ABSTRACT

Title of Document: THE FUNCTION OF MRN (MRE11-RAD50-NBS1) COMPLEX DURING WRN (WERNER) FACILITATED ATM (ATAXIA-TELANGIECTASIA MUTATED) ACTIVATION

Junhao Ma, Master of Science, 2009

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WRN (Werner) protein is a member of the RecQ family showing helicase and exonuclease activity. WRN protein may lose function upon mutation and causes Werner syndrome (WS) which is an autosomal recessive, cancer-prone and premature aging disease. ATM (Ataxia-Telangiectasia mutated) protein initiates a signaling pathway in response to DNA double strand breaks (DSBs). Genomic disorder ataxia-telangiectasia (A-T) is associated with defective ATM. WRN protein is involved in ATM pathway activation when cells are exposed to DSBs associated with replication fork collapse. Because the Mre11-Rad50-Nbs1 (MRN) complex, a sensor of DSBs, is known to interact with WRN and ATM, we investigated whether the MRN complex mediates the WRN-dependent ATM pathway activation. In this study, we employed short-hairpin RNA to generate WRN- and Nbs1-deficient U-2 OS (osteosarcoma) cells. Cells were treated with clastogens which induce collapsed replication forks,

thus provided proof for whether WRN facilitates ATM activation via MRN complex. This study serves as a basis for future investigation on the correlation between ATM, MRN complex and WRN, which will ultimately help understand the mechanism of aging and cancer.

THE FUNCTION OF MRN (MRE11-RAD50-NBS1) COMPLEX DURING WRN (WERNER) FACILITATED ATM (ATAXIA-TELANGIECTASIA MUTATED) ACTIVATION

By

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Dedication

To my daughter Aimi, my wife Yangming, my father and mother.

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Chapter 1: Literature review

1.1 The ATM-mediated DNA-damage response

Cancer is a major cause of death worldwide. In year 2007, cancer accounted for 7.9 million deaths which were around 13% of all deaths (WHO, 2009). Disequilibrium of genome stability in the form of genomic alteration, ranging from single-nucleotide substitutions to chromosomal aberrations, leads the way to cancer. Although DNA mutation is crucial to genetic variability in germ line, genomic alteration in somatic cells is malignant. As genome stability continues to be impaired, a series of genomic aberrations will occur. Cells possess a strict system to safeguard such changes (Levitt & Hickson, 2002).

Replication errors, chemical changes in DNA components and normal DNA transactions all cause sequence alteration. However, the greatest damage to DNA is caused by DNA altering agents (Hoeijmakers, 2001). DNA altering agents could be either endogenous (cellular metabolism products) or exogenous (environmental factors). These agents damage DNA and affect proper function of normal protein, which in turn transforms cells into malignant states. In response to the damages, cells defend themselves by choosing to either correct DNA alteration or initiate apoptosis (programmed cell death). The mechanism of how cells choose to survive or die attracts great research efforts. It is also related to the purpose of this study and will be discussed in this chapter.

1.1.1 DNA-damage response

DNA damage induced by endogenous or exogenous reagents causes different forms of lesions including formation of covalent adducts with DNA and oxidative damage to bases and backbone of DNA (Norbury and Hickson, 2001). Some of the lesions cause cell death while others have the consequences from cellular malfunction to malignant transformation. It is recently clearer that cells have a signaling network that encompasses different processes including DNA-damage repair (Shiloh, 2003), cell-cycle checkpoint (Amundson *et al.*, 2001; Sesto *et al.*, 2002) or cell apoptosis (Norbury and Hickson, 2001).

1.1.1.1 Cell-cycle checkpoints

Cell cycle checkpoints are signal transduction programs that link cell cycle phases in an error free manner. Cell cycle checkpoints preserve genomic stability by means of recognizing damage, initiating and coordinating repair in times of replication stress (Bartek *et al.*, 2004). During checkpoints, cell cycle progression is temporarily halted to allow damage repaired or cellular functions completed. There are four different DNA damage checkpoints that cells employ: G1-S, S-M, G2-M and S. The G1-S, S-M and G2-M arrest the cell cycle until damage is repaired whereas the replication is slowed in S phase checkpoint. (Figure 1.1 cell cycle and major DNA damage checkpoints).

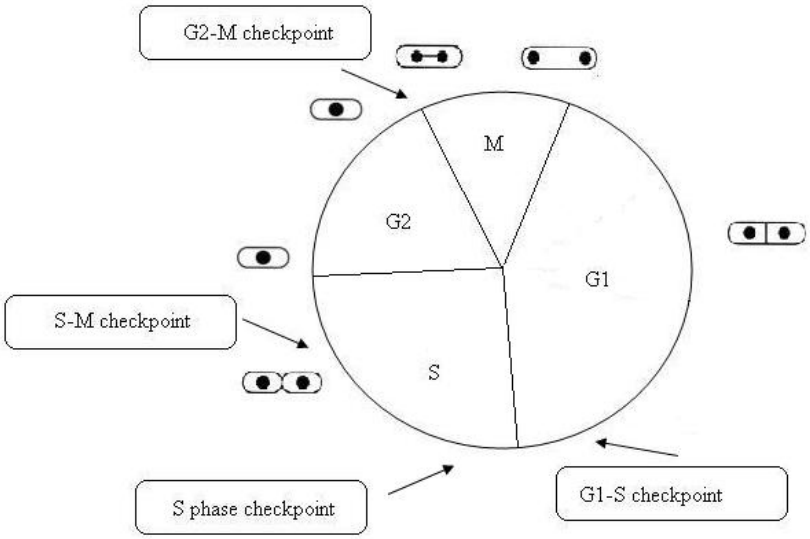


Figure 1.1 Cell cycle and DNA damage checkpoints. Cell spends time in G2 phase for normal cell growth. In M phase mitosis happens and two copies of DNA separate. In S phase nuclear division happens.

The G1-S checkpoint checks the integrity of genome for entry into the S phase and allows the cell to repair damage, if any, during G1 phase. Cells in G1 contain only one copy of the genome so that there is no correct template for damage repair. At this time error prone methods such as non-homologous end joining (NHEJ) are inevitable options and G1-S checkpoint can initiate cell apoptosis to avoid passing on errors.

The G2-M checkpoint halts cell mitosis under DNA damage pressure, ensuring that only correct genome copies are segregated in later phase. This checkpoint is also very

critical because two copies of genome make G2 a perfect time to repair damage.

Unlike the G2-M checkpoint, the S-M checkpoint arrests mitosis in another way. This checkpoint is initiated when replication discontinues due to nucleotide depletion or polymerase malfunction. This checkpoint ensures that replication is fully completed before chromosome segregation. Any attempt to segregate with uncompleted replication is stalled by this checkpoint.

The fourth checkpoint, the S phase checkpoint performs its task to slow down DNA replication in response to DNA damage. This stage of cell cycle is especially vulnerable as complicated metabolism and precise replication are required. Compared to the other three checkpoints, the S-phase checkpoint slows down rather than totally stops replication process in the presence of damage (Rowley *et al.*, 1999). Study on this checkpoint is one of the focuses of this research.

1.1.1.2 DNA double strand breaks

DNA double strand breaks (DSBs) are most dangerous and particularly effective among all kinds of DNA lesions to initiate finely-tuned DNA-damage response pathways whereas there is a highly conserved, sophisticated signaling network, existing from yeast to mammal, in response to DSBs (Shiloh, 2003). DSBs are extremely cytotoxic as they occur when the two complementary DNA double helix are broken at sufficiently close sites so that the two DNA ends are unable to keep

juxtaposed to each other by chromatin structure and base-pairing (Jackson, 2002). DSBs can follow normal genomic transactions (Bassing and Alt, 2004). However they are also introduced by oxygen radicals, ionizing radiation and radiomimetic chemicals.

Cells possess a complex signaling network in response to DSBs controlling a number of cellular systems. Activation of cell-cycle checkpoints is a part of the network. While cell cycle is temporarily halted or slowed down, significant changes take place in cells including gene expression, protein function and other physiological processes to assess DNA damage and initiate repair (Latella *et al.*, 2004). DNA repair mechanisms are another hallmark of the network. NHEJ (non-homologous end joining) and HR (homologous recombination) are the two main mechanisms used by eukaryotic cells to repair DSBs (Shiloh, 2006). NHEJ is a ligation process effective throughout the cell cycle and HR occurs between paired chromatids in the late S and G2 phases of the cell cycle.

1.1.1.3 The three-tiered signaling response to DSBs

There is increasing evidence showing that the DSB signaling cascade is composed of three kinds of proteins. ‘Sensors’ are proteins that sense DNA damage and convey signal to a group of proteins called ‘transducers’. These transducers act as signal magnifiers and in turn transmit the signal to functional proteins called ‘effectors’ in numerous pathways (McGowan and Russell, 2004).

Several typical and well-studied sensors are the mammalian MRN (Mre11-Rad50-Nbs1) complex, p53-binding protein 53BP1, tumor suppressor protein BRCA1 and mediator of DNA-damage checkpoint protein 1 (MDC1), all of which are recruited swiftly to DNA-damage site (Shiloh, 2006). In addition to their DNA processing function, they are all responsible for conveying early DNA-damage signal to downstream transducers. MRN complex is a target protein of this study and will be introduced in detail later.

The nuclear protein kinase ATM (ataxia-telangiectasia mutated) is a primary transducer of DSB signal. It in turn phosphorylates quite a number of downstream substrates in different damage response pathways (Kurz and Lees-Miller, 2004). The name comes from the genomic instability syndrome ataxia-telangiectasia (AT). Symptoms of this syndrome are immunodeficiency, radiation sensitivity, cerebellar degeneration and cancer predisposition, which unveils the relationship between maintenance of genome stability and cancer prevention (Shiloh, 2003). ATM belongs to a protein family named ‘The PI3K-like protein kinases (PIKKs). Mutated ATM gene was obtained from ataxia-telangiectasia syndrome patients in 1995 (Savitsky *et al.*, 1995). Cell lines from such patients are deficient in response to DSBs and show defect in almost all DSBs responses (Shiloh, 2001), which places ATM in an important place in the DSBs response network.

1.1.2 The PI3K-like protein kinases

Most PIKKs, as they are named, possess the lipid kinase phosphatidylinositol 3-kinase (PI3K) motifs. So far six family members have been found in mammal: ATM, ATR (Rad3-related), hSMG-1, mTOR (also known as FRAP), DNA-PK (the catalytic subunit of the DNA-dependent protein kinase) and TRRAP (Shiloh, 2006). Most of the family members also contain domain that are typical of serine/theronine kinase activity (Figure 1.2). Four of the PIKKs, DNA-PK, ATM, ATR and hSMG-1 are related to mammalian DNA damage response (Sesto *et al.* 2002, Abraham, 2001 and Durocher, 2001). DNA-PK and ATM response for the most part to DSBs whereas ATR and hSMG-1 response primarily to UV light induced replication arrest as well as to DSBs and stalled replication forks. ATM and ATR share some substrates in the downstream network in response to different forms of DSB inducers (Helt *et al.*, 2005).

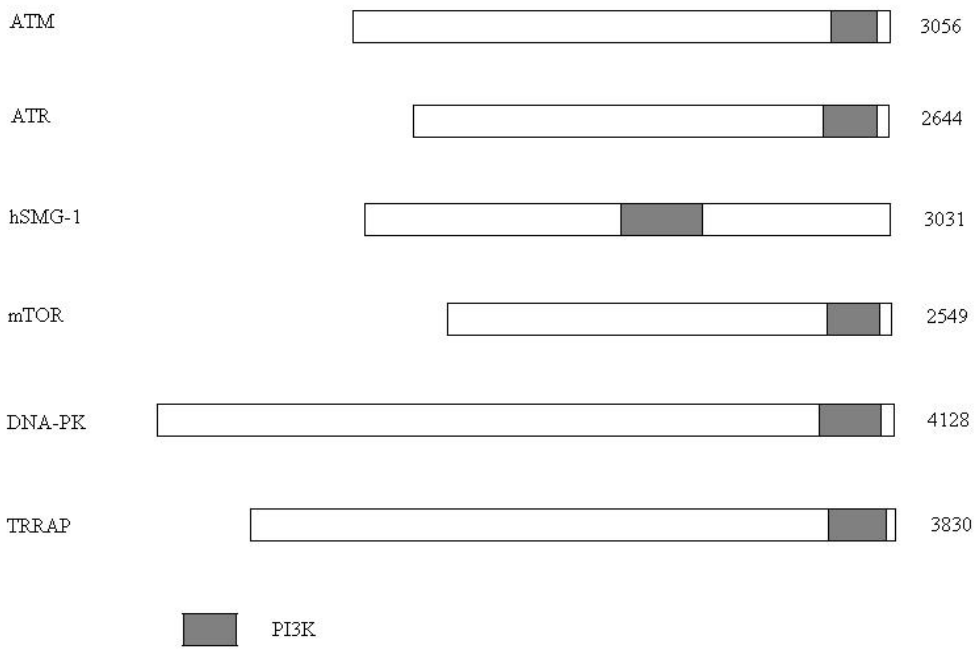


Figure 1.2 Mammalian PIKK family. The number of amino acids is indicated on the right. The PI3K domain contains the catalytic site for the family.

The functional relationship between ATM and ATR remains largely unknown. There is mounting evidence showing that ATM and ATR are triggered independently in response to DSBs and phosphorylate substrates in a redundant fashion (Shiloh, 2006). However, recent studies also show that part of ATR function as a DSB sensor requires ATM. ATR is dependent on ATM to bind to DSB sites and not until the binding occur can ATR phosphorylate downstream substrates. Other ATR functions such as response to UV light induced DNA lesions, however, do not require ATM assistance (Jazayeri *et al.*, 2006, Cuadrado *et al.*, 2006 and Adams *et al.*, 2006).

1.1.3 The role of ATM in the response to DSBs and replication stress

1.1.3.1 Sensors

When DSB forms, sensor proteins are first recruited to DNA damage sites. Understanding of the early stage of response is a key to understanding the whole repertoire. The MRN complex, as stated before, is the first recruit to the damaged sites (Lukas *et al.*, 2004 and Lou *et al.*, 2006). Then come MDC1 and 53BP1, in turn. MDC1 is responsible for conglomerating MRN complex and 53BP1 together to the damaged chromatin (Lukas *et al.*, 2004 and Bekker-Jensen *et al.*, 2005). Meticulous imaging analysis shows that ATM is spatially organized together with the MRN complex, MDC1 and 53BP1 in DSB flanking chromatin subcompartments (Bekker-Jensen *et al.* 2006).

