therefore, upon SNM1B depletion we observe increased ssDNA which RPA binds to, but a defect in Rad51 foci formation (Figures 3.6, 2.2A, 3.3, 3.2). Detection of Rad51 foci through immunofluorescence requires several kilobases of ssDNA, perhaps explaining why I did not observe induction of Rad51 foci upon shorter aphidicolin treatment, even though it has been shown that Rad51 is important in stabilizing stalled replication forks along with its roles at collapsed forks (Figure 3.6) [32]. Therefore, SNM1B may be responsible for generating a certain length of ssDNA overhang to initiate RPA displacement by Rad51.

Future studies to determine why SNM1B depletion results in defective Rad51 foci formation could utilize the Rad51 stabilizing mutant (K133R) which does not possess ATPase activity that is needed for Rad51 dissociation from DNA. Therefore, this mutant results in hyper-stable Rad51 filaments, and we may be able to analyze if this mutant Rad51 can rescue any phenotypes we have observed upon SNM1B depletion, including increased RPA foci formation and regions of ssDNA [9,33].

In this model we propose that SNM1B is important for facilitating the assembly of HR proteins to ssDNA regions at collapsed replication forks allowing for HR-mediated repair. It is important to remember that SNM1B may function differently in repairing DSBs that are induced by IR, during the repair of ICLs, collapsed forks that result from inefficient restart of aphidicolin-induced stalled forks, and during the repair of spontaneous replication-associated breaks. For example, SNM1B may be important for efficient ATM-dependent signaling in response to a DSB caused by IR, as has been previously suggested [34]. During the repair of ICLs, removal of the crosslink generates a break, and SNM1B could be involved in this step. Additionally, like explained above SNM1B could be important for recruitment of repair proteins to collapsed forks to initiate HR. Finally, SNM1B could have distinct functions in repairing DSBs at collapsed forks and restarting stalled forks, similar to components of the FA/BRCA network that have critical roles in both these processes (Figure 5.1, 5.2).

SNM1B is important in the resolution of replication stress in normal proliferating cells

We have observed that in unperturbed cells with SNM1B knockdown there is an increase in chromosomal aberrations, breaks at CFSs, regions of ssDNA, frequency of

stalled forks, and broken DNA. This increase in genome instability upon SNM1B deletion in normal proliferating cells can partly be attributed to telomere defects [35-37]. Furthermore, these striking phenotypes suggest that SNM1B plays a role in responding to spontaneous replication stress and perhaps even in normal DNA replication.

Replication forks can stall when encountering endogenous sources of stress including secondary structures, collision with transcription machinery, and protein:DNA adducts, for example [38]. It is important to note that some stalled forks resulting from endogenous sources of replication stress are different from aphidicolin-induced stalled forks. During spontaneous replication stress, there might be a physical block in progression of the replication fork, and therefore no generation of ssDNA. On the other hand, aphidicolin treatment results in long stretches of ssDNA as previously described. SNM1B has been shown to interact with PSF2 which is a component of the CMG helicase complex [39]. Several FA proteins have also been found to interact with PSF2 and have been shown to associate in a complex at replication forks in unperturbed cells [40]. Therefore, through its interaction with PSF2, SNM1B could be recruited to not only stalled replication forks but normal progressing forks.

SNM1B may have roles in removing secondary DNA structures that are difficult to replicate by working with other factors. A study found that SNM1B and TRF2 work with topoisomerase 2α to relieve topological stress of inner telomeres when telomeres are being replicated. They found that the binding of TRF2 and SNM1B to telomeres decreased the amount of TOP2 at telomeres during S phase. They concluded that SNM1B processes barriers at telomeres or prevents fork reversal [41]. It is known that TRF2 prefers binding to positive supercoiled DNA not just at telomeres, so TRF2 could potentially play a role through its association with SNM1B at other regions throughout the genome, not just at telomeres. Unregulated unwinding of the DNA double helix ahead of a stalled replication fork could result in more topological stress and positive supercoiling [42]. SNM1B could potentially be involved in relieving this strain as it does at telomeres.

BLM, WRN, and FANCJ are three helicases that SNM1B may potentially work in a complex with to process torsional strain or DNA secondary structures. FANCJ processes secondary structures at G-rich regions [43]. These need to be processed so

that ssDNA can form for efficient replication to occur. G-quadruplexes are resolved by the WRN helicase [44,45], and it was shown that DNA2 degrades reversed forks with WRN helicase [46]. Therefore, SNM1B could be involved with a helicase to process intermediate DNA structures and restart replication. Studies analyzing SNM1B association and colocalization with these helicases would provide insights into how SNM1B may be involved in preventing spontaneous replication stress.

