NBS1 DEFICIENCY PROMOTES GENOME INSTABILITY BY AFFECTING DNA DAMAGE SIGNALING PATHWAY AND IMPAIRING TELOMERE INTEGRITY

HOU YANYAN

NATIONAL UNIVERSITY OF SINGAPORE

2012

NBS1 DEFICIENCY PROMOTES GENOME INSTABILITY BY AFFECTING DNA DAMAGE SIGNALING PATHWAY AND IMPAIRING TELOMERE INTEGRITY

HOU YANYAN

(*Bachelor of Science, HUST*)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

NATIONAL UNIVERSITY OF SINGAPORE

2012

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my supervisor, Dr. Sherry Wang Xueying, from Department of Biochemistry, National University of Singapore. I was accepted as the first graduate student in Dr. Wang's lab two and a half years ago, which I feel extremely lucky and fortunate. Dr. Wang's enthusiasm to research and science infects me and motivates me all the time. Her encouragement, patience and advices are the source for me to overcome difficulties, get through the "dark times" and grow up as both a researcher and an individual. This thesis would not have been possible without her help in every aspect.

And a special thanks to my group member, Mr Toh Meng Tiak, for his help in many experiments.

I would also like to thank all of my lab members: Dr. Zhang Yong, Mr. Chai Juin Hsien, Miss Tay Ling Lee, Miss Dashayini Mahalingam, Miss Kong Chiou Mee and Miss Toh Ling Ling for their support, encouragement and invaluable insights throughout the course of this project.

Lastly and most importantly, I would like to thank my family members for their continuous moral support and encouragement which gives me strength to plod during my graduate study.

TABLE OF CONTENTS

SUMMARY

Nijmegen Breakage Syndrome (NBS), a rare autosomal recessive disorder typically caused by mutations in *NBS1* gene, is characterized by immunodeficiency and a strong predisposition to cancer. Studies revealed that NBS1 plays an important role in maintaining genome stability, but the underlying mechanism is controversial and elusive.

Our study used NBS cells derived from NBS patients with 657del5 mutation in *NBS1* gene as well as normal cells with wild type *NBS1* gene to examine the roles of NBS1 in maintaining genome stability. Our results showed that NBS1 was involved in ataxia-telangiectasia mutated (ATM)- and ataxia-telangiectasia and Rad3-related (ATR)-dependent DNA damage signaling pathways. NBS1 deficiency led to a decrease in the phosphorylation level of ATM and ATR as well as their downstream targets, including histone H2AX, p53, Chk1 and Chk2. The inefficiency in activating DNA damage signaling pathway led to multiple defects in cellular responses towards DNA damage. BrdU proliferation assay revealed a delay of NBS cells in inhibiting DNA synthesis after Doxorubicin (Dox) treatment. In addition, under high concentration of 1μM Dox, NBS cells exhibited 15% ~ 25% lower level of apoptosis compared to their normal counterparts, indicating a resistance to Dox treatment.

Accelerated telomere shortening was also observed in NBS fibroblasts, consistent with an earlier onset of cellular replicative senescence *in vitro*. This abnormality may be due to the shelterin protein telomeric binding factor 2 (TRF2) which was found to be upregulated in NBS fibroblasts. However, both accelerated telomere shortening and upregulation of TRF2 were not observed in NBS B-lymphocytes, although these cells

showed earlier occurrence of senescence-associated apoptosis. These results suggest that NBS1 deficiency exerts different regulatory effects on fibroblasts and B-lymphocytes even with the same type of gene mutation. Dysregulation of telomere shortening rate and TRF2 expression level in NBS fibroblasts led to frequent telomere end-to-end fusions and cellular aneuploidy.

Collectively, our results suggest a possible mechanism that NBS1 deficiency simultaneously affects ATM- and ATR-dependent DNA damage signaling and TRF2-regulated telomere maintenance, which synergistically leads to genomic abnormalities.

LIST OF TABLES

INTRODUCTION

MATERIALS AND METHODS

LIST OF FIGURES

INTRODUCTION

MATERIAL AND METHODS

RESULTS

DISCUSSION

APPENDICES

LIST OF ABBREVIATIONS

NBS: Nijmegen breakage syndrome

ATM: ataxia-telangiectasia Mutated

ATR: ataxia-telangiectasia and Rad3-related

ATLD: ataxia-telangiectasia-like disorder

DSB: double strand break

SSB: single strand break

FHA: forkhead-associated domain

BRCT: BRCA1 C-terminus domain

PI3K: phosphatidylinositol 3-kinase

PIKK: PI3K-like protein kinases

IR: ionizing radiation

MDC1: mediator of DNA damage checkpoint protein

53BP1: p53 binding protein 1

HU: hydroxyurea

Dox: doxorubicin

Ser: serine

Thr: threonine

NER: nucleotide excision repair

BER: base excision repair

RPA: replication protein A

PUMA: p53 upregulated modulator of apoptosis

BAX: BCL2-associated X protein

BrdU: 5-Bromo-2’-deoxy-uridine

PI: propidium iodide

PARP: poly-ADP-ribose-polymerase

PDLs: population doubling levels

TERT: telomerase reverse transcriptase

TER: telomerase RNA template

snoRNA: small nucleolar RNA

hnRNP: heterogeneous nuclear ribonucleoprotein

TRF1: telomeric repeat-binding factor 1

TRF2: telomeric repeat-binding factor 2

POT1: protection of telomeres 1

RAP1: the human ortholog of the yeast repressor/activator protein 1

TIN2: the TRF1- and TRF2-interacting nuclear protein 2

TPP1: the POT1-TIN2 organizing protein

XRS2: the ortholog of NBS1 in yeast

WRN: gene mutated in Werner syndrome

BLM: gene mutated in Bloom syndrome

PINX1: PIN1-interacting protein 1

TIFs: telomere dysfunction induced foci

HR: homologous recombination

NHEJ: non-homologous end joining

ALT: alternative lengthening of telomeres

E1A: the adenovirus early 1A region

RDS: radio-resistant DNA synthesis

DMEM: Dulbecco's modified eagle medium

MEM: minimum essential medium

FBS: fetal bovine serum

CCR: Coriell Cell Repositories

RPMI-1640: Roswell Park Memorial Institute-1640

NEAA: non-essential amino acid

HRP: horseradish peroxidase

LB: lysogeny broth

1. INTRODUCTION

1.1 NBS and NBS1 protein

NBS is a rare autosomal recessive disorder which was first delineated in 1981 by C. Weemaes and colleagues. NBS is characterized by immunodeficiency, microcephaly, growth retardation, congenital malformations and a strong predisposition to malignancies, especially to B-cell lymphoma (The International Nijmegen Breakage Syndrome Study Group 2000). The main causes of death in NBS patients are lymphoid malignancy and infectious complications of immunodeficiency (Resnick, Kondratenko et al. 2002). A study of 55 NBS patients showed that 40% of them developed cancer before 21 years old (The International Nijmegen Breakage Syndrome Study Group 2000).

The underlying gene mutated in NBS, *NBS1*, was cloned in 1998 with chromosomal location 8q21 (Varon, Vissinga et al. 1998). *NBS1* gene is 50 kb in size and consists of 16 exons. NBS1 is expressed ubiquitously and the expression level is higher in the testis (Kobayashi, Antoccia et al. 2004). Mutation screening of *NBS1* gene has identified six distinct mutations in NBS patients, including 657del5, 698del4, 835del4, 842insT, 1142delC and 976C>T (Varon, Vissinga et al. 1998). Among all these patients, 90% of them are homozygous for the 657del5 mutation. 657del5 mutation causes two truncated proteins because of premature termination at codon 219, a N-terminal and a C-terminal species with relative molecular weight of 26 KD and 70 KD respectively (Figure 1.1B) (Maser, Zinkel et al. 2001). The mutation of *NBS1* gene leads to pleiotropic phenotypes of NBS cells *in vitro*, such as hyper-sensitivity to ionizing radiation (IR), impaired cell

cycle checkpoints, decreased homologous recombination, accelerated telomere shortening and frequent chromosomal aberrations (Tauchi, Matsuura et al. 2002).

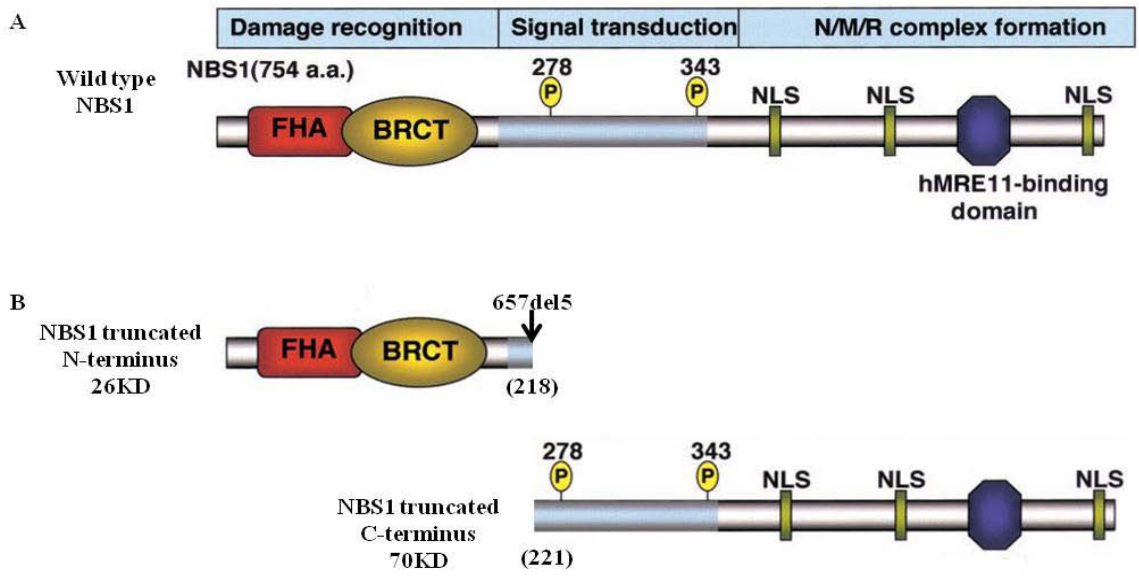

Figure 1.1 The structure of NBS1 (modified from (Tauchi, Matsuura et al. 2002)). A. Schematic diagram representing the wild type NBS1 structure. B. Schematic diagram representing the truncated NBS1 N-terminus and C-terminus structure caused by internal translation initiation due to 657del5 mutation.

The normal *NBS1* gene encodes a 754 amino acid protein that contains three functional regions (Figure 1.1A): the N-terminal DNA damage recognition region, the signal transduction region and the C-terminal MRE11 binding region (Kobayashi, Antoccia et al. 2004). The N-terminal DNA damage recognition region contains a forkhead-associated (FHA) domain and a BRCA1 C-terminus (BRCT) domain which are widely conserved in eukaryotes. FHA and BRCT domains involve in regulation of cell cycle checkpoints and DNA damage repair. The FHA domain is generally thought to mediate protein-protein

interactions (Durocher, Henckel et al. 1999). It is reported that the FHA/BRCT domain is essential for binding to the phosphorylated histone H2AX, following which the MRE11 and RAD50 are recruited to the vicinity of DNA damage foci (Kobayashi, Tauchi et al. 2002). The central region includes several SQ motifs that could be phosphorylated by ATM or ATR kinase in response to DNA damage, especially at serine (Ser) 278 and Ser343. Following phosphorylation, NBS1 undergoes a conformational change that makes NBS1 as an adaptor in DNA damage signaling pathway. Adaptor NBS1 positions NBS1-binding proteins in a manner such that could be phosphorylated by ATM/ATR (Yazhi, Zhao et al. 2006). Phosphorylation of NBS1 is essential to execute its downstream cellular functions, such as cell cycle checkpoint control and DNA damage repair (Iijima, Komatsu et al. 2004; Kobayashi, Antoccia et al. 2004; Zhang, Zhou et al. 2006). Mutation at the phosphorylation sites partially abrogates its cellular functions in DNA damage responses (Lim, Kim et al, 2000). The C-terminus of NBS1 contains the region that binds to MRE11. The binding of NBS1 to MRE11 is necessary for the recruitment of MRE11 and RAD50 from cytoplasm to nucleus, thus forming the MRN complex, a central player in many aspects of the cellular response towards DNA double strand breaks (DSBs) (Assenmacher and Hopfner 2004). In addition to MRE11, the C-terminus of NBS1 is able to attract other factors to DNA damage foci to amplify and propagate the original signal to multiple DNA damage response pathways (Bradbury and Jackson 2003).

1.2 MRN complex

MRN complex consists of three subunits, MRE11, RAD50 and NBS1. This complex is a main player in cellular response to DSBs in many aspects, including DSB detection and processing, DSB-activated cell cycle checkpoint and telomere maintenance (Assenmacher and Hopfner 2004). This broad range of cellular functions of MRN complex is explained by the multiple enzymatic and non-enzymatic activities of its components.

The MRE11 component is a nuclease with ssDNA endonuclease, 3’ to 5’ ssDNA exonuclease, dsDNA exonuclease and hairpin opening activities *in vitro* (Rupnik, Lowndes et al. 2010). These nuclease activities are dependent on the presence of NBS1 (Paull and Gellert 1999). RAD50 is a member of the Structural Maintenance of Chromosome family proteins with ATPase activity. The central region of RAD50 contains a large coiled-coil structure that allows itself fold back via a “hinge” region (Rupnik, Lowndes et al. 2010). The third component of MRN complex, NBS1, plays important roles in regulating complex functions. Firstly, NBS1 is required for the localization of MRE11 and RAD50 to nucleus. Secondly, NBS1 stimulates the activities of MRE11 and RAD50. Thirdly, NBS1 is also essential for the assembly of MRN complex at sites of DNA damage in nucleus (Carney, Maser et al. 1998; Horejsi, Falck et al. 2004; Rupnik, Lowndes et al. 2010).

Electron microscopy and scanning force microscopy revealed a striking architecture of MRN complex. The MRN complex exhibits as a bipolar structure with a head and two tails (Figure 1.2). The head is composed of two RAD50 ATPase domains along with a

MRE11 dimer. Although not directly imaged, NBS1 is suggested as part of the head and binds to MRE11 molecules by biophysical data (Assenmacher and Hopfner 2004). The tails presents as anti-parallel coiled-coil structure which can form interlocked hook bridges that might be important for MRN complex functions (Assenmacher and Hopfner 2004).

Figure 1.2 Structural model of the MRN complex (modified from (Assenmacher and Hopfner 2004)). MRE11 binds to RAD50, adjacent to the RAD50 ATPase domains. NBS1 is suggested binding to MRE11.

MRN complex is required to maintain genome stability. Null mutation of any component of MRN complex is lethal in higher eukaryotes (Luo, Yao et al. 1999; Yamaguchi-Iwai, Sonoda et al. 1999; Zhu, Petersen et al. 2001). Hypomorphic mutations in NBS1 and

MRE11 cause human genetic diseases, NBS and ataxia-telangiectasia like disease (ATLD), respectively (Matsuura, Tauchi et al. 1998; Stewart, Maser et al. 1999). Hypomorphic RAD50 mutant mice (RAD50 (S/S) mice) show growth defects and cancer predisposition, and die with complete bone marrow depletion as a consequence of hematopoietic stem cell failure (Bender, Sikes et al. 2002). Thus, disturbance of MRN complex activity has profound effects on genome stability, indicating the importance of this complex in maintaining the integrity of genome.

1.3 ATM and ATR kinases

ATM and ATR belong to a superfamily of protein kinases which contain a domain at their carboxyl termini with motifs that is characteristic of the lipid kinase phosphatidylinositol 3-kinase (PI3K), thus they are named 'PI3K-like protein kinases' (PIKKs). The mammalian members of PIKK family respond to various cellular stresses by phosphorylating other proteins in the corresponding pathways, therefore affecting numerous cellular processes depending on the spectrum of their targets (Shiloh 2003). ATM and ATR are at the central of DNA damage signaling pathways. About 25 substrates of ATM and ATR have been identified, and many of them have been revealed as candidates in DNA damage signaling pathway that play a role in cell cycle checkpoint, DNA damage repair or apoptosis (Matsuoka, Ballif et al. 2007).

The importance of ATM and ATR in DNA damage signaling pathway has been manifested in human genetic disorder ataxia-telangiectasia (A-T) and ATR-Seckle syndrome, which are caused by the mutation of *ATM* and *ATR* gene, respectively (Stiff, Reis et al. 2005). However, ATM and ATR have different functional roles as manifested by the pathological symptoms of A-T and ATR-Seckle syndrome (Table 1). The functional differences between ATM and ATR are also reflected in the genetically modified mice. ATM knockout mice are viable though infertile and growth-retarded (Xu, Ashley et al. 1996). In contrast, ATR knockout mice show early embryonic death in development subsequent to the blastocyst stage. ATR-null blastocyst cells only continue growth for 2 days before dying of caspase-dependent apoptosis (Brown and Baltimore 2000). These results indicate that ATR plays a vital role for normal cell growth, while

ATM is not essential for cell viability.

Although in the same family, ATM and ATR respond to different types of DNA damage stimuli. Due to this fact, it is generally thought that ATM and ATR orchestrate DNA damage response separately in response to specific types of DNA damage. While ATM mainly responds to DSBs, ATR primarily reacts to single strand breaks (SSBs) and stalled replication forks (Shiloh 2001; Matsuoka, Ballif et al. 2007). However, recent studies suggest that ATM- and ATR-mediated signaling pathways are highly interconnected. ATM and ATR communicate with each other to coordinate and modulate the cellular outputs in respond to DNA strand breaks and stalled replication forks (Hurley and Bunz 2007).

Many studies have revealed that NBS1 is involved in both ATM- and ATR-mediated DNA damage signaling pathways (Lim, Kim et al. 2000; Stiff, Reis et al. 2005). It is worth to note that the characteristics of NBS disease almost encompass those of A-T and ATR-Seckle (Table 1). Notably, A-T disease shares the clinical characteristics, such as hypersensitivity to IR, immunodeficiency and cancer predisposition, with NBS (Tauchi, Matsuura et al. 2002). Moreover, the cellular features of A-T cells also partly overlap with those of NBS cells, like chromosome instabilities, abnormal cell cycle checkpoints and accelerated telomere shortening (Kobayashi, Antoccia et al. 2004). Besides A-T disease, ATR-Seckle syndrome also shares several clinical symptoms with NBS, namely microcephaly and characteristic facial appearance (Stiff, Reis et al. 2005). The similarities between A-T/ATR-Seckle syndrome and NBS further imply that NBS1 and ATM/ATR work in the same or similar signaling pathway.

Table 1. Comparison of clinical signs with NBS, A-T, ATLD and ATR-Seckle syndrome

Clinical symptom	NBS	ATLD	A-T	ATR-Seckle syndrome
Ataxia	-	+	+	-
Growth retardation	+	NK	-	-
Characteristic facial appearance	+	-	-	+
Microcephaly	+	-	-	+
Hypersensitivity to IR	+	+	+	-
Immunodeficiency	+	-	+	-
Ovarian failure	+	NK	+	-
Mental retardation	-	-	-	+
Neuronal degeneration	-	NK	+	-
Telangiectasia	-	-	+	-
Cancer predisposition	+	NK	+	-
Cryptorchidism	-	-	-	+
Low birth weight	-	NK	-	+

‘+’ means clinical positive; ‘-’ means clinical negative, ‘NK’ means not known.

1.4 DNA damage response

DNA is susceptible to a multitude of damaging agents, including intracellular reactive metabolites and extracellular harmful factors, such as environmental chemicals, IR or UV light (Essers, Vermeulen et al. 2006). DNA damage caused by these damaging agents is a serious threat to cellular homeostasis as it compromises genome stability and integrity. Of the many types of DNA lesions, DSBs are particularly cytotoxic. If failed to be repaired, some of the DNA lesions may induce cell malignancy transformation (Shiloh 2006). Thus, cells have evolved a complex signaling network to regulate DNA damage response and maintain genome stability.

1.4.1 DNA damage sensing

DNA damage response begins with “sensor” proteins that sense DNA lesions/chromatin alterations after DNA damage induction. This process is characterized by rapid formation of DNA damage foci composed of recruited DNA damage response proteins (Shiloh 2006). The recruitment of these proteins follows a temporal order.

Histone H2AX is the first protein that is phosphorylated by ATM and possibly ATR shortly after induction of DSBs. The phosphorylated state of histone H2AX, γ-H2AX, immediately forms foci and co-localizes with other proteins that respond to DSBs, such as MRN complex (Kobayashi, Antoccia et al. 2004). MRN complex is the first candidate that is recruited to the sites of DSB foci (Tauchi, Matsuura et al. 2002). The recruitment of MRN complex follows two steps. Firstly, NBS1 interacts with γ-H2AX through the FHA/BRCT domain rather than directly binds to damaged DNA (Kobayashi, Tauchi et al. 2002). The interaction between NBS1 and γ-H2AX is essential for the following

recruitment of MRE11/RAD50 from cytoplasm to the vicinity of DSB damage sites, thus forming the functional MRN complex. In the second step, MRN complex switches to a mode of direct association with damaged DNA by the DNA binding region within MRE11/RAD50 (Tauchi, Matsuura et al. 2002; Kobayashi, Antoccia et al. 2004). However, it has also been reported that NBS1 recognition of DSB foci does not require the modification of H2AX (γ-H2AX). Using microbeam radiation, it was found that the recruitment of NBS1 to DNA damage sites was not impaired in $H2AX^{-/-}$ mice (Celeste, Fernandez-Capetillo et al. 2003). MDC1 (mediator of DNA damage checkpoint protein) and 53BP1 (p53 binding protein 1) are the following DSBs sensors that bind to DNA damage foci. The recruitment of additional proteins and the repeated protein-protein interaction stabilize the DSB foci and thus facilitate the transduction of damage signals to transducers (Shiloh 2006).

1.4.2 DNA damage mediating - ATM and ATR activation

Imaging analysis has demonstrated that ATM is also present at DSB foci together with MRN and other DSB damage sensors (Bekker-Jensen, Lukas et al. 2006), although the hierarchical association of ATM and MRN to the sites of damage foci has been rather elusive. Since NBS1 is known to be phosphorylated by ATM in response to DSB-inducing agents, ATM must function upstream of NBS1 (Lim, Kim et al. 2000). However, recent findings place NBS1 upstream of ATM and redefine NBS1 an activator in addition to a sensor (Shiloh 2006). It has been found that in response to DNA DSBs, MRN complex binds tightly to both DNA and ATM, implicating the role of MRN in the recruitment of ATM to damaged DNA (Matsuoka, Ballif et al. 2007). During this process,

dimeric ATM is autophosphorylated and become active monomers (Dupre, Boyer-Chatenet et al. 2006). But the remaining question is whether the recruitment of ATM to DSB foci must precede its activation. Further studies on the ATM activation mechanism will clarify this point.

ATR, which mainly responds to SSBs and stalled replication forks, is also found present together with MRN and BRCA1 at single-stranded DNA ends (Shiloh 2006). NBS1 is not only phosphorylated by ATM but also a downstream target of ATR (Stiff, Reis et al. 2005). However, whether NBS1 functions upstream of ATR and modulate its activation is not known. Recent findings suggest a positive role of NBS1 in the activation of ATR. In response to hydroxyurea (HU), a chemotherapeutic drug that induces replication stalling, the ATR-dependent phosphorylation of Chk1 and replication protein A (RPA) was defective in NBS1 deficient cells (Stiff, Reis et al. 2005; Manthey, Opiyo et al. 2007). Furthermore, the other ATR-dependent events, such as ubiquitination of FANCD2 and restart of stalled replication forks, were also impaired in NBS1 deficient cells (Zhou, Lim et al. 2006).

Recent data suggests that the activation of ATM and ATR could be affected by each other. In response to IR-induced DSBs, ATR is also robustly activated in addition to ATM. This activation of ATR is ATM-dependent (Cuadrado, Martinez-Pastor et al. 2006; Myers and Cortez 2006). ATM could induce the generation of RPA-coated single-stranded DNA, which is essential for the following recruitment of ATR to DSBs foci. Upon recruitment, ATR is subsequently activated by the DNA-protein structure, followed by the phosphorylation of its downstream target Chk1.

Understanding the convergence of ATM and ATR is taken one step further by showing that ATM is also activated in response to stimuli that are previously thought to activate ATR, such as UV and HU (Stiff, Walker et al. 2006). In addition, the activation of ATM is ATR-dependent without the requirement of γ-H2AX and 53BP1. ATM activation also leads to the phosphorylation and activation of its downstream target Chk2 to elicit cellular activities, such as cell cycle checkpoints.

Although the precise molecular events of ATM and ATR activation remain to be elucidated, growing evidence demonstrates a high degree of communication between these two kinases. ATM and ATR may function in an integrated molecular circuit to mediate diverse DNA damage signals and induce coordinated DNA damage response.

1.4.3 DNA damage effect - cell cycle checkpoint control

The survival of cells relies on faithful transmission of genetic information from parents to their progenies. This transmission requires not only accurate replication of DNA, but also the ability of cells surviving either spontaneous or induced DNA damage (Zhou and Elledge 2000). To preserve the stability of genome, cells have evolved the DNA damage repair and cell cycle checkpoint mechanisms to cope with DNA damage. These checkpoints verify whether the cellular activities at each phase of the cell cycle have been completed before cells progress to next phase. Three distinct checkpoints that have been identified and well studied are G1/S, intra-S and G2/M checkpoint.

The G1/S checkpoint is at the end of G1 phase, making the decision of whether the cell should enter S phase or delay S phase. The intra-S phase checkpoint is activated when cells are exposed to DNA damage-inducing agents that interfere with ongoing DNA

replication. Activated intra-S phase checkpoint inhibits replication and delay cell cycle progression through S phase. And the G2/M checkpoint is at the end of G2 phase which check several criteria to ensure that the cell is ready for mitosis. If all the criteria are reached, the cell initiates many cellular processes for the beginning of mitosis (Lamarche, Orazio et al. 2010).

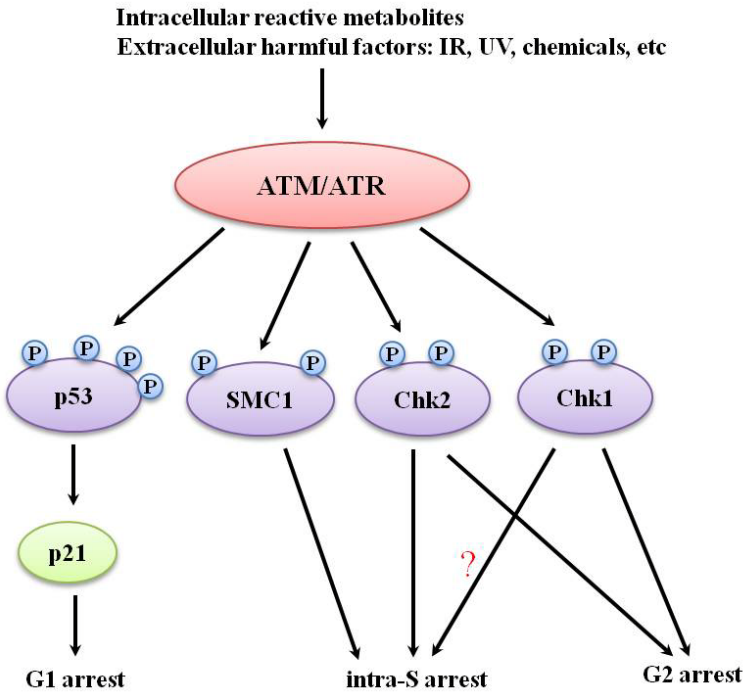

Figure 1.3 Major pathways of ATM/ATR-mediated cell cycle arrest, including G1 arrest, intra-S arrest and G2 arrest. The regulatory role of Chk1 on intra-S arrest remains to be elucidated.

ATM and ATR are the two protein kinases that phosphorylate numerous substrates to regulate cell cycle progression in response to DNA damage (Figure 1.3). The G1/S cell cycle checkpoint is mainly mediated by the activation and accumulation of p53 (Shiloh 2001). p53 could be phosphorylated by ATM and ATR at many different sites, including Ser 6, 9, 15, 46 and threonine (Thr) 18 (Yang, Xu et al. 2004). In particular, Ser15 is the

common site that could be phosphorylated by both ATM and ATR, which is important for its transactivating activity (Shiloh 2001; Yang, Xu et al. 2004). Activated p53 turns on the transcription of one important gene, *p21* (*WAF1, Cip-1*). p21 protein binds to several cyclin-Cdk complexes, which inhibits the complex activities and blocks cell cycle progression, resulting in G1 arrest (Levine 1997).

The intra-S cell cycle checkpoint is also controlled by several branches of ATM-mediated signaling pathways (Kastan and Bartek 2004). One branch involves the phosphorylation of NBS1 by ATM, a process that is required for the following ATM-mediated phosphorylation of cohesin protein SMC1 that is implicated in the activation of intra-S checkpoint (Yazdi, Wang et al. 2002). Another branch involves the activation of Chk2 by ATM. Activated Chk2 phosphorylates the cell cycle regulator CDC25A, leading to the poly-ubiquitination-mediated degradation of CDC25A. CDC25A degradation will ultimately lead to the inhibition of cyclin E/A-CDK2 kinase complexes. Since new replication origin firing requires the activity of CDK2 kinase to recruit into pre-replication complexes, inhibition of CDK2 kinase would finally block the DNA replication in S phase (Bartek, Lukas et al. 2004).

Studies show that ATR is also implicated in intra-S checkpoint (Luciani, Oehlmann et al. 2004). Slowing down the replication fork by DNA polymerase inhibitor aphidicolin strongly suppresses further initiation events and leads to intra-S cell cycle checkpoint. The intra-S checkpoint can be overcome by ATM/ATR kinase inhibitor, caffeine, or by ATR neutralizing antibodies, suggesting that the aphidicolin-induced checkpoint is ATR-dependent (Luciani, Oehlmann et al. 2004). However, depletion or inhibition of Chk1

does not abolish the intra-S checkpoint, indicating Chk1 is not involved in the signaling pathway that induces this checkpoint (Luciani, Oehlmann et al. 2004). Other studies have raised controversial viewpoints regarding the role of Chk1 in inducing intra-S checkpoint by showing that Chk1 mediates the degradation of Cdc25A and leads to intra-S checkpoint (Xiao, Chen et al. 2003).

In addition to regulating intra-S phase checkpoint, Chk2 is also known as a key regulator of the G2/M cell cycle checkpoint (Shiloh 2001). As a downstream target of ATM, Chk2 could be phosphorylated at Thr68 by ATM when exposed to DNA damage-inducing agents that cause DSBs. *In vitro*, Chk2 phosphorylates the members of Cdc25 family, particularly Cdc25C at Ser216. The phosphorylation of Cdc25C creates a binding site for 14-3-3 protein, leading to the formation of Cdc25C/14-3-3 complex, a process that sequesters Cdc25C in cytoplasm (Buscemi, Savio et al. 2001). The cytoplasmic Cdc25C fails to dephosphorylate and activate the cyclin-dependent nuclear kinase Cdc2, thus preventing mitosis and resulting in G2 arrest (Yang, Xu et al. 2004).

Chk1 is also linked to G2 arrest in response to DNA damage in several cell types (Yamane, Taylor et al. 2004; Wang, Li et al. 2008). It has been shown that Chk1 is partially responsible for lithium-induced G2 arrest in hepatocellular carcinoma cells SMMC-7721. Using Chk1 inhibitor SB218078 or Chk1 siRNA, or overexpression of the kinase dead Chk1 abrogates the G2 arrest induced by lithium (Wang, Li et al. 2008). Moreover, using Chk1 siRNA also destroys the G2 arrest induced by chemotherapeutic drug 6-thioguanine in Hela cells (Yamane, Taylor et al. 2004). Chk1 is also revealed to mediate G2 arrest in glioma cells in response to temozolomide treatment (Hirose,

Katayama et al. 2004). These data collectively suggests that Chk1 is also actively involved in the regulation of G2/M checkpoint.

1.4.4 DNA damage effect - apoptosis

Following the induction of DNA damage, another prominent route of cellular activities is apoptosis. Apoptosis could be induced by many DNA damaging agents that cause collapse of replication forks and/or DSBs (Kaina and Roos 2006). If these lesions fail to be repaired, they will trigger the apoptosis signaling to eliminate unwanted cells through at least two pathways, the extrinsic pathway and the intrinsic pathway.

Although rarely reported, ATM and ATR are also involved in mediating apoptosis signaling pathways by phosphorylating their downstream targets (Kaina and Roos 2006). p53 is the most extensively explored target that plays essential roles in modulating apoptosis. After activation, p53 regulates the apoptotic process primarily through intrinsic pathway that centers on mitochondria (Fridman and Lowe 2003).

p53 controls the transcription of pro-apoptotic genes in the Bcl-2 family, such as Bax (BCL-2-associated X protein), Puma (p53 upregulated modulator of apoptosis), Noxa and Bid. The net effect of transcription is to increase the ratio of pro-apoptotic to anti-apoptotic proteins, thereby favoring the release of apoptogenic factors from mitochondria, such as cytochrome *c*, AIF and SMAC/DIABLO (Kroemer and Reed 2000). The release of these factors from mitochondria to cytoplasm leads to the signaling cascade of caspases, the “executioner” of cell death, whereby promoting the occurrence of apoptosis (Kumar 2007). In addition to regulating the transcription of pro-apoptotic genes, p53 also activates the components that are involved in the apoptotic effector machinery, including

Apaf-1 and caspase 6, to potentiate cell death in the presence of cytochrome *c* (Fridman and Lowe 2003).

Another ATM downstream target c-Abl is also implicated in eliciting apoptosis in response to IR (Shaul 2000). The phosphorylation of c-Abl by ATM induces the activation of p73, a family member of p53 that is also linked to apoptosis (Shiloh 2001). In cells that are null for c-Abl, the apoptotic response to IR is impaired (Yuan, Huang et al. 1997). Moreover, overexpression of c-Abl in combination of p73 is sufficient to induce apoptosis in fibroblasts (Agami, Blandino et al. 1999).