In the past, Nbs1, a component of MRN complex as well as MDC1 and 53BP1 were assumed to be substrates of ATM phosphorylation (Kurz and Lees-Miller, 2004). Mounting new evidence illustrates a different picture: in addition to their effectors' role, they also acts as sensors that trigger transducer ATM. Thus a signal amplification model is established in a way that damage signal is enhanced by cyclic interaction between these proteins (Lavin, 2004).

1.1.3.2 ATM, the transducer

When it is inert, ATM protein exist in the form of dimmers or multimers which disassociate to active monomer form during DNA damage (Bakkenist and Kastan, 2003). The second step of activation is autophosphorylation on Ser1981. Both of the two steps require MRN. It has been found that the MRN complex physically finds ATM by C-terminal domain of the Nbs1 protein (Falck *et al.*, 2005 and You *et al.*, 2005). Of course, ATM activation also requires the contact of ATM-MRN conglomeration to DSB ends (Dupre *et al.*, 2006). In addition to the MRN cyclic interaction, there is another protein that is recently found to attract ATM to the damage site in a similar cyclic fashion. γH2AX, which is a phosphorylation substrate, is shown to bind to BRCT domain of MDC1, thus enabling MDC1 to hold on to ATM (Lou *et al.*, 2006). An ATM-MDC1 conglomeration is formed close to DNA flanking DSBs in such a way as to activate ATM and phosphorylate H2AX in the vicinity.

After the autophosphorylation process, ATM protein undergoes further modification in the forms of phosphorylation and acetylation. The complete activation process of ATM involves a large number of proteins, only a few of which have been investigated. Two phosphatase, PP2A and PP5 are reported to interact with ATM during its activation (Goodarzi *et al.*, 2004 and Ali *et al.*, 2004). WRN (Werner syndrome) protein has been recently reported to be involved in ATM activation in response to interstrand cross-link-induced DSBs (Cheng *et al.*, 2008). The WRN protein is also a target of this study and will be discussed in later chapters.

1.1.3.3 Downstream ATM effectors

There is a long list of known ATM effector proteins (Shiloh, 2006). However, the list is far from complete. The good news is the investigation of known proteins in the network leads us to understand the meticulously controlled strategies used by ATM.

The first strategy is that ATM target the same endpoint by means of different pathways. For example, at least five pathways mediated by ATM are involved in the intra-S checkpoint. The multifunctional Nbs1, component of MRN, is phosphorylated by ATM and involved in the checkpoint (Lim *et al.*, 2000). FANCD2 protein, which is phosphorylated on Ser222 is also found to be related to this checkpoint (Taniguchi *et al.*, 2002). In addition, BRCA1, SMC1 (structural maintenance of chromosome 1) protein, CHK1/CHK2 proteins are also activated by ATM and take care of several checkpoints (Kim *et al.*, 2002, Mailand *et al.*, 2000, Falck *et al.*, 2001 and Falck *et al.*, 2002).

Further more, another key strategy of ATM mediation is a same effector could be reached from different ATM mediated mechanisms in a redundant manner. For example, p53 protein, a main target of ATM activation is directly phosphorylated by ATM on Ser15 (Banin *et al.*, 1998). ATM also activates CHK2 as stated above which in turn phosphorylated p53 on Ser20 (McGowan 2002 and Bartek *et al.*, 2001). Other ATM dependent phosphorylation site of p53 on Ser9 and Ser 46 are also published (Saito *et al.*, 2002). ATM phosphorylates p53 inhibitor MDM2, preventing the nuclear export of p53-MDM2 complex and hence the stability of p53 (Khosravi *et al.*, 1999

and Maya *et al.*, 2001). Another p53 inhibitor Mdmx also undergoes ATM dependent phosphorylation which induces degradation of p53 (Pereg *et al.*, 2005 and Chen *et al.*, 2005).

Of course, numerous substrates of ATM are protein kinases that are themselves able to activate downstream effectors and are in control of branches of pathways. CHK2 is a prominent example. It is found to phosphorylate p53, BRCA1, CDC25A and CDC25C. CHK2 mediated phosphorylation of CDC25C lead to cytoplasmic sequestration of CDC25C, which prevents activation of a number of proteins such as CDK1 (McGowan 2002 and Bartek *et al.* 2001).

There is recently a new theory which may be complimentary to the existing effector model. The new theory (Goodarzi *et al.*, 2008) correlates dynamic changes of chromatin to ATM signaling. The architecture of euchromatin is in such a loose form that repair factors are easy to access and manipulate DSBs. However, in heterochromatin, the nucleosome flexibility is limited by heterochromatic factors like KAP-1, HP1, HDACs (Lavin *et al.*, 2005; Ziv *et al.*, 2006). ATM is required to phosphorylate these factors and decrease their interaction with heterochromatin, and therefore change condensed chromatin regions into open forms and allow the damage be repaired in an ATM dependent manner (Fernandez-Capetillo and Nussenzweig, 2008) .

Research on cellular response to DSBs remains the epicenter of understanding how

cells respond to DNA damage. Each of the three tiers of the signaling cascade provides ramification of research interests. In particular, elucidating how signal is passed on from MRN complex to ATM continues to be a major research direction. What is the conformation of this complex? How does the complex rush to the damage site? How does it change ATM from inert dimmers into activated forms? Some of the questions already have answers and some of them are still open. In next chapter of this review, some known and unknown aspects of MRN complex will be investigated.

1.2 The key players that mediates ATM pathway activation

Among all the proteins upstream or downstream ATM, some of them like p53 have been studied for decades, their function clarified, while the mechanism of some others, although essential in the signal pathways, remain largely unknown. This thesis study chose two factors, the MRN complex and WRN protein, from many and tries to further investigate their roles in the ATM pathway activation.

1.2.1 MRN

The MRN complex was first identified in budding yeast as repair proteins composed of three subunits. Among them, Mre11 and Rad50 are highly conserved in all taxonomic kingdoms while Nbs1 homologues are only identified in eukaryotes: Xrs2 in yeast and Nbs1 in vertebrate cells (van de Bosch *et al.*, 2003). The conservation degree between species suggests a crucial role in maintenance of genomic stability

(Lee *et al.*, 2003). Figure 1.3 illustrates that two Mre11s, two Rad50s and one Nbs1 form a 2:2:1 stoichiometric MRN complex. Rad50 and Nbs1 are not directly bound to each other but to Mre11 instead (Williams *et al.*, 2007).

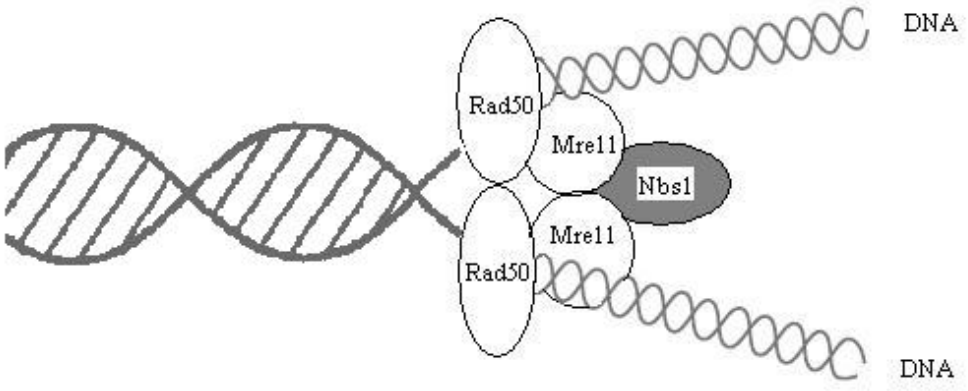


Figure 1.3 2:2:1 stoichiometric MRN complex structure. Two Mre11s, two Rad50s and one Nbs1 form a 2:2:1 stoichiometric MRN complex. Rad50 and Nbs1 are not directly bound to each other but to Mre11 instead

1.2.1.1 MRN, its subunits and the function as a sensor of DNA DSBs

As shown in Figure 1.4, Mre11 has five conserved phosphoesterase motifs forming the nuclease activity in its N-terminus and two DNA binding domains in its C-terminus. It also contains Nbs1 binding domain near its phosphoesterase motifs and Rad50 binding domains in the C-terminus (D'Amours and Jackson, 2002). Rad50 contains Walker A and Walker B motifs (ATPase domains) that bind together in the presence of ATP. In the middle of Rad50 there are CXXC sequences that bridge DNA. Between ATPase domains and CXXC sequences are coiled-coil regions. Rad50 binds

to Mre11 near Walker motifs (Williams *et al.*, 2007). Nbs1 contains ATM and Mre11 binding domains in the C-terminus and FHA (Forkhead Associated) and BRCT (Breast cancer C-Terminal) phosphopeptide interaction domains in the other end (D'Amours and Jackson, 2002). These two phosphorylation interaction domains are indispensible for DNA lesion recognition, S-phase checkpoint function and checkpoint phosphorylation of Nbs1 (Kobayashi *et al.*, 2004; Horejsi *et al.*, 2004).

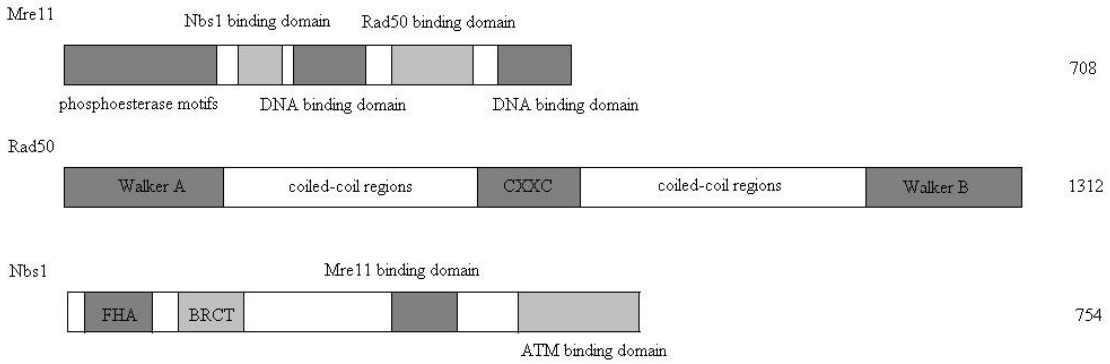


Figure 1.4 Known structural domains for the Mre11, Rad50 and Nbs1 proteins. The number of amino acids is indicated on the right.

There are mounting evidence showing that MRN complex is rapidly recruited to DNA damage site (Lavin, 2007) independent of ATM association (Mirzoeva and Petrini, 2001). It should also be noted that, during S phase, the complex binds to chromatin in the absence of DNA damage (Mirzoeva and Petrini, 2003). The dynamics of the binding is as follows: The coiled-coil regions of Rad50 fold in a shape to bring Walker A and B motifs in proximity to form a globular domain which binds DNA. The CXXC sequences as mentioned above dimerize by a Zn2+ ion which

consequently bridge two DNA molecule together (Hopfner *et al.*, 2002). Upon binding to DNA, the parallel orientation of the coiled-coil regions favors inter-complex association and inhibits intra-complex interaction (Moreno-Herrero *et al.*, 2005). Rad50 associates with Mre11 and Mre11 in turn associates with Nbs1 by the binding domains introduced in the leading paragraph. The association between Rad50 and Mre11 stimulates the exonuclease and endonuclease activities of Mre11 (Paull and gellert, 1998; Trujillo and Sung, 2001) while the binding between Nbs1 and Mre11 initiates the endonuclease ability of the latter (Paull and gellert, 1999).

It is evident that the initial event in recognizing and responding to DNA DSBs is the binding of MRN complex, which bridges broken ends together. However, there are other proteins that facilitate such binding. For example, MDC1, a large nuclear protein, was shown to physically couple the complex to damage site. Mutations in some conserved motifs of MDC1 disrupt the accumulation of the MRN complex at DNA DSBs sites in vivo (Spycher *et al.*, 2008).

1.2.1.2 Activation of ATM by MRN complex

It has been discussed briefly in the chapter 1 how ATM is recruited and rapidly activated. Here the complete investigation process will be introduced as well as some very important milestone experiments. These experiments are also very illustrating when this thesis research is designed.

In order to prove MRN complex is upstream of ATM and is required for ATM activation, NBS (Nijmegan Breakage Syndrome) and ATLD (Ataxia-Telangiectasia-like Disease) cells, both of which contain hypomorphic mutation in the complex subunits, are used as basis of studies. And ATM activation is determined either by autophosphorylation on Ser1981 or its capacity to phosphorylate downstream effectors in these studies. ATM activation was found to be retarded in both cells response to radiomimetic chemical neocarzinostatin treatment (Uziel *et al.*, 2003). It has also been shown that an Nbs1 construct retaining ATM and Mre11 binding site could stimulate ATM activation in most NBS mutation cells (Digweed *et al.*, 1999; Cerosaletti and Concannon, 2004; Falck *et al.*, 2005). When Nbs1 protein lacks ATM binding site, in this case the NbFR5ΔAtm expressed by NBS cells, ATM activation is dramatically reduced (Cerosaletti *et al.*, 2006).

Viral infection of cells was also used to investigate how MRN complex activates ATM. An end-joining of adenovirus genome resembles DNA damage. Results show that ATM is activated in end-joining regions (Carson *et al.*, 2003) and degradation of MRN complex decreases the level of ATM autophosphorylation.

There are also in vitro studies on the ability of MRN complex to activate ATM (Lee and Paull, 2004, 2005). Baculovirus expressing MRN components was used in these studies, and a direct activation of ATM was observed by immunoprecipitation. Surprisingly, those studies further found that Mre11/Rad50 could hook ATM to DNA damage sites, whereas it was impossible to activate ATM without Nbs1.

Consequently, there are emerging data suggesting that ATM is not recruited only by the C-terminus binding domain of Nbs1. In a study on Nbs1-knockout mice with BAC transgenes, a mutant carrying an Nbs1 transgene that lacks 20 amino acids in the C-terminus displayed normal ATM activation (Difilippantonio *et al.*, 2007). The lack of an Nbs1 function seems to be remediated by other interaction between MRN components and ATM. Another experiment based on a mouse model of Nbs1 lacking 24 amino acids in the C-terminal also showed normal ATM phosphorylation (Stracker *et al.*, 2007). These findings are supported by data that ATM contacts different part of the MRN complex (Lee and Paull, 2004; Stracker *et al.*, 2007), which points to a more complicated map of MRN complex mediated ATM activation.