Regulation of SNM1B functions

I have found that SNM1B plays critical roles in responding to aphidicolin-induced stalled replication forks and in maintaining genome stability. However, the mechanism through which SNM1B functions are regulated during these processes is not defined. I found that SNM1B forms foci after aphidicolin treatment (Figure 2.6), and the localization of many DNA repair proteins is regulated through post-translations modifications (PTMs). For example, FANCD2 and FANCI are monoubiquitinated by the FA core complex in response to DNA damage. This PTM is important for localization of FANCD2 and FANCI to repair foci [12,13]. Interestingly, we identified a lysine residue in SNM1B (K398) that was within a semi-conserved motif (K**K**QL) where FANCD2 (R**K**QL) and FANCI (R**K**AM) are monoubiquitinated (Figure 4.1A) [47].

I identified that this residue within SNM1B is important for localization of SNM1B to stalled forks induced by aphidicolin (Figure 4.2). Furthermore, I found that mutation of this residue (K398R) results in a defect in FANCD2 foci formation (Figure 4.1B,C). If this residue is a possible monoubiquitination site, when there is a defect in this PTM, SNM1B is not able to localize to the stalled fork and therefore downstream factors, like FANCD2, are not recruited as efficiently. Mass spectrometry analysis to identify if this residue is indeed monoubiquitinated can be performed. There may be other residues that are important for SNM1B localization or other PTMs that are relevant for SNM1B functions in the response to different damaging agents like MMC or IR. These additional PTMs (like phosphorylation or SUMOylation) can be identified through mass spectrometry as well. The functional relevance of these residues can be further investigated using site-directed mutagenesis of *SNM1B*.

There is also the possibility that this lysine reside is not modified but instead is important in mediating SNM1B interactions with other proteins. These protein interactions may be critical for the ability of SNM1B to localize to damaged DNA sites. Therefore, disruption of these potential protein interactions could result in decreased SNM1B localization to stalled forks and subsequent defects in recruitment of FANCD2 to the stalled replication fork.

Although the K398R SNM1B mutant had defects in foci formation to sites of stalled replication forks, I found that protein levels of this mutant SNM1B were similar to wildtype SNM1B (Figure 4.3A). Cellular fractionation experiments demonstrated that both WT-SNM1B and K398R SNM1B protein was found in the chromatin-associated fraction. I also observed that after aphidicolin treatment there was an increase in SNM1B protein in not only the chromatin fraction but in the cytoplasmic and nuclear fractions as well (Figure 4.3C). I have consistently observed that after aphidicolin treatment there is a two-fold induction of SNM1B protein in whole cell lysates. The K398R mutant was found to be similar to WT-SNM1B, suggesting that this mutant can associate with chromatin but is not localizing to sites of stalled replication forks (Figure 4.3A,B).

SNM1B protein levels have previously been reported to be low in cells, and I found that treatment with a proteasome inhibitor resulted in an increase in SNM1B protein levels (Figure 4.3D). Therefore, this demonstrates that SNM1B protein is regulated through proteasome degradation. Since I observed an increase in SNM1B protein after aphidicolin, this increase could mean that upon induction of stalled replication forks SNM1B protein levels are stabilized. I found that MMC treatment did not result in this increase in SNM1B protein levels (Supp. Fig. 4.1A). Analyzing other DNA damaging agents like HU and IR would provide insight into if this increase in protein levels is for a specific type of DNA damage. Another explanation is that SNM1B protein levels could be cell cycle regulated. Since aphidicolin treatment results in cells being arrested in S phase, there is an accumulation of S phase cells and this could explain the increase in SNM1B protein I observe. Analyzing SNM1B protein levels in synchronized cells would provide insights into this hypothesis.

One mechanism through which SNM1B protein can be stabilized is by protein interactions. One study found that the interaction of SNM1B with TRF2, a component of the shelterin complex, stabilized SNM1B protein levels. It was shown that upon TRF2 binding to SNM1B, polyubiquitination of SNM1B was inhibited therefore preventing degradation of SNM1B via the proteasome. Furthermore, it was demonstrated that expression of an SNM1B mutant that was not degraded resulted in increased cell death suggesting that SNM1B could be nucleolytically digesting DNA with no control [35,48].