1.4.5 DNA damage response as anti-cancer barrier

Accumulating evidence suggests that cancer is essentially a disease of genes (Hoeijmakers 2001). The initiation and progression of cancer involves a series of DNA mutations that inactivate tumor-suppressor genes and activate proto-oncogenes. The observation that many tumor-suppressor genes that are inactivated during the process of carcinogenesis are components of the DNA damage response network (Bartek, Lukas et al. 2007) reflects the significance of the integrity of DNA damage response in preventing cancer. Recently, DNA damage response has been proposed as an anti-cancer barrier in early human carcinogenesis (Bartkova, Horejsi et al. 2005).

ATM and ATR, as the central players in DNA damage response, serve as critical barriers to constrain tumor development. An investigation of the human tumor specimens from urinary bladder, lung, colon and breast shows phosphorylation of ATM, Chk2, p53, histone H2AX, as well as the 53BP1 foci (DiTullio, Mochan et al. 2002; Bartkova, Horejsi et al. 2005). Activation of ATM/ATR-mediated DNA damage pathways could

delay or prevent cancer in the early stage before malignant conversion. However, mutations in ATM/ATR signaling pathway might allow cell growth and limit cell death of the incipient cancer cells, thus increasing genomic instabilities and promoting tumor progression (Bartek, Lukas et al. 2007). Consistent with this viewpoint, mutation of *TP53*, the gene that encodes the tumor-suppressor protein p53, is found in 50% of human cancers (Toledo and Wahl 2006). Furthermore, the mouse model with targeted mutation of p53 (p53 $^{S18, 23A}$) develops a wide spectrum of tumors after 1 year latency, suggesting the role of wild type p53 in tumor suppression (Chao, Herr et al. 2006).

However, DNA damage response is not always activated in the early lesions of tumor. It has been reported that the activation of DNA damage response is observed in majority of human cancers, while not in testicular germ-cell tumors (Bartek, Lukas et al. 2007; Bartkova, Rajpert-De Meyts et al. 2007). This exception could be explained that the molecular events that drive the pathogenesis of testicular germ-cell tumors are unable to reach the threshold levels of DNA damage required for DNA damage response (Bartek, Lukas et al. 2007). The speculation may also provide some hints to the question of why the initial pre-malignant cells could grow and proliferate in the first place rather than being detected and eliminated by DNA damage response machinery. Another more likely explanation relies in the fact that not all oncogenic insults have the same ability to cause DNA damage, thus escaping from the surveillance of DNA damage response network. Examination of a variety of oncogenes shows that activation of the majority of oncogenes could evoke DNA damage responses, such as H-ras, c-Myc and E2F1 (Powers, Hong et al. 2004; Di Micco, Fumagalli et al. 2006; Pickering and Kowalik 2006; Reimann, Loddenkemper et al. 2007). However, a small subset of oncogenic events, such as

overexpression of proto-oncogene cyclin D1 and loss of tumor-suppressor gene p16ink4a, do not activate DNA damage responses (Bartek, Lukas et al. 2007).

As a barrier of cancer development, DNA damage response on the other hand provides pressure that favors the growth of cells with defects in the DNA damage signaling machinery. Therefore, cells with deficient DNA damage signaling are preferentially selected to survive and perpetuate rather than being eliminated, which finally contributes to cancer initiation. Many human diseases caused by mutations of the genes involved in DNA damage signaling machinery have illustrated this point by showing a strong predisposition to cancer, such as A-T, NBS and ATLD (Metcalfe, Parkhill et al. 1996; Williams, Williams et al. 2007).

Considering the importance of DNA damage signaling pathway in prevention of cancer development, it has become a target for cancer therapies. Conventional chemotherapy works by impairing the cell division of fast-proliferating cells, thus causing apoptosis. However, due to the potential mutations in DNA damage response machinery, cancer cells may favor cell cycle arrest rather than apoptosis, resulting in resistance to chemotherapeutic drugs. Therefore, choosing appropriate treatments to the cancer with specific cellular defects would have profound effects on outcome. Recent years, inhibitors of the proteins involved in DNA damage response pathway have been developed and used in a combination with other treatment strategies. For example, ATM inhibitors, KU55933 and CP466722, have been used to treat cancers and are effective in sensitizing cancer cells to IR (White, Choi et al. 2008). Chk1, the protein that is activated by ATR and induces intra-S and G2/M cell cycle arrest, is also a hot target in treating

cancers. Inhibitors of Chk1, such as UCN-01, XL844, PF-00477736 and AZD7762, are especially effective in cancer cells that are defective in G1/S cell cycle arrest (Ashwell and Zabludoff 2008; Ljungman 2009). Inhibitors that target other DNA damage signaling proteins, such as ATR, MRN complex, Chk2 and p53 have also been developed. They have been used to treat specific types of cancers to initiate cancer cell death rather than cell cycle arrest (Ljungman 2009).

1.5 The biology of telomeres

1.5.1 Telomere and telomerase

Telomeres are highly specialized nucleoprotein structures at chromosome ends composed of telomeric DNA and associated proteins (Blackburn 2001). Telomeric DNA consists of a stretch of tandem G-rich repeats (5-26 bp) oriented 5’ to 3’ toward the chromosomal terminus (McEachern, Krauskopf et al. 2000). In humans, the telomeric repeat sequence is 5’-TTAGGG-3’ and the length of telomeric tract ranges from 5 to 15 kb which is kept in a cell-type specific manner (Lingner and Hug 2006). Due to the “end-replication” problem, the extreme end of telomeric DNA is a 3’ single-strand overhang rather than a duplex. In mammalian cells, the single-strand 3’-overhang of telomeric DNA folds back into the duplex telomeric DNA to form a “T-loop”, a process which protects eukaryotic chromosome ends from chromosome fusion, recombination and telomeric degradation (Blackburn 2001).

The most common way to solve the “end-replication” problem occurs through telomerase, a specialized DNA polymerase that adds telomeric DNA repeats onto chromosome ends (Greider 1996). Telomerase is composed of two essential components, the protein component (TERT) and the RNA component (TER). The protein component contains the catalytic core of this enzyme, while the RNA component provides the template for telomeric DNA repeats (Blackburn 1992; Lingner and Hug 2006). In human, the telomerase RNA has a length of 450 nucleotides which contains the redundant template nucleotides 5’-CUAACCCUAAC-3’. The redundancy of the RNA template allows the base pairing of RNA with growing telomere during replication (Greider 1996).

In addition to TERT and TER, the biogenesis and assembly of active telomerase requires additional protein subunits to mediate its access to telomeres (Cong, Wright et al. 2002). So far, at least 13 proteins that associate with human telomerase (hTERT and hTER) have been identified, including the molecular chaperone p23 and p90, the DNA damage response regulator 14-3-3, the H/ACA snoRNA (small nucleolar RNA) binding proteins dyskerin, hNOP10, hNHP2 and hGAR1, and hnRNPs (heterogeneous nuclear ribonucleoproteins) C1, C2, A1 and UP1. The telomerase-associated proteins are thought to regulate telomerase activity and modulate the accessibility of telomerase to telomeres. However, the precise actions of most telomerase-associated proteins are still unknown and remain to be determined (Cong, Wright et al. 2002).

Telomerase-dependent telomere elongation occurs in S phase, while no elongation is observed in G1 phase of the cell cycle (Lingner and Hug 2006). The newly-synthesized telomeric DNA repeats will balance the loss of chromosome ends caused by the semi-conservative DNA replication (Collins 2006). If the balance is lost, cells will suffer from cumulative loss of telomere repeats and eventually become senescence as a response to DNA damage when telomeres are critically short (de Lange 2005).

However, telomerase is not a “housekeeping” enzyme that is found in all cell types. In most of human somatic cells, telomerase activity is distinguished during embryonic development, but only exists in several cell lineages, such as embryonic stem cells, germ cells, activated lymphocytes and almost all types of cancer cells (Shay and Bacchetti 1997; Collins and Mitchell 2002). The loss of telomerase activity in human somatic cells has been suggested as an anti-cancer mechanism (Shay and Wright 2005). Reactivation of

telomerase activity exists in approximately 90% of all human cancers (Shay and Bacchetti 1997). Ectopic expression of *hTERT* in cooperation with other two oncogenes, the simian virus 40 large-T oncoprotein and an oncogenic allele of H-RAS, could successfully convert normal human epithelial and fibroblast cells into tumorigenic cells (Hahn, Counter et al. 1999).

Telomerase activity is regulated at multiple levels, such as transcription, mRNA splicing, post-translational modification, transportation and localization, as well as assembly of active telomerase holoenzyme (Cong, Wright et al. 2002). But the regulation of *hTERT* gene transcription is the most important layer. In most situations, the *hTERT* expression level is the limiting factor and is closely correlated to telomerase activity in most cell types (Takakura, Kyo et al. 1999). Post-translational modification of hTERT, such as reversible phosphorylation, provides another important layer to control telomerase activity. Reversible phosphorylation of hTERT could regulate the protein structure and localization, thereby switching the active and inactive status of telomerase activity (Cong, Wright et al. 2002).

The mechanisms that regulate telomerase activity are still not fully understood. Identification of new telomerase-associated proteins may contribute to the discovery of the unidentified cellular functions of telomerase. Revealing the multiple layers that regulate telomerase activity would further aid the investigation of the functions of telomerase in telomerase elongation, immortalization as well as carcinogenesis.

1.5.2 Telomere and shelterin complex

In mammalian cells, the telomeric TTAGGG repeats associate with shelterin complex

that is composed of six telomere-specific proteins, including telomeric repeat binding factor 1 (TRF1), TRF2, protection of telomeres 1 (POT1), the human ortholog of the yeast repressor/activator protein 1 (RAP1), the TRF1- and TRF2-interacting nuclear protein 2 (TIN2) and the POT1-TIN2 organizing protein 1 (TPP1) (de Lange 2005). The specificity of shelterin complex to telomere is determined by three of its components, TRF1, TRF2 and POT1 (Palm and de Lange 2008). TRF1 and TRF2 directly bind to the duplex region of telomeres, whereas POT1 binds to the single-strand 3'-overhang. These three proteins are interconnected by the rest three components of shelterin, RAP1, TIN2 and TPP1, and form a stable complex binding to telomeres (de Lange 2005; Palm and de Lange 2008).

Shelterin complex is implicated in the protection of telomeres by affecting the structure of telomeres. The natural ends of telomeres are long single-strand 3'-overhangs. However, electron microscopy of the purified telomeric restriction fragments stabilized by psoralen and UV in both human and mouse cells showed that telomeres are presented as "T-loops" (Griffith, Comeau et al. 1999). Accumulating evidence suggests that the shelterin complex has DNA remodeling activities that are responsible for "T-loop" formation (de Lange 2005). At least three components of the shelterin complex have been identified with DNA remodeling activity, including TRF1, TRF2 and TIN2. TRF2 can remodel artificial telomeres into loops, although with low efficiency (Griffith, Comeau et al. 1999). TRF1, with the help of TIN2, can bend and pair telomeric DNA repeats *in vitro*, activities which might correspond to the folding of telomeres into "T-loops" *in vivo* (Bianchi, Smith et al. 1997).

By hiding into “T-loops”, telomeres are protected from being recognized as DSBs by DNA damage signaling machinery. Inhibition or loss of shelterin complex components would therefore jeopardize the integrity of telomeres and lead to DNA damage response at telomeric ends. Recent studies revealed that the canonical DNA damage signaling pathways are involved in protecting telomere integrity, particularly the ATM- and ATR-mediated pathways (de Lange 2005). It was firstly found that inhibition of TRF2 with a dominant negative version activates ATM kinase as well as its downstream target p53, and leads to p21-mediated G1/S cell cycle arrest (Karlseder, Broccoli et al. 1999). The mouse model with conditional deletion of TRF2 confirms this result by showing accumulated telomere dysfunction induced foci (TIFs) formed by 53BP1, γH2AX and phosphorylated ATM (Celli and de Lange 2005). In contrast to TRF2, depletion of POT1 activates ATR signaling pathway and ATR-dependent phosphorylation of Chk1. When inhibition of ATR by shRNA, the telomere damage response is significantly suppressed indicated by a pronounced decrease of TIFs (Denchi and de Lange 2007). Conditional deletion of TRF1 activates both ATM and ATR kinases and their substrates Chk1 and Chk2. Inhibition of kinase activities by ATM and ATR inhibitors rescues TIFs induced by TRF1 deletion, which further manifests the involvement of both ATM and ATR pathways in response to dysfunctional telomeres (Martinez, Thanasoula et al. 2009). TIFs formation has also been reported when TIN2 is inhibited (Kim, Beausejour et al. 2004). Collectively, these data argue the importance of shelterin complex components in protecting telomere integrity and repressing the DNA damage responses at telomeric ends.

As a consequence of telomere dysfunction, telomeric fusions are frequently observed in cells deprived of protection from shelterin complex. One type of telomeric fusions is non-

homologous end joining (NHEJ) which involves covalent fusions of the C-strand of one telomere and the G-strand of another thus creating a dicentric or circular chromosome (Smogorzewska, Karlseder et al. 2002). Inhibition or loss of shelterin components, such as TRF1, TRF2, POT1 and TPP1, leads to accumulated NHEJ in cells (de Lange 2005; Denchi and de Lange 2007; Martinez and Blasco 2010). Another type of telomeric fusions is homologous recombination (HR) in which DNA sequences are exchanged between similar or identical fragments. HR has been observed in cells with functional mutation of TRF2, named TRF2$^{\Delta B}$. TRF2$^{\Delta B}$ mutants are protected from NHEJ but show telomere truncations and contain circular extrachromosomal telomeric DNA (Cesare and Griffith 2004). HR between telomeres could result in generation of aberrant telomere length and lead to telomere deletions, inversions as well as translocations which are detrimental to cells (de Lange 2005). Thus, shelterin complex components play essential roles in preventing telomeric fusion-associated cell death.

Shelterin complex components are negative regulators of telomere length. Long-term overexpression of TRF1 leads to gradual telomere shortening without affecting the expression of telomerase in human sarcoma cell line HT1080 (vanSteensel and deLange 1997). Mouse model with transgenic TRF1 expression in the context of epithelial tissues (K5TRF1 mouse) has shorter telomeres in the epidermis compared to wild-type control. Moreover, K5TRF1 cells exhibit increased aberrant telomeric fusions, such as end-to-end fusions, telomere recombination and multitelomeric signals (Munoz, Blanco et al. 2009). Similar to TRF1, overexpression of TRF2 is also implicated in negative regulation of telomere length in both mouse and human cells (Smogorzewska, Van Steensel et al. 2000; Munoz, Blanco et al. 2005). The same effect of negative regulation of telomere length has

also been reported in cells with TIN2 or RAP1 overexpression (Kim, Beausejour et al. 2004; O'Connor, Safari et al. 2004). However, how shelterin components exert the negative effect in regulation of telomere length is far from being fully understood.

1.5.3 Other telomere associated proteins

In addition to shelterin complex, telomeres also bind to a large number of other proteins that are involved in DNA damage signaling and repair pathways (Table 2) (Munoz, Blanco et al. 2006). In particular, these proteins include MRN complex, Ku70/80 and ATM (Munoz, Blanco et al. 2006; Palm and de Lange 2008). Unlike shelterin complex, these proteins have non-telomeric functions and are typically more abundant at non-telomeric sites in nucleus or cytoplasm (Palm and de Lange 2008). The association of these proteins to telomeres suggests a role in protecting and maintaining telomere integrity. Ku70/80, the protein involved in NHEJ, is required for telomere localization to the nuclear periphery (Galy, Olivo-Marin et al. 2000), while loss of Ku70/80 function leads to striking recombinational activities near chromosomal ends (Baumann and Cech 2000). RAD50, MRE11and XRS2 (the ortholog of NBS1 in yeast) have also been implicated in the maintenance of telomeres. Mutation of any of these genes leads to pronounced telomere shortening in *S. cerevisiae* (Le, Moore et al. 1999). The role of ATM at telomeric ends is manifested by A-T disease in human that *ATM* gene mutation results in accelerated shortening of telomeres in A-T cells (Metcalfe, Parkhill et al. 1996).

Table 2. List of non-shelterin proteins associated with telomeres

Protein	Telomeric interaction	Telomeric function	Non-telomeric function	Implication in carcinogenesis
MRN	TRF2	Telomere length regulation; prevent telomeric end fusions	DSB sensor; HR repair	Mutations cause cancer onset
ATM	TRF1/ TRF2	Telomere length regulation; telomere integrity maintenance	DSB response	Mutations cause cancer onset
WRN	TRF2	Telomeric circles formation repression	DNA resolution; branch migration	Mutations cause cancer onset
BLM	TRF1/ TRF2	Prevention of telomeric fusions	HR repression; branch migration	Mutations cause cancer onset
DNA-PKcs	TRF1	Prevention of telomeric fusions	NHEJ	Tumor suppressor
Rad9/Rad 1/Hus1	TERT	Regulation of telomerase activity	Cell cycle checkpoints regulation	Rad9 is abundant in prostate cancer
Ku70/80	TRF2	Telomere length regulation	NHEJ; V(D)J recombination	Overexpressed in gastric cancer

XPF/ ERCC1	TRF2	Telomere length regulation; telomere overhang processing	NER; degradation of 3' tail ends	NK
Apollo	TIN2/ TRF2	Protect telomeres from DNA repair	5' exonuclease	NK
PINX1	TRF1/ TERT	Telomerase inhibitor	Chromosomal segregation	Tumor suppressor
Tankyrase	TRF1	Telomere length regulation	Role in mitosis	Overexpressed in many cancers
PARP1/2	TRF2	NK	ssDNA breaks repair; BER	PARP inhibitors sensitize cancer cell death
RAD51D	NK	Prevention of telomeric fusions	HR repair	Mutations cause cancer onset
ORC1	TRF2	NK	Replication initiation	NK

'NK' means not known. Abbreviations: NER: nucleotide excision repair; BER: base excision repair; WRN: gene mutated in Werner syndrome; BLM: gene mutated in Bloom syndrome; PINX1: PIN1-interacting protein 1; PARP: poly-ADP-ribose-polymerase; ORC1: origin recognition complex subunit 1. Selected references: MRN (Lamarche, Orazio et al. 2010); ATM (Pandita 2002); WRN (Li, Jog et al. 2008); BLM (Lillard-Wetherell, Machwe et al. 2004); DNA-PKcs, Tankyrases, PARP1/2, RAD51D (de Lange 2005); Ku70/80 (Ponnusamy, Alderson et al. 2008); XPF/ERCC1 (Wu, Mitchell et al. 2008), ORC1 (Noguchi, Vassilev et al. 2006).

Direct interaction between shelterin components, especially TRF2, and factors that are involved in DNA damage response has been observed. TRF2 serves as a protein hub at telomeric ends and interacts with a number of factors, such as MRN complex and ATM (Munoz, Blanco et al. 2006). The interaction indicates an interplay between DNA damage response and telomere integrity maintenance. Indeed, canonical DNA damage responses are activated when telomeres are deprived of protection from shelterin components (as discussed above in section **1.5.2**). In turn, shelterin components might also influence DNA damage response. It has been observed that overexpression of TRF2 inhibits ATM autophosphorylation at Ser1981 as well as the phosphorylation of its downstream targets, NBS1 and p53, after IR (Karlseder, Hoke et al. 2004).

1.5.4 Telomerase and shelterin in cancer and aging

In most normal human somatic cells, the low telomerase level is insufficient to maintain telomere length and support indefinite cell division. Therefore, these cells undergo gradual telomere attrition with age which eventually results in critically short telomeres and senescence, indicating a direct link between telomere length and cellular aging (Harley, Futcher et al. 1990). In many diseases that are associated with premature aging, short telomeres are observed, such as A-T, NBS, Werner syndrome and Bloom syndrome (Munoz, Blanco et al. 2006). In addition, short telomeres are also observed in late stage cancers, probably due to their long proliferation history (Blasco 2005).

Reactivation of telomerase is observed in more than 90% of human cancers (Shay and Bacchetti 1997), indicating that acquisition of telomerase is one of the essential steps in tumorigenesis. Some cancers that are not detectable of telomerase activity maintain

telomere length by another telomerase-independent mechanism, alternative lengthening of telomeres (ALT) (Henson, Neumann et al. 2002). Although with telomere lengthening mechanisms, tumors generally have shorter telomere length than their surrounding normal tissues which may eventually lead to cell death within tumors (Blasco 2005).

The impact of short telomeres in the whole organism has been manifested by telomerase-deficient mouse model. The first telomerase deficient mouse was generated by deletion of the mouse *TER* component (*mTER*) from germline. The mTER$^{-/-}$ mice are only viable for six months and suffer from a series of pathologies associated with loss of telomeric DNA repeats, including a reduction in proliferation potential, increased apoptosis, loss of fertility, decreased tissue regeneration and tissue atrophies (Blasco, Lee et al. 1997; Blasco 2005). Reintroduction of *mTER* gene into the mTER$^{-/-}$ mice prevents telomere shortening, premature aging and loss of organismal viability (Samper, Flores et al. 2001). These results suggest that an appropriate telomere length is necessary to maintain tissue homeostasis.

Recent studies show that shelterin components also play a role in cancer susceptibility even in the presence of normal telomerase activity. Aberrant expression of TRF1, TRF2, TIN2 and POT1 is observed in some human tumor types (Blasco 2005; Martinez and Blasco 2010). To study the function of shelterin components in cancer and aging, mouse models with genetic modification of various shelterin components have been generated. However, complete deletion of TRF1, TRF2, POT1a, TPP1 or TIN2 leads to early embryonic lethality (Martinez and Blasco 2010). Due to this fact, tissue specific conditional mouse models and transgenic mouse models have been generated recently to

study the potential roles of shelterin components in caner and aging (Martinez and Blasco 2010).

Conditional deletion of TRF1 in stratified epithelia (TRF1$^{\Delta/\Delta}$ K5-Cre mice) leads to perinatal death and multiple skin abnormalities, such as skin hyperpigmentation, skin morphogenesis and absence of mature hair follicles (Martinez, Thanasoula et al. 2009). p53 deletion in TRF1$^{\Delta/\Delta}$ K5-Cre mice rescues mice survival and most of the skin-related defects, indicating that the defects associated with TRF1 deletion are mediated by p53. *TRF1/p53* double null mice develop squamous cell carcinomas, suggesting a tumor suppressive effect of TRF1 (Martinez, Thanasoula et al. 2009). TPP1$^{\Delta/\Delta}$ K5-Cre mice show similar phenotypes as that observed in TRF1$^{\Delta/\Delta}$ K5-Cre mice (Tejera, d'Alcontres et al. 2010). As an ortholog of TRF1, TRF2 is also implicated in tumorigenesis. TRF2 transgenic mice (K5TRF2 mice) exhibit severe skin defects and an increased incidence of skin cancer (Munoz, Blanco et al. 2005). In line with this, an elevation of TRF2 is frequently observed in human skin carcinomas (Munoz, Blanco et al. 2005). However, conditional deletion of TRF2 in liver does not compromise mice viability and liver regeneration, probably due to the fact that liver regeneration occurs without cell division, thus circumventing the chromosome segregation problems caused by TRF2 deletion (Denchi, Celli et al. 2006).

Mouse contains two *POT1* orthologs, *POT1a* and *POT1b*. *POT1a* and *POT1b* have different roles revealed by single knockouts. While abrogation of *POT1a* results in embryonic lethality, *POT1b*-deficient mice survive to adulthood and show degenerative abnormalities, such as skin hyperpigmentation and bone marrow failure (Hockemeyer,

Daniels et al. 2006). Because of the embryonic lethality of TIN2 knockout mice, conditional or tissue-specific TIN2 knockout mice are required for further analysis of its *in vivo* function. RAP1 deficiency mouse models suggest that RAP1 is not required for viability but important in protection of telomeres from recombination (Sfeir, Kabir et al. 2010).

Understanding the roles of telomerase and shelterin complex in human diseases is essential for designing appropriate therapeutic strategies. In diseases associated with premature aging and shortened telomeres, reactivation of telomerase is one of the potential strategies. It has been reported that the telomere-elongation defect of NBS could be rescued by simultaneous reintroduction of *NBS1* and *hTERT* (Ranganathan, Heine et al. 2001). In cancers characterized by high telomerase activity, anti-telomerase is an important aspect for cancer therapy. However, targeting telomerase is challenging due to the lag period that anti-telomerase inhibitor takes to exert cytotoxic effects (Satyanarayana, Manns et al. 2004). The most likely use of anti-telomerase inhibitors is as an adjuvant strategy in combination with surgery (Shay, Zou et al. 2001). In addition, the fact that expression of shelterin components TRF1, TRF2 and TIN2 is altered in human cancers raises the potential of using them as therapeutic targets for cancer. Future investigation of the biology of shelterin components in diseases and cancers would certainly facilitate the exploration of their clinical usage.

1.6 Project rationale and aims

Accumulating evidence suggests that NBS1 is involved in both ATM- and ATR-dependent signaling pathways. In addition to being a downstream substrate that could be phosphorylated by either ATM or ATR in response to specific type of DNA lesions, NBS1 is also reported as an upstream regulator of ATM that influences ATM autophosphorylation. However, whether NBS1 is also an upstream regulator of ATR is not fully understood.

The involvement of NBS1 in cell cycle checkpoint is reported in several studies. In response to IR, NBS cells failed to induce intra-S checkpoint control (Tauchi, Matsuura et al. 2002). Defects in inducing G1/S and G2/M checkpoint have also been observed in NBS cells (Buscemi, Savio et al. 2001). However, other studies showed normal and proficient G1/S and G2/M checkpoint in spite of NBS1 deficiency (Antoccia, di Masi et al. 2002). The role of NBS1 in maintaining checkpoint integrity still remains controversial. Moreover, the influence of NBS1 deficiency in apoptosis is rarely reported and how NBS1 regulates DNA damage induced apoptosis is waiting to be elucidated.

Besides cell cycle checkpoint and apoptosis, NBS1 also plays a role in telomere maintenance (Lamarche, Orazio et al. 2010). In yeast, XRS2, the functional homolog of NBS1, is involved in telomerase-dependent telomere synthesis (Wu, Xiao et al. 2007). In human, NBS1 is associated with shelterin component TRF2 in S phase while not in other phases (Zhu, Kuster et al. 2000). The interaction of NBS1 and TRF2 in S phase suggests a role of NBS1 in telomere replication. Furthermore, it has been reported that NBS fibroblasts showed premature growth cessation in culture. But the mechanism of how

NBS1 deficiency affects telomere replication and attrition, therefore premature aging is far from fully established.

This study aims to examine the roles of NBS1 both in DNA damage signaling pathway and in maintaining telomere integrity. On one hand, the role of NBS1 as an upstream regulator of both ATM and ATR will be examined by using NBS cells derived from NBS patients with 657del5 mutation. The function of NBS1 in regulating DNA synthesis and apoptosis will also be examined after introduction of DNA damage by Dox treatment. On the other hand, the telomere shortening rate of NBS cells will be determined *in vitro* and compared with the age, gender and race-matched normal counterparts. If aberrant telomere shortening rate is observed in NBS cells, this study will further elucidate the underlying mechanism of how NBS1 affects the telomere shortening rate by looking into the potential changes in telomerase activity and shelterin complex in NBS cells.

Cancer predisposition is one of the characteristics of NBS disease. As telomere dysfunction has been implicated in carcinogenesis, this study will also examine the integrity of telomeres in NBS cells. If frequent telomere aberrations are observed in NBS cells, this study would provide new evidence to explain the high incidence of cancers in NBS patients from the point of telomeres.

2. MATERIALS AND METHODS

2.1 Cells

Table 3. List of fibroblasts and B-lymphocytes used in this study

	Disease	Cat. ID	Cell type	Immort-alization	Age	Sex	race
Pair1	Normal	AG09309	Fibroblast	NA	21	F	Caucasian
	NBS	GM07166	Fibroblast	NA	20	F	Caucasian
Pair2	Normal	GM00637	Fibroblast	SV40	18	F	Caucasian
	NBS	GM15989	Fibroblast	SV40	20	F	Caucasian
Pair3	Normal	AG14725	B-lymphocyte	EBV	11	M	Caucasian
	NBS	GM15814	B-lymphocyte	EBV	12	M	Caucasian
Pair4	Normal	GM22671	B-lymphocyte	EBV	28	F	Caucasian
	NBS	GM07078	B-lymphocyte	EBV	20	F	Caucasian
Normal fibroblast		GM01864	Fibroblast	NA	11	M	Caucasian

‘M’: male, ‘F’: female, ‘SV40’: Simian Virus 40, ‘EBV’: Epstein-Barr virus, ‘NA’: not applicable.

Table 4. List of cancer cells used in this study

Disease	Cat. ID	Cell type	Origin
Breast cancer MCF7	HTB-22	Epithelial	mammary gland
Colon cancer HCT116	CCL-247	Epithelial	colon

Cells used in this study were obtained from Coriell Cell Repositories (CCR) or ATCC. In each pair, the NBS cells were paired with normal cells under the criteria of age, gender and race. The NBS cell lines within each pair are homozygous for a deletion of 5 nucleotides in exon 6 of *NBS1* gene, called 657del5 mutation. Additionally, another three cell lines, including one normal fibroblast cell line GM01864, the human breast cancer cell line MCF7 and the human colon cancer cell line HCT116 were also used in particular experiments.

Fibroblast cell lines at CCR were established by outgrowth of undifferentiated mesodermal cells from a biopsy. The morphology of fibroblasts is spindle shaped or stellate. B-lymphocytes were isolated as peripheral blood mononuclear cells and transformed with Epstein-Barr virus. The B-lymphocytes are small round cells that grow as loose aggregates in suspension.

2.2 Cell culture

2.2.1 Cell culture conditions

The five fibroblast cell lines were cultured in Minimum Essential Medium Eagle (MEM, Gibco, Invitrogen) and the four B-lymphocytes cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Sigma-Aldrich). Both medium was supplemented with 15% fetal bovine serum (FBS, Gibco, Invitrogen), 1% L-Glutamine (Gibco, Invitrogen), 1% non-essential amino acid (NEAA, Gibco, Invitrogen) and 1% vitamin solution (Gibco, Invitrogen), 100 U/ml penicillin and streptomycin (Gibco, Invitrogen) and incubated in 37 °C under 5% CO_2. The two cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen) supplemented with 10% heat-inactivated FBS, 1% L-Glutamine and 100 U/ml penicillin and streptomycin.

2.2.2 Cell harvesting

Fibroblasts and cancer cells were harvested by trypsinization. Growing medium was removed and cells were washed with PBS once. 0.25% (w/v) Trypsin with 0.38 g/L EDTA (Gibco, Invitrogen) was added into cell culture dish which was then incubated in 5% CO_2, 37 ^{o}C incubator for 3 minutes. Cells were resuspended with MEM (for fibroblasts) or DMEM (for cancer cells) and centrifuge at 1000 × *g* for 5 minutes. The medium was aspirated and the pellet was washed with PBS twice. Cells were then used for following experiments.

B-lymphocytes were harvested in tubes and centrifuge at 1000 × *g* for 5 minutes. The medium was aspirated and the pellet was washed with PBS twice. Cells were then used

for following experiments.

2.2.3 Cell storage

For storage of cells, the pellet was resuspended with freezing medium composed of 10% DMSO (Sigma-Aldrich) and 90% FBS, and stored in cryovials (Thermo Scientific). Frozen cells were stored at -180 ^{o}C liquid nitrogen tank.

2.3 Western Blotting

2.3.1 Protein extraction and separation

Cell pellet was resuspended in 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 5 mmol/L EDTA, and 0.1% NP-40 with protease and phosphatase inhibitors (1 µg/ml Aprotinin, Leupeptin and Pepstatin, 1 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF). The lysate were centrifuged at 17,000 × *g* for 10 minutes before protein quantitation using Bradford assay (Biorad). The lysates were then mixed with reducing agent, 5 × loading buffer with 20 × DTT (Fermentas), and boiled at 95 ^{o}C for 5 minutes. The protein was run on appropriate SDS-PAGE gels and transferred to nitrocellulose membrane (Millipore). After transfer, the membrane was incubated with 5% skimmed milk, primary antibody and secondary antibody sequentially. Following incubation, the membrane was washed using Tris buffered saline- 0.1% (v/v) Tween 20 (TBST). Horseradish peroxidase (HRP) conjugated secondary anti-mouse or anti-rabbit IgG antibodies were used. Immunostaining was detected using ECL Plus Detection Regent (GE healthcare).

2.3.2 Antibodies

Table 5. List of antibodies used in this study

Antibody name	Company	Cat. No.	Antibody isotype	Molecular weight (KD)
NBS1	Cell signaling	#3002	Rabbit	95

ATM	Novus Biologicals	NB100-104	Rabbit	370
ATM-pS1981	Rockland	#200-301-400	Mouse	370
ATR	Cell signaling	#2790	Rabbit	250
ATR-pS428	Cell signaling	#2853	Rabbit	300
Chk2	Cell signaling	#2662	Rabbit	62
Chk2-pT68	Cell signaling	#2661	Rabbit	62
Chk1	Cell signaling	#2345	Rabbit	56
Chk1-pS317	Cell signaling	#2349	Rabbit	56
p53	Cell signaling	#9286	Mouse	53
p53-pS15	Cell signaling	#9284	Rabbit	53
γH2AX-pS146	Novus Biologicals	NBP1-19255	Mouse	14
p21	Cell signaling	#2946	Mouse	21
Cleaved caspases 3	Cell signaling	#9664	Rabbit	17,19
PARP	Abcam	#9542	Rabbit	24, 116
TRF1	Abcam	Ab14397	Mouse	55

POT1	Abcam	Ab47082	Rabbit	71
RAP1	Bethyl laboratories	A300-306A	Rabbit	60
TIN2	Abcam	Ab13791	Mouse	39
TPP1	Abnova	H00065057-M02	Mouse	86
TOPBP1	Calbiochem	PC743	Rabbit	180
α-tubulin	Sigma-Aldrich	T9026	Mouse	50
HRP-GAPDH	Cell signaling	#3683	Rabbit	37
HRP- β-actin	Abcam	Ab20272	Mouse	42

2.4 5-Bromo-2'-deoxy-uridine (BrdU) Labeling & Detection (Roche, Cat. No. 11444611001)

Cells were cultured in 96-well flat bottom plates (Thermo Scientific) in a density of 10,000 cells per well and incubate for 24 hours. 10 µM BrdU labeling solution and 1 µM Dox were added to culture medium at the same time and cells were incubated for either 10 hours or 22 hours. Before removing the culture medium, suspension cells were spin down for 10 minutes at 300 × *g* in a centrifuge. Cells were dried to the bottom of the 96-well plate for approximately 2 hours at 60 ^{o}C. Then, cells were fixed with 200 µl precooled fixative (70% ethanol p.a. in 0.5 M HCl) per well for 30 minutes at -30 ^{o}C. Fixative was removed and cells were washed 3 times with 250 µl wash medium (PBS containing 10% FBS) per well. Cells were Incubated with 100 µl nuclease working solution per well for 30 minutes at 37 ^{o}C water bath. Nuclease working solution was removed and cells were washed 3 times with 250 µl wash medium containing 10% FBS per well. 100 µl anti-BrdU-POD, Fab fragments, working solution was added per well for 30 minutes at 37 ^{o}C. Cells were washed 3 times with 250 µl washing buffer after antibody conjugate was removed. 100 µl peroxidase substrate was added per well. Cells were incubated at room temperature until positive samples show a green color, and is clearly distinguishable from the color of pure peroxidase substrate (2-30 minutes). Extinction of the samples was measured in a microplate reader at 405 nm with a reference wave-length at approximately 490 nm.