1.2.1.3 Nbs1 and its role in the DNA damage response

Previously, it was mentioned that Nbs1 plays a role in the cyclic pathway of ATM mediated DSBs response, which is Nbs1 both serves as a sensor activating ATM and an ATM effector as well. It is why Nbs1 is chosen as the target protein of this thesis research. Here Nbs1 is picked out for more detailed introduction for the sake of understanding the rationale of research design.

Nijmegan Breakage Syndrome (NBS) is a recessive autosomal instability disorder characterized by growth retardation, immunodeficiency and cancer predisposition (Varon *et al.*, 1998). The Nbs1 gene which mutated in NBS was isolated from

chromosome 8q21-24 (Matsuura *et al.*, 1998; Carney *et al.*, 1998). The gene is 50kb in size and is transcripted to two mRNAs, 2.6kb and 4.8kb, respectively. Nbs1 gene encodes a protein of 754 amino acids which displays a low homology to yeast Xrs2 at both ends. As stated before, unlike Mre11 or Rad50, homologs of Nbs1 have not been identified in archaebacteria or prokaryotes and are very unique only to eukaryotic cells.

The protein contains an N-terminus region, a central region and a C-terminus region. FHA and BRCT domains in the N-terminus are very conserved in eukaryotes and both related to cell cycle checkpoints or DNA repair. The FHA motif recognizes phosphorylation of the target protein (Durocher *et al.*, 1999). The C-terminus region binds to Mre11 and has three possible nuclear localization signal sequences (Kobayashi *et al.*, 2004). There are several SQ motifs in the central region of Nbs1. The serine residues at 278 and 343 are phosphorylated by ATM, which plays an essential role in the intra-S checkpoint (Wu *et al.*, 2000; Tauchi *et al.*, 2000).

It has already been discussed in the previous sessions how Nbs1 take part in the MRN mediated ATM activation in response to DNA DSBs. However, Nbs1 has multiple roles which exceed beyond the range of ATM activation and will be introduced respectively here.

Interaction with chromatin

10% of human histone H2A protein is the histone variant H2AX which is evenly distributed in chromatin. The phosphorylated form of H2AX, the γ-H2AX interacts with MRN complex through Nbs1 (Kobayashi *et al.*, 2004). Evidences show that the direct interaction of γ-H2AX with Nbs1 is through the FHA/BRCT domains (Kobayashi *et al.*, 2002; Cerosaletti and Concannon, 2003). This interaction is very important for the MRN complex form foci in response to DSBs as well as for other damage response proteins containing BRCT domains (Stewart *et al.*, 2003; Bassing *et al.*, 2002).

HR repair and inter-chromosomal translocation

Non-homologous end joining (NHEJ) and homologous recombination (HR) are two major pathways to repair radiation-induced DSBs. It has been shown in Nbs1-defient chicken cells that Nbs1 plays an indispensible role in HR rather than NHEJ repair (Tauchi *et al.*, 2002). This result was also confirmed in mice study that NBS cells are deficient in meiotic recombination and HR repair (Bassing *et al.*, 2002).

Whereas Nbs1 is not required in NHEJ, increased chromosomal translocations are observed in NBS cells, which is consistent with Nbs1 mutant mice study (Kobayashi *et al.*, 2004). These data suggest that Nbs1 protein functions in suppression of inter-chromosomal recombination.

DNA damage checkpoints

In vertebrates, cell cycle progression will be temporarily halted in response to DSB inducing agents until DNA damage is repaired. At least three pathways have been reported participating in the intra-S cell cycle checkpoint, two of which are Nbs1 dependent: the ATM/Nbs1/SMC1 pathway (Kim *et al.*, 2002; Yazdi *et al.*, 2002) and the ATM/FANCD2 pathway (Nakanishi *et al.*, 2002). In the first pathway, Nbs1 phosphorylates SMC1 on Ser278 and Ser343. In the second pathway, the FANCD2 phosphorylation requires the phosphorylation of Nbs1 on Ser343 by ATM. There is evidence showing that it is Mre11 binding domain instead of FHA/BRCT domains of Nbs1 taking part in the efficient induction of the intra-S checkpoint (Stewart *et al.*, 2003; Goldberg *et al.*, 2003). G1 and G2 checkpoints may involve Nbs1, however the reports are controversial and the mechanism behind them remains unsolved (Kobayashi *et al.*, 2004).

1.2.3 WRN

There are a number of human disorders that belongs to a category of diseases called human premature aging disorders. These patients prematurely develop a variety of conditions which shows clinical characteristics seen in the normal aging process, for example, malignant neoplasms, type II diabetes mellitus and ocular cataracts (Yu *et al.*, 1996). Therefore, it is quite understandable that studying human premature aging disorders cast light on many aging process in humans.

Werner syndrome (WS) is a rare autosomal recessive disorder that belongs to this disease category. WS patients display remarkable aging symptoms in early stage of life starting around puberty. Those symptoms are similar to those of more elderly individuals. In addition, some geriatric disorders such as hypertension or Alzheimer's disease are not seen in WS. Moreover, WS displays symptoms that are not age related including reduced fertility, hypogonadism and short stature. Thus, WS has been regarded as a perfect model to study human premature aging disorders as well as to understand the mechanism of normal aging. The 162kDa Werner protein, known as WRN, is encoded by the gene defective in WS (Yu *et al.*, 1996). In this chapter, the biochemical and catalytic characteristics of this protein will be introduced. Then the focus of the introduction falls into how WRN functions in DNA damage response pathways.

1.2.3.1 WRN and the RecQ family of DNA helicases

The WRN protein belongs to a category of DNA helicases called the RecQ family. This widely expressed protein family are required for the maintenance of genome integrity (Mohaghegh and Hickson, 2001). Family members have been identified throughout eukaryotes while five of them are present in humans. This family is characterized by a highly conserved ~400 amino acids helicase domain (Hickson, 2003). For some 'long form' family members, there are flanking domains on both sides of the central helicases domain which are not quite conserved while other family members, 'short form', only comprise the conserved helicases domain (see

Figure 1.5 the human RecQ helicases family).

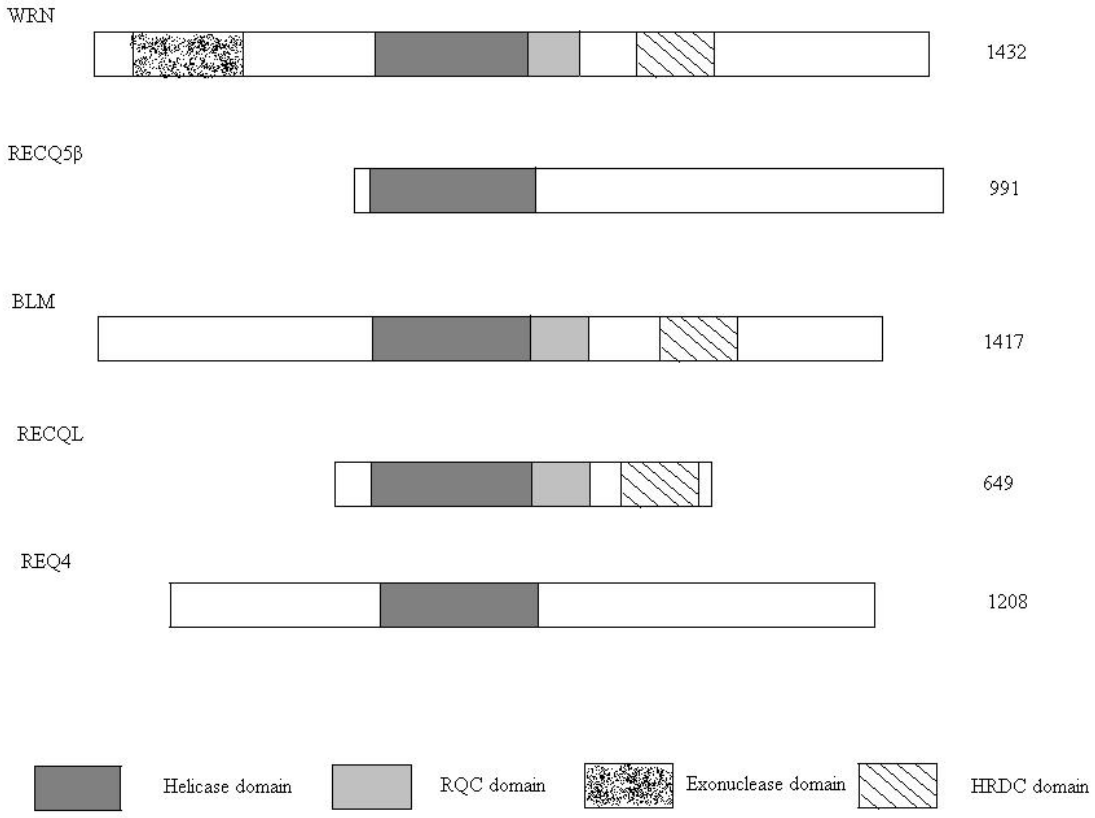


Figure 1.5 The human RecQ helicases family. The number of amino acids is indicated on the right.

The RecQ family are considered to be ‘caretaker’ tumor suppressors because studies on mutants find out that defects in the genes generate autosomal recessive disorders and an elevated incidence of cancer (Ellis *et al.*, 1995; Yu *et al.*, 1996; Kitao *et al.*, 1999). RecQ helicases are found to have dynamic subnuclear localizations and bind to numerous partners under different cell growth conditions. Thus, they have multiple roles in DNA replication as well as in telomere maintenance (Hickson, 2003). The WRN protein is a very unique RecQ member for it comprises both conserved and

flanking domains and participates in a wide range of cellular activities.

The WRN gene is located at position 8p12 on chromosome (Yu *et al.*, 1996). The WRN protein possesses a DNA-dependent ATPase activity that unwinds double strand DNA in the 3'→5' direction (Shen *et al.*, 1998). Besides, it is the only known protein of this family to possess a 3'→5' exonuclease activity (Shen *et al.*, 1998). This activity is initiated at a duplex blunt if the substrate also contains bubble, loop or Holiday junction (Shen and Loeb, 2000). Like other family members, the conserved RecQ helicase domain is located centrally. There is a putative transcription activation domain between the helicase and exonuclease domains. Thus the helicase and exonuclease activities are physically separated from each other and don't functionally interfere with each other. The C-terminal region accommodates nuclear localization element (Fry, 2002) and the RecQ conserved RQC domain containing localization signal-dependent nuclear targeting sequence. In addition, a HRDC (Helicase and RNaseD C-terminal) domain considered to participating DNA binding also exists in the C-terminal region (Bernstein *et al.*, 2003). Please refer to Figure 1.5 above for a schematic representation of the WRN domains.

1.2.3.2 Multiple roles for WRN

Thanks to its bifunctional nature, the WRN protein acts as intermediates in DNA replication, telomere maintenance and DNA damage response. Given the large number of its protein partners, the cellular functions of WRN is still far from clear.

The pathways incorporating WRN could only be delineated partially based on some well studied WRN interactors (Opresko *et al.*, 2003 also see Figure 1.6 for in vivo functions of WRN and interactors).

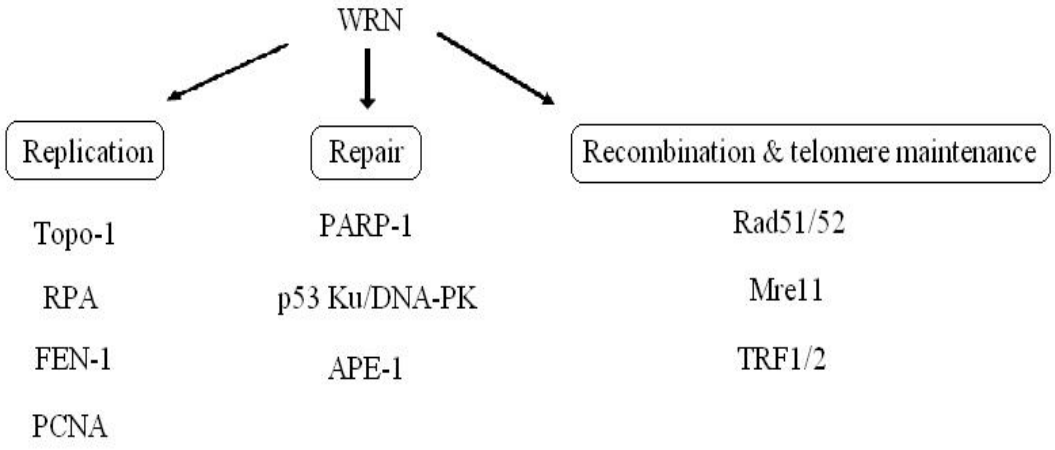


Figure 1.6 WRN and interactors. These interactors take part in a variety of DNA metabolism pathways and a precise map of WRN cellular function is still not clear.

WRN is a part of the 17S DNA replication complex and interacts with topoisomerase I (Lebel *et al.*, 1999). WRN interacts with topoisomerase I through the two terminals and facilitates topoisomerase I to relax negatively supercoiled DNA, which explains why WS cells are sensitive to camptothecin, a DNA topoisomerase I inhibitor, during G2 and S phase (Laine *et al.*, 2003). WRN associates with replication protein A (RPA) which is necessary for DNA replication (Wold, 1997). RPA facilitates WRN to unwind longer substrates while WRN alone can only unwind much shorter partial duplexes (Brosh *et al.*, 1999). RPA may help WRN to cope with rotational rigidity during unwinding process by restraining it at the single-strand/double strand junction of the DNA template (Garcia *et al.*, 2004). Furthermore, WRN associates with the

major replicative DNA polymerase δ. Studies show that the C-terminal region of WRN interacts with the p50 and p125 subunits of DNA polymerase δ and also co-localizes with the two subunits in the nucleolus (Szekely *et al.*, 2000). Another replication protein that associates WRN is the FEN-1 protein (Brosh *et al.*, 2001). WRN interacts with FEN-1 through its RQC domain as mentioned above. This interaction greatly enhances nucleolytic activity of FEN-1 (more than 80 fold). The cleavage function of FEN-1 is also increased through combination with WRN.