This provides further evidence as to why SNM1B protein levels are kept low and also as to why SNM1B nuclease activity needs to be tightly controlled during its functions in repairing stalled replication forks. This study however did not address the fact that SNM1B has functions beyond its role at telomeres and concluded that when SNM1B is not TRF2 bound it is degraded. However, when SNM1B is needed for something other that telomere processing, like restarting a stalled fork or repairing a collapsed fork, it could be stabilized through another mechanism. Perhaps another protein regulates SNM1B protein levels upon entrance into S phase or upon induction of stalled replication forks. One possibility is that SNM1B protein could be cell cycle regulated where it is induced at a certain point within the cell cycle, then further regulated via protein interactions that promote protein stabilization, and then localization to sites of stalled forks is regulated separately, potentially through other protein interactions or PTMs.

Since SNM1B protein levels are expressed as such low levels in the cell, it is very difficult to detect endogenous SNM1B with the available antibodies. Therefore, all these experiments have been conducted by ectopic expression of V5 tagged SNM1B. One way to analyze if endogenous SNM1B protein levels behave similarly could be to use the CRISPR/Cas9 genome editing system to tag the endogenous SNM1B locus [49,50]. In our cellular system, transcription of SNM1B is driven by a constitutive CMV promoter. Therefore, it would also be important to analyze endogenous mRNA transcript levels of SNM1B throughout the cell cycle and upon aphidicolin treatment.

<u>SNM1B has critical functions during the resolution of replication stress which can have implications for human disease.</u>

Nucleases are critical to the maintenance of genome stability. Defects in nucleases and other DNA repair proteins lead to deleterious chromosomal rearrangements that can lead to human diseases like Fanconi anemia or cancer [38]. A truncation mutant of SNM1B was identified in a patient with a severe form of dyskeratosis congenita, Hoyeraal-Hreidarsson syndrome, who exhibited phenotypes including microcephaly, bone marrow failure, and developmental defects. Cells expressing this truncation mutant had defects in telomere functions but did not exhibit hypersensitivity to ICL inducing agents, providing further evidence that SNM1B has separate functions in telomere protection and the DDR [51]. The *SNM1B* locus is emerging as being mutated in a variety of cancer types [52]. In one study, SNM1B was mutated in half of primary mediastinal B cell lymphomas, and the *SNM1B* locus has been mapped at a chromosomal breakpoint associated with Wilms tumor [53,54]. Defects in SNM1B functions in processing aberrant DNA intermediates at stalled forks to facilitate replication restart or in repairing collapsed forks could lead to an accumulation of deleterious oncogenic chromosomal rearrangements.

We showed in Chapter 2 that SNM1B depletion results in increased breaks at common fragile sites (CFSs) in both unperturbed and aphidicolin treated cells (Figure 2.8). These results demonstrate that SNM1B is important in preventing instability at these specific sites that are susceptible to replication-induced genomic damage. It has also been shown that deletions and duplications in the genome, copy number variants (CNVs), can arise from defects in replication [55-59]. Replication stress induced by aphidicolin, hydroxyurea (HU), or low dose IR produces CNVs [60-62]. Interestingly, certain hotspots for CNVs overlap with CFSs and are present at areas of active transcription of large genes [63]. These results suggest that areas of the genome where transcription of large genes and replication occur simultaneously are more prone to breaks, which can lead to deleterious CNVs. However, the proteins involved in these processes and the mechanistic details have not been uncovered. Since SNM1B is important in preventing breaks at CFSs, analyzing any changes in frequency of CNVs upon SNM1B depletion would provide insights into not only the mechanism behind formation of these rearrangements but also provide insight into if SNM1B has a role in resolving replication stress induced by the collision of replication and transcription machinery.

Since CNVs can result from aphidicolin, IR, and HU, determining how SNM1B depletion affects HU induced stalled replication forks is a potential future area of investigation. Another source of replication stress is over expression of oncogenes which drives cells into S-phase as described in Chapter 1. Therefore, it would be interesting to determine if the hyper-proliferation and hyper-replication of these cells in S phase affects SNM1B protein levels.

In conclusion, in this thesis I have found that the SNM1B DNA nuclease has critical roles in the response to spontaneous and induced stalled replication forks. Together, my findings demonstrate that SNM1B functions downstream of ATR-dependent signaling in the response to stalled replication forks. SNM1B is likely important in processing aberrant DNA intermediates thereby providing binding sites for downstream repair proteins in the FA/BRCA network to localize and facilitate the restart of replication fork progression. Furthermore, SNM1B also has critical roles in responding to endogenous sources of replication stress that occur during every S phase of the cell cycle. I have also begun to uncover how SNM1B functions during these repair processes are regulated. SNM1B is an important player in the prevention of potentially deleterious rearrangements resulting from replication-associated genomic damage. By further defining the functions of SNM1B nuclease activity in facilitating complete replication of the genome, we can understand the cellular mechanisms that are in place to maintain genome stability and prevent chromosomal anomalies including potential oncogenic translocations.

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