2.5 FITC Annexin V Apoptosis Detection (BD Pharmingen, Cat. No. 556570)

Cells were seeded with a density of 1×10^5 cells/ml in 10 ml medium, 1 day prior Dox treatment. Dox concentration and incubation period was decided based on experimental design. Cells were collected by centrifugation at 500 × *g* for 5 minutes. Cells were washed 2 times with cold PBS and resuspended in 1 × Binding Buffer at a concentration of 1×10^6 cells/ml. 100 µl of the solution (1×10^5 cells) was transferred to a 5 ml culture tube containing 5 µl of FITC Annexin V and 5 µl propidium iodide (PI). Cells were incubatesd for 15 minutes at room temperature in the dark. After incubation, 400 µl of 1 × Binding Buffer was added to each tube. Just before doing flow cytometry, cells were transferred to the flow tube through the filter (60 µm). Samples were analyzed within 1 hour by flow cytometry (BD LSR II Flow Cytometer).

2.6 TeloTAGGG Teloere Length Assay (Roche, Cat. No. 12209136001)

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). Cells (maximum 5×10^6) were centrifuged for 5 minutes at 300 × *g*. Pellet was resuspended in 200 µl PBS and with 20 µl proteinase K. Subsequently, 200 µl Buffer AL was added (without added ethanol) into the cell mixture. Mixture was mixed thoroughly by vortexing 5-10 seconds and incubated at 56 ^{o}C for 10 minutes. After incubation, 200 µl ethanol (96-100%) was added to the sample and thoroughly mixed by vortexing. The mixture was transferred into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 minute at 6,000 × *g*. After centrifugation, flow-through and the collection tube were discarded. DNeasy Mini spin column was replaced in a new collection tube. 500 µl Buffer AW1 was added before centrifugation for 1 minute at 6,000 × *g*. Flow-through and collection tube were discared again. DNeasy Mini spin column was replaced in a new collection tube. 500 µl Buffer AW2 was added before centrifugation for 3 minutes at 20,000 × *g* to dry the DNeasy membrane. Flow-through and collection tube were discared and the DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube. 30-50 µl Buffer AE was added directly onto the DNeasy membrane. Column was incubated at room temperature for 1 minute, then centrifuged for 1 minute at 6,000 × *g* to elute. DNA concentration was determined using Nanodrop (Thermo Scientific). The extracted genomic DNA was stored at -80 ^{o}C freezer.

Genomic DNA (1-2 µg) was digested by enzyme Hinf I and Rsa I for 2 hours at 37 ^{o}C. To stop the reaction, 5 µl of gel electrophoresis loading buffer was added with quick-spin of the reaction vial. Separation of digested DNA was done by 0.8% agarose gel

electrophoresis at 5 V/cm in 1 × TAE buffer until the bromophenol blue tracking dye is separated about 10 cm from the starting well. Gel was submerged in HCl solution and agitated for 5-10 minutes at room temperature, until the bromophenol blue stain changes color to yellow. Gel was rinsed 2 times with water before submerged in the denaturation solution and neutralization solution sequentially for 2 × 15 minutes. Gel was rinsed 2 times with water after each submergence. Digested DNA was blotted from the gel to nylon membrane by capillary transfer using 20 × SSC as a transfer buffer overnight. After southern transfer, transferred DNA on the wet blotting membrane was fixed by UV-crosslinking (120 mJ). Membrane was washed 2 times with 2 × SSC. For prehybridization, blot was submerged in prewarmed DIG Easy Hyb and incubated for 30-60 minutes at 42 ^{o}C. Prehybridization solution was discarded and hybridization solution was added to the membrane immediately. Membrane was incubated for 3 hours at 42 ^{o}C before washed twice with stringent wash buffer I for 5 minutes, followed by wash with stringent buffer II for 15-20 minutes at 50 ^{o}C. Membrane was rinsed in 100 ml 1 × washing buffer for 1-5 minutes. Then membrane was incubated in 100 ml freshly prepared 1 × blocking solution for 30 minutes. After blocking, membrane was incubated in 500-100 ml Anti-DIG-AP working solution for 30 minutes. Membrane was washed twice with 1 × washing buffer for 15 minutes before incubated in 100 ml of 1 × detection buffer for 2-5 minutes. Detection buffer was discarded and excess liquid was removed from the membrane by placing the membrane, DNA side up, on a sheet of absorbent paper. Wet membrane was immediately placed, DNA side facing up, on an opened hybridization bag and approximately 40 drops substrate solution was very quickly applied to the membrane. Substrate solution was carefully spread homogeneously over

the membrane without trapping air bubbles. Membrane was incubated for 5 minutes before exposed to X-ray film for appropriate time (10 minutes-24 hours) to get optimal result.

2.7 β-galactosidase Staining (US Biological, Cat. No. G1041-76)

Normal and NBS fibroblasts were cultured to the population doubling level (PDL) as required. PDL = 3.32(log (total viable cells at harvest/total viable cells at seed)). Growth medium was removed from the cells. The plate was rinsed 1 time with PBS. 1 ml of 1 × Fixative Solution was added to each 35 mm well to fix cells for 10-15 minutes at room temperature. The plate was rinsed two times with PBS. 1 ml of the β-galactosidase staining solution was added and the plate was incubated at 37 ^{o}C overnight in incubator. The next day, while the staining solution is still on the plate, the cells were checked under a microscope for the development of blue color.

2.8 Growth curve study

NBS as well as normal B-lymphocytes were cultured and split 1:3 every other day. Cell number was counted every time when splitting. Cells were seeded for growth in flasks (Thermo Scientific) with a density of 1×10^5 cells/ml medium. Cells were cultured for 18 days until massive cell death was observed in NBS B-lymphocytes.

2.9 Telomerase activity assay (XpressBio, Cat. No. XT-100)

Count cells and take 1×10^6 cells to use. Spin down cells and wash with PBS for 2 times. Resuspend cell pellet in 50 µl TeloExpress Lysis Buffer and incubate on ice for 30 minutes. Spin the sample at 12,000 × *g* for 3 minutes at 4 °C. Transfer supernatant to a fresh microcentrifuge tube. Respin sample at 12,000 × *g* for 20 minutes at 4 °C. Transfer supernatant to another fresh microcentrifuge tube. Then quantify protein concentration by Bradford assay. Dilute sample to a final concentration of 1.1 µg/µl with TeloExpress Lysis Buffer (5 µl in total). Add 1 µl sample into PCR Reaction Mixture (15 µl TeloExpress Master Mix and 9 µl RNase-free Water for one reaction) and mix well in PCR tubes. Place the tubes in the real-time PCR instrument (Qiagen, Rotor-Gene Q) and start the program. Use the real-time PCR instrument's software to plot threshold cycle and determine the telomerase activity of samples in reference to the standard curve.

2.10 RT-PCR

Total RNA was extracted using RNeasy kit with on-column DNase digestion (Qiagen) with slight modifications. Cell pellet was washed with PBS twice before Trizol (Invitrogen) lysis of samples. Pipet up and down for at least six times before centrifugation at 12,000 × *g* for 10 minutes at 4 ^{o}C. After centrifugation, transfer the supernatant into a new 1.5 ml microcentrifuge tube and add appropriate amount of chloroform to each tube (trizol : chloroform = 5:1). Shake by hands for 15 seconds before incubation for 2-3 minutes at room temperature. Centrifuge at 12,000 × *g* for 15 minutes at 4 ^{o}C to separate different layers. Transfer the upper colorless layer into a new 1.5 ml microcentrifuge tube. Add 1 volume of 70% ethanol to the colorless lysate and mix well by pipetting. Transfer up to 700 µl of the sample to an RNeasy spin column placed in a 2 ml collection tube and centrifuge for 15 seconds at 8,000 × *g*. Discard the flow-through and add 350 µl Buffer RW1 to the column and centrifuge for 15 seconds at 8,000 × *g*. Discard the flow-through, add 80 µl DNase I incubation mixture directly to the column membrane and place on bench top for 15 minutes. Then add 350 µl Buffer RW1 to the RNeasy spin column and centrifuge for 15 seconds at 8,000 × *g*. Add 500 µl Buffer RPE to the column after discarding flow-through and centrifuge at 8,000 × *g* for 15 seconds. The same step was repeated with centrifugation for 2 minutes. Place the RNeasy spin column in a new 1.5 ml microcentrifuge tube and add 30-50 µl RNase-free water directly onto the column membrane. Incubate at room temperature for 1 minute and centrifuge at 8,000 × *g* for another 1 minute to elute. Determine the total RNA concentration using Nanodrop. The extracted mRNA was stored at -80 ^{o}C freezer.

One step RT-PCR was performed using the One Step RT-PCR kit (Qiagen) following manufacturer's protocol. mRNA was transcribed and amplified following the program as described using Thermal Cyclers PCR machine: DNA synthesis for 30 minutes at 50 ^{o}C, followed by initial PCR activation step for 15 minutes at 95 ^{o}C. The three-step cycling profile is as follows: Denaturation at 94 ^{o}C for 30 seconds, Annealing at 55 ^{o}C for 30 seconds and Extension at 72 ^{o}C for 1 minute for 30 cycles. Final extension is at 72 ^{o}C for 10 minutes, followed by 4 ^{o}C forever. The primers for TRF2 are: 5'-TGCTCAAGTTCTA CTTCCACGA-3' and 5'-TTGATAGCTGATTCCAGTGGTG-3'. PCR products were run on 2% agarose gel and viewed under UV Gel Doc (BioRad).

2.11 Cytogenetic analysis of metaphase spreads

Normal and NBS fibroblasts were cultured to late passages. Metaphase spreads were prepared as described by the Jeppesen's protocol (Jeppesen 2000) with slight modifications. Cells were first arrested at metaphase by incubation with 0.1 µg/ml colcemid (Gibco Invitrogen) under normal culture conditions for 8-12 hours depending on the cell growth rates. Cells were then harvested by trypsinization. Hypotonic KCl solution (75 mM) was then added to cells for 10 minutes at 37 ^{o}C for swelling. Following which, 5×10^4 cells were diluted with the KCl solution containing 0.1% Tween 20 (v/v) and cytocentrifuged at 1000 rpm for 5 minutes onto glass microscopic slides. After cytocentrifugation, slides were allowed to dry for a few minutes before being transferred to KCM solution (120 mM NaCl, 10 mM Tris HCl pH 7.5, 0.5 mM EDTA, 0.1% (v/v) Triton X-100) for 15 minutes at room temperature to solubilize cellular membranes. Antibody incubations were carried out in KCM with 10% normal serum to block non-specific binding and slides were washed with KCM between primary and secondary antibody incubations. After incubation, slides were fixed with KCM containing 4% formaldehyde and finally washed with distilled water for 5 minutes before mounting with Vectashield with DAPI (Vectorlabs). Slides were then observed using Olympus Fluoview 1000 confocal microscopy system.

2.12 Transfection, virus production and cell infection

The following plasmids were used for transfection and virus production.

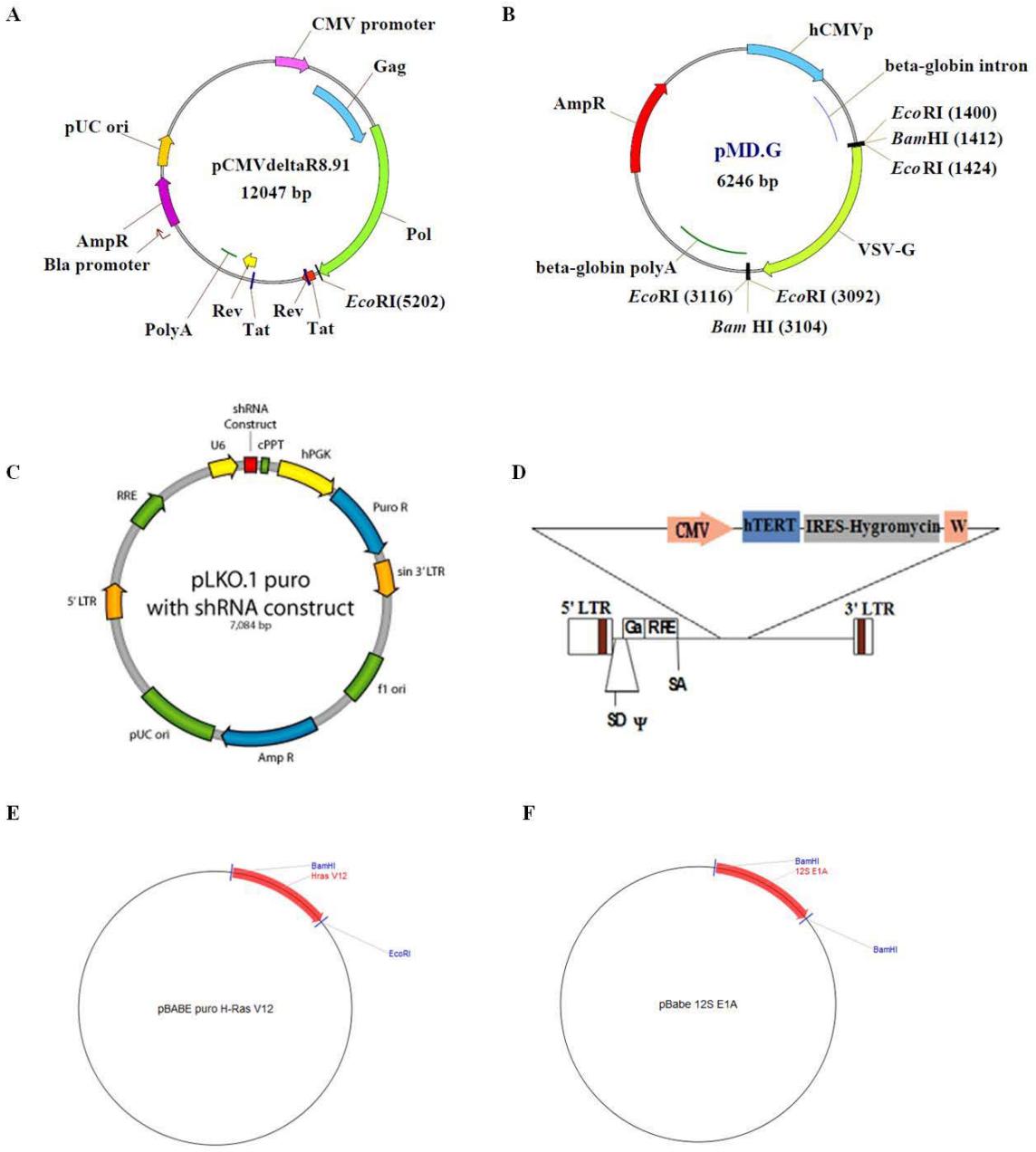

Figure 2.1 Plasmid constructs used for virus production. A. lentiviral packaging plasmid pCMV. B. Lentiviral enveloping plasmid pMD.G. C. Lentiviral shRNA NBS1, Addgene plasmid 1864. D. Lentiviral CMV hTERT. E. Retroviral pBABE H-RAS V12, Addgene plasmid 9051. F. Retroviral pBABE E1A (the adenovirus early 1A region), Addgene plasmid 18742.

2.12.1 Transformation and amplification of plasmids

Competent *Escherichia coli* cells were used for transformation. Thaw cells from -80 °C on wet ice. Add 1 µl plasmid into tubes containing competent cells (50 µl/tube) and tap tube to mix. Incubate the mixture on ice for 15 minutes. After incubation, put the mixture tube to 42 °C water bath for 90 seconds for heat shock. Place back the tube on ice for 3 minutes. Add 200 µl lysogeny broth (LB) medium (without ampicillin) to the tube and shake the tube for 30 minutes at 30 °C. Take out the mixture in tube and spread onto LB plates (with ampicillin). Let plates dry at room temperature for 2 minutes before incubation for 24 hours at 32 °C for colony growth. Pick colonies into liquid LB medium (with ampicillin) to amplify the plasmids by shaking at 30 °C for 16 hours.

Plasmid was then extracted from cells and purified using QIAprep Spin Miniprep Kit (Qiagen). Bacteria pellet was resuspended in 250 µl Buffer P1 in a microcentrifuge tube. Following this step, 250 µl Buffer P2 was added and mixed thoroughly by inverting the tube for 4-6 times. Before centrifugation, 350 µl Buffer N3 was added to mixture and mixed immediately by inverting the tube 4-6 times. Then centrifuge the tube for 10 minutes at 13,000 rpm. Apply the supernatants to the QIAprep spin column by pipetting and centrifuge for 30-60 seconds. Discard the flow-through and add 0.75 ml Buffer PE to wash the column by centrifuging for 30-60 seconds. Discard the flow-through and centrifuge for an additional 1 minute to remove the residual wash buffer. After centrifugation, place the column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB or nuclease-free water to the center of each column and let it stand for 1 minute before centrifuging for 1 minute. The extracted plasmid DNA was stored at -

30 °C freezer.

2.12.2 Lentivirus production

Plasmids with interested genes (12 μg), virus enveloping plasmid pMD.G (4 μg) and virus packaging plasmid pCMV (8 μg) were co-transfected into 293T cells cultured in 10 cm dishes using Lipofectamine (Invitrogen) method. Medium that is used for transfection was Opti-MEM reduced serum medium (Invitrogen). Six or eight hours post transfection, medium was changed to DMEM. Virus was harvested in two batches (at 24 and 48 hours) and filtered using 0.45 μm filters. The virus were then stored at -80 °C in 1.5 ml aliquots.

2.12.3 Retroviral production

12 μg of plasmids were transfected into Pheonix cells cultured in 10 cm dishes using Lipofectamine method. Medium that is used for transfection was Opti-MEM reduced serum medium (Invitrogen). Six or eight hours post transfection, medium was changed to Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen) supplemented with 10% heat-inactivated FBS (without penicillin and streptomycin). Virus was harvested in two batches (at 24 and 48 hours) and filtered using 0.45 μm filters. The virus were then stored at -80 °C in 1.5 ml aliquots.

2.12.4 Cell infection

For cell infection, 1.5 ml virus with 0.5 ml DMEM (without penicillin and streptomycin) and 2 μl polybrene (8 mg/ml) were added to cells cultured in 6-well plates with ~70% confluency. Cells were incubated at normal culture condition for 24 hours before double infection was performed. After 2 days of infection, remove medium with virus particles

and add fresh DMEM medium and culture for 24 hours before selection with appropriate antibiotics.

2.13 Soft agar assay/Anchorage-independent growth assay

Infected cells were seeded into each well of a 24-well plate with a density of 1×10^5 cells/well. The cells were embedded into 0.3% (w/v) noble agar (Sigma-Aldrich) in DMEM supplemented with 16.66% FBS, over a substratum of 0.6% noble agar in DMEM supplemented with 10% FBS. Fresh medium was added weekly and the agar plate was observed for colony formation for 6 weeks. At the end of 6 weeks, the colonies from each well were counted under microscope. Tumorigenicity of different samples was assessed by comparing the average number of colonies observed from 8 wells.

3. RESULTS

3.1 NBS1 deficiency does not affect the expression of MRE11 and RAD50

In this study, cells derived from NBS patients who have typical 657del5 mutation of the *NBS1* gene were used. As controls, normal cells with wild type *NBS1* gene were also employed and paired with NBS cells under the criteria of age, gender and race for a more reliable comparison. As shown, the wild type NBS1 protein was only expressed in normal cells but not in NBS cells (Figure 3.1A), which corresponds to the notion that the 657del5 mutation abolishes the expression of full length NBS1 protein (Maser, Zinkel et al. 2001). We further checked the expression level of another two components that consist of MRN complex, MRE11 and RAD50, in NBS cells as well as in normal cells. Results showed that although NBS1 deficiency, the expression of MRE11 and RAD50 was not affected (Figure 3.1B).

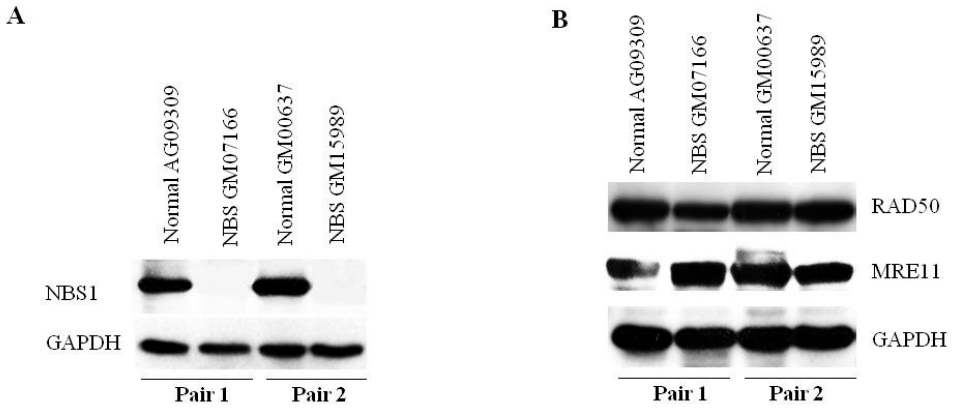

Figure 3.1 NBS1 deficiency does not affect the expression of MRE11 and RAD50. A. The expression of NBS1 protein in NBS fibroblasts as well as in age, race and gender-matched normal fibroblasts. The four cell lines were classified into two pairs, nominated as Pair 1 and Pair 2. GAPDH serves as the loading control. B. the expression of MRE11 and RAD50 in NBS fibroblasts as well as in normal fibroblasts. GAPDH serves as the loading control.

3.2 NBS1 deficiency affects ATM phosphorylation

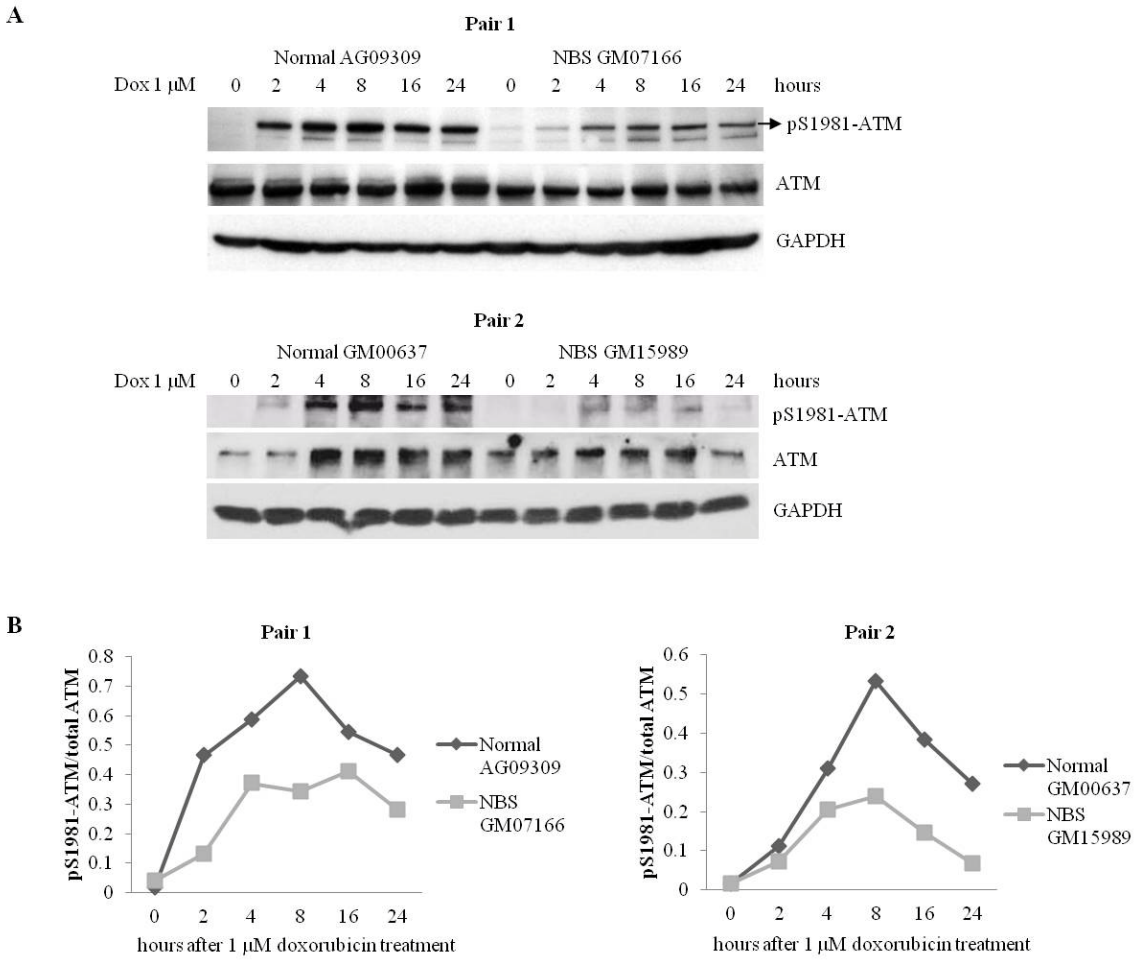

Figure 3.2 NBS1 deficiency affects ATM phosphorylation. A. The expression and phosphorylation level of ATM in NBS fibroblasts as well as in normal fibroblasts. Cells were treated with 1 μM Dox and collected at the time points indicated. Arrow indicates the band that represents pS1981-ATM. GAPDH serves as the loading control. B. The immunoblots in A were scanned and quantitated by densitometer, and the phosphorylation level of ATM was normalized to its total protein level.

To determine if NBS1 deficiency affects the phosphorylation of ATM, these two pairs of fibroblasts (Pair 1 and Pair 2) were then subjected to 1 μM Dox treatment and the phosphorylation level of ATM at Ser1981 was examined at different time points by

western blot. Results showed that ATM was quickly activated in normal cells and reached the highest level in 8 hours after Dox treatment (Figure 3.2A). However, in NBS cells, ATM phosphorylation was severely impaired, exhibited by a much lower level than that in normal counterparts (Figure 3.2B). Although ATM phosphorylation level decreased dramatically in NBS cells, there was still a basal level detectable (Figure 3.2A), indicating that NBS1 deficiency does not fully abolish ATM phosphorylation.

3.3 NBS1 deficiency affects the phosphorylation of ATM downstream targets

If NBS1 deficiency affects ATM activation, whether the activation of ATM downstream targets is also affected is the question that we want to address next. H2AX, p53 and Chk2 are three important ATM downstream substrates that are involved in DNA damage response (Varon, Vissinga et al. 1998). The phosphorylation statuses of these three proteins were also examined by western blot. Results showed that the phosphorylation of H2AX at Ser139 and phosphorylation of p53 at Ser15 were also severely affected in NBS cells (Figure 3.3A). In normal cells, these two proteins were quickly phosphorylated to a high level and the high phosphorylation level was maintained for all the rest time points detected. But in NBS cells, the phosphorylation level was significantly decreased (Figure 3.3A). Moreover, the total level of p53 was also affected in NBS cells, suggesting a possibility that NBS1 deficiency compromises p53 stability. The defects in phosphorylation of p53 were also observed in human breast cancer cells MCF7 with NBS1 knockdown (Figure S1). Surprisingly, the phosphorylation level of Chk2 at Thr68 was not reduced in NBS cells, but only exhibited a delay in activation in pair 2. As shown, Chk2 was activated and reached a high level within 2 hours in normal cells, but was activated in NBS cells at a much later time point of 8 hours (Figure 3.3B). Taken together, these results suggest that NBS1 deficiency could affect the phosphorylation of ATM downstream targets, leading to either a lower phosphorylation level or a delayed activation.

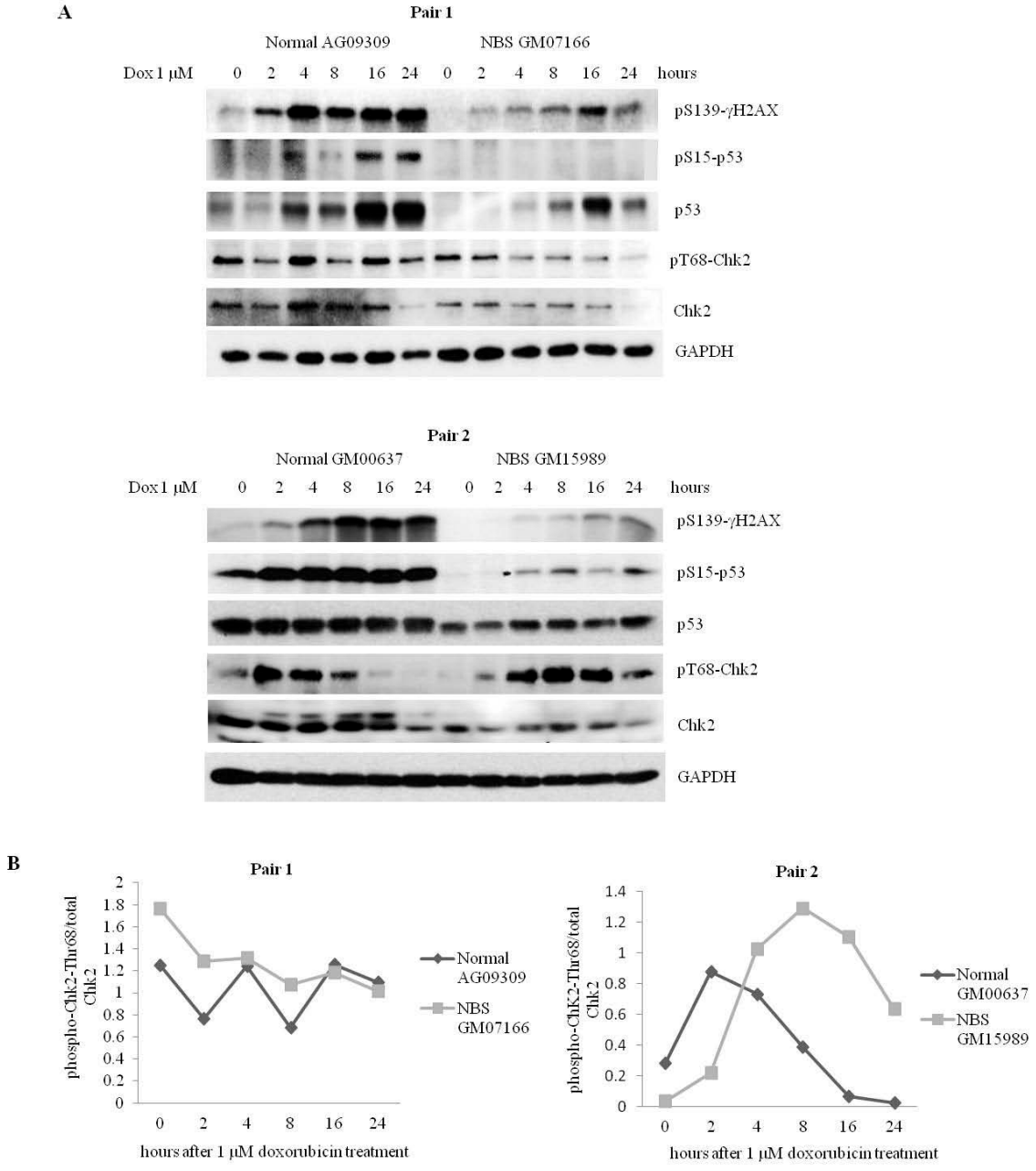

Figure 3.3 NBS1 deficiency affects the phosphorylation of ATM downstream targets. A. The phosphorylation of ATM downstream targets, including histone H2AX, p53 and Chk2. Cells were treated with 1 μM Dox and collected at the time points indicated. GAPDH serves as the loading control. B. The immunoblots of Chk2 and pT68-Chk2 were scanned and quantitated by densitometer, and the phosphorylation level of Chk2 was normalized to its total protein level.