The second important role for WRN is maintenance of genomic stability in the presence of DNA damage. Not only the WRN/FEN-1 complex discussed above is involved in non-homologous DNA end joining (NHEJ) (Wu *et al.*, 1999), a Ku-DNA-PK complex has also been reported to interact with WRN in repairing DNA DSBs caused by ionizing radiation, genotoxic chemicals and physiological oxidative stress (Featherstone and Jackson, 1999). The complex comprises of DNA-PKcs and Ku70/Ku80 heterodimer. The heterodimer interacts with the both the C and N terminal of WRN. It is possible that the Ku activates WRN to remove some replication blocks (Ozgenc and Loeb, 2005) and also DNA-PKcs to phosphorylate WRN and negatively regulate its end processing during NHEJ (Opresko *et al.*, 2003). In addition, WRN and Ku70/Ku80 participate in the formation of a cellular trimeric complex with poly (ADP-ribose) polymerase-1 known as PARP-1 (Li *et al.*, 2004). PARP-1 regulates WRN exonuclease activity and participates in the early response to DNA damage.

Biochemical and genetic evidence suggest that WRN participate in the homologous recombinational (HR) repair pathway as well as the base excision repair (BER) which are both very important in response to DNA damage. WRN participation in HR is dependent on Rad51 (Opresko *et al.*, 2003). Direct physical interaction between WRN and the MRN complex containing Rad51 has been found (Cheng *et al.*, 2004). BER is an essential pathway to cope with DNA lesions such where WRN may also participates. It forms a complex with apurinic/apyrimidinic endonuclease (APE-1), a key regulator in early BER (Ahn *et al.*, 2004). The helicase activity of WRN is required for DNA polymerase β strand displacement synthesis on a nicked BER intermediate (Harrigan *et al.*, 2006).

Loss of telomeric DNA triggers replicative senescence. WS cell culture experiment shows WS fibroblast cells are more exposed for telomere fragment lost than normal fibroblast cells (Orren, 2006). The WRN/Ku, WRN/DNA-PK and WRN/MRN complex interaction are not only important of DNA damage response but also essential in telomere metabolism (Opresko *et al.*, 2003). In addition, WRN protein is found to co-localize with relomeric repeat binding proteins TRF1 and TRF2 (Yeager *et al.*, 1999). RQC domain of WRN has been reported to physically interact with TRF2 (Opresko *et al.*, 2002). The WRN exonuclease digestion of the telomeric repeats is limited by the association of TRF2 and TRF1 (Opresko *et al.*, 2004). Recent evident shows both enzymatic activities of WRN are needed to suppress telomeric-circle formation in normal cells which express telomerase reverse transcriptase (Li *et al.*, 2008).

1.2.3 ATM/MRN and ATM/WRN in response to DNA damage

Although the interactions between ATM/MRN and ATM/WRN in the presence of DNA damage are not clarified very clearly, understanding the so far known interactions shed light on the hypothesis and design of this thesis study.

1.2.3.1 ATM/MRN complex interdependence model in response to DNA damage

As discussed above, it could be conclude that the MRN complex and ATM are interdependent in recognizing and signaling DNA DSBs. Berkovich *et al.* (2007) provided a relatively clearer picture of the dynamics of proteins involved in this event (Figure 1.7). A binding of the MRN complex to damaged DNA happens initially followed by recruitment of ATM in the break and surrounding area in an Nbs1 dependent manner, whereas inactive ATM is unable to bind to DNA DSBs. At least three autophosphorylation sites (Ser1981, Ser 367 and Ser1893) have been found to be inherent post translational parts of the activation mechanism. As ATM is displaced from the break site, downstream effector proteins are recruited to repair the DSBs.

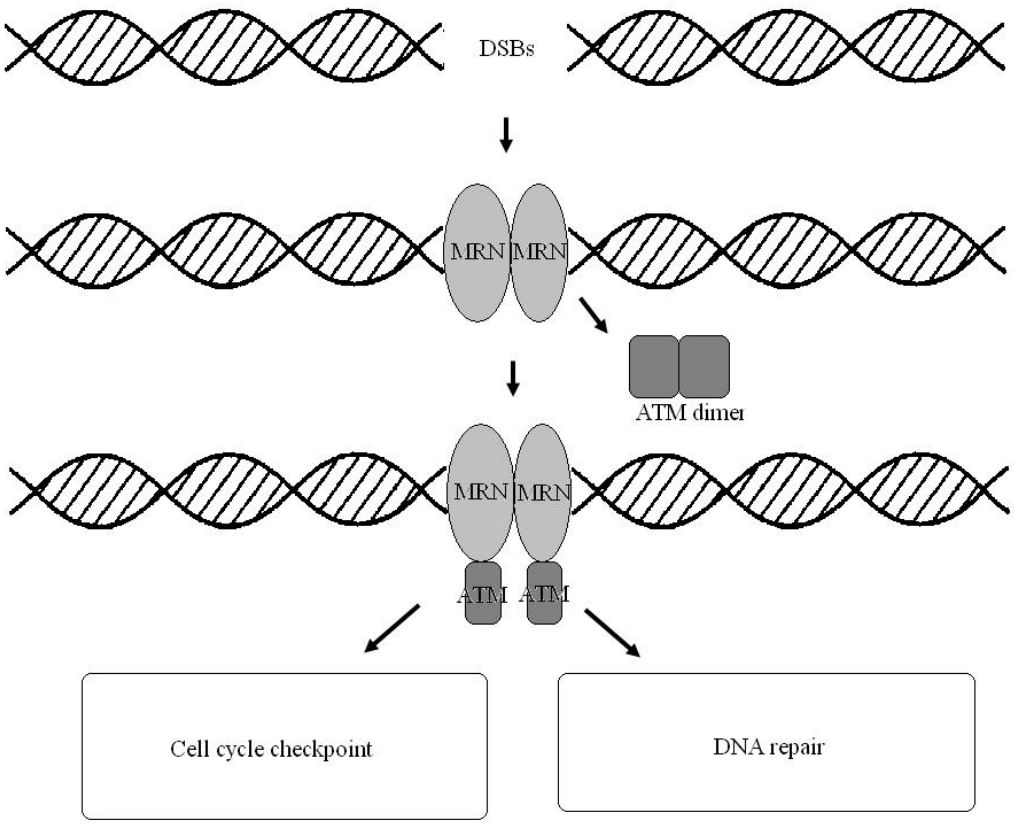


Figure 1.7 Early events in DSB response. The MRN complex is the first to be recruited to DSB site followed by stimulation of ATM dimmers. After binding to MRN, ATM initiates its effectors and downstream responses.

1.2.3.2 ATM/WRN interdependence model in response to DNA damage

According to the previous chapters, ATM and WRN are proved to be key regulators in response to DNA damage independently. Also there is report suggesting that RecQ helicases may be implicated in ATM activation (Davalos *et al.*, 2004). There is still limited evidence supporting the interaction between ATM and WRN. It was not until recently that depletion of WRN interferes with ATM activation as well as ATM

downstream effectors in response to DNA interstrand cross-link-induced DSBs (Cheng *et al.*, 2008). Using the same cell models, this study also found WRN and ATM cooperates in a PUVA-induced intra-S-phase checkpoint. Taken together, WRN is required for ATM activation under the condition of replication-dependent DSBs while resolve recombination intermediates on the other hand. Post-translational phosphorylation of WRN regulates the multiple roles of this protein (Sidorova et al., 2008).

1.2.4 Mutations in ATM, MRN, and WRN in humans

In this chapter, all known mutations in the three protein/protein complex will be reviewed to provide a more vivid and historic picture of this research study.

Before ATM gene was found in 1995, researcher used to propose that AT disease was caused by multiple genes because this gene is so pleiotropic in its clinical features. Patients were categorized into four complementation groups based on clinical characterization while researchers thought they might be determined by four different genes (Jaspers *et al.*, 1988). Further study found that there was only one gene behind all four complementation groups. This gene was found on chromosome 11q22-23 by positional cloning (Savitsky *et al.*, 1995). The complete sequence of ATM (150,000 nt; 66 exons) was found by the same research group (Savitsky *et al.*, 1995). It was also found that loss of ATM function in AT patients is due to different alternations which caused premature truncations or in frame amino acids deletions consequently (Table 1.1). Over 250 mutations throughout this large gene have been reported so far and

researchers have been trying to establish phenotype-genotype relationship in emerging ATM mutation because mutation status in ATM alleles is an important determinant of cellular and clinical features (Austen *et al.*, 2007; Broeks *et al.*, 2008).

Mutation	Phenotype	ATM protein levels %
875C→T	Slow progression of classic AT symptom	Trace
3576G→A	Extended longevity	5
5435del3	Late onset of ataxia	12
6047A→G	Slow progression of classic AT symptom	4
7271T→G	Extended life span	100
9139C→T	Decreased radiosensitivity	16
8030A→G	Mild neurological features	<10
8494C→T	Slow progression of classic AT symptom	11

Table 1.1 Phenotype and genotype relationships of AT variants (Rotman and Shiloh, 1998; Chun and Gatti, 2004)

Given the relationship between ATM and Nbs1 protein, NBS disease share a variety of clinical features with AT in such a degree that NBS was once thought to be a variant of AT (Shiloh 1997). Studies later revealed that some then-AT diseases were actually caused by another gene located on chromosome 8q21 (Saar *et al.*, 1997). Nbs1 gene was soon identified by positional cloning to have a 4386bp cDNA sequence and a molecular mass of 95kDa (Matsuura *et al.*, 1998; Varon *et al.*, 1998). Although more than a dozen mutations were already screened at the same time when

Nbs1 gene was identified, unlike ATM mutations, research has been focused on several dominant ones. The 657del5, the I171V and the R215W mutations are believed to be responsible for the majority of NBS cases (Nowak *et al.*, 2008). These mutations of the Nbs1 gene were all found to increase cancer susceptibility (Bogdanova *et al.*, 2008; Roznowski *et al.*, 2008; Masi *et al.*, 2008).

Although different degree of ATM mutation could be used to explain AT type syndromes which might be milder or severer than classic AT disease, some AT like syndromes are not caused by ATM mutation (Hernandez *et al.*, 1993). ATLD, Just like NBS though rarer, is one kind of AT like disease that is caused by mutation in another gene: Mre11 (Stewart *et al.*, 1999). ATLD gives rise to slower progress of AT symptom in early stage of development and does not cause telangiectasia. Table 1.2 shows the difference between AT, NBS and ATLD in detail. Two mutations: 350 A→G and 1897C→T, are responsible for ATLD (Taylor and Byrd, 2004). Because Mre11 mutation induced ATLD is very rare, there is only very limited report on new mutations in ATLD patient. Only two 630G→C mutation cases were reported in Arabian population (Alsbeih *et al.*, 2008) whereas there are reports showing new mutation in cultured cells lines (Wen *et al.*, 2008).

Clinical features	AT	ATLD	NBS
Telangiectasia	+	-	-
Ataxia	+	+	-
Reduced immunoglobulin levels	+	-	+
Abnormal eye movements	+	+	-

Microcephaly	-	-	+
Craniofacial abnormalities	-	-	+
Congenital malformations	-	-	+
Dysarthria	+	+	-
Raised serum AFP level	+	-	-

Table 1.2 Clinical features of AT, ATLD and NBS (Taylor and Byrd, 2004). + presence of feature, - absence of feature.

As a partial model of human aging, investigation into WRN gene has long drawn research interests. The location of the gene was initially narrowed to chromosome 8p12 by linkage analysis (Goto *et al.*, 1992). A WRN gene was identified also by positional cloning and composed of 1432 amino acids (Yu *et al.*, 1996). Like ATM protein, the large size of WRN gene makes possible a high number of mutations including nonsense, frameshift or insertion/deletion. However, all those mutations have only two kinds of truncated protein products which either fail to localize to the nucleus properly or become unstable in enzymatic activities (Opresko *et al.*, 2007; Ozgenc and Loeb, 2005).

1.3 Hypothesis and concept of this thesis work

The idea of this research study was illustrated by a 2008 paper on WRN and ATM interaction in response to DNA DSBs (Cheng *et al.*, 2008). In this paper, WRN was found to be required for ATM activation in response to agents that produce interstrand cross links DSBs. Considering the interaction between WRN/ATM/MRN complex,

we hypothesize that WRN might stimulate ATM activity with the help of MRN complex.

In designing the experiments, we chose to knockdown Nbs1 expression with Nbs1 short-hairpin RNA (shRNA) because Nbs1 serves as an central role in the MRN complex and Nbs1 shRNA is available from another research group in Australia and proven to be quite effective (Zhong *et al.*, 2007). Meanwhile, WRN knockdown cell line and its control are already available in our lab. Thus, all the experiments are based on four cell lines as shown in Table 1.3.

	NW	WRN	NC	CON
Nbs1	-	+	-	+
WRN	-	-	+	+

Table 1.3 four cell lines established from WRN and Nbs1 shRNA. + normal gene expression; - knocked down gene expression.). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

First, western blot analysis is used to confirm the genetic characteristics of the four lines. Pictures of cell morphology are taken and compared. Then cell survival assays are conducted to compare the growing ability of four different lines.

DNA damaging agents Camptothecin (CPT) hydroxyurea (HU), both producing collapsed replication forks and replication dependent DSBs during S-phase (Pommier

et al.; Lundin *et al.*, 2002), are selected to treat the four cell lines in a timely manner according to Cheng et al's paper (Cheng *et al.*, 2008). Aphidicolin (Aph) is selected for control treatments because it inhibits replication by inhibiting DNA polymerase therefore also halts cell cycle progression in early S phase appearing similar to the effects of CPT or HU (Saleh-Gohari *et al.*, 2005).

In completing this research, we hope to be able to investigate two questions: Whether MRN complex is involved in WRN mediated S phase checkpoint and whether the MRN complex is required for WRN mediated ATM activity.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Bacterial strain, cell strain and vectors

Bacteria	**Description**	**References**
Escherichia coli DH5α	a recombinant-deficient amber suppressing strain used as host of gene cloning	Hanahan, 1983
Cell	**Description**	**References**
U-2 OS	an osteosarcoma cell line	Cheng *et al.*, 2006
Plasmid vectors	**Description**	**References**
p*Silencer*^{TM} 3.1-H1 hygro vector	vector expressing WRN shRNA	Ambion Inc.
pCIpur vector	vector expressing Nbs 1 shRNA	Zhong *et al.*, 2007

Table 2.1 Bacterial strain, cell strain and vectors

2.1.2 shRNA sequences

WRN

5’-TGAAGAGCAAGTTACTTGA-3’ (Cheng *et al.*, 2006)

Nbs1

5’-GGGAGAAATGTGAATTCAAACGTGTGCTGTCCGTTGAGTTCACGTTTC-

TTCCTTTTT-3’ (Zhong *et al.*, 2007)

2.1.3 Chemicals and reagents

The major chemicals and reagents used in this research are listed in Appendix I.