3.4 NBS1 deficiency also affects ATR phosphorylation and the phosphorylation of ATR downstream target Chk1

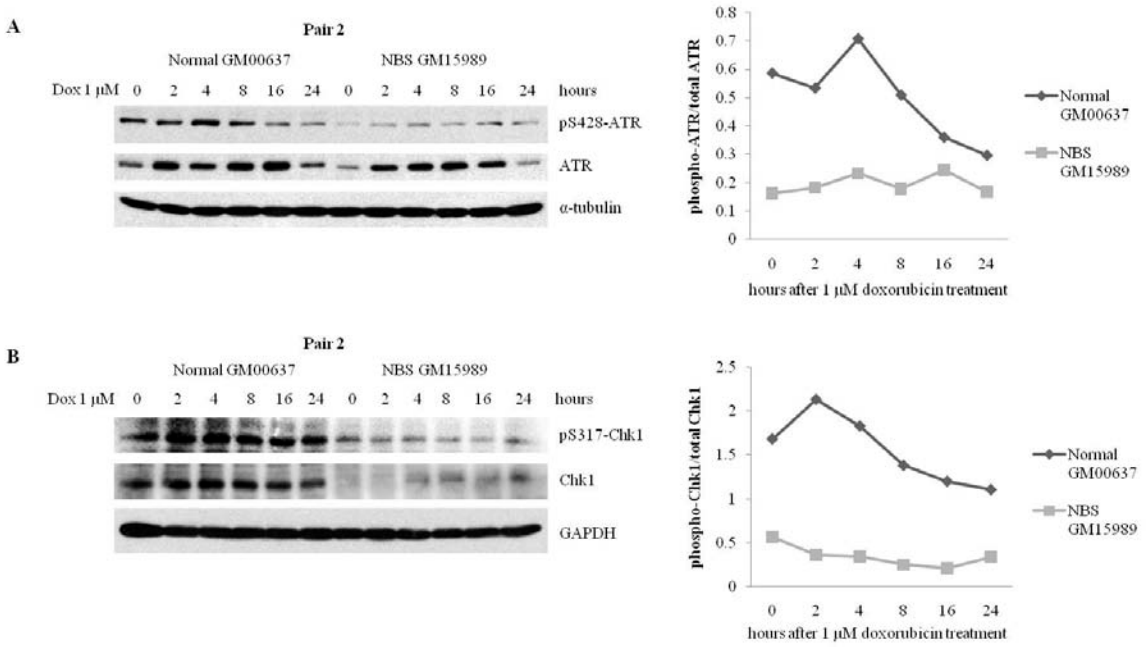

Figure 3.4 NBS1 deficiency affects the phosphorylation of ATR as well as its downstream target Chk1. A. The expression and phosphorylation of ATR in Pair 2. Cells were treated with 1 μM Dox and collected at the time points indicated. α-tubulin serves as the loading control. The immunoblots of ATR and pS428-ATR were scanned and quantitated by densitometer, and the phosphorylation level of ATR was normalized to its total protein level. B. The phosphorylation of ATR downstream target Chk1 in Pair 2. Cells were treated with 1 μM Dox and collected at the time points indicated. Phosphorylation of Chk1 at Ser317 was detected. GAPDH serves as the loading control. The immunoblots of Chk1 and pS317-Chk1 were scanned and quantitated by densitometer, and the phosphorylation level of Chk1 was normalized to its total protein level.

The phosphorylation status of ATR upon Dox treatment was also investigated. As shown by western blot (Figure 3.4A), normal cells exhibited ATR phosphorylation at Ser428 even without Dox treatment. When exposed to 1 μM Dox, the phosphorylation level in normal cells increased and reached a peak in 4 hours. But NBS cells only showed a subtle increase when subjected to Dox and the phosphorylation level was much lower than that in normal counterparts (Figure 3.4A). This result suggests that NBS1 deficiency also

compromises the phosphorylation of ATR. In addition to ATR, we also observed a decrease in the induction level of TOPBP1 (Figure S2). Since TOPBP1 is crucial in activation of ATR and the initiation of ATR-dependent signaling pathway (Kumagai, Lee et al. 2006; Choi, Lindsey-Boltz et al. 2009), we suggested that the decrease in ATR phosphorylation was due to the reduction in TOPBP1 level. Chk1, a direct downstream target of ATR (Zhao and Piwnica-Worms 2001), also showed impaired phosphorylation at Ser317 in NBS cells (Figure 3.4B). Collectively, NBS1 deficiency affects both ATR and ATR-dependent phosphorylation of downstream substrate.

3.5 NBS1 deficiency delays inhibition of DNA synthesis after DNA damage occurs

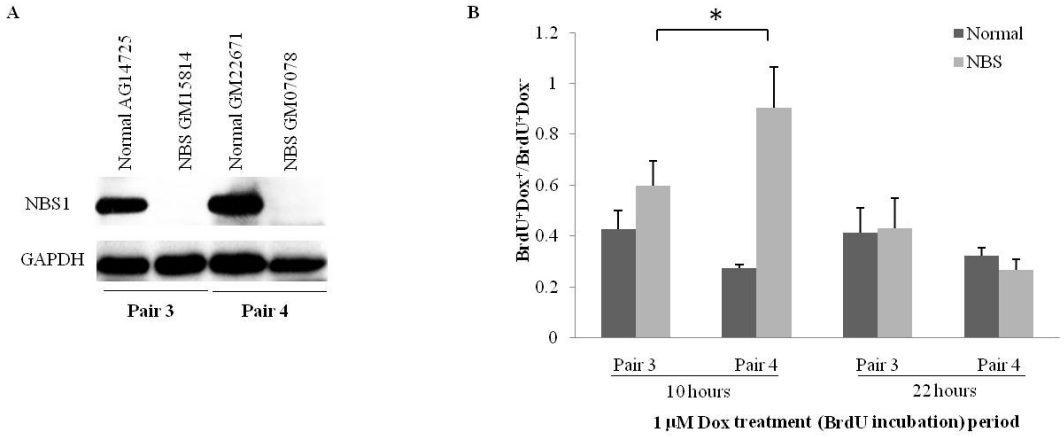

Figure 3.5 NBS1 deficiency delays inhibition of DNA synthesis after DNA damage occurs. A. The expression of NBS1 protein in NBS B-lymphocytes as well as in age, race and gender-matched normal B-lymphocytes. The four cell lines were classified into two pairs, nominated as Pair 3 and Pair 4. GAPDH serves as the loading control. B. Cells were seeded into 96-well plate and after culturing for 2 days, cells were treated with 1 μM Dox and 10 μM BrdU at the same time for either 10 or 22 hours. The bar represents the ratio of Dox-treated $BrdU^+$ cells to untreated $BrdU^+$ cells. Data are mean ± S.D. from triplicates. (*, $P \leq 0.05$)

DNA damage response could lead to inhibition of DNA synthesis to stop the propagation of "bad" cells with DNA lesions. We next investigated the potential roles of NBS1 in eliciting inhibition of DNA synthesis when DNA is damaged. Since pair 2 fibroblasts are transformed with simian virus 40 which would render G1/S checkpoint inactive and therefore affect the number of cells entering S phase for DNA synthesis (Petrini, Attwooll et al. 2009), we used additional 2 pairs of B-lymphocytes for analysis of DNA synthesis status (Pair 3 and Pair 4). As shown in the western blot, full length NBS1 was only expressed in normal cells but not in NBS cells (Figure 3.5A). Furthermore, similar as the phenotypes shown in NBS fibroblasts, the ATM-dependent phosphorylation events, such as phosphorylation of histone H2AX, p53 and Chk2, were also impaired in NBS B-

lymphocytes (Figure S3).

BrdU incorporation assay was employed to assess the proliferation profile of cells after 1 μM Dox treatment for either 10 or 22 hours. From this result, we found that the cell proliferation was suppressed after Dox treatment in both normal and NBS cells, exhibited by the ratio of $BrdU^{+}Dox^{+}$ cells to $BrdU^{+}Dox^{-}$ cells less than 1 (Figure 3.5B). Although suppression of cell proliferation was observed in both normal and NBS cells, at 10 hours, NBS cells showed a lesser degree of arrest than the normal cells, indicated by a higher $BrdU^{+}Dox^{+}$ to $BrdU^{+}Dox^{-}$ cells ratio. It was only after 22 hours of Dox treatment, did the NBS cells exhibit a similar degree of arrest as their normal counterparts (Figure 3.5B). This result indicates the suppression of proliferation in NBS cells is not as efficient as that in normal cells, suggesting a delay in inhibition of DNA synthesis in NBS cells.

3.6 NBS1 deficiency affects the initiation of apoptosis

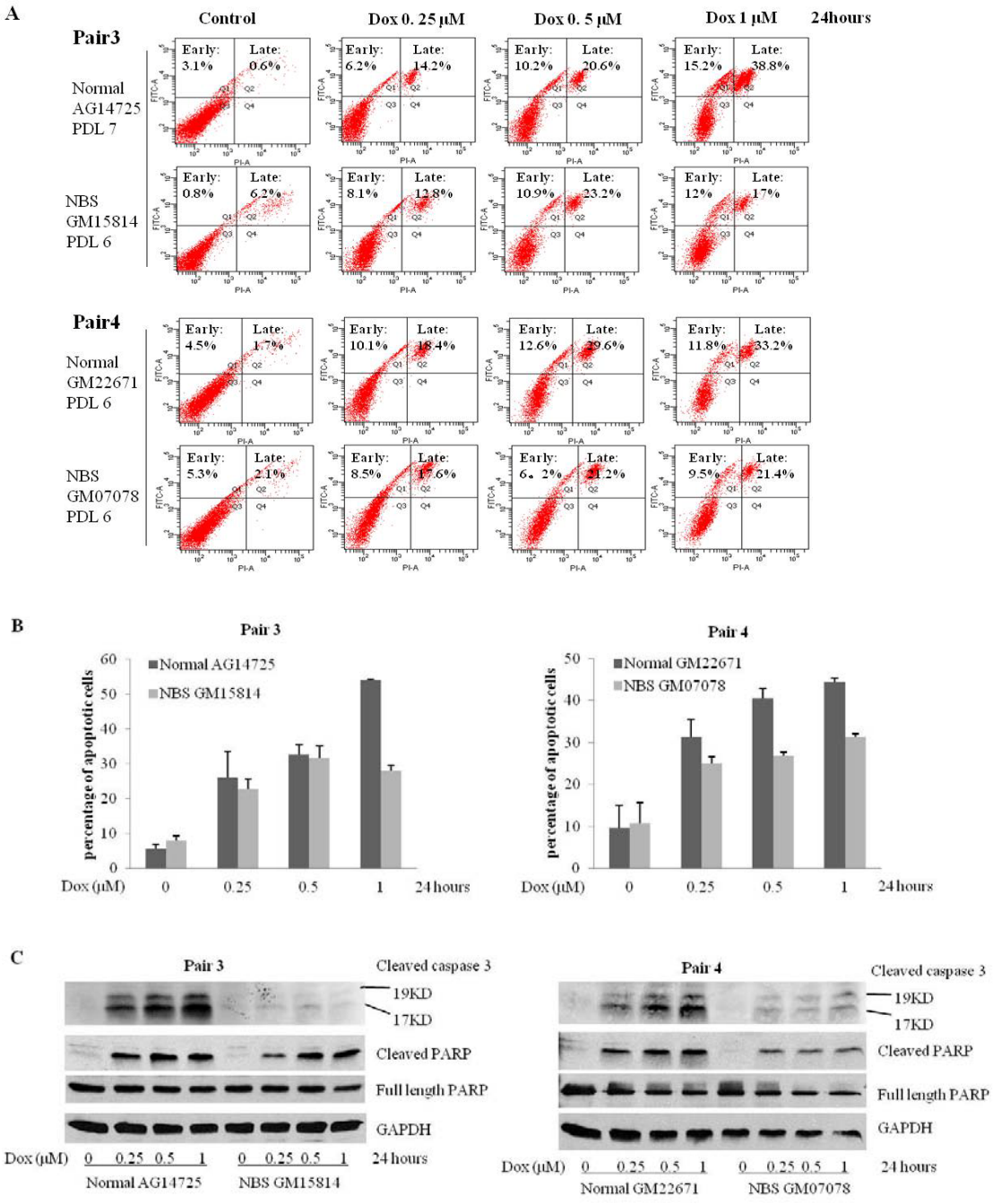

Figure 3.6 NBS1 deficiency affects the initiation of apoptosis. A. FITC Annexin V apoptosis assay. B-lymphocytes were treated with Dox at the indicated concentrations for 24 hours. The number of apoptotic cells was analyzed by flow cytometry. B. Quantitation of the percentage of apoptotic cells (including early and late apoptotic cells) shown in A. Data are mean ± S.D. from 3 independent experiments. C. Western blot analysis of apoptosis-related proteins, including caspases 3 and PARP. Cells were treated with Dox at the indicated concentration for 24 hours.

Another cellular event of DNA damage response is to initiate apoptosis when DNA damage is beyond repair. Cells treated with different concentration of Dox for 24 hours were harvested and subjected to flow cytometry analysis. Results showed that NBS cells had comparable apoptosis level to normal cells under lower concentration of Dox treatment. When the concentration of Dox was increased to a high concentration of 0.5 μM (in pair 4) or 1 μM (in pair 3), normal cells exhibited elevated level of apoptosis. However, the apoptosis level in NBS cells remained low as that under lower concentrations of Dox treatments (Figure 3.6A & B), indicating that NBS cells were defective in inducing apoptosis when cells were exposed to high dosage of Dox. Western analysis of apoptosis associated markers showed that cleaved caspases 3 almost diminished in NBS cells. However, as a direct downstream target of caspases 3 (Simbulan-Rosenthal, Rosenthal et al. 1998), PARP only exhibited a minor decrease in its cleaved form in NBS cells (Figure 3.6C). This is probably due to the low level of cleaved caspases 3 in NBS cells. The low efficiency in cleavage of these proteins may be responsible for the defects of NBS cells in initiation of apoptosis under high concentration of Dox treatment.

3.7 NBS1 deficiency promotes telomere shortening and an earlier onset of senescence in fibroblasts

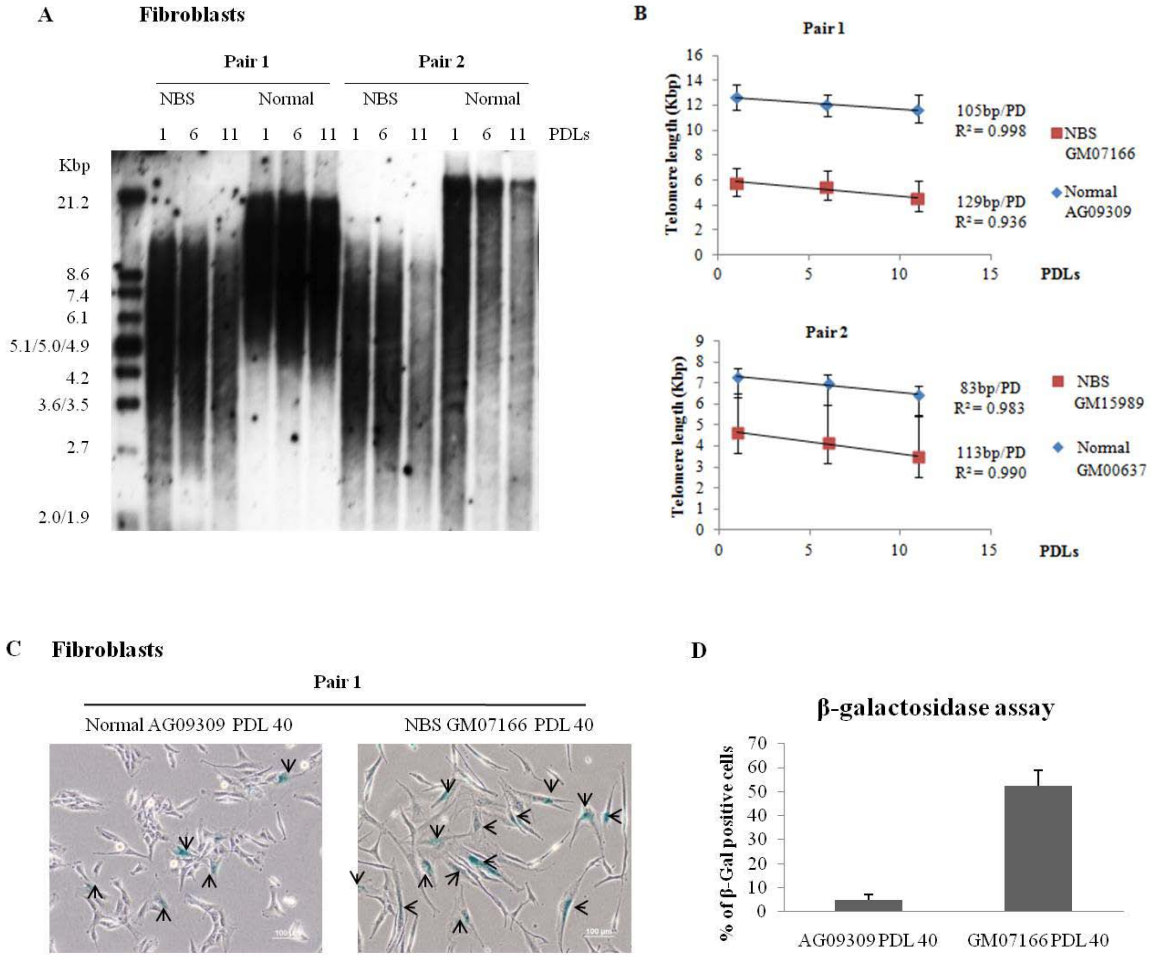

Figure 3.7 NBS1 deficiency leads to accelerated telomere shortening and an earlier onset of senescence in NBS fibroblasts. A. Measurement of the telomere restriction fragment length. Genomic DNA isolated from normal and NBS fibroblasts at indicated PDLs were analyzed. B. Telomere shortening rate in normal and NBS fibroblasts. Data are mean ± S.D. from duplicate experiments. Telomere shortening rate (slope of the regression line) and Spearman's regression coefficient are indicated. C. Cellular senescence assay in fibroblasts using β-galactosidase staining. Arrows indicate senescent cells. D. Bars represent the percentage of β-galactosidase positive cells. Data are mean ± S.D. from 5 images each.

Premature aging has been observed in NBS fibroblasts *in vitro* (Ranganathan, Heine et al.

2001). Premature cellular senescence could be elicited by accelerated telomere shortening. We therefore asked whether NBS1 deficiency elicits premature aging through regulating telomere attrition rate. Telomere length of the two pairs of fibroblasts was tested by the Terminal Restriction Fragment southern blot. Result showed that the telomere length of NBS fibroblasts was generally shorter than that of age-matched normal fibroblasts, which probably represents the long-term accumulative effect of an accelerated telomere shortening rate of NBS cells *in vivo*. When comparing the telomere attrition rate with each replication cycle *in vitro*, we found that NBS fibroblasts indeed had a higher telomere shortening rate compared to that in normal fibroblasts (Figure 3.7A). For each replication cycle, the telomere shortening rate of NBS fibroblasts was around 30 bp faster than that of its respective normal counterparts (Figure 3.7B). This result strongly indicates that NBS1 plays a role in telomere length maintenance and the deficiency of NBS1 leads to faster telomere attrition.

We performed β-galactosidase assay to study the senescence status of normal as well as NBS fibroblasts *in vitro*. Cells were cultured to the same PDL and stained, and the cells stained blue were counted as senescent cells. Consistent with the accelerated telomere shortening, NBS fibroblasts exhibited a significantly higher percentage of cells undergoing senescence compared to normal cells with the same PDLs (Figure 3.7C & D). These results suggest that NBS cells have a larger population of cells with critically short telomeres.

3.8 NBS1 deficiency leads to an earlier onset of cell death in B-lymphocytes

Progressive telomere shortening in the absence of telomerase would eventually trigger DNA damage responses and lead to replicative senescence or senescence-associated apoptosis (Blasco 2005). In this regard, accelerated telomere shortening could not only lead to earlier onset of senescence, but also lead to earlier occurrence of senescence-associated apoptosis in cells. In order to examine the role of NBS1 in induction of senescence-associated apoptosis, the two pairs of B-lymphocytes were cultured *in vitro* till late passages and subjected to flow cytometry analysis. Results showed that NBS B-lymphocytes had a higher percentage of cells undergoing apoptosis compared to normal counterparts when cultured to similar or even lower PDLs (Figure 3.8A & B), indicating NBS1 deficiency leads to earlier and more severe senescence-associated apoptosis.

This result was corroborated by microscopy images of NBS B-lymphocytes which showed prevalent cell debris under normal culture conditions, while normal B-lymphocytes remained spherical even at higher PDLs, suggesting a healthy growing status (Figure 3.8C). The growth curve demonstrated the trend of replication and apoptosis status of NBS B-lymphocytes over 18 days. As shown (Figure 3.8D), the growth rate of NBS B-lymphocytes is comparative to that of normal cells at the early days of cell culture. But at later days, the number of NBS B-lymphocytes ceased increasing and even started to decrease, probably indicating the onset of cellular senescence and cell death in these cells. By contrast, normal B-lymphocytes only had a slight decrease in cell growth rate even at later days, suggesting a healthy cell replication and proliferation status.

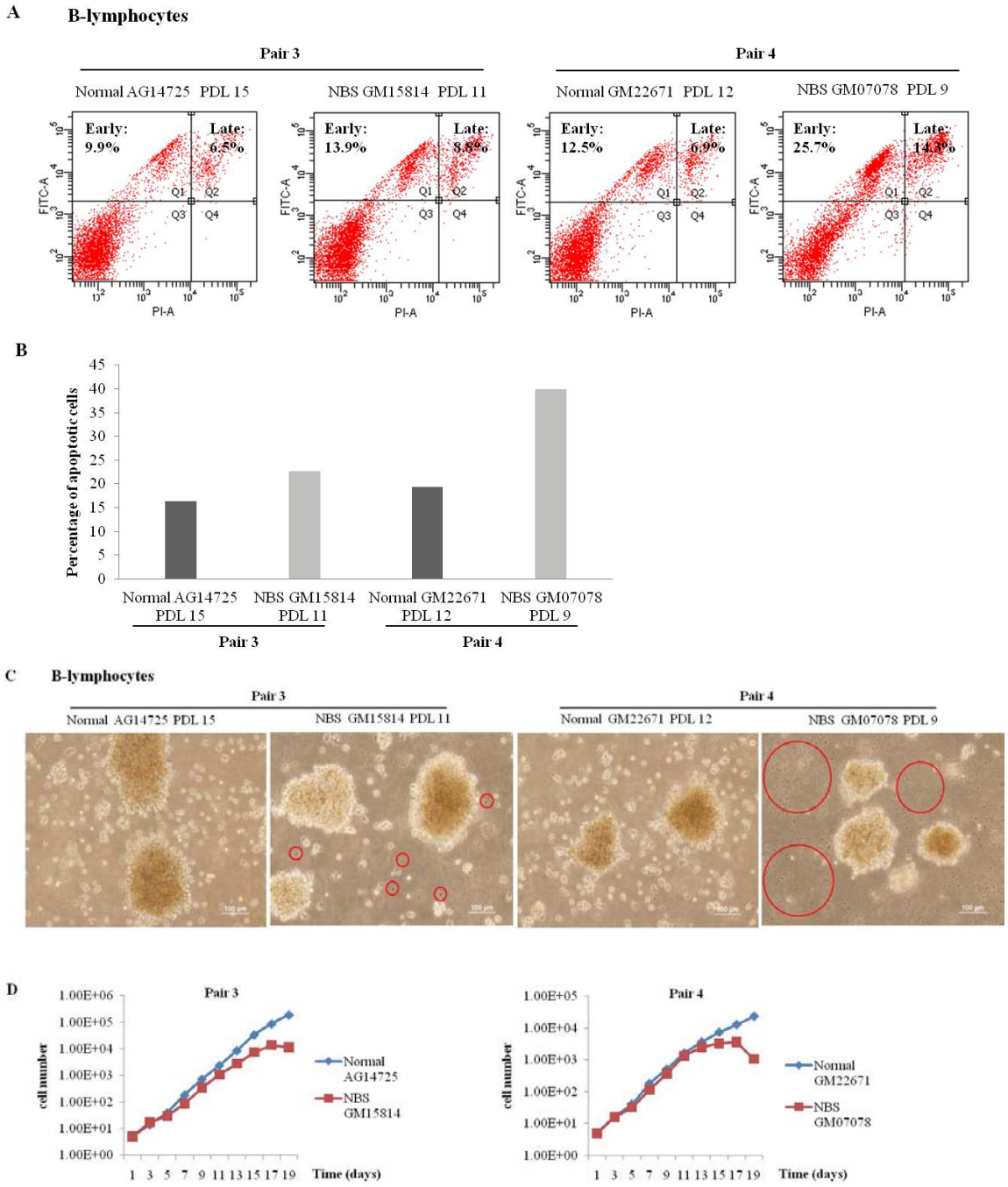

Figure 3.8 NBS1 deficiency leads to an earlier onset of cell death in B-lymphocytes. A. FITC Annexin V apoptosis assay. B-lymphocytes were cultured to late passages under normal culture condition without drug treatment. The number of apoptotic cells was analyzed by flow cytometry. B. Quantitation of the percentage of apoptotic cells (including early and late apoptotic cells) shown in A. C. Cellular morphologies of B-lymphocytes at late passages. Circles enclose the dead cell debris. D. Growth curve of B-lymphocytes. Cells were split and counted every other day over 18 days. The accumulative cell number was shown against days.

3.9 Accelerated telomere shortening is not observed in NBS B-lymphocytes

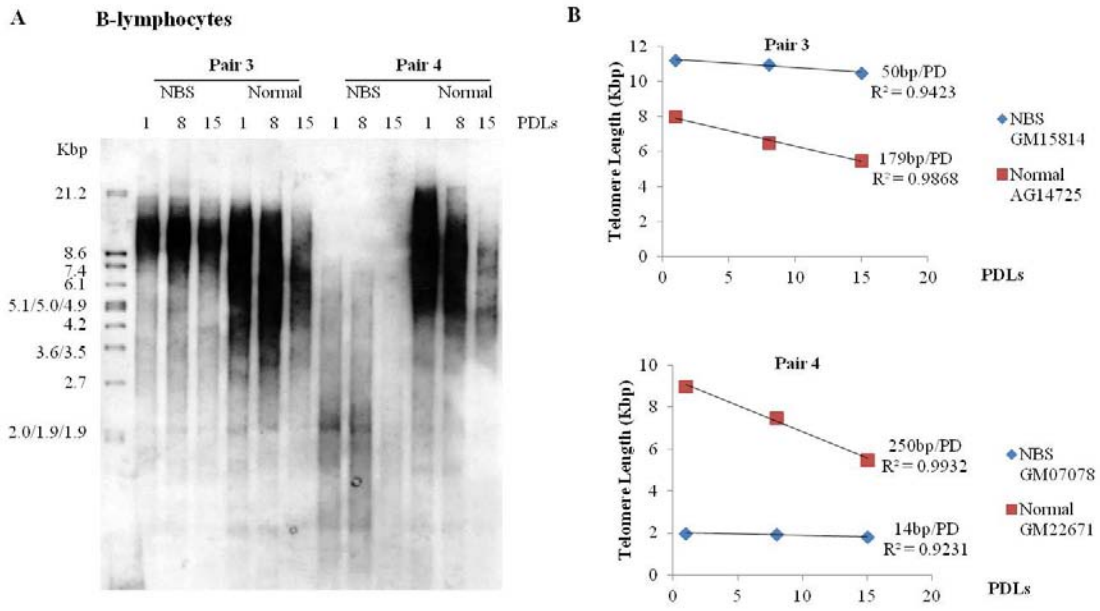

Figure 3.9 NBS1 deficiency does not lead to accelerated telomere shortening in NBS B-lymphocytes. A. Measurement of the telomere restriction fragment length. Genomic DNA isolated from normal and NBS B-lymphocytes at indicated PDLs were analyzed. B. Telomere shortening rate in normal and NBS B-lymphocytes. Telomere shortening rate (slope of the regression line) and Spearman's regression coefficient are indicated.

The earlier onset of senescence-associated apoptosis could be driven by accelerated telomere attrition which would result in the faster generation of cells with critically short telomeres. Therefore, telomere length of the two pairs of B-lymphocytes was tested by the Terminal Restriction Fragment southern blot. However, the shortened telomeres were only observed in the NBS B-lymphocytes of Pair 4, but not in the one of Pair 3 (Figure 3.9A). Furthermore, when comparing the telomere shortening rate with each replication cycle, a lower rather than a higher telomere shortening rate was detected in both NBS B-lymphocytes (Figure 3.9B), suggesting that NBS1 deficiency does not cause accelerated

telomere shortening in B-lymphocytes. The earlier occurrence of senescence-associated apoptosis observed in NBS B-lymphocytes is probably due to other mechanisms. Moreover, this result suggests that the extremely short telomere length observed in NBS B-lymphocytes of Pair 4 is not because of accelerated telomere attrition, but probably due to their long proliferation history in NBS patients before isolated.

3.10 NBS1 deficiency does not affect telomerase activity

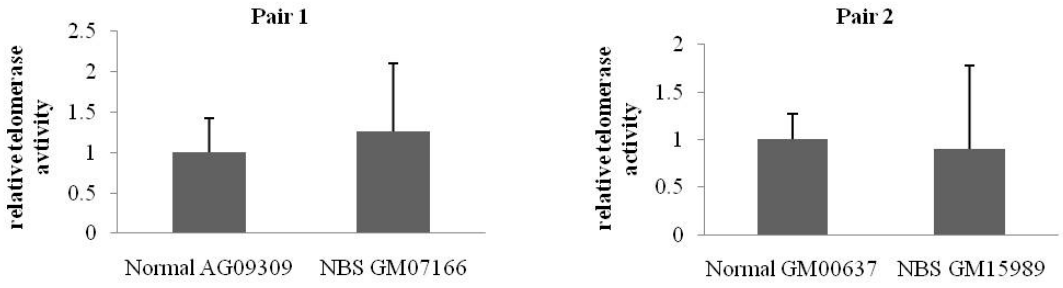

Figure 3.10 Real-time PCR for relative telomerase activity in NBS versus normal fibroblasts. Cells with similar PDLs were lysed in TeloExpress lysis buffer and the supernatant containing telomerase was used for subsequent Real-time PCR analysis. Data are mean ± S.D. from 2 biological repeats.

Telomere length is maintained by the activity of telomerase. Although it is generally thought that human primary fibroblasts lack telomerase activity, transient expression of telomerase has been reported in human fibroblasts (Masutomi, Yu et al. 2003). We next investigated whether telomerase is involved in the regulation of telomere shortening rate in NBS fibroblasts. Telomerase activity of NBS fibroblasts was compared with normal counterparts by real-time PCR. Results showed that NBS fibroblasts have comparative telomerase activity to normal counterparts (Figure 3.8), suggesting that the accelerated telomere shortening is not due to the changes in telomerase activity.

3.11 NBS1 deficiency leads to upregulation of TRF2 in fibroblasts

Shelterin complex proteins protect the telomere integrity, but it also has been claimed that these proteins are negative regulators for telomere length (de Lange 2005). We next looked into the different components of shelterin complex and found that the cellular level of TRF2 was upregulated in NBS fibroblasts (Figure 3.9A). However, the expression of other shelterin components, including TRF1, RAP1 and POT1, did not show obvious changes (Figure 3.9B). RT-PCR confirmed this result by showing an upregulation of TRF2 at mRNA level (Figure 3.9C). The overabundance of TRF2 at telomere ends may negatively regulate telomere length, resulting in accelerated telomere shortening in NBS fibroblasts.

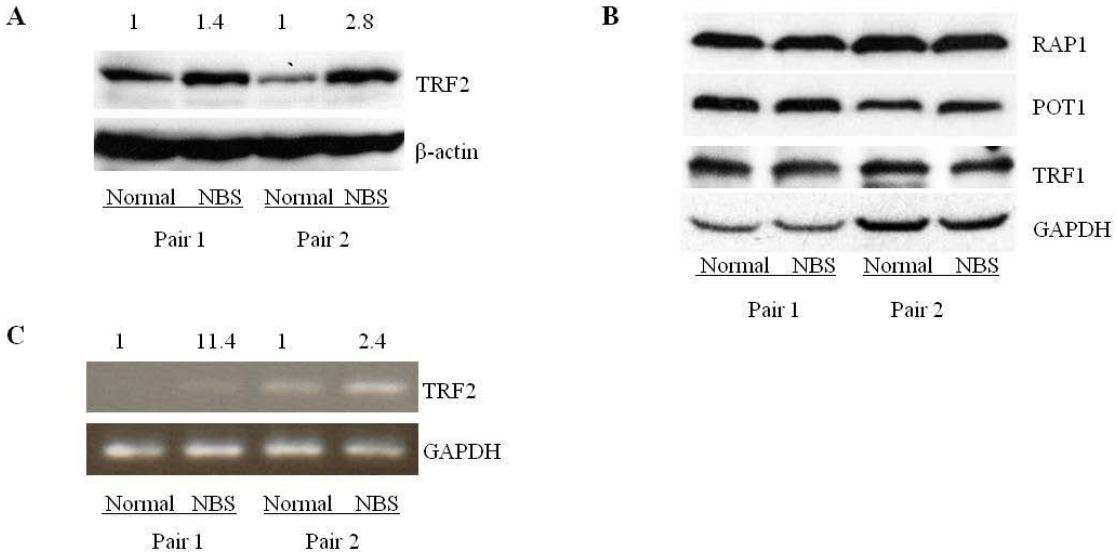

Figure 3.11 NBS1 deficiency leads to upregulation of TRF2. A. Western blot analysis of the TRF2 protein level in NBS fibroblasts as well as in normal counterparts with similar PDLs. The numbers above the blot indicate its fold difference measured by densitometer with normal cell's TRF2 protein level being set at a reference value of 1. β-actin serves as the loading control. B. Western blot analysis of the other shelterin complex proteins in NBS and normal fibroblasts, including TRF1, POT1 and RAP1. GAPDH serves as the loading control. C. RT-PCR analysis of the TRF2 mRNA level in NBS and normal fibroblasts. mRNA was extracted from fibroblasts with similar PDLs and used in one-step RT-PCR analysis. The numbers above the blot indicate its fold difference measured by densitometer with normal cell's TRF2 protein level being set at a reference value of 1.

3.12 TRF2 level is not affected in NBS B-lymphocytes

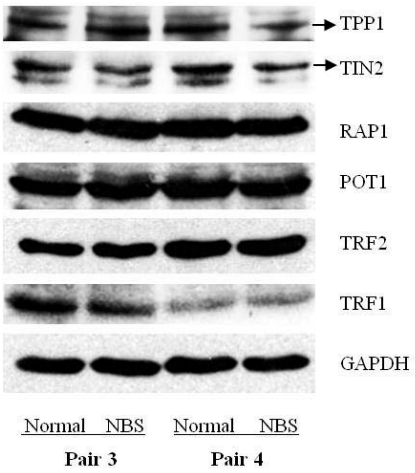

Figure 3.12 NBS1 deficiency does not affect the expression level of TRF2 in B-lymphocytes. Western blot analysis of the protein levels of all the six components of shelterin complex, including TRF1, TRF2, POT1, RAP1, TIN2 and TPP1 in NBS B-lymphocytes as well as in normal ones with similar PDLs. GAPDH serves as the loading control.

The expression levels of shelterin components in NBS B-lymphocytes were also detected by western blots. However, TRF2 protein level was not altered in NBS B-lymphocytes within each pair. And the expression levels of the rest five components of shelterin complex, including TRF1, POT1, RAP1, TPP1 and TIN2, also did not show alterations in the condition of NBS1 deficiency (Figure 3.12). These results further manifest that the same type of NBS1 mutation would cause different defects at telomeric ends in fibroblasts and B-lymphocytes.

3.13 NBS1 deficiency potentiates chromosome instabilities in NBS fibroblasts

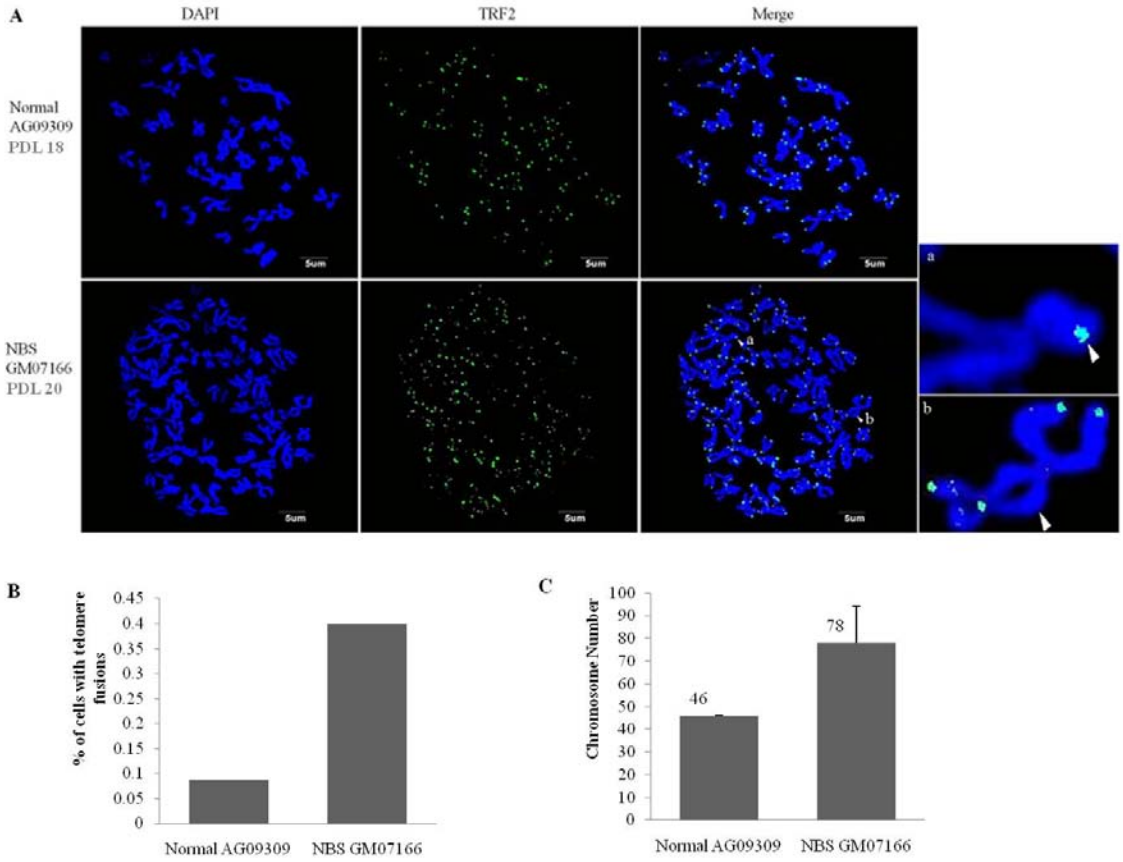

Figure 3.13 NBS1 deficiency leads to chromosome instabilities. A. Metaphase spreads of Pair 1 fibroblasts were stained with antibodies against TRF2 (green) and visualized by immunofluorescence. DNA was stained with DAPI (blue). Arrows point to telomeric end fusions. The insets (a and b) are representatives of telomeric fusions. B. Bars represent the percentage of cells that are positive with telomeric fusions. The total cell number is 25. C. Bars represent the average number of chromosomes enumerated from the metaphase spreads. Data are mean ± S.D. from 25 spreads each.

The accelerated telomere shortening and dysregulation of shelterin complex components may jeopardize the stability of telomeres in NBS cells. To evaluate the integrity of telomeres of NBS cells, we performed cytogenetic analysis of metaphase spread to investigate directly at the chromosome ends. As shown, prevalent telomere associations

were observed in NBS fibroblasts (Figure 3.13A), exhibited by telomeres of different or the same chromosomes exist in unusually close proximity. Although very rare, telomere fusions were also observed in normal cells (Figure 3.13B). Telomere associations affect the chromosome separation during mitosis, resulting in aneuploid cells. We found that most of the normal cells retain 46 chromosomes during culture *in vitro*, although few of them showed abnormal chromosome numbers that slightly deviate from 46 (Figure 3.13C). In contrast, NBS cells showed an average chromosome number of 78 which significantly deviates from the normal chromosome number, suggesting that the continued replication of NBS cells *in vitro* leads to more severe genome instabilities.

3.14 NBS1 deficiency does not promote malignant transformation of fibroblasts *in vitro*

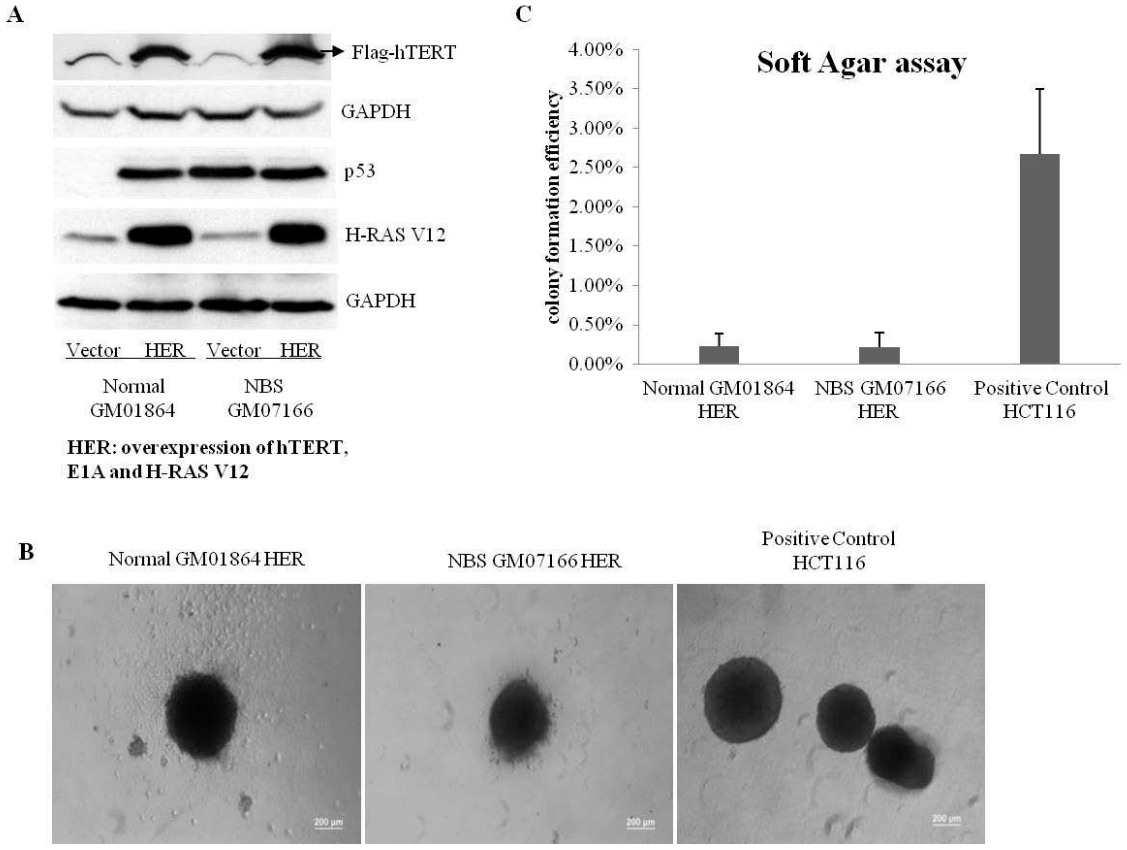

Figure 3.14 NBS1 does not promote malignant transformation of fibroblasts *in vitro*. A. Western blot analysis of the protein level of hTERT, H-RAS and p53 in normal as well as in NBS fibroblasts infected with virus particles containing *hTERT*, *E1A* and *H-RAS V12* gene. Arrow indicates the band of Flag-hTERT. B. The representative images of colonies formed in soft agar plates containing transformed cells. C. Quantification of the colony formation efficiency of different cell lines. Human colon cancer cells HCT116 serve as the positive control. The colony formation efficiency equals the average number of colonies in one well divided by the total cells seeded. Data are mean ± S.D. from 8 wells each.

DNA damage response and telomere integrity maintenance are two important aspects in preventing malignant transformation of cells in the early stage of cancer (Bartek, Lukas et al. 2007). Having observed defects in both of these aspects in NBS fibroblasts, it seems to

be apparent that NBS fibroblasts are more prone to malignant transformation than normal cells. To test this hypothesis, soft agar assay which detects and measures the morphological transformation of cells was performed. Normal as well as NBS fibroblasts were infected with viruses containing plasmid hTERT, H-RAS V12 and E1A, simultaneously. The successfully transfected clones are subjected to soft agar assay. Due to the difficulty in getting transfected clones of AG09309, another normal fibroblast GM01864 was used as control of NBS fibroblast GM07166. As shown (Figure 3.14A), hTERT and H-RAS V12 were successfully transfected into cells. As E1A overexpression leads to accumulation of p53 (Querido, Teodoro et al. 1997), we also detected the protein level of p53 to reflect the transfection efficiency of E1A. However, p53 level was only upregulated in normal fibroblasts but not in NBS fibroblasts, suggesting that E1A-induced p53 accumulation may be NBS1-dependent. The transfected cells were then seeded into agar plates and incubated for 6 weeks. Colonies were observed in both of the transfected cell lines although with a lower efficiency compared to the positive control HCT116, suggesting that transfected cells had less tumorigenicity (Figure 3.14B). Surprisingly, no obvious difference in the colony formation efficiency was observed when comparing NBS to normal fibroblasts (Figure 3.14C). This result indicates that NBS1 deficiency does not promote the malignant transformation of fibroblasts even though impairing the DNA damage response signaling pathway and telomere integrity.

4. DISCUSSION

4.1 NBS1 deficiency affects the DNA damage response

The *NBS1* gene encodes a 95 KD protein (Difilippantonio and Nussenzweig 2007). The 657del5 mutation of this gene leads to a frame shift and premature termination at codon 219 which abolishes the expression of full length NBS1 (Maser, Zinkel et al. 2001). It is predicted that the premature termination would result in the expression of two truncated proteins, the 26 KD N-terminus and the 70 KD C-terminus (Tauchi, Matsuura et al. 2002). The NBS1 antibody used in this study recognizes C-terminal residues of human NBS1. However, the 70KD C-terminus was not observed (data not shown). Maser and colleagues also reported that only the 26 KD fragment, but not the 70 KD one, was found in NBS fibroblasts (Maser, Zinkel et al. 2001). The 70 KD C-terminus of NBS1 contains the region that binds to MRE11 which is necessary for the nuclear localization of MRE11/RAD50 and the formation of functional MRN complex (Kobayashi, Antoccia et al. 2004). Our results showed that although the absence of NBS1 C-terminus in NBS fibroblasts, the expression level of another two components of MRN complex were not affected.

It has been proved in *Xenopus* egg extracts that the C-terminus of NBS1 is essential for recruiting ATM to damaged DNA where its subsequent autophosphorylation occurs (You, Chahwan et al. 2005). Our results demonstrated that in the absence of full length NBS1 and its C-terminus, ATM phosphorylation at Ser1981 was diminished in NBS cells when exposed to Dox treatment. This result indicates that NBS1 is not only a downstream substrate of ATM (Lim, Kim et al. 2000), but also serves as an upstream regulator that

mediates the phosphorylation and activation of ATM. However, NBS cells still retain a low level of ATM phosphorylation under Dox treatment. We suggest that ATM autophosphorylation exists in a low basal level in cells that are under DNA damage even without functional NBS1. NBS1 serves as an amplifier for ATM activity which facilitates ATM to reach a threshold maximal activity when DNA damage occurs (Horejsi, Falck et al. 2004).

Lying in the crossroad of DNA damage signaling pathway, ATM mediates diverse responses through phosphorylation on different downstream targets. Histone H2AX is one of them. We found that NBS1 deficiency severely affects the phosphorylation of histone H2AX at Ser139 in response to Dox treatment. p53 is another ATM target which plays multifaceted role in DNA damage response. We also observed that p53 phosphorylation is seriously impaired in NBS cells. Although the phosphorylation of ATM downstream substrates H2AX and p53 was severely affected, we could still observe a basal level of phosphorylation and activation of these proteins. This result suggests that NBS1 deficiency does not fully abolish the phosphorylation of ATM targets, probably due to the existence of a basal level of ATM phosphorylation. However, the activation of Chk2 was apparently normal though slightly delayed in NBS cells under Dox treatment. Like p53, Chk2 could also be phosphorylated by ATM and functions in cell cycle arrest. The phosphorylation of Chk2 brings its catalytic domain into the close proximity of another Chk2 molecule that allows auto-trans-phosphorylation to occur (Oliver, Knapp et al. 2007). In NBS cells, although impaired, ATM activation was still present at basal levels. It could be explained that the basal level of activated ATM is sufficient to elicit initial phosphorylation of Chk2 which creates conditions for its following auto-trans-

phosphorylation. But this process may take longer time than the direct phosphorylation of Chk2 by ATM, so that NBS cells showed a delay in Chk2 phosphorylation. Although pair 1 and pair 2 generally exhibited similar trends, differences between these 2 pairs did exist, such as p53 expression level and Chk2 phosphorylation level. The differences between pair 1 and pair 2 may due to the transformation of SV40 in pair 2 cells. SV40 may activate DNA damage signaling pathway even without Dox treatment, causing differential protein expression and phosphorylation profiles between pair 1 and pair 2.

As a DNA intercalating agent, Dox not only causes DSBs, but also inhibits cell proliferation and DNA synthesis by generating stalled replication forks (Kim, Lee et al. 2009) which could be repaired by ATR-dependent signaling pathway (Stiff, Reis et al. 2005). Indeed, we found that when subjected to Dox treatment, ATR phosphorylation level increased in normal cells. However, the phosphorylation event was impaired when NBS1 is deficient. This result suggests that NBS1 is not only an upstream regulator of ATM, but also functions upstream of ATR.

Recent years, several studies showed an essential role of TOPBP1 in activating ATR and eliciting ATR-dependent signaling events in both human cells and *Xenopus* egg extracts (Kumagai, Lee et al. 2006; Mordes, Glick et al. 2008; Choi, Lindsey-Boltz et al. 2009). It was further shown that NBS1 interacts with TOPBP1, a process that is essential for TOPBP1 to activate ATR (Yoo, Kumagai et al. 2009). In human NBS1-2A mutants (mutations within BRCT domain), TOPBP1 failed to bind to NBS1 which further affects ATR-dependent signaling processes, such as phosphorylation of Chk1 (Yoo, Kumagai et al. 2009). In line with this, our study showed a reduction in TOPBP1 expression level

when NBS1 is deficient, which is probably responsible for the impaired activation of ATR in NBS fibroblasts. Although the direct interaction between NBS1 and TOPBP1 has been reported, how NBS1 regulates the expression level of TOPBP1 remains to be determined.

As a direct downstream substrate of ATR (Liu, Guntuku et al. 2000), Chk1 phosphorylation level was also affected in NBS fibroblasts (Figure 3.4B). In addition, the total protein level of Chk1 was also severely compromised, suggesting that NBS1 deficiency affects the stability of Chk1.

All together, our results suggest that NBS1 is an upstream regulator of both ATM and ATR kinase. The deficiency of NBS cells in producing full length NBS1 renders them inefficient in activating these two kinases as well as their downstream substrates upon DNA damage. But either a lower level of phosphorylation or a delayed activation of proteins still exists in NBS cells, indicating that NBS1 deficiency could only partially affect the DNA damage signaling pathway.

As an initial response when DNA damage occurs, normal cells with intact DNA damage signaling pathway would arrest to allow DNA damage to be repaired (Kastan and Bartek 2004). Different cell cycle arrest mechanisms are required in response to DNA damage in a cell-type and DNA-damage specific manner. All three types of cell cycle arrest, including G1/S, intra-S and G2/M arrest, have been reported in human cells treated with Dox although in a cell-type specific manner (Robles, Buehler et al. 1999; Lee, Youn et al. 2005; Bar-On, Shapira et al. 2007). For example, human MCF7 cells exhibited G1/S and G2/M arrest when subjected to Dox treatment (Bar-On, Shapira et al. 2007), whereas

normal human F65 cells only showed G2/M arrest under the same drug treatment (Lee, Youn et al. 2005). How the kinases in DNA damage signaling pathway select and activate different substrates, thereby mediates cell-type specific responses, are still not fully resolved. But mutation of the genes in DNA damage signaling pathway will be one of the causes that affect the DNA damage response profile.

NBS1 is involved in several signaling pathways that mediate cell cycle arrest. Mutation of *NBS1* at ATM phosphorylation site (S343A mutation) resulted in a failure of S-phase arrest in response to IR in 293T cells (Lim, Kim et al. 2000). This phenomenon is known as radio-resistant DNA synthesis (RDS), in which cells continue DNA synthesis in the presence of IR-induced DNA damage. RDS was also observed in NBS cells with 657del5 mutation (Kobayashi, Antoccia et al. 2004). Dox has similar effects with IR in terms of the damages that they could generate in cells (Lee, Youn et al. 2005). Consistent with previous reports, our data showed that in response to Dox treatment for 10 hours, NBS cells were deficient in inhibiting DNA synthesis. However, this defect was diminished after 22-hour treatment with Dox, by when NBS cells exhibited successful inhibition of DNA synthesis as that observed in normal cells. This result indicates that NBS1 deficiency may delay the intra-S phase arrest, but does not abolish it.

At least three parallel pathways involved in intra-S phase checkpoint have been reported, including the ATM/NBS1/SMC1 pathway (Yazdi, Wang et al. 2002), the ATM/CHK2/ CDC25A/CDK2 pathway (Falck, Petrini et al. 2002) and the ATM/FANCD2 pathway (Nakanishi, Taniguchi et al. 2002). Although it was previously thought that only the ATM/NBS1/SMC1 pathway is NBS1-dependent (Kobayashi, Antoccia et al. 2004), the

recent recognition of NBS1's function as an upstream regulator of ATM suggest a dependency on NBS1 in all the three pathways. However, as shown by our study, NBS1 deficiency did not fully abolish the phosphorylation of ATM and ATM-mediated downstream events. In particular, the phosphorylation of Chk2 was only delayed but not impaired in the phosphorylation level in the condition of NBS1 deficiency. This result probably provided an explanation for the delayed inhibition of DNA synthesis observed in NBS cells.

Another cellular event that responds to DNA damage is apoptosis. Activation of apoptosis signaling pathway serves as an essential mechanism in removing damaged cells to maintain genome stability. One of the most important pathways that mediate apoptosis is the ATM-Chk2-p53 pathway. Mutation or deletion of the genes involved in apoptosis signaling pathways would lead to defects in inducing apoptosis. A-T cells that are defective in *ATM* gene are more sensitive to IR and exhibit less apoptosis after IR than normal cells (Duchaud, Ridet et al. 1996). In addition, thymocytes derived from *ATM* knockout mice also exhibit a lower apoptosis level following IR than the corresponding wild type mice, indicating the importance of ATM in triggering apoptosis (Westphal, Rowan et al. 1997). Chk2 could phosphorylate p53 at additional Ser residues, including Ser15 and Ser20. It has been demonstrated that in cells expressing dominant negative Chk2 and mice with deficient Chk2, a defect in apoptosis was observed, suggesting Chk2 also plays a role in mediating apoptosis in response to DNA damage (Rogoff, Pickering et al. 2004). NBS1 has also been reported participating in apoptosis pathway. It has been shown that the DNA-damage induced apoptosis level is significantly reduced in NBS cells in response to IR (Tauchi, Iijima et al. 2008).

Using annexin V apoptosis assay, we also observed a defect of NBS cells in inducing apoptosis. However, this defect only existed in cells exposed to high concentration of Dox treatment, but not in cells under low concentration. The concentration of Dox may be proportional to the amounts of DNA lesions caused. Under low concentration of Dox, small amount of DNA lesions are generated in cells. And as shown earlier, although the phosphorylation of ATM and the phosphorylation events elicited by ATM were either impaired or delayed in NBS cells, they still exhibited a basal level of phosphorylation or delayed activation. We speculate that the activated basal-level proteins are sufficient to encounter the small scale lesions caused by low concentration of Dox, but not enough to deal with the massive DNA damage caused by high concentration of Dox. This result suggests that the partially affected ATM signaling pathway in NBS cells could retain the apoptotic event to some degree but could not fully restore it when cells are under large scale of DNA damage.

Caspases are the central components mediating apoptosis (Riedl and Shi 2004). Among them, caspases 3 is a frequently activated death executioner which catalyzes the specific cleavage of various key cellular proteins (Porter and Janicke 1999). Activation of caspases 3 is through cleavage by other initiator caspases (Li, Nijhawan et al. 1997). Our results showed that NBS1 deficiency severely affects the cleavage of caspases 3 in response to Dox treatment at different concentrations. However, this inefficiency in cleavage and activation of caspases 3 did not significantly affect the cleavage of its downstream targets, such as PARP. Besides caspase 3, caspase 7 was also reported as an upstream regulator of PARP. It is possible that caspase 7 activity was not affected in NBS cells and contributed to the cleavage of PARP. Also, the unaffected PARP could be

due to the basal level of cleaved caspase 3 in NBS cells. However, the molecular mechanism of how NBS1 mediates the apoptotic signal to caspases therefore inducing apoptosis still remains to be elucidated.

Taken together, our results suggested that NBS1 was involved in ATM/ATR-midiated DNA damage signaling pathway. Deficiency of NBS1 affected ATM/ATR phosphorylation as well as their downstream effectors, leading to defects in apoptosis and DNA synthesis (Figure 4.1).

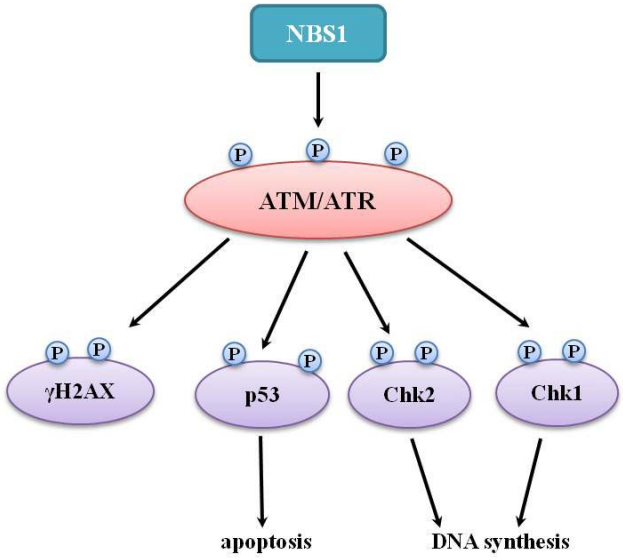

Figure 4.1 Model of NBS1's role in regulating ATM/ATR-mediated DNA damage signaling pathways.

4.2 NBS1 deficiency compromises telomere integrity

NBS1 have been shown to directly bind to telomeres (Zhu, Kuster et al. 2000; Dimitrova and de Lange 2009). The binding of NBS1 to telomeres suggests a role of this protein in protecting telomere integrity. Besides NBS1, many other proteins that are involved in DNA damage response are found associated with telomeres, such as ATM and the other two components of MRN complex, MRE11 and RAD50 (Munoz, Blanco et al. 2006). Many of the telomere-associated proteins are mutated in human genomic instability syndromes that are characterized by premature aging and shortened telomeres (Blasco 2005). Premature aging is always attributed to accelerated telomere shortening of cells. For example, A-T cells that are derived from A-T patients featured by premature aging show accelerated shortening of telomeres (Metcalfe, Parkhill et al. 1996). Our study using NBS fibroblasts derived from NBS patients with 657del5 mutation showed that NBS fibroblasts also have shorter telomeres than normal counterparts. When comparing the telomere shortening rate *in vitro,* we observed that NBS fibroblasts have a higher telomere attrition rate with each replication cycle. This result extends our recognition of NBS's role at telomeric ends.

However, the accelerated telomere attrition was not observed in NBS B-lymphocytes with the same mutation type of *NBS1*. One possible explanation to account for the different effects of NBS1 mutation in NBS fibroblasts and NBS B-lymphocytes is the fact that the expression of NBS1 truncated fragments, the 26 KD N-terminus and the 70 KD C-terminus, are differentially expressed in these two types of cells. Although the 26 KD fragment is expressed in both NBS fibroblasts and B-lymphocytes, the 70 KD fragment is

only detected in B-lymphocytes but not in fibroblasts (Maser, Zinkel et al. 2001). The 70 KD fragment physically interacts with MRE11/RAD50 complex and is essential for their nuclear localization and MRN complex formation (Desai-Mehta, Cerosaletti et al. 2001). This process may be important for the recruitment of MRN complex from cytoplasm to telomeres to partially restore its function at telomeric ends, such as regulation of telomere shortening rate. But this possibility has not been tested and other causes that may lead to the difference in telomere shortening rate between NBS fibroblasts and B-lymphocytes need to be determined.

Accelerated telomere shortening will result in the earlier occurrence of critically short telomeres which would further elicit cellular senescence (Hezel, Bardeesy et al. 2005). We observed premature cellular senescence in NBS fibroblasts, as showed by β-galactosidase assay, which corroborates with the finding of accelerated telomere attrition in these cells. Although NBS B-lymphocytes did not show telomere shortening defects, we did observe an earlier onset of senescence-associated apoptosis in these cells under normal cell culture conditions. The occurrence of senescence-associated apoptosis could be induced by shortened and unstable telomeres (Blasco 2005). Thus, it is possible that NBS1 deficiency could lead to telomeric abnormalities other than accelerated telomere shortening, even though with the presence of the 70 KD C-terminus.

Telomerase is required to extend telomere length and prevent telomere attrition in most cell types, except cancer cells that use ALT mechanism (Henson, Neumann et al. 2002). Thus, lack of telomerase activity may be the cause for the accelerated telomere shortening in NBS fibroblasts. However, our result that NBS and normal fibroblasts

showed similar level of telomerase activity excludes this possibility, indicating that the accelerated attrition of telomeres is caused by other mechanisms.

As mentioned earlier, A-T cells that are mutated in *ATM* gene also exhibited accelerated telomere shortening (Metcalfe, Parkhill et al. 1996). It has been proposed that this defect in A-T cells is caused by a decreased accessibility of telomerase to telomeres (Wu, Xiao et al. 2007). ATM, as a protein kinase, can phosphorylate TRF1, a process that will reduce the binding ability of TRF1 to telomeres. The reduction in TRF1 binding level at telomeric ends facilitates the assembly of telomerase to telomeres and leads to telomerase-dependent telomere elongation (Wu, Xiao et al. 2007). Therefore, *ATM* mutation would exert a negative effect in the telomere elongation by reducing the accessibility of telomerase to telomeres, which may further lead to accelerated telomere shortening observed in A-T cells. With regard to the close relationship between NBS1 and ATM, it is possible that NBS1 also protects telomere from accelerated telomere shortening through the regulation of the accessibility of telomerase to telomeres.

This model provides some hints to study the accelerated telomere shortening in A-T and NBS cells not only from the aspect of telomerase but also from the area of shelterin complex. The speculation of the relationship between shelterin complex and accelerated telomere shortening is strengthened by the fact that shelterin complex components are negative regulators of telomere length (vanSteensel and deLange 1997; Smogorzewska, Van Steensel et al. 2000; O'Connor, Safari et al. 2004; de Lange 2005; Munoz, Blanco et al. 2009). TRF1 and TRF2 are the two most frequently investigated shelterin components at telomeric ends. It has been well established that TRF1 and TRF2 expression level plays

an important role in determining telomere shortening rate (vanSteensel and deLange 1997; Richter, Saretzki et al. 2007). Overexpression of TRF1 or TRF2 leads to accelerated telomere shortening *in vitro* and premature aging *in vivo* (Munoz, Blanco et al. 2006; Munoz, Blanco et al. 2009).

Our results showed that TRF2 was upregulated at both mRNA and protein level in fibroblasts with NBS1 deficiency. However, the expression level of TRF1 was not affected by NBS1 deficiency but maintained at the similar level as that in normal fibroblasts. The upregulation of TRF2 may contribute to the accelerated telomere shortening observed in NBS fibroblasts. TRF2 has also been reported as a substrate of ATM with a phosphorylation site at Thr188 (Huda, Tanaka et al. 2009). It is possible that the phosphorylated TRF2 has a similar mode with the phosphorylated TRF1 which would dissociate from telomeres and facilitate telomerase-dependent telomere elongation (Figure 4.2A). However, NBS1 deficiency affects ATM phosphorylation, which may further impair the phosphorylation of TRF2 by ATM. Un-phosphorylated TRF2 might accumulate to a high level and remain associated with telomeres, thereby preventing the access of telomerase to telomeres and leading to accelerated telomere shortening (Figure 4.2B).

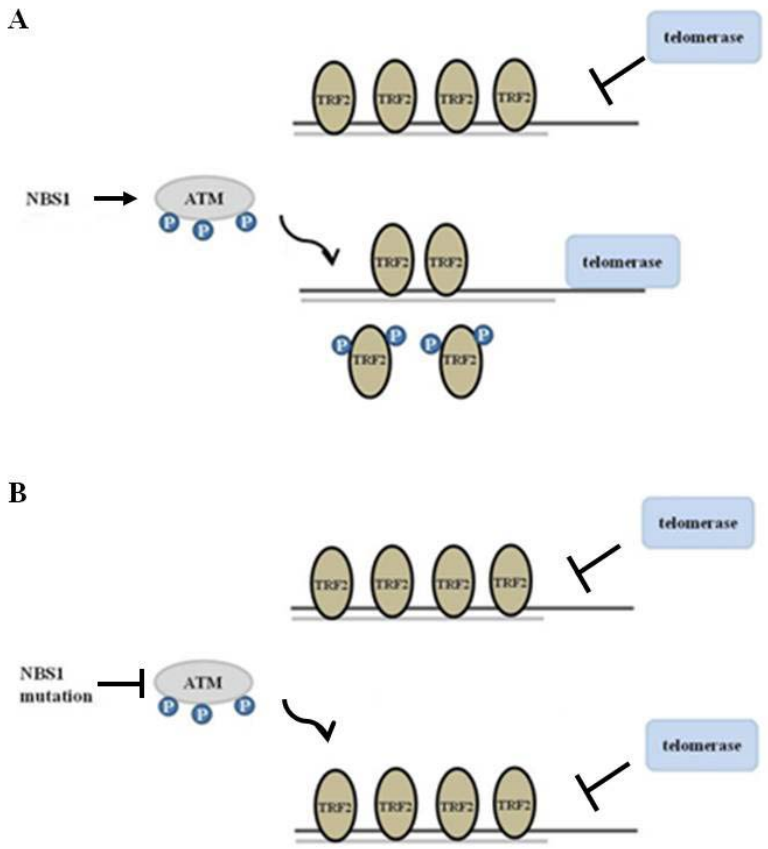

Figure 4.2 Model for NBS1- and ATM-mediated phosphorylation of TRF2 in modulating telomerase-dependent telomere elongation. A. NBS1 mediates the optimal phosphorylation of ATM, a process that contributes to the phosphorylation of TRF2. Phosphorylated TRF2 dissociates from telomeres which facilitates the access of telomerase to telomeric ends and leads to telomerase-dependent telomere elongation. B. When NBS1 is mutated, the process of ATM auto-phosphorylation is affected, leading to incompetence in TRF2 phosphorylation. Unphosphorylated TRF2 remains associated with telomeres, thereby preventing the access of telomerase to telomeres and telomerase-dependent telomere elongation.

Recently, a new feedback loop between p53 and TRF2 during cellular senescence has been reported. Cellular senescence activates the canonical DNA damage signaling pathway that engages p53 to initiate replicative senescence or senescence-associated apoptosis (Deng, Chan et al. 2008). During this process, activated p53 induces the

expression of Siah1, a p53-inducible ubiquitin ligase that is capable of ubiquitinating TRF2 and leads to proteasomal-mediated degradation of TRF2 (Figure 4.3A) (Fujita, Horikawa et al. 2010). However, NBS1 deficiency affects the activation of p53, which may subsequently influence the induction of Siah1. It has been shown that inhibition of Siah1 stabilizes TRF2 and results in TRF2 accumulation (Fujita, Horikawa et al. 2010). If NBS1 deficiency affects Siah1 level, TRF2 degradation would also be affected. As a result, an accumulated higher TRF2 level would be expected in NBS cells. It is possible that the access of telomerase to telomeres is blocked due to the accumulated TRF2 level, contributing to the accelerated telomere shortening (Figure 4.3B).