2.1.4 Buffer, solution and gel

Buffer, solution and gel were prepared according to the formulation listed in Appendix II.

2.1.5 Commercial kits

The major commercial kits used in this research are listed in Appendix III.

2.1.6 Equipments and facilities used

All equipments and facilities were provided by Department of Nutrition and Food Science, University of Maryland, College Park and United States Department of Agriculture. The inventory is shown Appendix IV.

2.1.7 Growth medium

The growth medium used in this research is listed in Appendix V.

2.2 Methods

2.2.1 Transformation of the plasmid into DH5α competent cell

The plasmid was obtained from lab and dissolved in TE buffer (Promega) with a concentration of 40 ng/μl.

The competent cell was prepared using a protocol modified from a standard procedure (Sambrook *et al.*, 1989). DH5a was inoculated into 5 ml LB medium and shaken at 250 rpm, 37 ℃ overnight to obtain a starter culture. On the next day, 4 ml starter culture was added to 400 ml LB medium and grew until optimal density 600 nm (O.D. 600) reached 0.4. The culture was chilled on ice for 10 min and centrifuged at 1600 g for 7 min at 4 ℃. The cell pellet was washed once and suspended in 80 ml pre-chilled 60 mM $CaCl_2$ solution. The suspension was chilled on ice for 30 min and then centrifuged at 1100g for 5 min at 4 ℃. Finally, the pellet was resuspended in 8 ml pre-chilled 60 mM CaCl2 solution. Aliquots of 200 μl were transferred into sterile 1.5 ml microfuge tubes and stored at -80 ℃ for use.

Right before transformation, -80 ℃ competent cell stock was thawed on ice. A total 200ng plasmid DNA was added to 100 μl competent cells. The mixture was kept on ice for 20 min and subjected to a heat shock at 42 ℃ for 2 min. The cells were immediately rescued by adding 0.5 ml LB medium and incubation at 37 ℃ for 1 hour with shaking at 300 rpm. The resuspension was evenly spread on LB agar plate supplemented with 100 μl/ml ampicillin and incubated at 37 ℃ overnight.

2.2.2 Screening of right recombinants

White colonies were identified as positive recombinants. Positive recombinants were inoculated into 5 ml LB medium with shaking at 250 rpm, 37 ℃ overnight for the purpose of plasmid preparation. Plasmid DNA was isolated using the PureYield^{TM} plasmid Maxiprep System (Promega). The procedures were according to the manufacturer's manual.

2.2.3 Culturing, stocking and initiating U-2 OS cells.

All U-2 OS cells were cultured in DMEM medium 37 ℃ and 5% CO2 in an incubator. Medium was changed every three days or when cells were 100% confluent. To obtain a stock, the DMEM medium was aspirated the cells were washed with 10% PBS once. Trypsin was then added into cells and cells were kept at 37 ℃ for trypsin digestion. After 5 min, culture medium was added into digested cells to stop enzymatic reaction. A cell suspension was obtained here for protein extraction, flow cytometric analysis or stocking. 150 μl DMSO was added into aliquots of 1.5 ml medium and mixed well. The mixture was immediately transferred to a stock box filled with 2-proponol at -80 ℃ overnight. The cell stocks were then kept in liquid nitrogen tank for storage. Upon initiation, the cell stocks were thawed at 37 ℃ in water bath and then centrifuged at 5000 rpm for 5min. The DMSO and medium mixture was poured out and fresh DMEM medium was added. After pipetting up and down several times, the cells were transferred to flasks or dishes for future culturing.

2.2.4 Transfection of U-2 OS cell

WRN and WRN control cell lines were obtained from the cell line stock of Cheng lab. These two lines were established according to Cheng *et al.*'s paper (Cheng *et al.*, 2006).

This protocol was modified from the Qiagen's manual. Approximately 8×10^5 of WRN or WRN control cells were seeded into a 60mm dish in 5 ml of DMEM growth medium with 1:250 supplemented Hygromycin B. The cells were incubated at 37 ℃ and 5% CO2 in an incubator overnight to reach 80% confluent. On the day of transfection, 3μg of plasmid DNA in TE buffer was added into DMEM medium without FBS and antibiotics to a final volume of 150 μl. Another 25 μl of PolyFect Transfection Reagent (Qiagen) was added. The mixture was vortexed for 10 sec and incubated at room temperature for 10 min to allow complex formation. Meanwhile, the DMEM medium was aspirated from the 60mm dishes and the cells were washed with 2 ml 10% PBS once. 3 ml DMEM medium was then added into the dishes. 1 ml DMEM medium was added into the reaction tube and mixed well. The mixture was immediately transferred to the 60 mm dishes. Puromycin was added into the total volume of the growth medium to a concentration of 0.4 μg/ml. The dishes were swirled gently to ensure even distribution of the complexes. The cells were then incubated at 37 ℃ and 5% CO2 in an incubator to allow gene expression.

2.2.5 Screening of the right recombinants

Recombinants were incubated in DMEM medium supplemented with 0.4 μg/ml puromycin at 37 ℃ and 5% CO_2 in an incubator for 1 week to allow gene expression. When the non-recombinants were killed by puromycin, 500 living cells were seeded per 100 mm dish to form single colonies. After two weeks, single colonies were isolated and harvested by clone rings. Monoclones were seeded onto 6 well plates while Hygromycin B and puromycin were used alternately in DMEM medium to maintain a selection pressure. After about 1 month, recombinants were enough in number to be screened by Western blotting analysis.

This Western blotting protocol was modified from the abcam's manual. The cells were prepared according to 2.2.1 to form a cell suspension. The suspension was then transfered into 2 ml tube and centrifuged at 3,000 rpm and 4 ℃ for 5 min. The supernatant was discarded and 1 ml of 10% PBS was added to wash. The suspension was again centrifuged at 3,000 rpm and 4 ℃ for 5 min. The supernatant was aspirated completely and an ice chilled lysis mix consisting of 100 μl lysis buffer, 0.5 μl PMSF and 2 μl 50X proteinase inhibitor solution was added. After pipetted up and down several times, the reaction mix was placed on ice for 15 min and centrifuged at 20,000 g and 4 ℃ for 20 min. The supernatant containing protein was aspirated and chilled in liquid nitrogen for 2 hours and stored at -80 ℃ for later use.

To determine the protein concentration, a mixture of BCA™ protein assay reagent A (100 μl), B (2μl) and protein solution (5 μl) was place at 37 ℃ for 30 min to allow the reaction to happen. The concentration was then analyzed by a FLUOstar OPTIMA microplate reader.

25 μg protein from each sample was mixed with 5×loading buffer and 2-mercaptoethanol (5% of loading buffer). The mixture was boiled at 95 ℃ for 5 min to denature the protein. The denatured protein was then added into Criterion™ precast gel for electrophoresis at 200 volts for 50 min. After the electrophoresis, the gel was placed in ice cold transfer buffer for 3 min to equilibrate. A PVDF transfer member was cut into appropriate size and soaked in methanol for 2 min. The gel and PVDF transfer member were then sandwiched between sponge and paper after ensuring no air bubbles formed between the layers. The sandwich was submerged into pre-chilled transfer buffer at 100 volts for 1 hour. The member was rinsed by TBS-T once and submerged in blocking buffer to prevent non-specific binding for 1 hour at room temperature under agitation.

The member was rinsed for 5 min three times in TBS-T at room temperature then incubated with primary antibody at 4 ℃ overnight. The Nbs1 antibody was diluted into blocking buffer at a ratio of 1:1,000. The WRN and Tublin antibody were both diluted into blocking buffer at a ratio of 1:5,000. After overnight incubation, the member was rinsed for 10 min, three times in TBS-T then incubated with secondary antibody at room temperature for 1 hour. The anti-mouse secondary antibody was

diluted into blocking buffer at a ratio of 1:1,0000. After that, the member was rinsed for 10 min, three times in TBS-T then incubated with SuperSignal™ Chemiluminescent Substrate for signal detection. X-ray films and automated x-ray developer were used here.

2.2.6 Cell Survival assay

5,000 cells of each cell line were seeded onto 6-well plates cultured in DMEM medium corresponding antibiotics (Hygromycin B or Puromycin) 37 ℃ and 5% CO2 in an incubator. After 6 days, cells were resuspended according to 2.2.3 and counted using a hemocytometer. A total three trials were conducted and each trial consisted of 3 repeats.

2.2.7 Cell cycle progression assay

All treatments were applied when cells reached 30% confluent. For CPT treatment, CPT was added into the medium to a final dilution of 1 μM. The cells were subject to treatment for 3 hours. The medium was then aspirated and the cells were rinsed in PBS once. Fresh medium was added afterwards to restore cell growth at 37 ℃ and 5% CO2 in an incubator. Cells were harvest according to 2.2.3 at 0, 4, 8, 16, 24 or 48 hours after removal of treatment.

For HU treatment, HU was added into the medium to a final dilution of 0.5 mM. The cells were subject to treatment for 24 hours. The medium was then aspirated and the

cells were rinsed in PBS once. Fresh medium was added afterwards to restore cell growth at 37 ℃ and 5% CO2 in an incubator. Cells were harvest according to 2.2.3 at 0, 2, 4, 8 or 24 hours after removal of treatment.

For Aph treatment, Aph was added into the medium to a final dilution of 1 μg/ml. The cells were subject to treatment for 24 hours. The medium was then aspirated and the cells were rinsed in PBS once. Fresh medium was added afterwards to restore cell growth at 37 ℃ and 5% CO2 in an incubator. Cells were harvest according to 2.2.3 at 0, 4, 6, 8 or 16 hours after removal of treatment.

The harvested cells were centrifuged at 1,500 rpm for 5 min at 4 ℃. The supernatant was discarded and the pellet was washed with cold PBS once. The suspension was centrifuged at 1,500 rpm for 5 min at 4 ℃. The supernatant was discarded but about 200 μl PBS was kept in the tube. The cells were pipetted up and down several times to be resuspended into single cell forms. 3 ml cold (-20 ℃) 70% ethanol was added dropwise into the cell suspension while vortexing. The cells were store at -20 ℃ for staining.

Upon staining, the cells were spinned down at 1,500 rpm for 5 min at 4 ℃. The supernatant was discarded and the pellet was washed with cold PBS once. The suspension was centrifuged at 1,500 rpm for 5 min at 4 ℃. The supernatant was discarded but about 100 μl PBS was kept in the tube. 900 μl of 1×Propidium Iodide

containing RNase was added and the mixture was incubated in dark for 1 hour at room temperature using the Cellular DNA Flow Cytometric Anaylysis Reagent Set according to the manufacturer's manual. The mixture was scanned by a FACSCaliber Flow Cytometer (BD Biosciences) with program Cell Quest Pro. Cell cycle modeling was performed using ModFit (Verity).

Chapter 3: Results

3.1 Establishing positive Nbs-1 knockdown recombinants

3.1.1 Nbs1 expression levels of positive recombinants

Western blotting analysis was used here to confirm positive recombinants obtained according to 2.2.4. Tublin primary antibody was used as control.

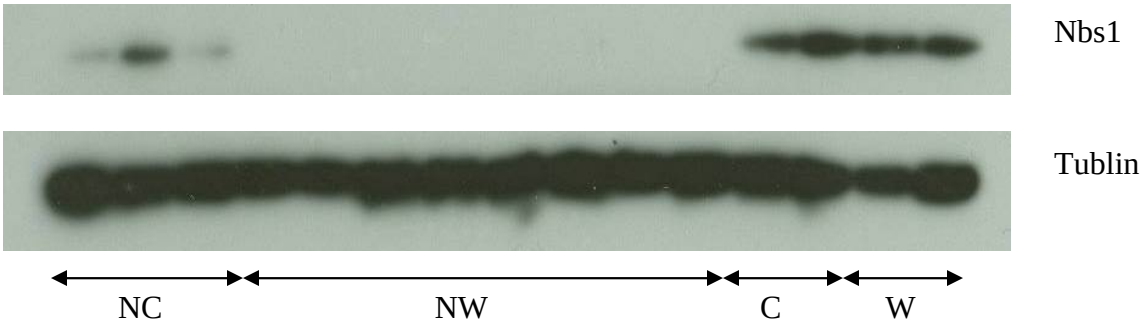


Figure 3.1 Western blotting analysis of the expression profile of the Nbs1 gene in four cell lines. Tublin expressions were used as control. NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

According to Western Blotting result, Nbs1 expression was successfully decreased in both NC and NW cell lines. One monoclone of NC and NW was selected for further analysis.

3.1.2 Morphology of positive recombinants

Pictures were taken for the cells under optic microscope when the four lines of cells reached about 40% confluent. The magnification was 20x.

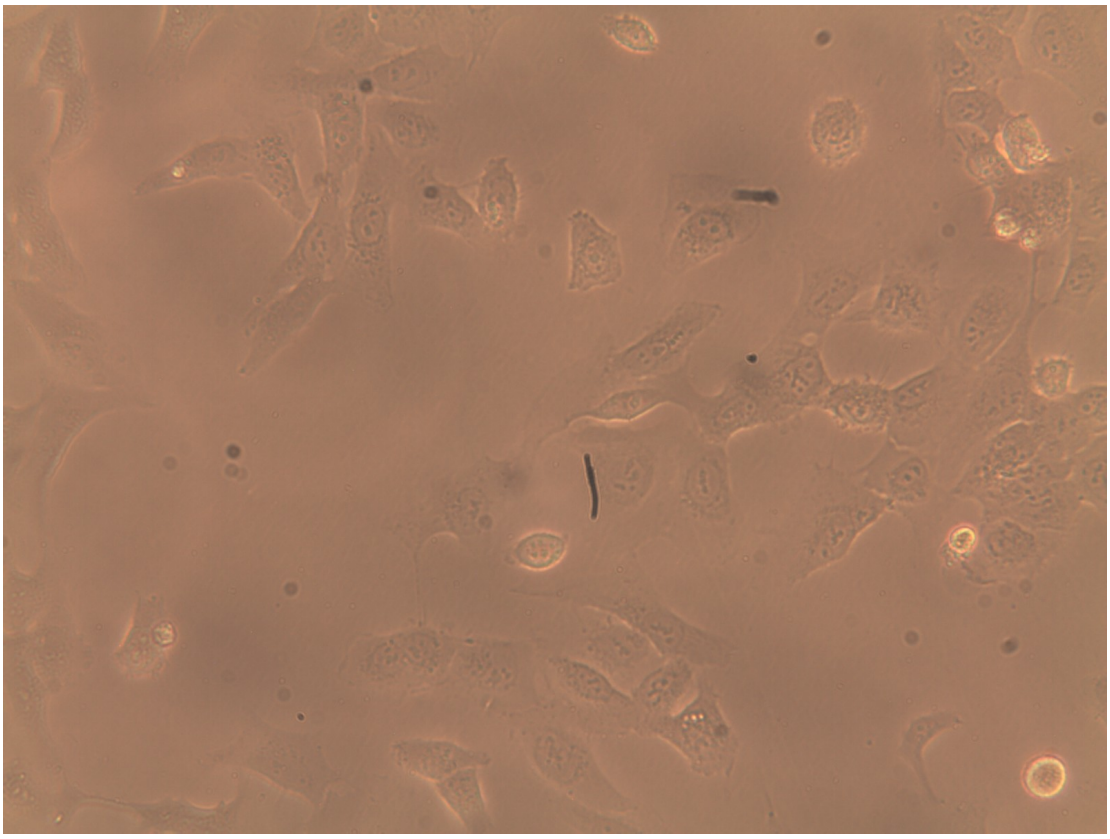


Figure 3.2 WRN (W) cells. They are compact and in a regular rectangular form like /WRN (NW) cells.