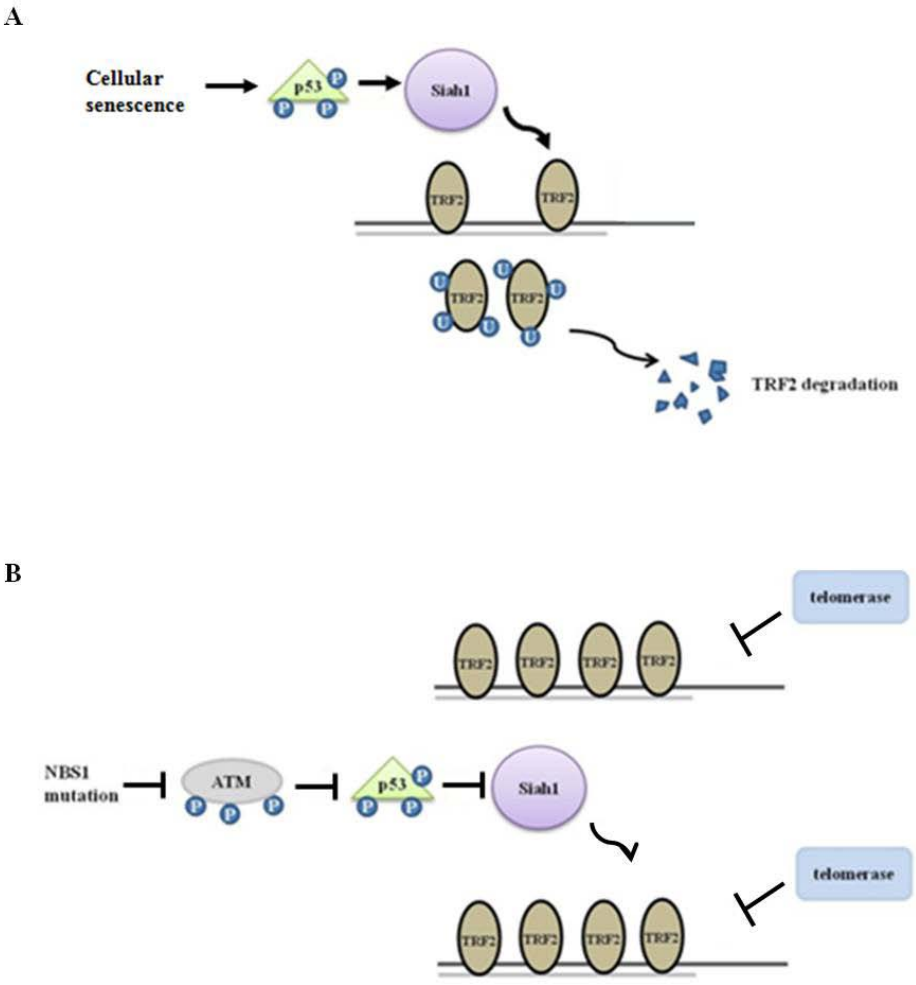

Figure 4.3 Model for p53-dependent ubiquitination of TRF2 in modulating telomerase-dependent telomere elongation. A. p53 is activated during cellular senescence. Activated p53 induces the expression of Siah1, a ubiquitin ligase that is capable of ubiquitinating TRF2. Ubiquitinated TRF2 is subjected to proteasomal-mediated degradation. B. When NBS1 is mutated, the optimal phosphorylation of ATM is affected, which further affects the phosphorylation and activation of p53. As a result, Siah1 induction level is also impaired, leading to TRF2 accumulation at telomeres rather than being degraded. Accumulation of TRF2 prevents the the access of telomerase to telomeres and telomerase-dependent telomere elongation.

Why NBS1 deficiency selectively upregulates TRF2 but not TRF1 also remains unknown. We speculate that the difference between TRF1 and TRF2 in terms of their functions at telomeric ends may be the leading cause for the differential regulation of their expression

level in NBS fibroblasts. Although TRF1 and TRF2 have similar function and binding mode to telomeric DNA, TRF2 plays an important role in T-loop formation that protects telomere integrity (Nishimura, Hanaoka et al. 2005). Furthermore, TRF2 has been shown to interact with MRN complex and maintain telomere integrity in a combination effort with ATM (Zhu, Kuster et al. 2000; Dimitrova and de Lange 2009). The difference between TRF1 and TRF2 may be the reason why TRF2 is affected in the condition of NBS1 deficiency, but not TRF1.

In line with the observation that NBS1 deficiency does not lead to accelerated telomere shortening in NBS B-lymphocytes, we also did not observe the upregulation of TRF2 as well as other shelterin components in NBS B-lymphocytes. Unveiling the interaction of TRF2 and NBS1 as well as their functions at telomeric ends will partially answer the question of why the telomere shortening rate of NBS fibroblasts and NBS B-lymphocytes are differentially regulated even though with the same type of gene mutation.

TRF2 is essential in maintaining the correct structure at telomere termini and preventing telomeres from end-to-end fusions (van Steensel, Smogorzewska et al. 1998). It has been shown that the dominant negative allele of TRF2 would induce telomere end-to-end fusions in metaphase and anaphase cells (van Steensel, Smogorzewska et al. 1998). Consistent with previous studies, our result showed that in the absence of functional NBS1, NBS fibroblasts that are characterized by dysregulated TRF2 level also showed frequent telomere end-to-end fusions. The abnormalities at telomere ends might cause abnormal chromosomal segregation, thus leading to aberrant chromosome number observed in NBS fibroblasts.

4.3 NBS1 deficiency promotes genome instabilities and is implicated in carcinogenesis of lymphoid cells

NBS1 deficiency affects ATM- and ATR-mediated DNA damage signaling pathway. ATM and ATR are the two master regulators in DNA damage response network by signaling to control cell cycle checkpoints, DNA synthesis, DNA repair and apoptosis (Cimprich and Cortez 2008). Therefore, NBS1 deficiency would have an impact on these cellular activities, as observed in our study by BrdU assay and FITC Annexin V apoptosis assay. Improper response towards DNA damage may allow the continual growth of cells with DNA damage rather than being arrested, repaired or even “killed”, thus leading to genome instabilities.

NBS1 deficiency also affects telomere integrity. As observed, NBS1 deficiency leads to frequent telomere end-to-end fusions and aneuploidy of cells in NBS fibroblasts, similar phenotypes as shown in A-T cells (Pandita 2002). End-to-end fusions might be triggered by critically short telomeres which are supposed to lead to replicative senescence. However, the process of senescence shares many features with classic DNA DSB response (Hezel, Bardeesy et al. 2005) and requires functional DNA damage response machinery. NBS1 deficiency affects DNA damage response, therefore may also affect the process of replicative senescence and result in telomere end-to-end fusions.

Dysregulation of both DNA damage response and telomere integrity has been involved in cancer initiation and progression (Stewart and Weinberg 2006; Luijsterburg and van Attikum 2011). Since NBS1 deficiency leads to abnormalities in both of these two aspects, it is natural to link NBS1 deficiency to carcinogenesis. Indeed, NBS patients that

are mutated in *NBS1* gene are characterized by cancer predisposition, especially to B-cell lymphoma and T-cell lymphoblastic lymphoma/leukaemia (The International Nijmegen Breakage Syndrome Study Group 2000), indicating that NBS1 deficiency preferentially promotes the malignant transformation of B- and T-cell.

Although fibroblasts are always thought as static entity during cancer initiation and progression, recent studies showed that fibroblasts progress together with cancer cells and affect the morphology of tumors (Kalluri and Zeisberg 2006; Tsellou and Kiaris 2008). Thus, the possibility of NBS1 deficiency in promoting malignant transformation of fibroblasts was also tested *in vitro* by soft agar assay. However, the result suggested that NBS1 deficiency does not increase the tumorigenicity of NBS fibroblasts regardless of the affected DNA damage response network and compromised telomere integrity.

Based on our results, we propose that NBS1 deficiency promotes the malignant transformation of lymphoid cells, thus leading to lymphoma/leukemia, in two ways. First of all, NBS cells with disrupted DNA damage responses license the continual growth and survival of cells regardless of genomic abnormalities, which presents a cellular setting that predisposes bad cells to sustain, accumulate and perpetuate, leading to carcinogenesis. On the other hand, NBS1 deficiency speeds up the process towards replicative senescence or senescence-associated apoptosis of NBS cells. Senescence and senescence-associated apoptosis process requires proper DNA damage response which is compromised due to *NBS1* mutation. As a consequence, the natural process of senescence and senescence-associated apoptosis would also be affected, leading to unprotected short telomeres and genomic instabilities, which finally contributes to carcinogenesis (Figure

4.4).

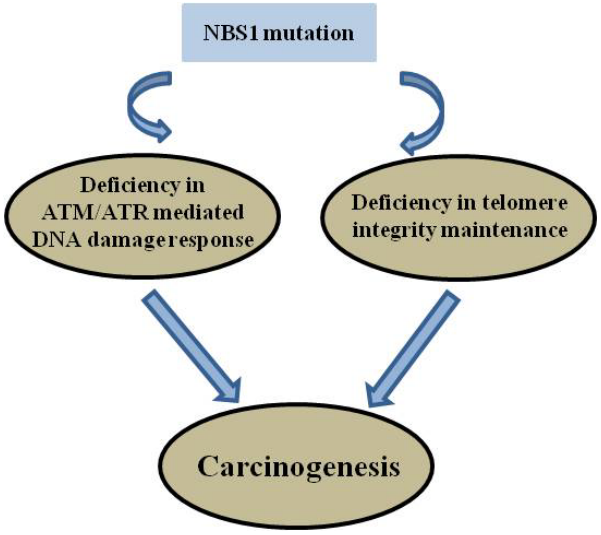

Figure 4.4 Model for NBS1 deficiency-initiated malignant transformation of lymphoid cells.

5. CONCLUSIONS

This study examined the roles of NBS1 in protecting genome stability from the aspect of maintaining DNA damage response network and telomere integrity. Our work suggests that 657del5 mutation of *NBS1* gene affects the DNA damage response network in both NBS fibroblasts and B-lymphocytes, leading to abnormal cellular responses. Furthermore, our work demonstrated that NBS1 gene mutation compromises the telomere integrity. We provided solid evidence that NBS fibroblasts have a higher telomere shortening rate *in vitro* in NBS fibroblasts. Moreover, we found that TRF2 expression was upregulated in NBS fibroblasts, which is an important clue for studying the underlying mechanism of accelerated telomere shortening in future. However, accelerated telomere shortening and upregulated TRF2 level were not observed in NBS B-lymphocytes, indicating NBS1 mutation has different effects at the telomeric ends of fibroblasts and B-lymphocytes.

Also, our results provided possible explanations to the high incidence of cancer in NBS patients. Since dysregulation of DNA damage response network and telomere integrity has been implicated in carcinogenesis, we propose that NBS patients are predisposed to cancer not only due to defects in repairing DNA damage but also owing to defects in maintaining telomere integrity.

6. FUTURE WORK

6.1 Reintroduction of wild-type NBS1 into NBS fibroblasts and examination of the DNA damage response and telomere shortening rate in these cells

As NBS1 mutation affects DNA damage response and telomere integrity in NBS fibroblasts, reintroduction of wild-type NBS1 to reach an expression level that close to normal counterparts in NBS fibroblasts may protect cells from these deficiencies. We have tried to infect NBS fibroblasts with wild-type NBS1 in our study. However, due to the low infection efficiency, the majority of cells died after selection with mammalian cell culture selective agent. The alive cells remained were hard to expand and get stable overexpressed clones. In the future, we will modify the infection protocol to achieve high infection efficiency.

After we get the stable overexpressed clones with wild-type NBS1, the ATM/ATR mediated DNA damage response pathway will be examined. We postulate that ATM and ATR as well as their downstream targets will exhibit normal phosphorylation and activation in response to Dox treatment after reintroduction of wild-type NBS1. In addition, the DNA synthesis status and apoptosis profile will be examined by BrdU assay and Annexin V Apoptosis Detection, respectively. Moreover, the wild-type NBS1 overexpressed cells will be cultured to a long period and the telomere shortening rate will be determined and compared with normal fibroblasts using TeloTAGGG Telomere Length Assay. We expect that the wild-type NBS1 overexpressed NBS fibroblasts have comparative telomere shortening rate with normal fibroblasts. Furthermore, the telomere integrity will be examined by metaphase spread analysis. Due to the existence of

telomeric end fusions in NBS fibroblasts prior to reintroduction of wild-type NBS1, we speculate that telomereic end fusions are still observable in wild-type NBS1 overexpressed cells.

6.2 To study the underlying mechanism of NBS1 deficiency-induced TRF2 upregulation and accelerated telomere shortening in NBS fibroblasts

As the upregulation of TRF2 and accelerated telomere shortening was observed in NBS fibroblasts, we next want to understand how NBS1 deficiency leads to these two phenotypes and whether there is a causal relationship between TRF2 upregulation and accelerated telomere shortening.

Two hypotheses will be tested. The first hypothesis is that NBS1 deficiency compromises ATM-dependent phosphorylation of TRF2 as well as the dissociation of TRF2 from telomeres, resulting in the accumulation of unphosphorylated TRF2 at telomeric ends which leads to accelerated telomere shortening. As the first step, the efficiency of the recruitment of ATM to telomeres will be determined by confocal immunofluorescence in NBS vs. normal fibroblasts. Confocal immunofluorescence will also be employed to check the co-localization of TRF2 and ATM. Secondly, the phosphorylation level of TRF2 will be examined using western blot. If TRF2 phosphorylation is ATM-dependent, we would expect a higher phosphorylation level of TRF2 in normal fibroblasts compared to NBS fibroblasts. In the third step, we would transfect normal fibroblasts with a dominant negative allele of TRF2 with mutations at the ATM phosphorylation site. Confocal immunofluorescence will be performed to compare the TRF2 localization site in TRF2 wild-type fibroblasts ($NBS1^{+/+}$ $TRF2^{+/+}$) and TRF2 mutant fibroblasts ($NBS1^{+/+}$

TRF2$^{\Delta/\Delta}$). If TRF2 phosphorylation facilitates its dissociation from telomeres, we would expect dispersed TRF2 in nucleus but not restricted at telomeric ends in NBS1$^{+/+}$ TRF2$^{+/+}$ cells, while in NBS1$^{+/+}$ TRF2$^{\Delta/\Delta}$ cells, TRF2 mainly localizes at the telomeric ends. This step confirms the dissociation of TRF2 from telomeres after its phosp horylation by ATM. Lastly, the NBS1$^{+/+}$ TRF2$^{\Delta/\Delta}$ fibroblasts will be cultured to a long period. DNA will be extracted from cells with different PDLs and subjected to TeloTAGGG Telomere Length Assay. The telomere shortening rate of NBS1$^{+/+}$ TRF2$^{\Delta/\Delta}$ fibroblasts will be compared to that of NBS1$^{+/+}$ TRF2$^{+/+}$ cells. If accelerated telomere shortening is also observed in NBS1$^{+/+}$ TRF2$^{\Delta/\Delta}$ cells, we can establish a model of NBS1 deficiency induced accumulation of TRF2 and accelerated telomere shortening as hypothesized.

The second hypothesis is based on the model of p53-dependent induction of Siah1 and ubiquitination of TRF2. As p53 phosphorylation and activation is impaired by NBS1 deficiency, we hypothesize that Siah1 induction and TRF2 ubiquitination will also be affected in NBS fibroblasts, leading to accumulated TRF2 at telomeric ends and accelerated telomere shortening. To test this hypothesis, western blot will be used to detect the expression level of Siah1 in NBS fibroblasts vs. normal fibroblasts. A lower level of Siah1 is expected in NBS fibroblasts compared to that in normal counterparts. To prove that the impaired induction of Siah1 is the cause of the upregulated TRF2 level in NBS fibroblasts, we will overexpress Siah1 in NBS fibroblasts and check the resulted TRF2 level in the second step. Expectedly, we would see a lower TRF2 level in the Siah1 overexpressed NBS fibroblasts. Lastly, the Siah1 overexpressed fibroblasts will be cultured to a long period. DNA will be extracted from cells with different PDLs and subjected to TeloTAGGG Telomere Length Assay. The telomere shortening rate of Siah1

overexpressed NBS fibroblasts will be compared to untransfected cells. If a lower telomere shortening is also observed in Siah1 overexpressed cells, we can conclude that the accelerated telomere shortening observed in NBS fibroblasts is due to impaired induction of Siah1 and ubiquitination of TRF2.

6.3 To study the role of the 70 KD C-terminus of NBS1 at telomeric ends in NBS B-lymphocytes

As reported, the 70 KD C-terminus of NBS1 is expressed in NBS B-lymphocytes (Maser, Zinkel et al. 2001). The 70 KD fragment contains the region that interacts with MRE11 and is essential for the recruitment of MRE11 from cytoplasm to nucleus to form the functional MRN complex (Kobayashi, Antoccia et al. 2004). In the future, we want to explore the role of the 70 KD fragment at telomeric ends in NBS B-lymphocytes. To confirm the existence of the 70 KD fragment in NBS B-lymphocytes, antibody that specifically recognizes the C-terminus of NBS1 will be used in western blot and confocal immunofluorescence. Confocal immunofluorescence will be subsequently used to visualize the localization of MRE11 and RAD50 in NBS B-lymphocytes. If MRN complex could be partially restored in nucleus with the presence of 70 KD fragment, it may interact with shelterin complex to exert its roles at telomeric ends. The interaction and co-localization of MRN complex and TRF2 will also be examined by co-immunoprecipitation and confocal immunofluorescence.

The interaction of MRN complex with TRF2 may be essential to control the TRF2 cellular level, therefore regulating the telomere shortening rate. Thus, the two hypotheses (see section **6.1**) will also be examined to reveal the cellular activities of the 70 KD C-

terminus in NBS B-lymphocytes. In this way, we will have a clear understanding of why the same type mutation of *NBS1* exerts different effect at telomeric ends in NBS fibroblasts and B-lymphocytes.

6.4 To examine the telomere integrity and malignant transformation of NBS B-lymphocytes

NBS patients are prone to B-cell lymphoma (The International Nijmegen Breakage Syndrome Study Group 2000), indicating that B cells are prone to malignant transformation in the condition of NBS1 mutation. Soft agar assay will be performed to test this hypothesis in NBS vs. normal B-lymphocytes. Expectedly, we will observe a higher ratio of malignant transformation in NBS B-lymphocytes than that in normal counterparts.

Chromosomal instabilities, such as telomere abnormalities, can promote the malignant transformation (Michor 2005). Therefore, metaphase spread will also be performed in NBS B-lymphocytes to examine the telomere integrity and find out potential telomere end-to-end fusions.

7. REFERENCES

Nijmegen Breakage Syndrome Study Group (2000). "Nijmegen breakage syndrome. The International Nijmegen Breakage Syndrome Study Group." Arch Dis Child **82**(5): 400-406.

Agami, R., G. Blandino, et al. (1999). "Interaction of c-Abl and p73 alpha and their collaboration to induce apoptosis." Nature **399**(6738): 809-813.

Antoccia, A., A. di Masi, et al. (2002). "G2-phase radiation response in lymphoblastoid cell lines from Nijmegen breakage syndrome." Cell Proliferation **35**(2): 93-104.

Ashwell, S. and S. Zabludoff (2008). "DNA damage detection and repair pathways--recent advances with inhibitors of checkpoint kinases in cancer therapy." Clinical cancer research : an official journal of the American Association for Cancer Research **14**(13): 4032-4037.

Assenmacher, N. and K. P. Hopfner (2004). "Mre11/Rad50/Nbs1: Complex Activities." Chromosoma **113**(4): 157-166.

Bar-On, O., M. Shapira, et al. (2007). "Differential effects of doxorubicin treatment on cell cycle arrest and Skp2 expression in breast cancer cells." Anti-cancer drugs **18**(10): 1113-1121.

Bartek, J., C. Lukas, et al. (2004). "Checking on DNA damage in S phase." Nature Reviews Molecular Cell Biology **5**(10): 792-804.

Bartek, J., J. Lukas, et al. (2007). "DNA damage response as an anti-cancer barrier - Damage threshold and the concept of 'conditional haploinsufficiency'." Cell Cycle **6**(19): 2344-2347.

Bartkova, J., Z. Horejsi, et al. (2005). "DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis." Nature **434**(7035): 864-870.

Bartkova, J., E. Rajpert-De Meyts, et al. (2007). "DNA damage response in human testes and testicular germ cell tumours: biology and implications for therapy." International journal of andrology **30**(4): 282-291; discussion 291.

Baumann, P. and T. R. Cech (2000). "Protection of telomeres by the Ku protein in fission yeast." Molecular Biology of the Cell **11**(10): 3265-3275.

Bekker-Jensen, S., C. Lukas, et al. (2006). "Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks." Journal of Cell Biology **173**(2): 195-206.

Bender, C. F., M. L. Sikes, et al. (2002). "Cancer predisposition and hematopoietic failure

in Rad50(S/S) mice." Genes & Development **16**(17): 2237-2251.

Bianchi, A., S. Smith, et al. (1997). "TRF1 is a dimer and bends telomeric DNA." Embo Journal **16**(7): 1785-1794.

Blackburn, E. H. (1992). "Telomerases." Annual Review of Biochemistry **61**: 113-129.

Blackburn, E. H. (2001). "Switching and signaling at the telomere." Cell **106**(6): 661-673.

Blasco, M. A. (2005). "Telomeres and human disease: Ageing, cancer and beyond." Nature Reviews Genetics **6**(8): 611-622.

Blasco, M. A., H. W. Lee, et al. (1997). "Telomere shortening and tumor formation by mouse cells lacking telomerase RNA." Cell **91**(1): 25-34.

Bradbury, J. M. and S. P. Jackson (2003). "The complex matter of DNA double-strand break detection." Biochemical Society Transactions **31**(Pt 1): 40-44.

Brown, E. J. and D. Baltimore (2000). "ATR disruption leads to chromosomal fragmentation and early embryonic lethality." Genes & Development **14**(4): 397-402.

Buscemi, G., C. Savio, et al. (2001). "Chk2 activation dependence on Nbs1 after DNA damage." Molecular and Cellular Biology **21**(15): 5214-5222.

Carney, J. P., R. S. Maser, et al. (1998). "The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: Linkage of double-strand break repair to the cellular DNA damage response." Cell **93**(3): 477-486.

Celeste, A., O. Fernandez-Capetillo, et al. (2003). "Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks." Nature Cell Biology **5**(7): 675-U651.

Celli, G. B. and T. de Lange (2005). "DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion." Nature Cell Biology **7**(7): 712-U110.

Cesare, A. J. and J. D. Griffith (2004). "Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops." Molecular and Cellular Biology **24**(22): 9948-9957.

Chao, C., D. Herr, et al. (2006). "Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression." EMBO Journal **25**(11): 2615-2622.

Choi, J. H., L. A. Lindsey-Boltz, et al. (2009). "Cooperative activation of the ATR checkpoint kinase by TopBP1 and damaged DNA." Nucleic Acids Research **37**(5):

1501-1509.

Cimprich, K. A. and D. Cortez (2008). "ATR: an essential regulator of genome integrity." Nature reviews. Molecular cell biology **9**(8): 616-627.

Collins, K. (2006). "The biogenesis and regulation of telomerase holoenzymes." Nature Reviews Molecular Cell Biology **7**(7): 484-494.

Collins, K. and J. R. Mitchell (2002). "Telomerase in the human organism." Oncogene **21**(4): 564-579.

Cong, Y. S., W. E. Wright, et al. (2002). "Human telomerase and its regulation." Microbiology and Molecular Biology Reviews **66**(3): 407-+.

Cuadrado, M., B. Martinez-Pastor, et al. (2006). "ATM regulates ATR chromatin loading in response to DNA double-strand breaks." Journal of Experimental Medicine **203**(2): 297-303.

de Lange, T. (2005). "Shelterin: the protein complex that shapes and safeguards human telomeres." Genes & Development **19**(18): 2100-2110.

de Lange, T. (2005). "Shelterin: the protein complex that shapes and safeguards human telomeres." Genes & Development **19**(18): 2100-2110.

Denchi, E. L., G. Celli, et al. (2006). "Hepatocytes with extensive telomere deprotection and fusion remain viable and regenerate liver mass through endoreduplication." Genes & Development **20**(19): 2648-2653.

Denchi, E. L. and T. de Lange (2007). "Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1." Nature **448**(7157): 1068-1071.

Deng, Y. B., S. S. Chan, et al. (2008). "Telomere dysfunction and tumour suppression: the senescence connection." Nature Reviews Cancer **8**(6): 450-458.

Desai-Mehta, A., K. M. Cerosaletti, et al. (2001). "Distinct functional domains of nibrin mediate Mre11 binding, focus formation, and nuclear localization." Molecular and Cellular Biology **21**(6): 2184-2191.

Di Micco, R., M. Fumagalli, et al. (2006). "Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication." Nature **444**(7119): 638-642.

Difilippantonio, S. and A. Nussenzweig (2007). "The NBS1-ATM connection revisited." Cell Cycle **6**(19): 2366-2370.

Dimitrova, N. and T. de Lange (2009). "Cell Cycle-Dependent Role of MRN at

Dysfunctional Telomeres: ATM Signaling-Dependent Induction of Nonhomologous End Joining (NHEJ) in G(1) and Resection-Mediated Inhibition of NHEJ in G(2)." Molecular and Cellular Biology **29**(20): 5552-5563.

DiTullio, R. A., Jr., T. A. Mochan, et al. (2002). "53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer." Nature cell biology **4**(12): 998-1002.

Duchaud, E., A. Ridet, et al. (1996). "Deregulated apoptosis in ataxia telangiectasia: Association with clinical stigmata and radiosensitivity." Cancer Research **56**(6): 1400-1404.

Dupre, A., L. Boyer-Chatenet, et al. (2006). "Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex." Nat Struct Mol Biol **13**(5): 451-457.

Durocher, D., J. Henckel, et al. (1999). "The FHA domain is a modular phosphopeptide recognition motif." Molecular Cell **4**(3): 387-394.

Essers, J., W. Vermeulen, et al. (2006). "DNA damage repair: anytime, anywhere?" Current Opinion in Cell Biology **18**(3): 240-246.

Falck, J., J. H. Petrini, et al. (2002). "The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways." Nature Genetics **30**(3): 290-294.

Fridman, J. S. and S. W. Lowe (2003). "Control of apoptosis by p53." Oncogene **22**(56): 9030-9040.

Fujita, K., I. Horikawa, et al. (2010). "Positive feedback between p53 and TRF2 during telomere-damage signalling and cellular senescence." Nature Cell Biology **12**(12): 1205-U1196.

Galy, V., J. C. Olivo-Marin, et al. (2000). "Nuclear pore complexes in the organization of silent telomeric chromatin." Nature **403**(6765): 108-112.

Greider, C. W. (1996). "Telomere length regulation." Annual Review of Biochemistry **65**: 337-365.

Griffith, J. D., L. Comeau, et al. (1999). "Mammalian telomeres end in a large duplex loop." Cell **97**(4): 503-514.

Hahn, W. C., C. M. Counter, et al. (1999). "Creation of human tumour cells with defined genetic elements." Nature **400**(6743): 464-468.

Harley, C. B., A. B. Futcher, et al. (1990). "Telomeres shorten during ageing of human fibroblasts." Nature **345**(6274): 458-460.

Henson, J. D., A. A. Neumann, et al. (2002). "Alternative lengthening of telomeres in mammalian cells." Oncogene **21**(4): 598-610.

Hezel, A. F., N. Bardeesy, et al. (2005). "Telomere induced senescence: End game signaling." Current Molecular Medicine **5**(2): 145-152.

Hirose, Y., M. Katayama, et al. (2004). "Cooperative function of Chk1 and p38 pathways in activating G2 arrest following exposure to temozolomide." Journal of neurosurgery **100**(6): 1060-1065.

Hockemeyer, D., J. P. Daniels, et al. (2006). "Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres." Cell **126**(1): 63-77.

Hoeijmakers, J. H. (2001). "Genome maintenance mechanisms for preventing cancer." Nature **411**(6835): 366-374.

Horejsi, Z., J. Falck, et al. (2004). "Distinct functional domains of Nbs1 modulate the timing and magnitude of ATM activation after low doses of ionizing radiation." Oncogene **23**(17): 3122-3127.

Huda, N., H. Tanaka, et al. (2009). "DNA Damage-Induced Phosphorylation of TRF2 Is Required for the Fast Pathway of DNA Double-Strand Break Repair." Molecular and Cellular Biology **29**(13): 3597-3604.

Hurley, P. J. and F. Bunz (2007). "ATM and ATR: components of an integrated circuit." Cell Cycle **6**(4): 414-417.

Iijima, K., K. Komatsu, et al. (2004). "The Nijmegen breakage syndrome gene and its role in genome stability." Chromosoma **113**(2): 53-61.

Jeppesen, P. (2000). "Immunofluorescence in cytogenetic analysis: method and applications." Genetics and Molecular Biology **23**(4): 1107-1114.

Kaina, B. and W. P. Roos (2006). "DNA damage-induced cell death by apoptosis." Trends in Molecular Medicine **12**(9): 440-450.

Kalluri, R. and M. Zeisberg (2006). "Fibroblasts in cancer." Nature reviews. Cancer **6**(5): 392-401.

Karlseder, J., D. Broccoli, et al. (1999). "p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2." Science **283**(5406): 1321-1325.

Karlseder, J., K. Hoke, et al. (2004). "The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response." PLoS biology **2**(8): E240.

Kastan, M. B. and J. Bartek (2004). "Cell-cycle checkpoints and cancer." Nature **432**(7015): 316-323.

Kim, H. S., Y. S. Lee, et al. (2009). "Doxorubicin exerts cytotoxic effects through cell cycle arrest and Fas-mediated cell death." Pharmacology **84**(5): 300-309.

Kim, S., C. Beausejour, et al. (2004). "TIN2 mediates functions of TRF2 at human telomeres." Journal of Biological Chemistry **279**(42): 43799-43804.

Kobayashi, J., A. Antoccia, et al. (2004). "NBS1 and its functional role in the DNA damage response." DNA Repair (Amst) **3**(8-9): 855-861.

Kobayashi, J., H. Tauchi, et al. (2002). "NBS1 localizes to gamma-H2AX foci through interaction with the FHA/BRCT domain." Current Biology **12**(21): 1846-1851.

Kroemer, G. and J. C. Reed (2000). "Mitochondrial control of cell death." Nature Medicine **6**(5): 513-519.

Kumagai, A., J. Lee, et al. (2006). "TopBP1 activates the ATR-ATRIP complex." Cell **124**(5): 943-955.

Kumar, S. (2007). "Caspase function in programmed cell death." Cell Death and Differentiation **14**(1): 32-43.

Lamarche, B. J., N. I. Orazio, et al. (2010). "The MRN complex in double-strand break repair and telomere maintenance." Febs Letters **584**(17): 3682-3695.

Le, S., J. K. Moore, et al. (1999). "RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase." Genetics **152**(1): 143-152.

Lee, S. M., B. Youn, et al. (2005). "Gamma-irradiation and doxorubicin treatment of normal human cells cause cell cycle arrest via different pathways." Molecules and Cells **20**(3): 331-338.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-331.

Li, B., S. P. Jog, et al. (2008). "WRN controls formation of extrachromosomal telomeric circles and is required for TRF2DeltaB-mediated telomere shortening." Molecular and cellular biology **28**(6): 1892-1904.

Li, P., D. Nijhawan, et al. (1997). "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade." Cell **91**(4): 479-489.

Lillard-Wetherell, K., A. Machwe, et al. (2004). "Association and regulation of the BLM helicase by the telomere proteins TRF1 and TRF2." Human molecular genetics **13**(17): 1919-1932.

Lim, D. S., S. T. Kim, et al. (2000). "ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway." Nature **404**(6778): 613-617.

Lingner, J. and N. Hug (2006). "Telomere length homeostasis." Chromosoma **115**(6): 413-425.

Liu, Q. H., S. Guntuku, et al. (2000). "Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint." Genes & Development **14**(12): 1448-1459.

Ljungman, M. (2009). "Targeting the DNA damage response in cancer." Chemical reviews **109**(7): 2929-2950.

Luciani, M. G., M. Oehlmann, et al. (2004). "Characterization of a novel ATR-dependent, Chk1-ndependent, intra-S-phase checkpoint that suppresses initiation of replication in Xenopus." Journal of Cell Science **117**(25): 6019-6030.

Luijsterburg, M. S. and H. van Attikum (2011). "Chromatin and the DNA damage response: The cancer connection." Molecular oncology **5**(4): 349-367.

Luo, G. B., M. S. Yao, et al. (1999). "Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation." Proceedings of the National Academy of Sciences of the United States of America **96**(13): 7376-7381.

Manthey, K. C., S. Opiyo, et al. (2007). "NBS1 mediates ATR-dependent RPA hyperphosphorylation following replication-fork stall and collapse." Journal of Cell Science **120**(23): 4221-4229.

Martinez, P. and M. A. Blasco (2010). "Role of shelterin in cancer and aging." Aging Cell **9**(5): 653-666.

Martinez, P., M. Thanasoula, et al. (2009). "Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice." Genes & Development **23**(17): 2060-2075.

Maser, R. S., R. Zinkel, et al. (2001). "An alternative mode of translation permits production of a variant NBS1 protein from the common Nijmegen breakage syndrome allele." Nature Genetics **27**(4): 417-421.

Masutomi, K., E. Y. Yu, et al. (2003). "Telomerase maintains telomere structure in normal

human cells." Cell **114**(2): 241-253.

Matsuoka, S., B. A. Ballif, et al. (2007). "ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage." Science **316**(5828): 1160-1166.

Matsuura, S., H. Tauchi, et al. (1998). "Positional cloning of the gene for Nijmegen breakage syndrome." Nature Genetics **19**(2): 179-181.

McEachern, M. J., A. Krauskopf, et al. (2000). "Telomeres and their control." Annual Review of Genetics **34**: 331-358.

Metcalfe, J. A., J. Parkhill, et al. (1996). "Accelerated telomere shortening in ataxia telangiectasia." Nature Genetics **13**(3): 350-353.

Metcalfe, J. A., J. Parkhill, et al. (1996). "Accelerated telomere shortening in ataxia telangiectasia." Nature Genetics **13**(3): 350-353.

Michor, F. (2005). "Chromosomal instability and human cancer." Philosophical Transactions of the Royal Society B-Biological Sciences **360**(1455): 631-635.

Mordes, D. A., G. G. Glick, et al. (2008). "TopBP1 activates ATR through ATRIP and a PIKK regulatory domain." Genes & Development **22**(11): 1478-1489.

Munoz, P., R. Blanco, et al. (2006). "Role of the TRF2 telomeric protein in cancer and ageing." Cell Cycle **5**(7): 718-721.

Munoz, P., R. Blanco, et al. (2009). "TRF1 Controls Telomere Length and Mitotic Fidelity in Epithelial Homeostasis." Molecular and Cellular Biology **29**(6): 1608-1625.

Munoz, P., R. Blanco, et al. (2005). "XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer." Nature Genetics **37**(10): 1063-1071.

Myers, J. S. and D. Cortez (2006). "Rapid activation of ATR by ionizing radiation requires ATM and Mre11." The Journal of biological chemistry **281**(14): 9346-9350.