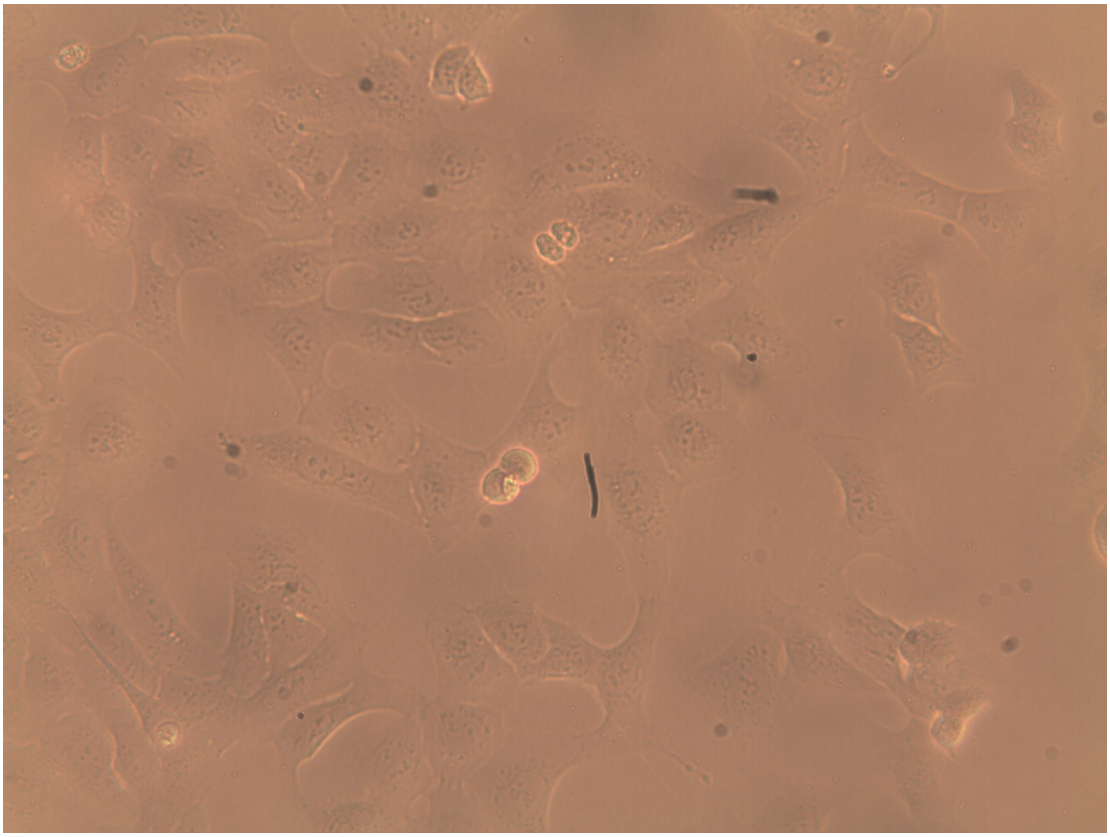


Figure 3.3 WRN control (C) cells. These cells are inflated and extending their membrane to each other to form synapse like pointing.

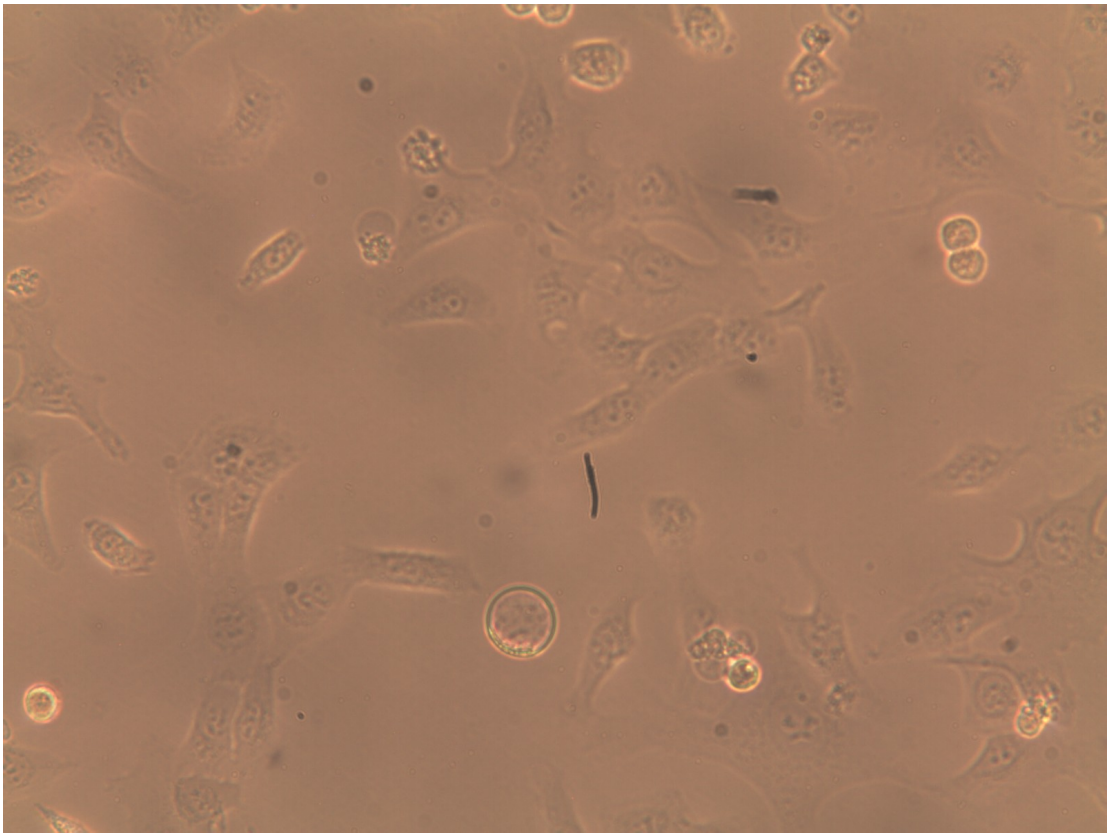


Figure 3.4 Nbs1/WRN (NW) cells. They are compact and in a regular rectangular form like WRN (W) cells.

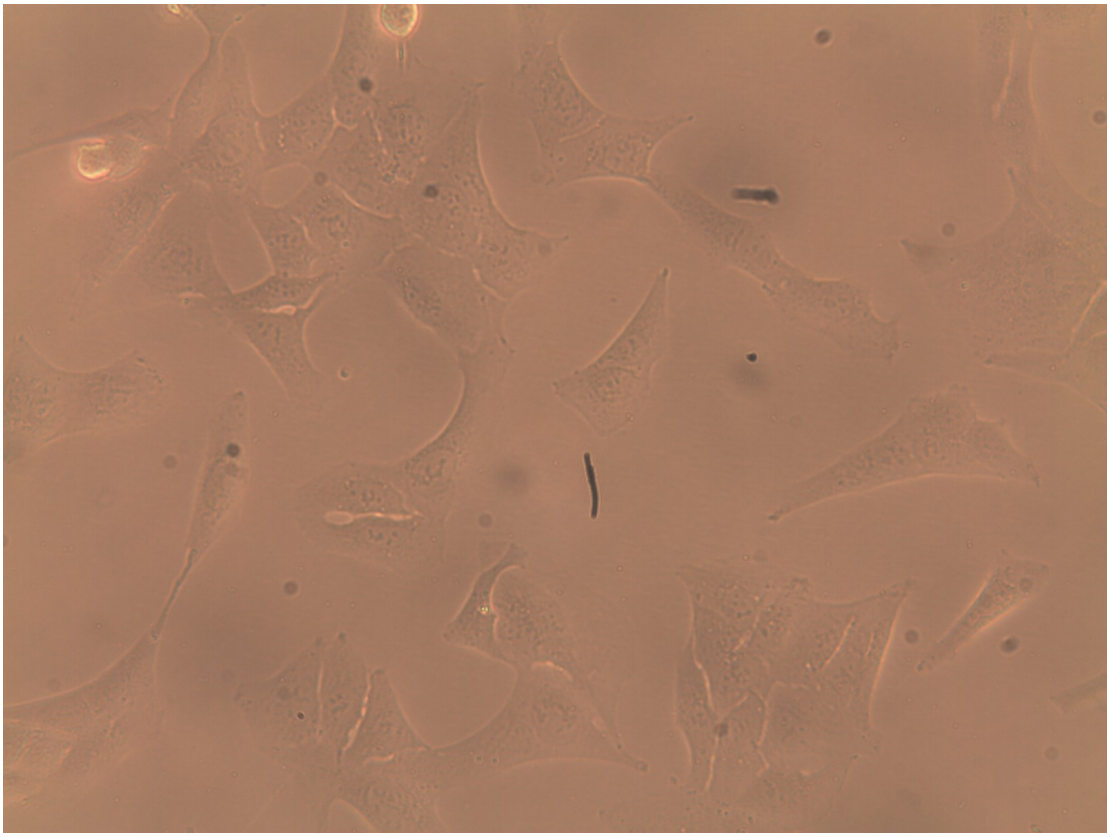


Figure 3.5 Nbs1/WRN control (NC) cells. These cells are even bigger and more expanded. Their member is so extended outwards to form a very sharp shape of spear.

It is very obviously that W and NW cells are quite similar in shape. They are compact and in a regular rectangular form. C cells, however, are inflated and extending their membrane to each other to form synapse like pointing whereas NC cells are even bigger and more expanded. Their member is so extended outwards to form a very sharp shape of spear.

3.2 Cell Survival assay

Cell survival assay was conducted in accordance with 2.2.6.

	Trial 1	Trial 2	Trial 3	Average
W	55666.67	50666.67	58333.33	54888.89
C	20333.33	19333.33	16666.67	18777.78
NW	60833.33	50000	64000	58277.78
NC	11666.67	16000	16166.67	14611.11

Table 3.1 Cell number after 6 days of growth. NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

Table 3.1 could also be displayed in a chart as shown in Figure 3.6 below.

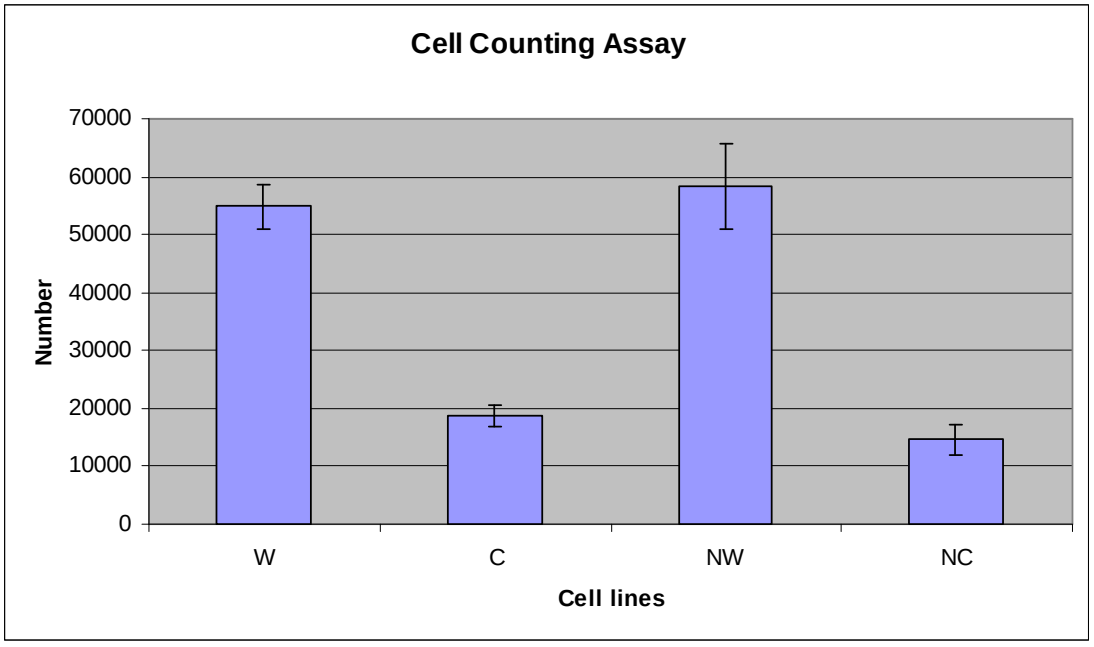


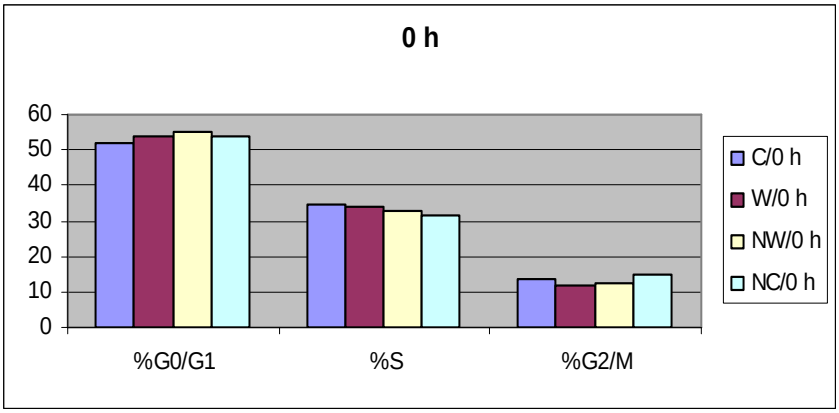
Figure 3.6 Cell number after 6 days of growth. NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

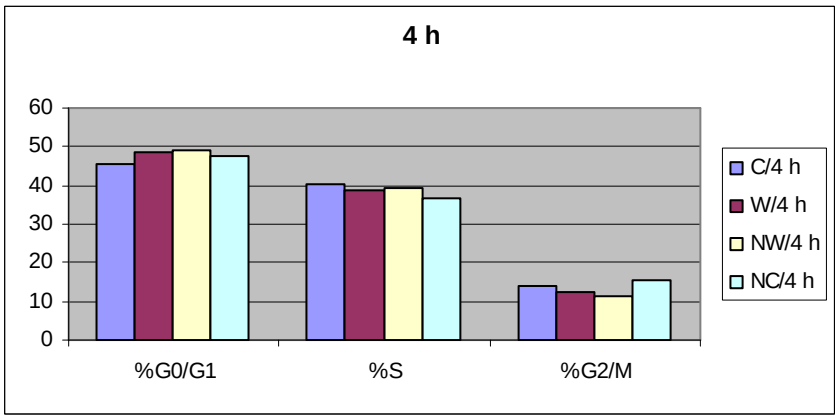
Two cell lines: W and NW grew at similar speed. The number rose from 500 in the beginning to about 50,000 to 60,000 in the end. However, another two lines, C and NC, although growing at similar speed as each other, only reach less than 20,000 cells after 6 days, which were 1/3 to ¼ of the numbers of W and NW.