Nakanishi, K., T. Taniguchi, et al. (2002). "Interaction of FANCD2 and NBS1 in the DNA damage response." Nature Cell Biology **4**(12): 913-920.

Nishimura, Y., S. Hanaoka, et al. (2005). "Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities." Protein Science **14**(1): 119-130.

Noguchi, K., A. Vassilev, et al. (2006). "The BAH domain facilitates the ability of human Orc1 protein to activate replication origins in vivo." The EMBO journal **25**(22): 5372-5382.

O'Connor, M. S., A. Safari, et al. (2004). "The human Rap1 protein complex and modulation of telomere length." Journal of Biological Chemistry **279**(27): 28585-28591.

Oliver, A. W., S. Knapp, et al. (2007). "Activation segment exchange: a common mechanism of kinase autophosphorylation?" Trends Biochem Sci **32**(8): 351-356.

Palm, W. and T. de Lange (2008). "How Shelterin Protects Mammalian Telomeres." Annual Review of Genetics **42**: 301-334.

Pandita, T. K. (2002). "ATM function and telomere stability." Oncogene **21**(4): 611-618.

Paull, T. T. and M. Gellert (1999). "Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex." Genes & Development **13**(10): 1276-1288.

Petrini, J. H. J., C. L. Attwooll, et al. (2009). "The Mre11 Complex and the Response to Dysfunctional Telomeres." Molecular and Cellular Biology **29**(20): 5540-5551.

Pickering, M. T. and T. F. Kowalik (2006). "Rb inactivation leads to E2F1-mediated DNA double-strand break accumulation." Oncogene **25**(5): 746-755.

Ponnusamy, S., N. L. Alderson, et al. (2008). "Regulation of telomere length by fatty acid elongase 3 in yeast. Involvement of inositol phosphate metabolism and Ku70/80 function." The Journal of biological chemistry **283**(41): 27514-27524.

Porter, A. G. and R. U. Janicke (1999). "Emerging roles of caspase-3 in apoptosis." Cell Death and Differentiation **6**(2): 99-104.

Powers, J. T., S. K. Hong, et al. (2004). "E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis." Molecular Cancer Research **2**(4): 203-214.

Querido, E., J. G. Teodoro, et al. (1997). "Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis." Journal of Virology **71**(5): 3526-3533.

Ranganathan, V., W. F. Heine, et al. (2001). "Rescue of a telomere length defect of Nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit." Curr Biol **11**(12): 962-966.

Reimann, M., C. Loddenkemper, et al. (2007). "The Myc-evoked DNA damage response

accounts for treatment resistance in primary lymphomas in vivo." Blood **110**(8): 2996-3004.

Resnick, I. B., I. Kondratenko, et al. (2002). "Nijmegen breakage syndrome: Clinical characteristics and mutation analysis in eight unrelated Russian families." Journal of Pediatrics **140**(3): 355-361.

Richter, T., G. Saretzki, et al. (2007). "TRF2 overexpression diminishes repair of telomeric single-strand breaks and accelerates telomere shortening in human fibroblasts." Mechanisms of Ageing and Development **128**(4): 340-345.

Riedl, S. J. and Y. Shi (2004). "Molecular mechanisms of caspase regulation during apoptosis." Nature reviews. Molecular cell biology **5**(11): 897-907.

Robles, S. J., P. W. Buehler, et al. (1999). "Permanent cell cycle arrest in asynchronously proliferating normal human fibroblasts treated with doxorubicin or etoposide but not camptothecin." Biochemical pharmacology **58**(4): 675-685.

Rogoff, H. A., M. T. Pickering, et al. (2004). "Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2." Molecular Cell Biology **24**(7): 2968-2977.

Rupnik, A., N. F. Lowndes, et al. (2010). "MRN and the race to the break." Chromosoma **119**(2): 115-135.

Samper, E., J. M. Flores, et al. (2001). "Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc-/- mice with short telomeres." EMBO reports **2**(9): 800-807.

Satyanarayana, A., M. P. Manns, et al. (2004). "Telomeres, telomerase and cancer: an endless search to target the ends." Cell Cycle **3**(9): 1138-1150.

Sfeir, A., S. Kabir, et al. (2010). "Loss of Rap1 induces telomere recombination in the absence of NHEJ or a DNA damage signal." Science **327**(5973): 1657-1661.

Shaul, Y. (2000). "c-Abl: activation and nuclear targets." Cell Death and Differentiation **7**(1): 10-16.

Shay, J. W. and S. Bacchetti (1997). "A survey of telomerase activity in human cancer." European Journal of Cancer **33**(5): 787-791.

Shay, J. W. and W. E. Wright (2005). "Senescence and immortalization: role of telomeres and telomerase." Carcinogenesis **26**(5): 867-874.

Shay, J. W., Y. Zou, et al. (2001). "Telomerase and cancer." Human molecular genetics **10**(7): 677-685.

Shiloh, Y. (2001). "ATM and ATR: networking cellular responses to DNA damage." Current Opinion in Genetics & Development **11**(1): 71-77.

Shiloh, Y. (2003). "ATM and related protein kinases: Safeguarding genome integrity." Nature Reviews Cancer **3**(3): 155-168.

Shiloh, Y. (2006). "The ATM-mediated DNA-damage response: taking shape." Trends in Biochemical Sciences **31**(7): 402-410.

Simbulan-Rosenthal, C. M., D. S. Rosenthal, et al. (1998). "Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis." Journal of Biological Chemistry **273**(22): 13703-13712.

Smogorzewska, A., J. Karlseder, et al. (2002). "DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2." Current Biology **12**(19): 1635-1644.

Smogorzewska, A., B. Van Steensel, et al. (2000). "Control of human telomere length by TRF1 and TRF2." Molecular and Cellular Biology **20**(5): 1659-1668.

Stewart, G. S., R. S. Maser, et al. (1999). "The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder." Cell **99**(6): 577-587.

Stewart, S. A. and R. A. Weinberg (2006). "Telomeres: Cancer to human aging." Annual Review of Cell and Developmental Biology **22**: 531-557.

Stiff, T., C. Reis, et al. (2005). "Nbs1 is required for ATR-dependent phosphorylation events." Embo Journal **24**(1): 199-208.

Stiff, T., S. A. Walker, et al. (2006). "ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling." EMBO Journal **25**(24): 5775-5782.

Takakura, M., S. Kyo, et al. (1999). "Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells." Cancer Research **59**(3): 551-557.

Tauchi, H., K. Iijima, et al. (2008). "NBS1 regulates a novel apoptotic pathway through Bax activation." DNA Repair **7**(10): 1705-1716.

Tauchi, H., S. Matsuura, et al. (2002). "Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability." Oncogene **21**(58): 8967-8980.

Tejera, A. M., M. S. d'Alcontres, et al. (2010). "TPP1 Is Required for TERT Recruitment, Telomere Elongation during Nuclear Reprogramming, and Normal Skin Development in Mice." Developmental Cell **18**(5): 775-789.

Toledo, F. and G. M. Wahl (2006). "Regulating the p53 pathway: in vitro hypotheses, in vivo veritas." Nature Reviews Cancer **6**(12): 909-923.

Tsellou, E. and H. Kiaris (2008). "Fibroblast independency in tumors: implications in cancer therapy." Future Oncology **4**(3): 427-432.

van Steensel, B., A. Smogorzewska, et al. (1998). "TRF2 protects human telomeres from end-to-end fusions." Cell **92**(3): 401-413.

vanSteensel, B. and T. deLange (1997). "Control of telomere length by the human telomeric protein TRF1." Nature **385**(6618): 740-743.

Varon, R., C. Vissinga, et al. (1998). "Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome." Cell **93**(3): 467-476.

Wang, X. M., J. Li, et al. (2008). "Involvement of the role of Chk1 in lithium-induced G2/M phase cell cycle arrest in hepatocellular carcinoma cells." Journal of Cellular Biochemistry **104**(4): 1181-1191.

Westphal, C. H., S. Rowan, et al. (1997). "atm and p53 cooperate in apoptosis and suppression of tumorigenesis, but not in resistance to acute radiation toxicity." Nature Genetics **16**(4): 397-401.

White, J. S., S. Choi, et al. (2008). "Irreversible chromosome damage accumulates rapidly in the absence of ATM kinase activity." Cell Cycle **7**(9): 1277-1284.

Williams, R. S., J. S. Williams, et al. (2007). "Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template." Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire **85**(4): 509-520.

Wu, Y., T. R. Mitchell, et al. (2008). "Human XPF controls TRF2 and telomere length maintenance through distinctive mechanisms." Mechanisms of ageing and development **129**(10): 602-610.

Wu, Y., S. Xiao, et al. (2007). "MRE11-RAD50-NBS1 and ATM function as co-mediators of TRF1 in telomere length control." Nat Struct Mol Biol **14**(9): 832-840.

Xiao, Z., Z. Chen, et al. (2003). "Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents." The Journal of biological chemistry **278**(24): 21767-21773.

Xu, Y., T. Ashley, et al. (1996). "Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma." Genes & Development **10**(19): 2411-2422.

Yamaguchi-Iwai, Y., E. Sonoda, et al. (1999). "Mre11 is essential for the maintenance of chromosomal DNA in vertebrate cells." EMBO Journal **18**(23): 6619-6629.

Yamane, K., K. Taylor, et al. (2004). "Mismatch repair-mediated G2/M arrest by 6-thioguanine involves the ATR-Chk1 pathway." Biochemical and Biophysical Research Communications **318**(1): 297-302.

Yang, L., Z. P. Xu, et al. (2004). "ATM and ATR: Sensing DNA damage." World Journal of Gastroenterology **10**(2): 155-160.

Yazdi, P. T., Y. Wang, et al. (2002). "SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint." Genes & Development **16**(5): 571-582.

Yoo, H. Y., A. Kumagai, et al. (2009). "The Mre11-Rad50-Nbs1 Complex Mediates Activation of TopBP1 by ATM." Molecular Biology of the Cell **20**(9): 2351-2360.

You, Z., C. Chahwan, et al. (2005). "ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1." Mol Cell Biol **25**(13): 5363-5379.

Yuan, Z. M., Y. Y. Huang, et al. (1997). "Regulation of DNA damage-induced apoptosis by the c-Abl tyrosine kinase." Proceedings of the National Academy of Sciences of the United States of America **94**(4): 1437-1440.

Zhang, Y., J. Q. Zhou, et al. (2006). "The role of NBS1 in DNA double strand break repair, telomere stability, and cell cycle checkpoint control." Cell Research **16**(1): 45-54.

Zhao, H. and H. Piwnica-Worms (2001). "ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1." Molecular and cellular biology **21**(13): 4129-4139.

Zhou, B. B. S. and S. J. Elledge (2000). "The DNA damage response: putting checkpoints in perspective." Nature **408**(6811): 433-439.

Zhou, J. Q., C. U. K. Lim, et al. (2006). "The role of NBS1 in the modulation of PIKK family proteins ATM and ATR in the cellular response to DNA damage." Cancer Letters **243**(1): 9-15.

Zhu, J., S. Petersen, et al. (2001). "Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice." Current Biology **11**(2): 105-109.

Zhu, X. D., B. Kuster, et al. (2000). "Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres." Nature Genetics **25**(3): 347-352.

8. APPENDICES

Supplementary Figure 1

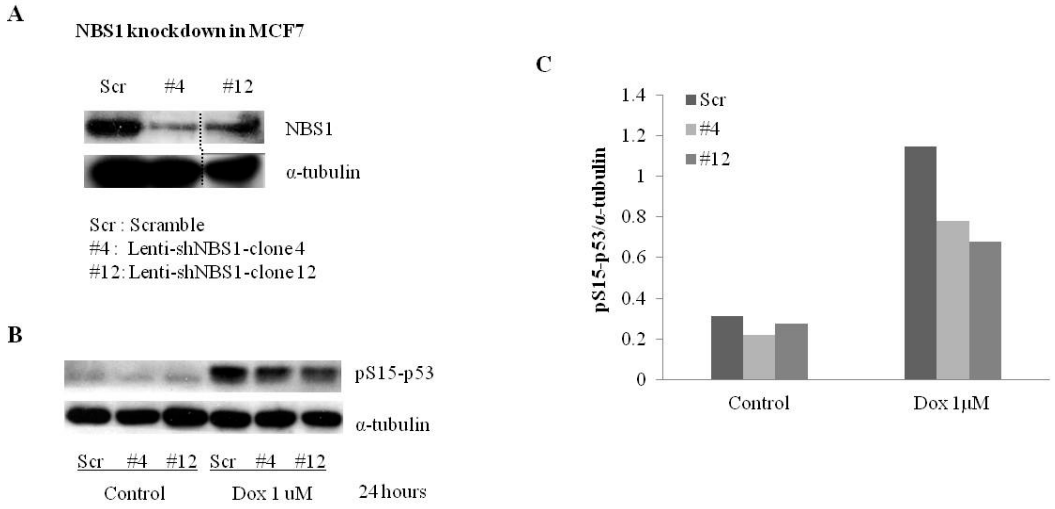

Figure S1. NBS1 knockdown in human breast cancer cells MCF7. A. Western blot analysis of the protein level of NBS1 after knockdown by shRNA (sequence: 5'-AAAACTGCAGAAAAA GCAAGCAGATACATGGGATTTTCTCTTGAAAAATCCCATGTATCTGCTTGCGGTGTTTC GTCCTTTCCACAAG-3'). 2 clones (clone 4 and clone 12) showed similar knockdown effect. α-tubulin serves as the loading control. B. Western blot analysis of the phosphorylation level of p53 in MCF7 cells with NBS1 knockdown. Cells were treated with 1 μM Dox for 24 hours. α-tubulin serves as the loading control. C. Western blots in B were scanned and quantified by densitometer. The phosphorylation level of p53 at Ser15 was normalized to the loading control α-tubulin.

Supplementary Figure 2

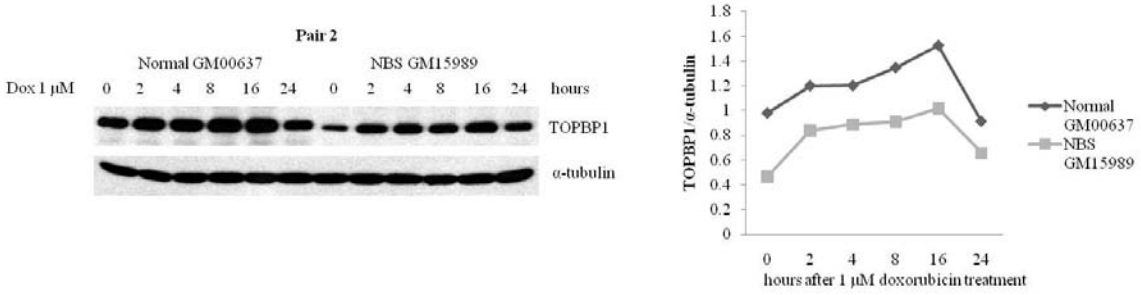

Figure S2. NBS1 deficiency affects the expression level of TOPBP1. Western blot analysis of the TOPBP1 protein level in NBS as well as normal fibroblasts. Cells were treated with 1 μM Dox and collected at the time points indicated. α-tubulin serves as the loading control.

Supplementary Figure 3

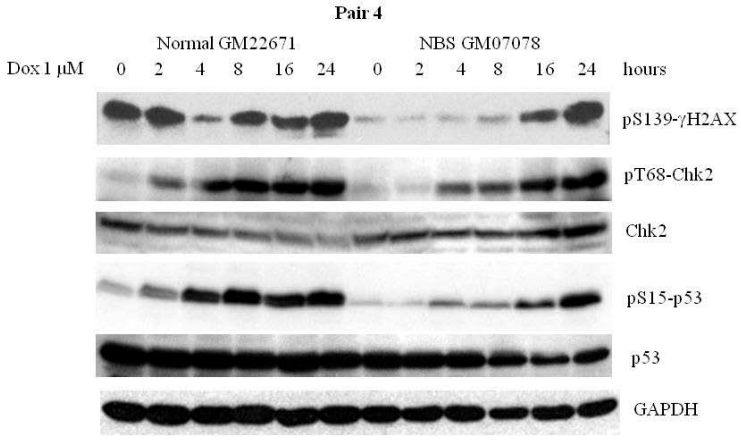

Figure S3. NBS1 deficiency also affects the DNA damage signaling pathway in B-lymphocytes. Western blot analysis of the phosphorylation level of ATM downstream targets, including histone H2AX, p53 and Chk2, in NBS as well as normal B-lymphocytes. Cells were treated with 1 μM Dox and collected at the time points indicated. GAPDH serves as the loading control.

CELL BIOCHEMISTRY & FUNCTION

CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct (2011)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/cbf.1840

NBS1 deficiency promotes genome instability by affecting DNA damage signaling pathway and impairing telomere integrity

Yan Yan Hou, Meng Tiak Toh and Xueying Wang*

Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Studies revealed that Nijmegen Breakage Syndrome protein 1 (NBS1) plays an important role in maintaining genome stability, but the underlying mechanism is controversial and elusive. Our results using clinical samples showed that NBS1 was involved in ataxia-telangiectasia mutated (ATM)-dependent pathway. NBS1 deficiency severely affected the phosphorylation of ATM as well as its downstream targets. BrdU proliferation assay revealed a delay of NBS cells in inhibiting DNA synthesis after Doxorubicin (Dox) treatment. In addition, under higher concentrations of Dox, NBS cells exhibited a much lower level of apoptosis compared to their normal counterparts, indicating a resistance to Dox treatment. Accelerated telomere shortening was also observed in NBS fibroblasts, consistent with an early onset of cellular replicative senescence *in vitro*. This abnormality may be due to the shelterin protein telomeric binding factor 2 (TRF2) which was found to be upregulated in NBS fibroblasts. The dysregulation of telomere shortening rate and of TRF2 expression level leads to telomere fusions and cellular aneuploidy in NBS cells. Collectively, our results suggest a possible mechanism that NBS1 deficiency simultaneously affects ATM-dependent DNA damage signaling and TRF2-regulated telomere maintenance, which synergistically lead to genomic abnormalities.

KEY WORDS—NBS1; ATM; DNA damage; telomere; TRF2; genome instability

INTRODUCTION

Nijmegen Breakage Syndrome (NBS) is a rare human genetic disorder characterized by immunodeficiency and a strong predisposition to cancer.[1] The underlying gene mutated in NBS, *NBS1*, was cloned in 1998 and since then human NBS1 protein has emerged as a player in the cellular response to DNA damage, especially to double strand breaks (DSBs).[2] In response to DSBs, NBS1 was found to have a close relationship with another DNA damage-related protein ATM,[3] the gene that is mutated in the ataxia-telangiectasia (A-T) disease.[4]

ATM is a member of the phosphoinositol 3-kinase-like kinase (PIKK) family.[5] ATM has a wide range of downstream targets, including DNA damage sensors, mediators, transducers as well as effectors.[6] NBS1 has been identified as a DNA damage sensor which could be phosphorylated by ATM in response to ionizing radiation (IR) that generates DSBs.[5] On the other hand, several other studies have placed NBS1 as an upstream regulator of ATM.[7–9]

The activation of ATM leads to the phosphorylation of a plethora of downstream substrates, such as p53, histone H2AX and Chk2.[10] The activation of these downstream targets results in cellular responses, such as cell cycle checkpoint controls, DNA damage repair and apoptosis.[11] The deficiency in either ATM or its downstream substrates would lead to defective cellular responses. It has been shown that A-T cells that are deficient in ATM exhibited defective G1/S, intra-S and G2/M cell cycle transition.[6] NBS1, as a downstream target of ATM, is also involved in the cell cycle arrest and apoptosis pathways. In response to IR, NBS cells exhibited radio-resistant DNA synthesis, indicating a failure in inducing intra-S checkpoint control.[12] Defects in inducing G1 or G2 arrest have also been reported in NBS cells.[13] However, other studies showed normal and proficient G1 and G2 checkpoint in spite of NBS1 deficiency.[14] The role of NBS1 in maintaining checkpoint integrity still remains controversial. Moreover, the influence of NBS1 deficiency on apoptosis is rarely reported and how NBS1 regulates DNA damage induced apoptosis is waiting to be elucidated.

Besides cell cycle checkpoint and apoptosis, NBS1 also plays a role in telomere maintenance.[15] In yeast, Xrs2, the functional homolog of NBS1, is involved in telomerase-dependent telomere synthesis.[16] In human, NBS1 is associated with telomeres in a cell-cycle regulated manner.[17] It has been reported that NBS fibroblasts showed premature growth cessation in culture. But how NBS1 deficiency leads to this phenomenon is not well studied. Shelterin complex serves as another mechanism to maintain telomere integrity by associating with telomeres and burying the telomeric ends into t-loops, thus preventing them from being recognized as

*Correspondence to: Xueying Wang, 8 Medical Drive, MD4A, #02-04, National University of Singapore, Singapore, 117597.
E-mail: bchwxy@nus.edu.sg

Received 2 August 2011
Revised 3 November 2011
Accepted 10 November 2011

DSBs.[18] NBS1 has been shown to interact with one of the components of shelterin complex, TRF2.[19] However, whether the interaction between NBS1 and TRF2 has an effect on telomere maintenance is still not known.

This study aims to examine the roles of NBS1 both in DNA damage signaling pathway and in maintaining telomere integrity. On one hand, we found that NBS1 deficiency affected ATM-mediated DNA damage signaling pathway and its subsequent cellular events, such as DNA proliferation and apoptosis. On the other hand, we observed accelerated telomere shortening and an earlier onset of senescence in NBS cells. Moreover, our group for the first time found that NBS1 deficiency is related to an upregulation of TRF2, which suggests an important clue for studying the accelerated telomere shortening in the future. This study also provided evidence that frequent telomere abnormalities exist in NBS cells. As telomere dysfunction has been implicated in carcinogenesis, this study extends our recognition of the high incidence of cancers in NBS patients.

MATERIALS AND METHODS

Cells and culture conditions

Cells were obtained from Coriell Cell Repositories (pair1: AG09309 & GM07166, pair2: GM00637 & GM15989, pair3: AG14725 & GM15814, pair4: GM22671 & GM07078) and cultured in either MEM or RPMI with 15% FBS, 1% L-Glutamine, 1% P/S, 1% NEAA and 1% vitamin solution, and incubated at 37 °C under 5% CO_2. The NBS cell lines within each pair are homozygous for a deletion of 5 nucleotides in exon 6 of *NBS1* gene (657del5 mutation).

Western blot and antibodies

Cells were harvested for protein lysate. Briefly, cells were resuspended in 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 5 mmol/L EDTA, and 0.1% NP40 containing protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 14,000 rpm for 10 min, and samples were run on SDS-PAGE gels. Western blotting was performed with the following antibodies: ATM, γH2AX (Novus Biologicals); ATM pS1981 (Rockland); NBS1, p53, p53 pS15, Chk2, Chk2 pT68, cleaved caspases3 (Cell Signaling); PARP, TRF1, POT1 (Abcam); TRF2 (BD Biosciences); RAP1 (Bethyl Laboratories); Horseradish peroxidase (HRP)-conjugated mouse anti-GAPDH (Cell Signaling), HRP-conjugated mouse anti-β actin (Abcam), or mouse anti-α tubulin (Sigma-Aldrich) were used as loading controls. Immunostaining was detected using ECL Plus Detection Reagent (GE Healthcare).

FITC Annexin V apoptosis assay

Cells were harvested after 24 hours treatment with Dox under the concentration of 0.25 μM, 0.5 μM or 1 μM. The apoptosis level was detected using the protocol as described by the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen). The data was analyzed using BD FACS Diva software.

BrdU assay

Cells were harvested after either 10 hours or 22 hours treatment with 1 μM Dox. The percentage of $BrdU^+$ cells was determined using the protocol described by the 5-Bromo-2'-deoxy-uridin labeling and detection kit III (Roche).

Telomere length assay

DNA was extracted from the cells using a genomic purification kit (PureLink, Invitrogen). Telomere length analysis was carried out using a non-radioactive TeloTAGGG Telomere Length Assay (Roche) as described.

β-Galactosidase staining

Normal and NBS fibroblasts were cultured to the population doubling level (PDL) indicated. Cells undergoing senescence were detected using the protocol as described by the β-Galactosidase Staining Kit (US Biological).

Cytogenetic analysis of metaphase spreads

Normal and NBS fibroblasts were cultured to late passages. Metaphase spreads were prepared as described by the Jeppesen's protocol.[20]

Telomerase activity assay

Telomerase activity was quantified using Telomeric Repeat Amplification Protocol (TRAP) as described by the TeloExpress Quantitative Telomerase Detection Kit (XpressBio). Telomerase activity in each sample was calculated based on the comparison with the Ct values of a standard curve generated from 10-fold dilutions of telomerase control (TC) oligo with known copy numbers of the telomeric repeats.

RT-PCR

One step RT-PCR was performed using the Qiagen One Step RT-PCR kit following manufacturer's protocol. The primers for TRF2 are: 5'-TGCTCAAGTTCTACTTCCACGA-3' and 5'-TTGATAGCTGATTCCAGTGGTG-3'. PCR products were run on 2% agarose gel and viewed under UV Gel Doc (BioRad).

RESULTS

NBS1 deficiency affects ATM phosphorylation and ATM-dependent phosphorylation of multiple downstream targets

In this study, cells derived from NBS patients who have typical 657del5 mutation of the *NBS1* gene were used. As controls, normal cells with wild type *NBS1* gene were also employed and paired with NBS cells under the criteria of age, gender and race for a more reliable comparison. To determine if NBS1 deficiency affects the phosphorylation of ATM, two NBS fibroblasts as well as their normal counterparts were used (Pair 1 and Pair 2). As shown, the wild type NBS1 protein was only expressed in normal cells but not in NBS cells (Figure 1A). Cells were then subjected to

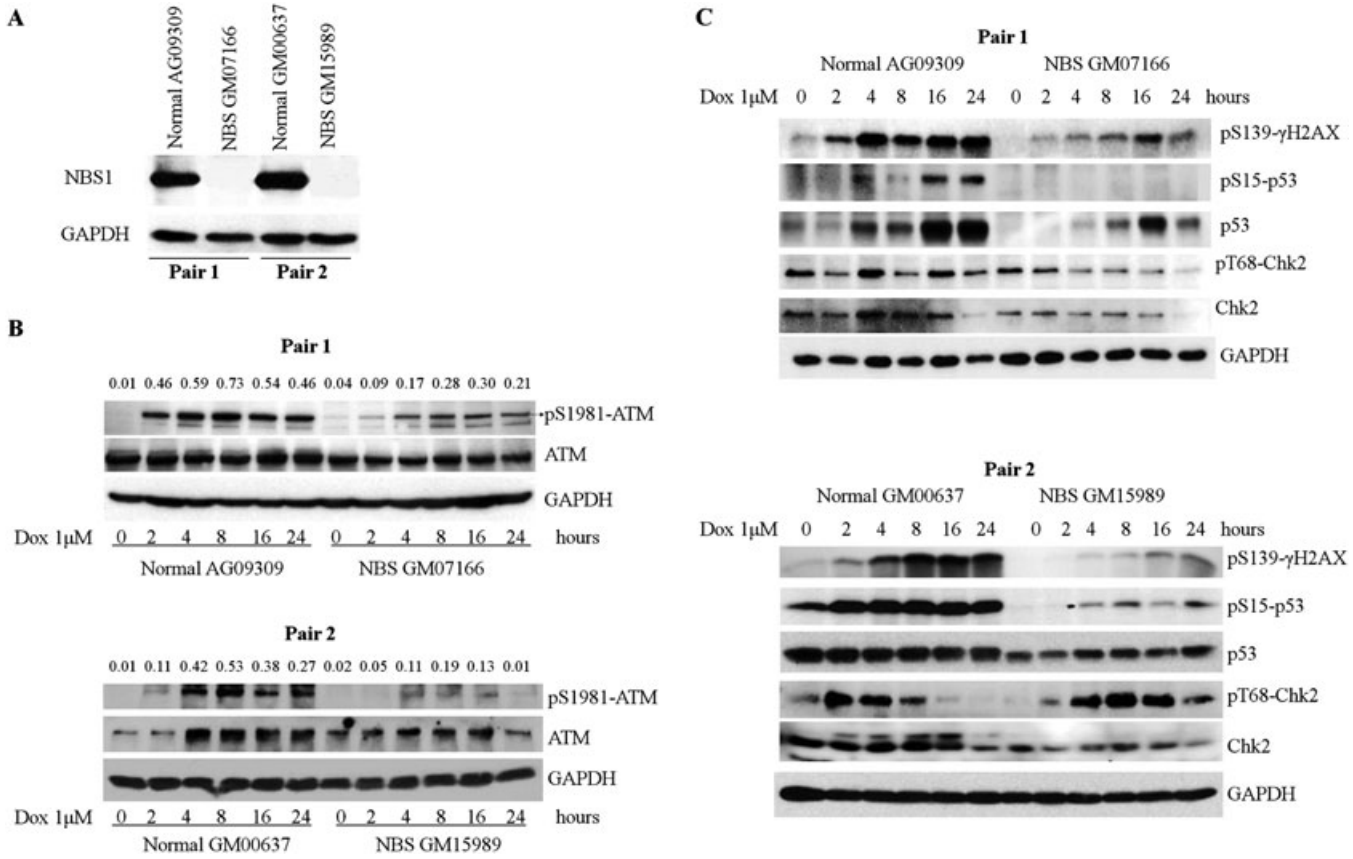

Figure 1. NBS1 deficiency affects ATM phosphorylation and the phosphorylation of multiple ATM downstream targets. A. The expression of NBS1 protein in NBS fibroblasts as well as in age, race and gender-matched normal cells. The four cell lines were classified into two pairs, nominated as pair 1 and pair 2. B. The expression and phosphorylation of ATM. Cells were treated with 1 μM Dox and collected at the time points indicated. The numbers above the blot indicate the level of pS1981-ATM normalized to the total ATM level measured by densitometer. C. The phosphorylation of ATM downstream targets, including H2AX, p53 and Chk2. Cells were treated with 1 μM Dox and collected at the time points indicated.

1 μM Dox treatment and the phosphorylation level of ATM at Ser1981 was examined at different time points by western blot. Results showed that ATM was quickly activated in normal cells and reached the highest level in 8 hours after Dox treatment (Figure 1B). However, in NBS cells, ATM phosphorylation was severely impaired, exhibited by a much lower level than that in normal counterparts (Figure 1B). Although ATM phosphorylation level decreased dramatically in NBS cells, there was still a detectable basal level of phosphorylated ATM (Figure 1B) indicating that NBS1 deficiency does not fully abolish ATM phosphorylation.

If NBS1 deficiency affects ATM activation, whether the activation of ATM downstream targets is also affected is the question that we want to address next. H2AX, p53 and Chk2 are three important ATM downstream substrates which are involved in DNA damage responses.[10] The phosphorylation statuses of these three proteins were also examined by western blot. Results showed that the phosphorylation of H2AX at Ser139 and phosphorylation of p53 at Ser15 were also severely affected in NBS cells under 1 μM Dox treatment (Figure 1C). In normal cells, these two proteins were quickly phosphorylated to a high level and the high phosphorylation level was maintained for all the rest time points detected. But in NBS cells, the phosphorylation level was significantly decreased (Figure 1C). Moreover, the total level of p53 was also affected in NBS cells, suggesting a possibility that NBS1 deficiency compromises p53 stability. Surprisingly, the phosphorylation level of Chk2 at Thr68 was not reduced in NBS cells, but only exhibited a delay in activation. As shown, Chk2 was activated and reached a high level within 2 hours in normal cells, but was activated in NBS cells at a much later time point around 8 hours under 1 μM Dox treatment (Figure 1C). Taken together, these results suggest that NBS1 deficiency could affect the phosphorylation of ATM downstream targets, leading to either a lower phosphorylation level or a delayed activation of ATM targets.

NBS1 deficiency delays inhibition of DNA synthesis after DNA damages occur

One of the cellular events of DNA damage response is to inhibit DNA synthesis to stop the propagation of "bad" cells

with DNA lesions. We next investigated the potential roles of NBS1 in eliciting inhibition of DNA synthesis when DNA is damaged. Since pair 2 fibroblasts are transformed with SV40 which would render G1/S checkpoint inactive and therefore affect the number of cells entering S phase for DNA synthesis,[21] we used additional 2 pairs of B-lymphocytes (Pair 3 and Pair 4) for analysis of DNA synthesis status. As shown in the western blot, full length NBS1 was only expressed in normal cells but not in NBS cells (Figure 2A). We performed BrdU incorporation assay to access the proliferation profile of cells after 1 μM Dox treatment for either 10 or 22 hours. From this result, we found that the cell proliferation was suppressed after Dox treatment in both normal and NBS cells, exhibited by the ratio of $BrdU^+$ Dox^+ cells to $BrdU^+Dox^-$ cells less than 1 (Figure 2B). Although suppression of cell proliferation was observed in both normal and NBS cells, at 10 hours, NBS cells showed a lesser degree of arrest than the normal cells, indicated by a higher $BrdU^+Dox^+$ to $BrdU^+Dox^-$ cells ratio. It was only after 22 hours of Dox treatment, did the NBS cells exhibit a similar degree of arrest as their normal counterparts (Figure 2B). This result indicates the suppression of proliferation in NBS cells is not as efficient as that in normal cells, suggesting a delay in inhibition of DNA synthesis in NBS cells.

NBS1 deficiency affects the initiation of apoptosis

Another cellular event of DNA damage response is to initiate apoptosis when DNA damage is beyond repair. Cells treated with different concentrations of Dox for 24 hours were harvested and subjected to flow cytometry analysis. Results showed that NBS cells had comparable apoptosis level to normal cells under lower concentration of Dox treatment. When the concentration of Dox was increased to a high concentration of 1 μM, normal cells exhibited elevated level of apoptosis. But apoptosis level in NBS cells remained low as that under lower concentrations of Dox (Figure 3A, B), indicating that NBS cells were defective in inducing apoptosis when cells were exposed to high dosage of Dox. Western analysis of apoptosis associated markers showed that cleaved caspase3 almost diminished in NBS cells. However, as a direct downstream target of caspase3, Poly-ADP-ribose-polymerase (PARP) only exhibited a minor decrease in its cleaved form in NBS cells (Figure 3C). This is probably due to the low level of cleaved caspase3 in NBS cells. The low efficiency in cleavage of these proteins may be responsible for the defects of NBS cells in initiation of apoptosis under high concentration of Dox treatment.

NBS1 deficiency promotes telomere shortening and an earlier onset of senescence

Premature aging has been observed in NBS fibroblasts *in vitro*.[22] Premature cellular senescence could be elicited by accelerated telomere shortening. We therefore asked whether NBS1 deficiency elicits premature aging through regulating telomere attrition rate. Telomere length of the two pairs of fibroblasts was tested by the Terminal Restriction Fragment southern blot. Result showed that the telomere length of NBS cells was generally shorter than that of age-matched normal cells. When comparing the telomere attrition rate, we found that NBS cells showed a higher telomere shortening rate compared to that in normal cells *in vitro* (Figure 4A). For each replication cycle, the telomere shortening rate of NBS cells is around 30 bp faster than that of its respective normal counterparts (Figure 4B). This result strongly indicates that NBS1 plays a role in telomere length maintenance and the deficiency of NBS1 leads to faster telomere attrition. We performed β-galactosidase assay to study the senescence status of normal as well as NBS fibroblasts *in vitro*. Cells were cultured to the same PDL and stained, and the cells stained blue were counted as senescent cells. Consistent with the accelerated telomere shortening, NBS fibroblasts exhibited a significantly higher percentage of cells undergoing senescence compared to normal cells with the same PDLs (Figure 4C, D). These results suggest that NBS cells have a larger population of cells with critically short telomeres.

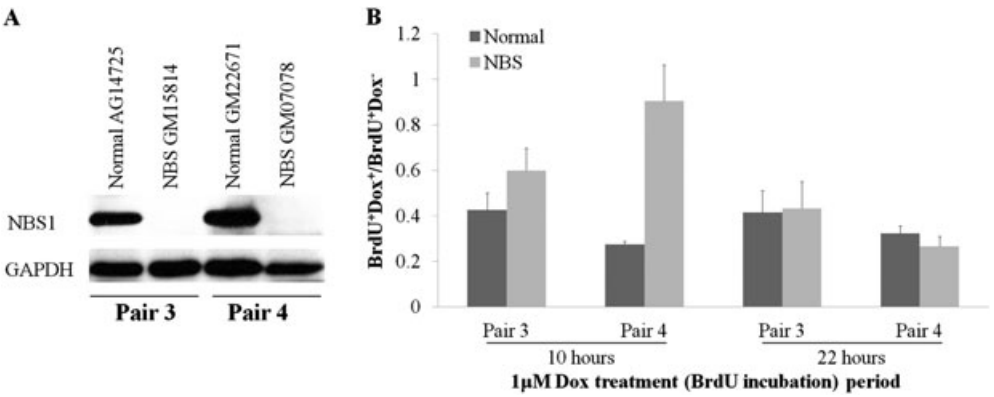

Figure 2. NBS1 deficiency delays inhibition of DNA synthesis after DNA damages occur. A. The expression of NBS1 protein in NBS B-lymphocytes as well as in age, race and gender-matched normal cells. The four cell lines were classified into two pairs, nominated as pair 3 and pair 4. B. BrdU incorporation assay. Cells were seeded onto 96-well plate and after culturing for 2 days, cells were treated with 1 μM Dox and 10 μM BrdU at the same time for either 10 or 22 hours. The bar represents the ratio of Dox-treated $BrdU^+$ cells to untreated $BrdU^+$ cells.

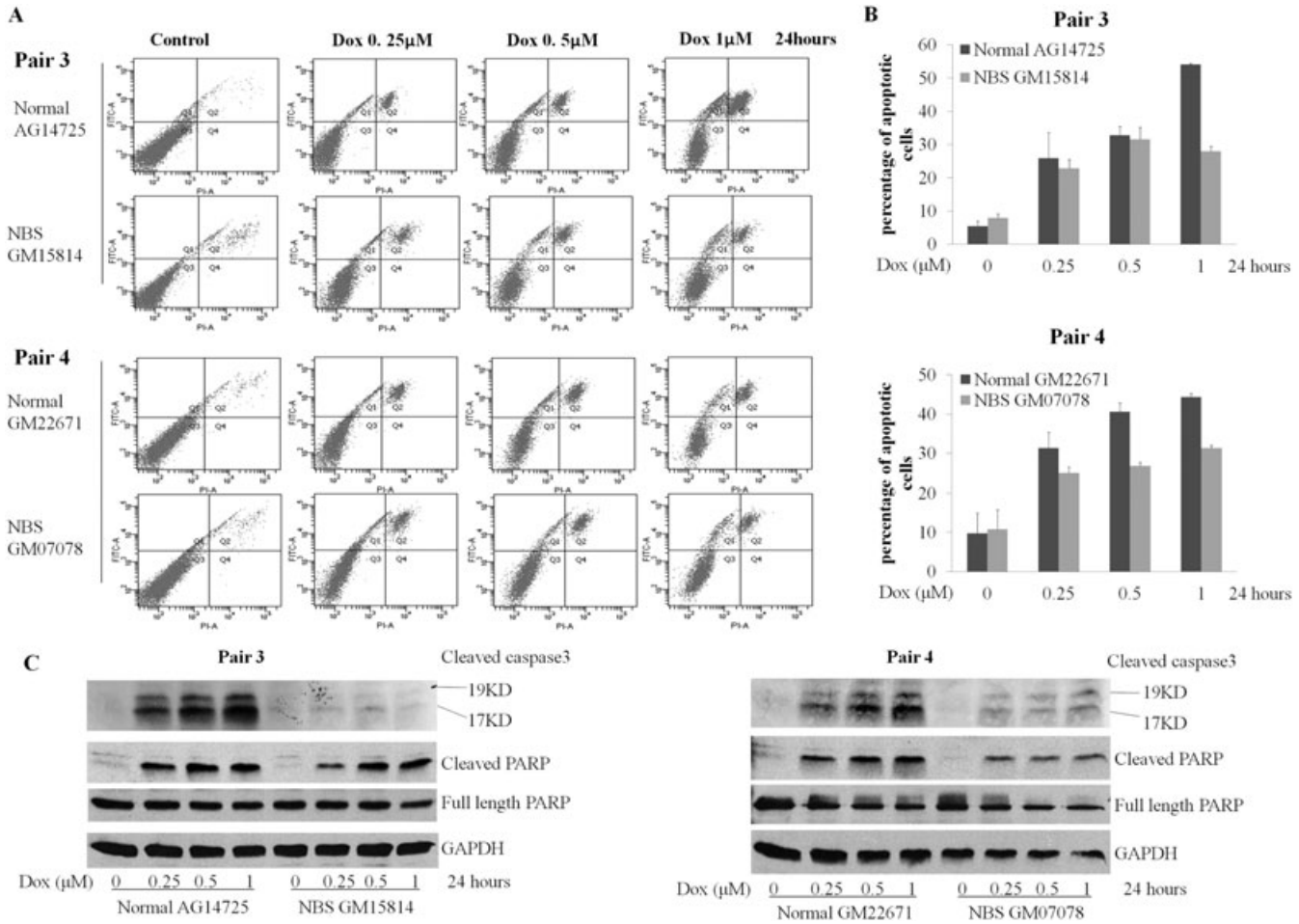

Figure 3. NBS1 deficiency affects the initiation of apoptosis. A. FITC Annexin V apoptosis assay. B-lymphocytes were treated with Dox at the indicated concentrations for 24 hours. The number of apoptotic cells was analyzed by flow cytometry. B. Quantitation of the percentage of apoptosis cells in A. C. Western blot analysis of apoptosis-related proteins, including cleaved caspase3 and PARP.

NBS1 deficiency does not affect telomerase activity but upregulates TRF2

Telomere length is maintained by the activity of telomerase. We questioned whether the accelerated telomere shortening is due to decreased telomerase activity in NBS cells. By real time PCR, we found that NBS1-deficient fibroblasts have comparative telomerase activity as control cells (Figure 5A), which suggests that the accelerated telomere shortening is not due to decreased telomerase activity. Shelterin complex proteins protect the telomere integrity, but it also has been claimed that these proteins are negative regulators for telomere length.[18] We next looked into the different components of shelterin complex and found that the cellular level of TRF2 was upregulated in NBS cells (Figure 5B). However, the expression of other components, including TRF1, RAP1 and POT1, did not show obvious changes (Figure 5C). RT-PCR further showed an upregulation of TRF2 at mRNA level (Figure 5D). The overabundance of TRF2 at telomere ends may negatively regulate telomere length, resulting in accelerated telomere shortening in NBS cells.

NBS1 deficiency promotes genome instability

The accelerated telomere shortening and dysregulation of shelterin complex components may jeopardize the stability of telomeres in NBS cells. To evaluate the integrity of telomeres of NBS cells, we performed cytogenetic analysis of metaphase spread to look directly at the chromosome ends. As shown, prevalent telomere associations were observed in NBS cells (Figure 6A), exhibited by telomeres of different or the same chromosomes exist in unusually close proximity. Although very rare, telomere fusions were also observed in normal cells (Figure 6B). Telomere associations affect the chromosome separation during mitosis, resulting in aneuploid cells. We found that most of the normal cells retain 46 chromosomes during culture *in vitro*, although

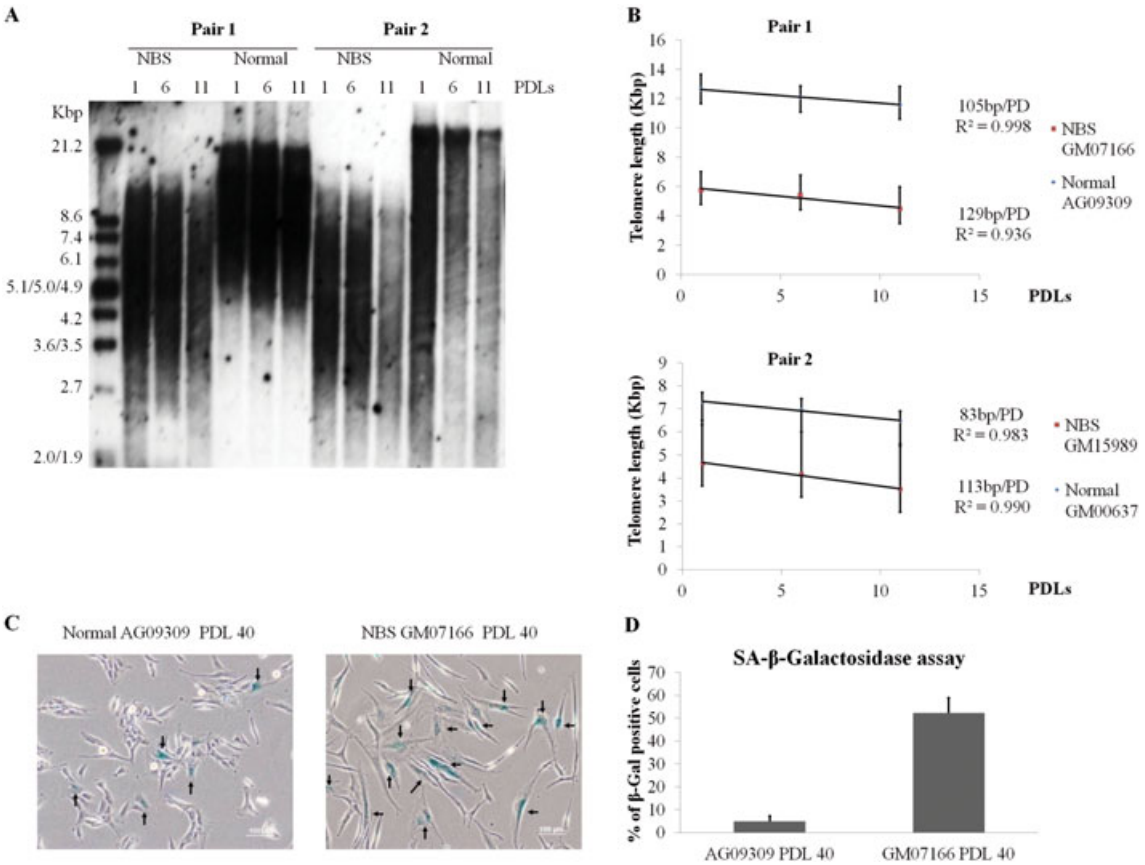

Figure 4. NBS1 deficiency leads to accelerated telomere shortening in NBS fibroblasts. A. Measurement of telomere restriction fragment length. Genomic DNA isolated from normal and NBS fibroblasts at indicated PDLs was analyzed. B. Telomere shortening rate in normal and NBS fibroblasts. Data are mean S.D. from duplicate experiments. Telomere shortening rate (slope of the regression line) and Spearman's regression coefficient are indicated. C. Cellular senescence assay using β-galactosidase staining. Arrows indicate senescent cells. D. Bars represent the percentage of β-galactosidase positive cells. Data are mean ± S.D. from 5 images each.

few of them showed abnormal chromosome numbers that slightly deviate from 46 (Figure 6C). However, NBS cells showed an average chromosome number of 78 which significantly deviates from the normal chromosome number, suggesting that the continued replication of NBS cells *in vitro* leads to more severe genome instabilities.

DISCUSSION

The *NBS1* gene encodes a 95KD protein.[23] 657del5 mutation of this gene leads to a frame shift and premature termination at codon 219 which abolishes the expression of the full length NBS1 protein. It is predicted that the premature termination would result in the expression of two truncated proteins, the 26KD N-terminus and the 70KD C-terminus.[12] However, only the 26KD fragment, but not the 70KD one, is found in NBS fibroblasts.[24] Our study using the antibody which recognizes the C-terminal residues of human NBS1 also did not detect the 70KD C-terminus band (data not shown). It has been proved in *Xenopus* egg extracts that the C-terminus of NBS1 is essential to recruit ATM to damaged DNA where its subsequent autophosphorylation happens.[25] Our results showed in the absence of both full length NBS1 and its C-terminus, ATM phosphorylation at Ser1981 was diminished in NBS cells when exposed to Dox treatment. This result strongly indicates that NBS1 serves as an upstream regulator of ATM. However, NBS cells still retain a low level of ATM phosphorylation under Dox treatment. We suggested that ATM autophosphorylation exists in a low level in cells that are under DNA damage even without functional NBS1. NBS1 serves as an amplifier for ATM activity which facilitates ATM to reach a threshold maximal activity when DNA damages occur.

Besides ATM, the phosphorylation of ATM downstream targets, including Histone H2AX and p53, was also severely affected. But NBS1 deficiency does not fully abolish the phosphorylation of these targets, probably due to the existence of a basal level of ATM phosphorylation. However, the activation of Chk2 was apparently normal though slightly delayed in NBS cells under Dox treatment. Like p53, Chk2 could also be phosphorylated by ATM and functions in cell cycle arrest. The phosphorylation of Chk2 brings its catalytic domain into the close proximity of another Chk2 molecule

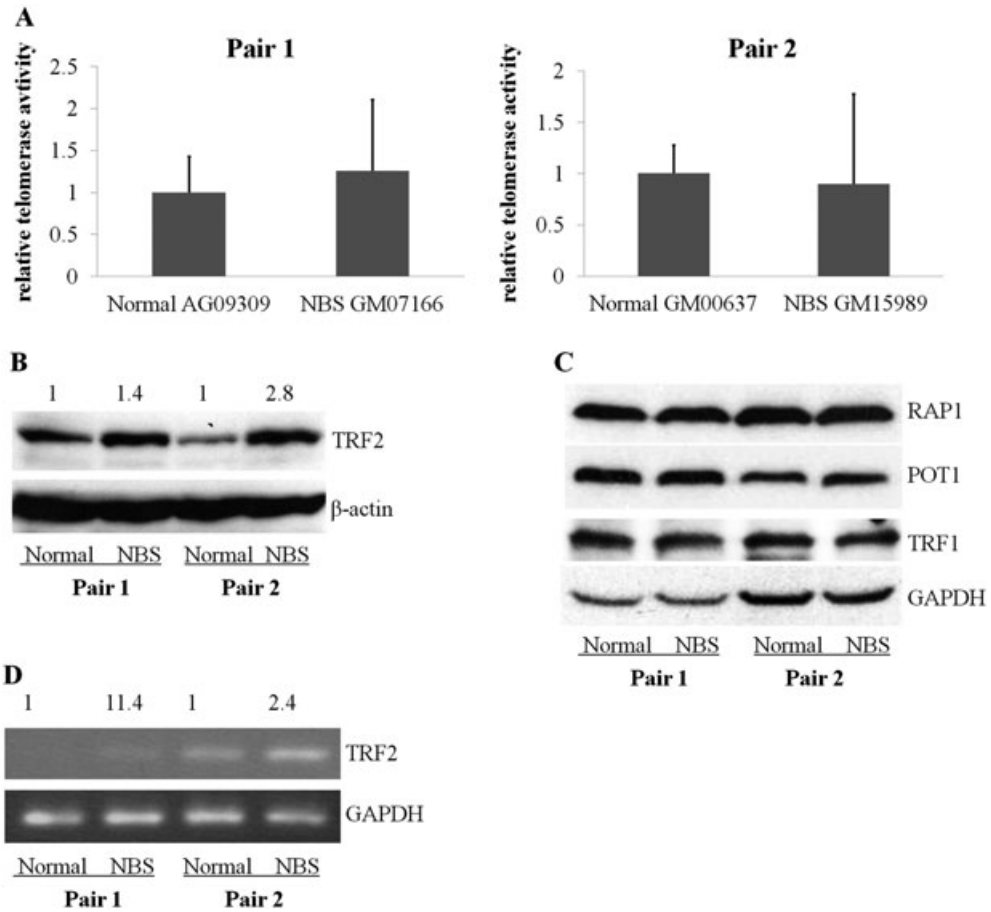

Figure 5. NBS1 deficiency upregulates TRF2. A. Real-time PCR for relative telomerase activity in NBS versus normal fibroblasts. B. Western blot analysis of the TRF2 protein level in NBS and normal fibroblasts. The numbers above the blot indicate its fold difference measured by densitometer with normal cell's TRF2 protein level being set at a reference value of 1. C. Western blot analysis of the other shelterin complex proteins in NBS and normal fibroblasts, including TRF1, POT1 and RAP1. D. RT-PCR analysis of the TRF2 mRNA level in NBS and normal fibroblasts. The numbers above the image indicate its fold difference measured by densitometer with normal cell's TRF2 mRNA level being set at a reference value of 1.

that allows auto-trans-phosphorylation to occur.[26] In NBS1 deficient cells, ATM activation was still present at basal levels. It could be explained that the basal level of activated ATM is sufficient to elicit initial phosphorylation of Chk2 which creates conditions for its following auto-trans-phosphorylation. But this process may take longer time than the direct phosphorylation of Chk2 by ATM, thus NBS cells had a delayed Chk2 phosphorylation.

As an initial response to DNA damages, normal cells with intact DNA damage signaling pathway would arrest to allow DNA damage to be repaired.[27] Our results showed that the proliferation rate of NBS cells was not as efficiently inhibited as that of normal cells when they were treated for 10 hours. But this difference was diminished after 22-hour treatment. By then, NBS cells showed comparable proliferation rate to normal cells. This result indicates that NBS1 deficiency may delay the checkpoint control, but does not abolish it.

Using annexin V apoptosis assay, we showed that NBS1 deficient cells exhibited defects in inducing apoptosis under higher concentration of Dox treatment, while these cells showed normal apoptosis level under lower concentration of Dox. The concentration of Dox may be proportional to the amounts of DNA lesions caused. Under lower concentration, small amount of DNA lesions are generated in cells. And as shown earlier, although the phosphorylation of ATM and the phosphorylation events elicited by ATM were either impaired or delayed in NBS1 deficient cells, there were still basal levels of activated proteins at later time points. We speculate that the activated basal-level proteins are sufficient to encounter the small scale DNA lesions but not enough to deal with larger scale DNA damage caused by higher concentration of Dox. This result suggests that the partially affected ATM and ATR signaling pathway in NBS cells could retain the apoptotic event to some degree but could not fully restore it when under large scale of DNA damage.

Evidence suggests that NBS1 binds to telomeres and is implicated in telomere length maintenance. Besides NBS1, many proteins that are crucial for maintaining genome stability are found associated with human telomeres, including ATM, the other two subunits of MRN complex, MRE11 and RAD50, WRN (gene mutated in Werner syndrome) and BLM (gene mutated in Bloom syndrome).[19] The presence

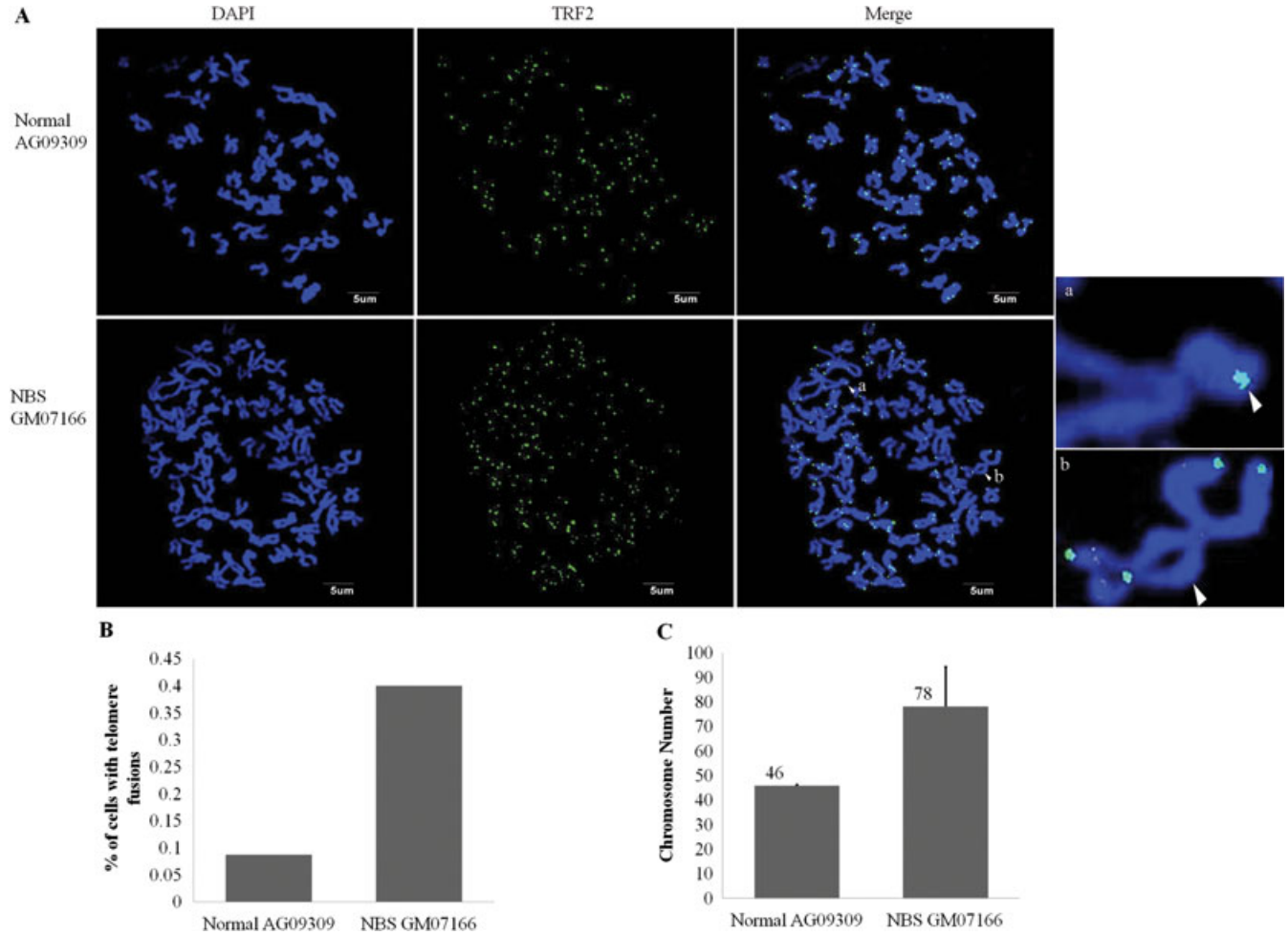

Figure 6. NBS1 deficiency promotes genome instability. A. Metaphase spreads of Pair 1 fibroblasts were stained with antibodies against TRF2 (green) and visualized by immunofluorescence. DNA was stained with DAPI (blue). Arrows point to telomeric end fusions. The insets (a and b) are representatives of telomere fusions. B. Bars represent the percentage of cells that are positive with telomere fusions. The total cell number is 25. C. Bars represent the average number of chromosomes enumerated from the metaphase spreads. Data are mean ± S.D. from 25 spreads each.

of these proteins at telomeric ends indicates a role of them in regulating telomere length and maintaining telomere integrity. Mutations of certain telomere associated genes would cause diseases that are characterized by premature aging, a clinical symptom that is probably linked to accelerated telomere shortening.[28] Our results showed that *NBS1* mutation also led to accelerated telomere shortening. At or around the same age, NBS cells exhibited shorter telomere length compared to normal cells. Moreover, we examined the telomere shortening rate *in vitro* and found that NBS cells had a higher telomere attrition rate with each population doubling. The accelerated telomere attrition probably leads to premature senescence of NBS cells, which was observed in our study by β-galactosidase assay. AT cells that are mutated in *ATM* gene also exhibited accelerated telomere shortening.[29] It has been suggested that ATM phosphorylates TRF1, a negative regulator of telomere length, thus reduces the binding of TRF1 to telomeres.[30] The reduction in TRF1 binding level at telomeric ends facilitates the assembly of telomerase to telomere and leads to telomerase-dependent telomere elongation.[30] Therefore, *ATM* mutation would exert a negative effect in the telomere elongation, which may be the cause for accelerated telomere shortening observed in AT cells. With regard to the close relationship between NBS1 and ATM, it is possible that NBS1 protects telomere from accelerated telomere shortening through the interplay with ATM.

It has been well established that TRF2 expression levels play an important role in determining telomere shortening rate.[31,32] Like TRF1, TRF2 is also recognized as a negative regulator of telomere length.[33] Overexpression of TRF2 leads to accelerated telomere shortening *in vitro* and premature aging *in vivo*.[19] Our results showed that TRF2 was upregulated at both mRNA and protein levels in NBS1 deficient cells. The upregulation of TRF2 may also contribute to the accelerated telomere shortening observed in NBS cells. But how NBS1 deficiency leads to upregulation of TRF2 is not known. In this study, we did not observe the

upregulation in TRF1 level. Although TRF1 and TRF2 have similar function and binding mode to telomeric DNA, TRF2 plays an important role in T-loop formation that protects telomere integrity.[34] The difference between TRF1 and TRF2 may be the cause that only TRF2 is affected in the condition of NBS1 deficiency, but not TRF1.

Telomere attrition causes replicative senescence,[35] a cellular process that shares many features with the classic DNA DSB damage responses.[36] NBS1 deficiency disrupts the cellular signaling network, therefore affects the normal process of cellular senescence and results in aberrant telomere associations. Our study clearly demonstrated aberrant telomere fusions in NBS fibroblasts with 657del5 mutation, suggesting genomic instabilities within these cells.

NBS1 deficiency has been implicated in carcinogenesis. 40% of NBS patients developed cancers before the age of 21 years old, especially B-cell lymphoma.[1] The high incidence of getting cancer manifests the importance of NBS1 in maintaining genome stability by mediating DNA damage response and protecting telomere integrity. On one hand, NBS cells with disrupted DNA damage responses license the continual growth and survival of cells regardless of genomic abnormalities, which presents a cellular setting that predisposes bad cells to sustain, accumulate and perpetuate, leading to carcinogenesis. On the other hand, accelerated telomere shortening speeds up the process towards replicative senescence. But checkpoints defects because of NBS1 deficiency would jeopardize the normal process of cellular senescence, thus leading to telomere abnormalities. Our work provides solid evidence that NBS fibroblasts have a higher telomere shortening rate *in vitro*. Moreover, we found that TRF2 expression was upregulated in NBS fibroblasts, which is an important clue for studying the underlying mechanism of accelerated telomere shortening in future. Also, our results from the aspect of telomere abnormalities provided possible explanations to the high incidence of cancer in NBS patients. Since telomere dysfunction has also been implicated in carcinogenesis, we propose that NBS patients are predisposed to cancer not only due to defects in repairing DNA damage but also because of defects in maintaining telomere integrity.

CONFLICT OF INTERESTS

The authors have declared that there is no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dashayini Mahalingam, Jane Wong See Mei, Tay Ling Lee, Ru Jianghua and Tan Wei Han for technical assistance. We also thank Drs. Zhang Yong and Gregory Bellot for critical reading of the manuscript. This work is supported by funding from the Academic Research Fund (AcRF) Tier 1 Faculty Research Committee (FRC) grant, National University of Singapore; and also from the grant NMRC/EDG/0058/2009, National Medical Research Council, Singapore.

REFERENCES

1. Nijmegen breakage syndrome. The International Nijmegen Breakage Syndrome Study Group. *Arch Dis Child* 2000; **82**(5): 400–406.
2. Kobayashi J, Antoccia A, Tauchi H, *et al*. NBS1 and its functional role in the DNA damage response. *DNA Repair (Amst)* 2004; **3**(8-9): 855–861.
3. Savitsky K, Bar-Shira A, Gilad S, *et al*. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 1995; **268**(5218): 1749–1753.
4. Kim ST, Lim DS, Canman CE, *et al*. Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* 1999; **274**(53): 37538–37543.
5. Gatei M, Young D, Cerosaletti KM, *et al*. ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nat Genet* 2000; **25**(1): 115–119.
6. Kurz EU, Lees-Miller SP. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair (Amst)* 2004; **3**(8-9): 889–900.
7. Lee JH, Paull TT. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 2004; **304**(5667): 93–96.
8. Horejsi Z, Falck J, Bakkenist CJ, *et al*. Distinct functional domains of Nbs1 modulate the timing and magnitude of ATM activation after low doses of ionizing radiation. *Oncogene* 2004; **23**(17): 3122–3127.
9. Difilippantonio S, Celeste A, Fernandez-Capetillo O, *et al*. Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. *Nat Cell Biol* 2005; **7**(7): 675–U56.
10. Varon R, Vissinga C, Platzer M, *et al*. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell* 1998; **93**(3): 467–476.
11. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev* 2001; **11**(1): 71–77.
12. Tauchi H, Matsuura S, Kobayashi J, *et al*. Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability. *Oncogene* 2002; **21**(58): 8967–8980.
13. Buscemi G, Savio C, Zannini L, *et al*. Chk2 activation dependence on Nbs1 after DNA damage. *Mol Cell Biol* 2001; **21**(15): 5214–5222.
14. Antoccia A, di Masi A, Maraschio P, *et al*. G2-phase radiation response in lymphoblastoid cell lines from Nijmegen breakage syndrome. *Cell Prolif* 2002; **35**(2): 93–104.
15. Lamarche BJ, Orazio NI, Weitzman MD. The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett* 2010; **584**(17): 3682–3695.
16. Wu Y, Xiao S, Zhu XD. MRE11-RAD50-NBS1 and ATM function as co-mediators of TRF1 in telomere length control. *Nat Struct Mol Biol* 2007; **14**(9): 832–840.
17. Zhu XD, Kuster B, Mann M, *et al*. Cell-cycle-regulated association of RAD50/MRE11/ NBS1 with TRF2 and human telomeres. *Nat Genet* 2000; **25**(3): 347–352.
18. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005; **19**(18): 2100–2110.
19. Munoz P, Blanco R, Blasco MA. Role of the TRF2 telomeric protein in cancer and ageing. *Cell Cycle* 2006; **5**(7): 718–721.
20. Jeppesen P. Immunofluorescence in cytogenetic analysis: method and applications. *Genet Mol Biol* 2000; **23**(4): 1107–1114.
21. Petrini JHJ, Attwooll CL, Akpinar M. The Mre11 Complex and the Response to Dysfunctional Telomeres. *Mol Cell Biol* 2009; **29**(20): 5540–5551.
22. Ranganathan V, Heine WF, Ciccone DN, *et al*. Rescue of a telomere length defect of Nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit. *Curr Biol* 2001; **11**(12): 962–966.
23. Difilippantonio S, Nussenzweig A. The NBS1-ATM connection revisited. *Cell Cycle* 2007; **6**(19): 2366–2370.
24. Maser RS, Zinkel R, Petrini JH. An alternative mode of translation permits production of a variant NBS1 protein from the common Nijmegen breakage syndrome allele. *Nat Genet* 2001; **27**(4): 417–421.
25. You Z, Chahwan C, Bailis J, *et al*. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol Cell Biol* 2005; **25**(13): 5363–5379.
26. Oliver AW, Knapp S, Pearl LH. Activation segment exchange: a common mechanism of kinase autophosphorylation? *Trends Biochem Sci* 2007; **32**(8): 351–356.

27. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004; **432**(7015): 316–323.
28. Blasco MA. Telomeres and human disease: Ageing, cancer and beyond. *Nat Rev Genet* 2005; **6**(8): 611–622.
29. Metcalfe JA, Parkhill J, Campbell L, *et al*. Accelerated telomere shortening in ataxia telangiectasia. *Nat Genet* 1996; **13**(3): 350–353.
30. Wu Y, Xiao S, Zhu XD. MRE11-RAD50-NBS1 and ATM function as co-mediators of TRF1 in telomere length control. *Nat Struct Mol Biol* 2007; **14**(9): 832–840.
31. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002; **295**(5564): 2446–2449.
32. Richter T, Saretzki G, Nelson G, *et al*. TRF2 overexpression diminishes repair of telomeric single-strand breaks and accelerates telomere shortening in human fibroblasts. *Mech Ageing Dev* 2007; **128**(4): 340–345.
33. Smogorzewska A, Van Steensel B, Bianchi A, *et al*. Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol* 2000; **20**(5): 1659–1668.
34. Nishimura Y, Hanaoka S, Nagadoi A. Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities. *Protein Sci* 2005; **14**(1): 119–130.
35. Martens UM, Chavez EA, Poon SSS, *et al*. Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Exp Cell Res* 2000; **256**(1): 291–299.
36. Hezel AF, Bardeesy N, Maser RS. Telomere induced senescence: End game signaling. *Curr Mol Med* 2005; **5**(2): 145–152.