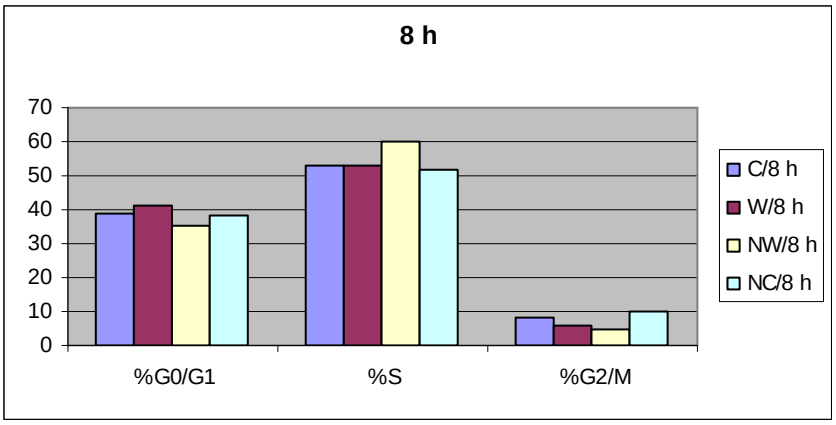
3.3 Flow Cytometric Analysis

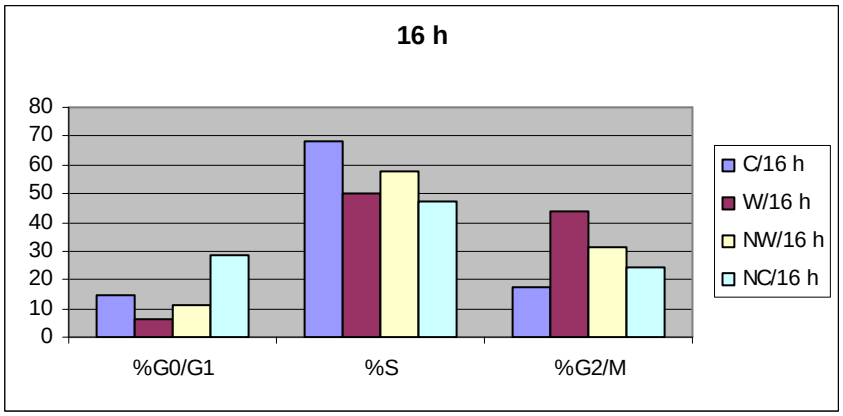
3.3.1 CPT treatment

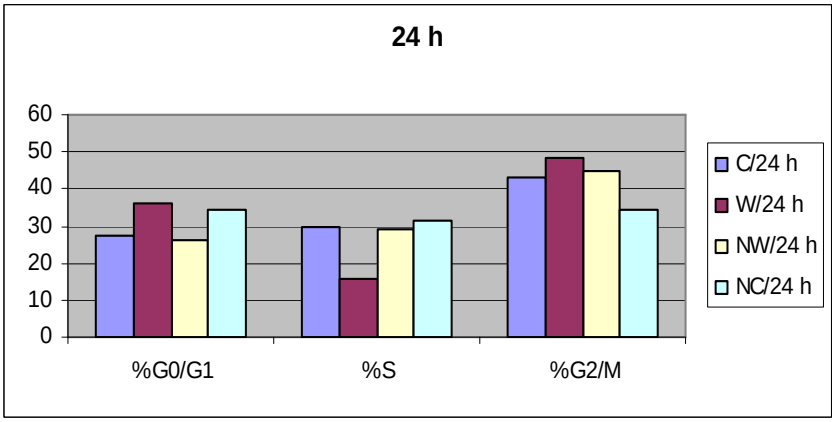
CPT treatment was applied in accordance to 2.2.7.






8 h
70
60
50
40
30
20
10
0
%G0/G1
%S
%G2/M
C/8 h
W/8 h
NW/8 h
NC/8 h


16 h
80
70
60
50
40
30
20
10
0
%G0/G1
%S
%G2/M
C/16 h
W/16 h
NW/16 h
NC/16 h


24 h
60
50
40
30
20
10
0
%G0/G1
%S
%G2/M
C/24 h
W/24 h
NW/24 h
NC/24 h

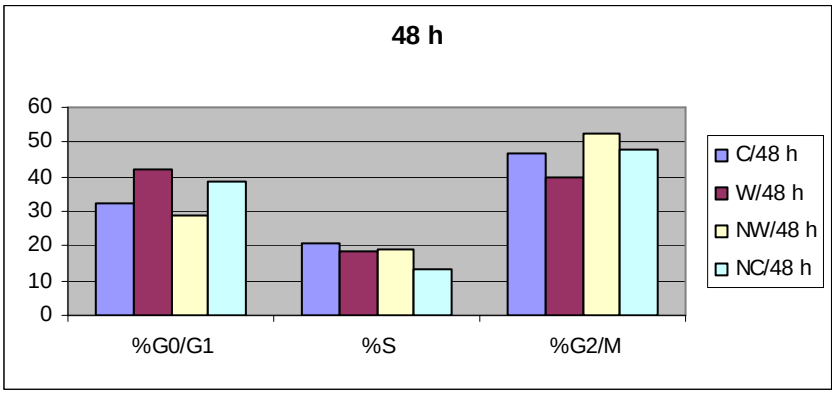
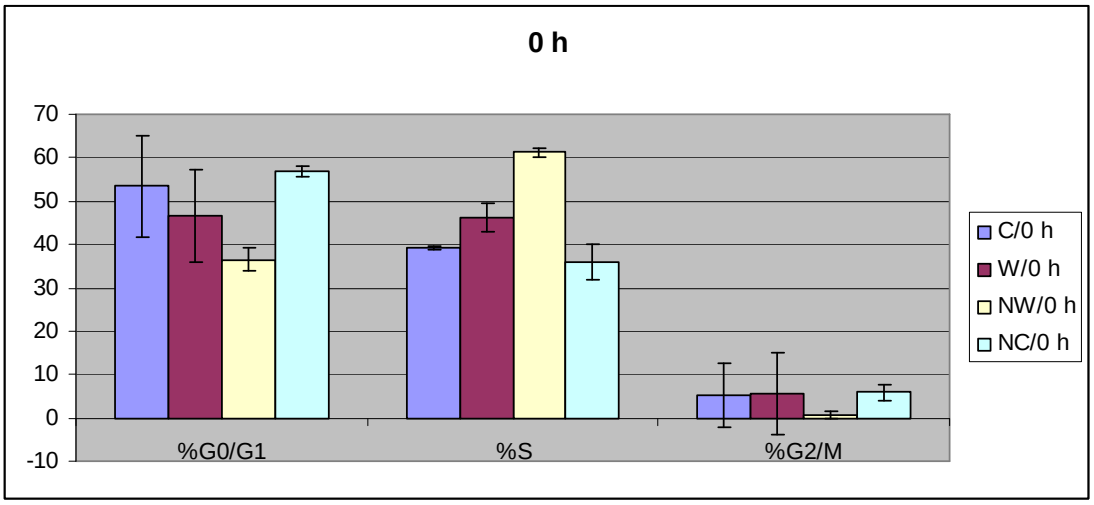


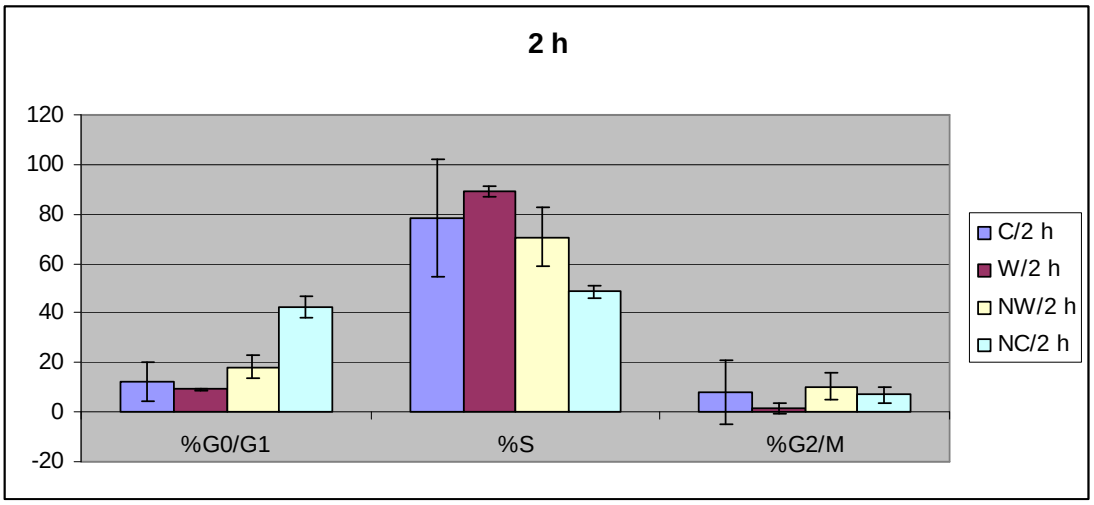
Figure 3.7 Column chart showing quantification of cell cycle profile of CPT treatment (n=1). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

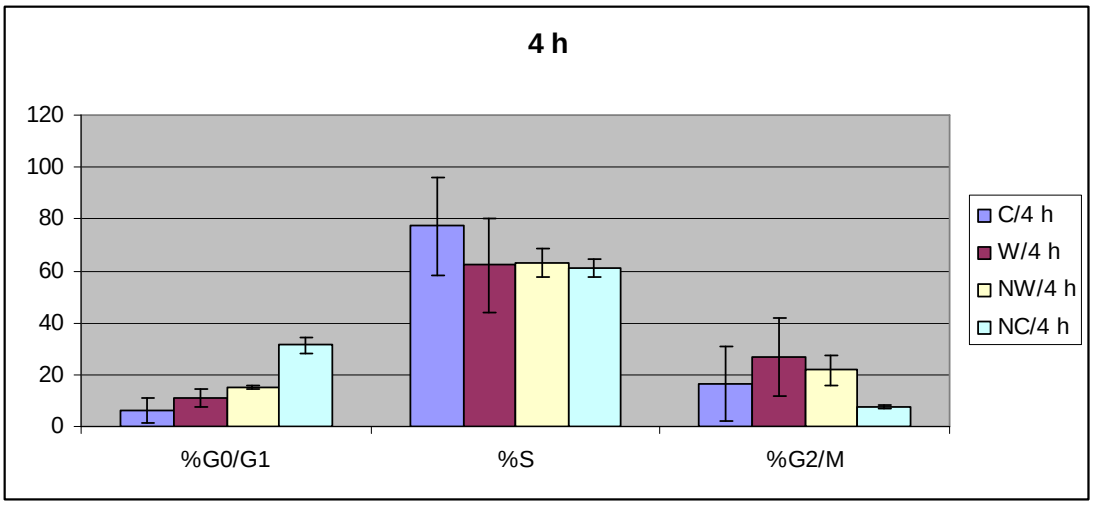
By 0 h after treatment, all four lines displayed similar pattern. The majority of cells were arrested at G0/G1 phase. By 4 h after treatment, a portion of cells progressed into S phase while all four lines still showed similar pattern. By 8 h after treatment, more cells of the four lines were in S phase than those in G0/G1 phase. By 16 h after treatment, cells began to show difference in progression pattern. Most C cells were still arrested in S phase while almost half of W cells appeared at G2/M phase. NW and NC cells left S phase at a speed faster than C cells but slower than W cells. By 24 h after treatment, far less W cells were still arrested in S phase than the other three lines while by 48 h, the four lines displayed similar profiles again.

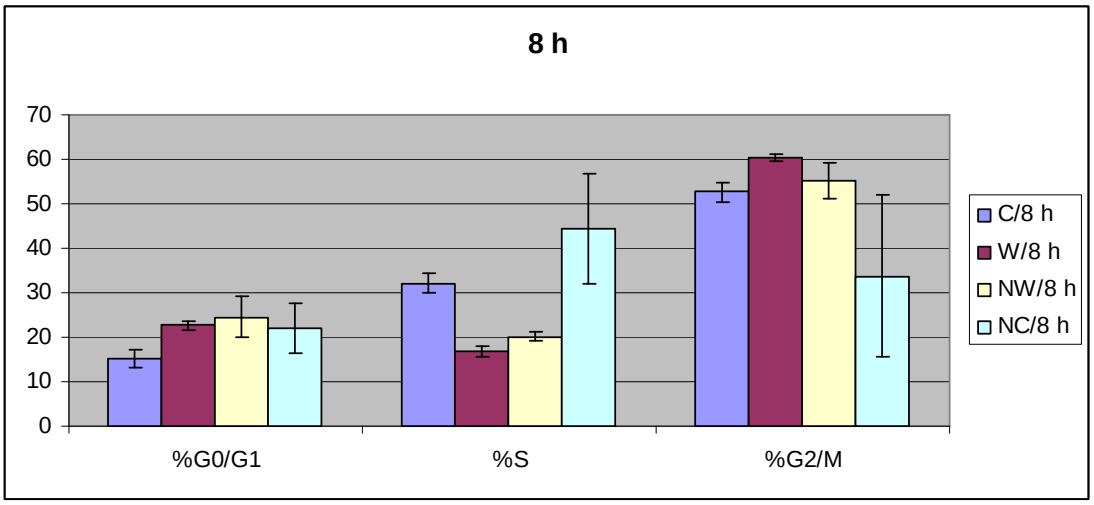
3.3.2 HU treatment

HU treatment was applied in accordance to 2.2.7.






4 h
120
100
80
60
40
20
0
%G0/G1
%S
%G2/M
C/4 h
W/4 h
NW/4 h
NC/4 h


8 h
70
60
50
40
30
20
10
0
%G0/G1
%S
%G2/M
C/8 h
W/8 h
NW/8 h
NC/8 h

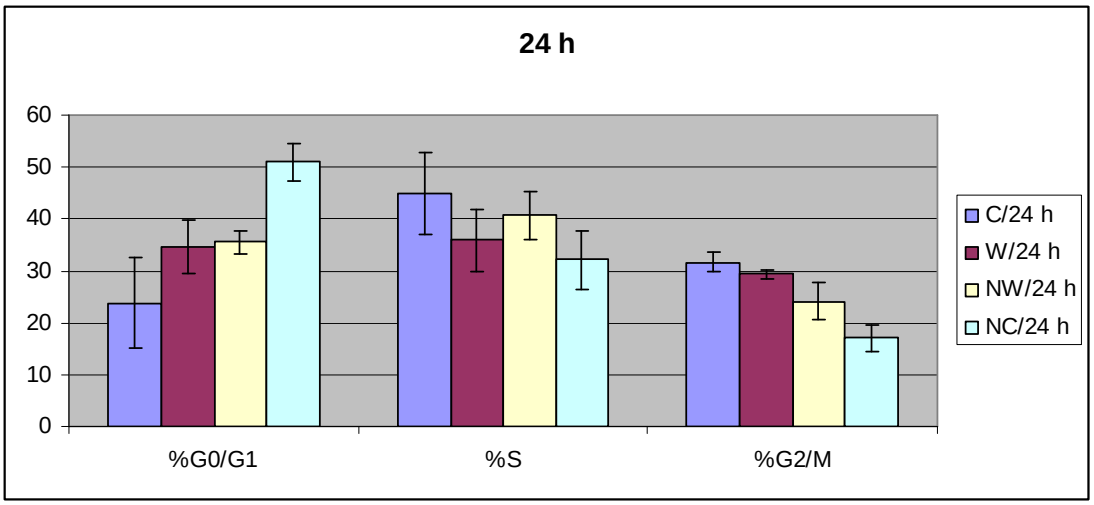
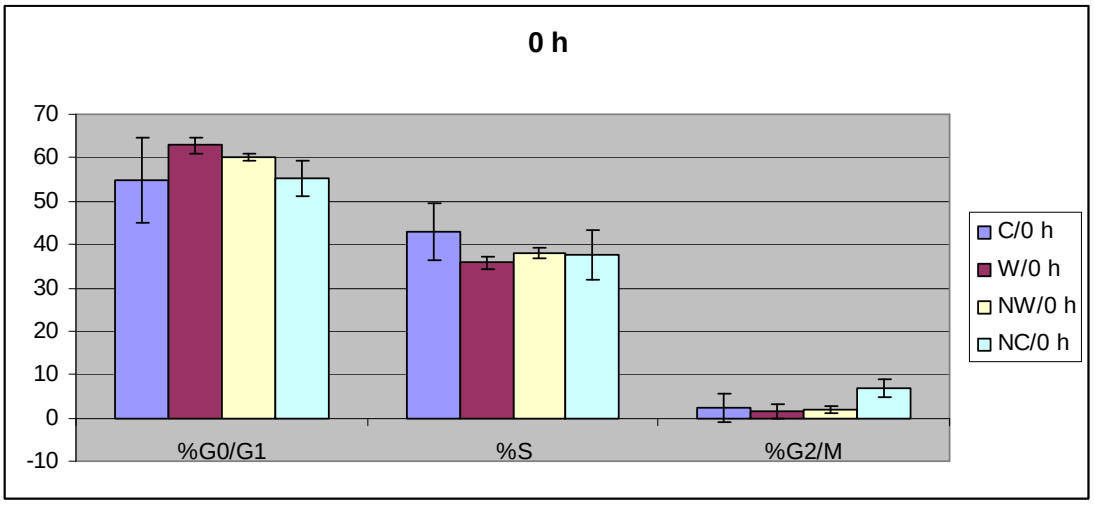


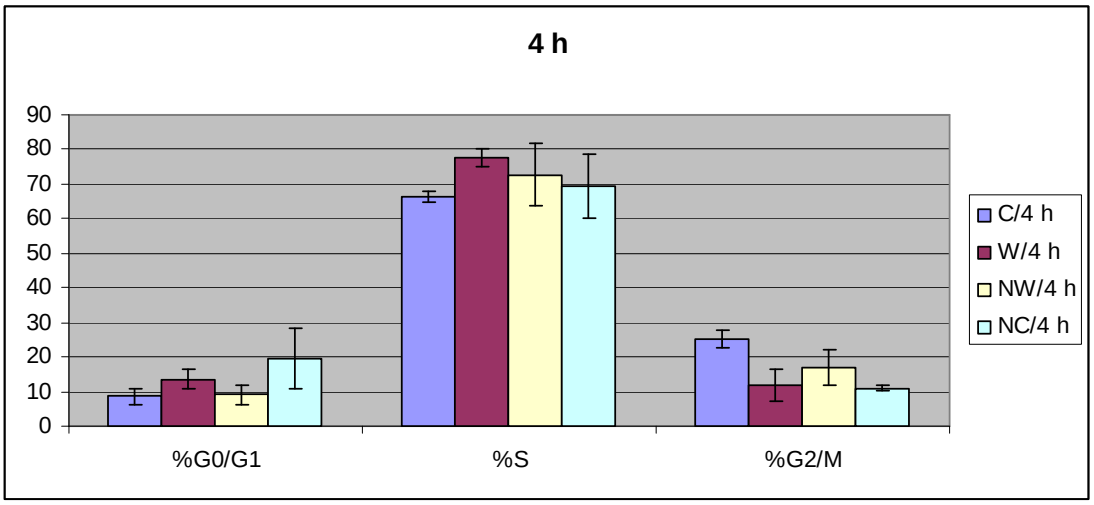
Figure 3.8 Column chart showing quantification of cell cycle profile of HU treatment (n=3). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

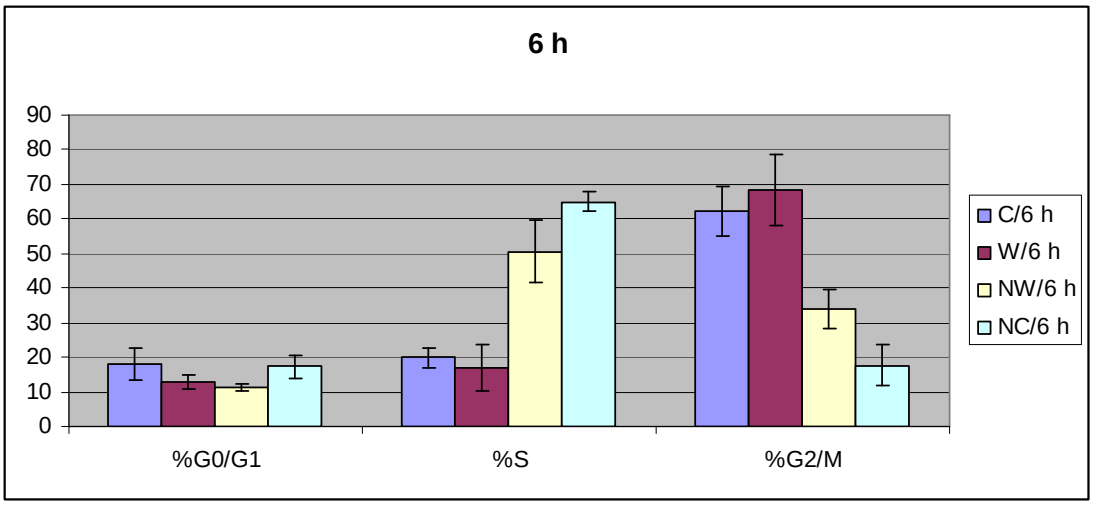
By 0 h after treatment, all four lines displayed similar pattern although most NC cells appeared at G0/G1 phase. The majority of cells were arrested at G0/G1 and S phase. By 2 h after treatment, most cells progressed into S phase while all four lines still showed similar pattern. By 4 h after treatment, cells began to show difference in progression pattern. Most C, NW and NC cells were still arrested in S phase while almost half of W cells appeared at G2/M phase. Compared to the 2 h chart, the change in W cells was most obvious and great. NW and NC cells entered G2/M phase at a speed even slower than C. By 8 h and 24 h after treatment, the four lines displayed similar profiles again.

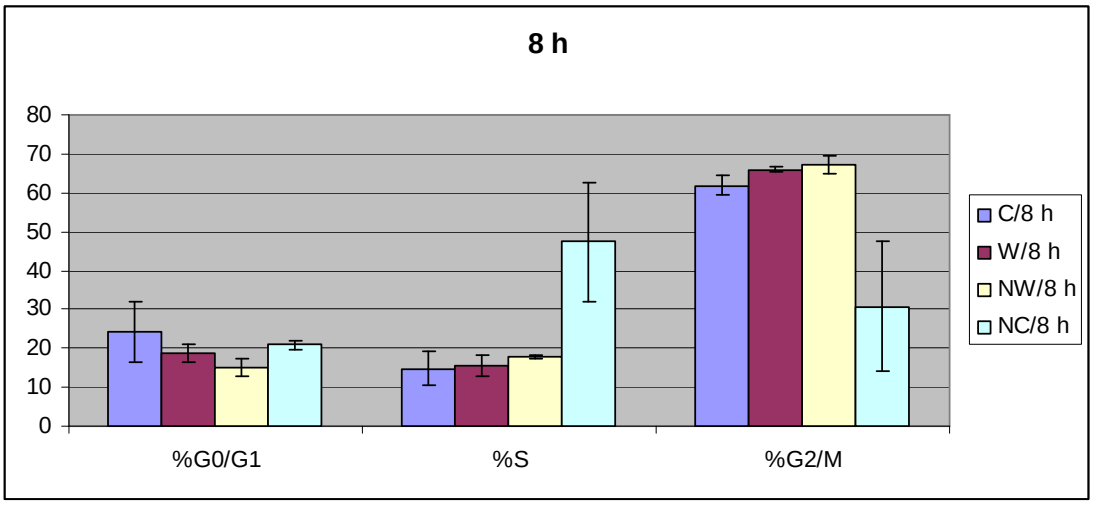
3.3.3 Aph treatment

Aph treatment was applied in accordance to 2.2.7.






6 h
90
80
70
60
50
40
30
20
10
0
%G0/G1
%S
%G2/M
C/6 h
W/6 h
NW/6 h
NC/6 h


8 h
80
70
60
50
40
30
20
10
0
%G0/G1
%S
%G2/M
C/8 h
W/8 h
NW/8 h
NC/8 h

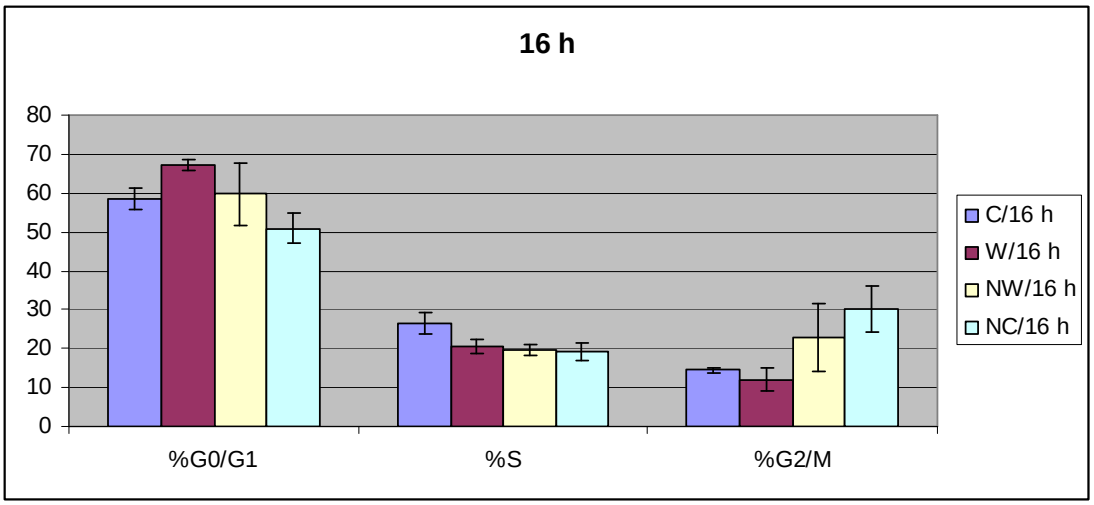


Figure 3.9 Column chart showing quantification of cell cycle profile of Aph treatment (n=3). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

By 0 h after treatment, all four lines displayed similar pattern that the majority of cells appeared at G0/G1 and S phase. By 4 h after treatment, most cells progressed and were arrested in S phase while all four lines still showed similar pattern. By 6 h after treatment, cells began to show difference in progression pattern. Most C and W progressed into G2/M phase at a similar speed while NC and NW cells were still arrested in S. By 8 h and 16 h after treatment, the four lines displayed similar profiles again.

Chapter 4: Discussion

4.1 Role of WRN and Nbs1 protein in the promotion of tumor cell growth

Inhibition of WRN expression was reported to strongly impair growth of several cancer cell lines including breast and ovarian adenocarcinoma, cervical and lung

carcinoma and endometrial leiomyosarcoma but excluding U-2 OS cells (Opresko *et al.*, 2007). Given the bifunctional nature, WRN protein is proposed to promote cell proliferation by reducing DNA damage and replicative stress. This function of WRN also benefits the growth of oncogenic cells. Interestingly, it was suggested in another research that restoration of WRN expression had tumor suppressing effect. The loss of WRN function was thought to activate alternating lengthening of telomerase (ALT) mechanism and this mechanism exists commonly in sarcomas and induces tumor formation in mouse models (Agrelo *et al.*, 2006).

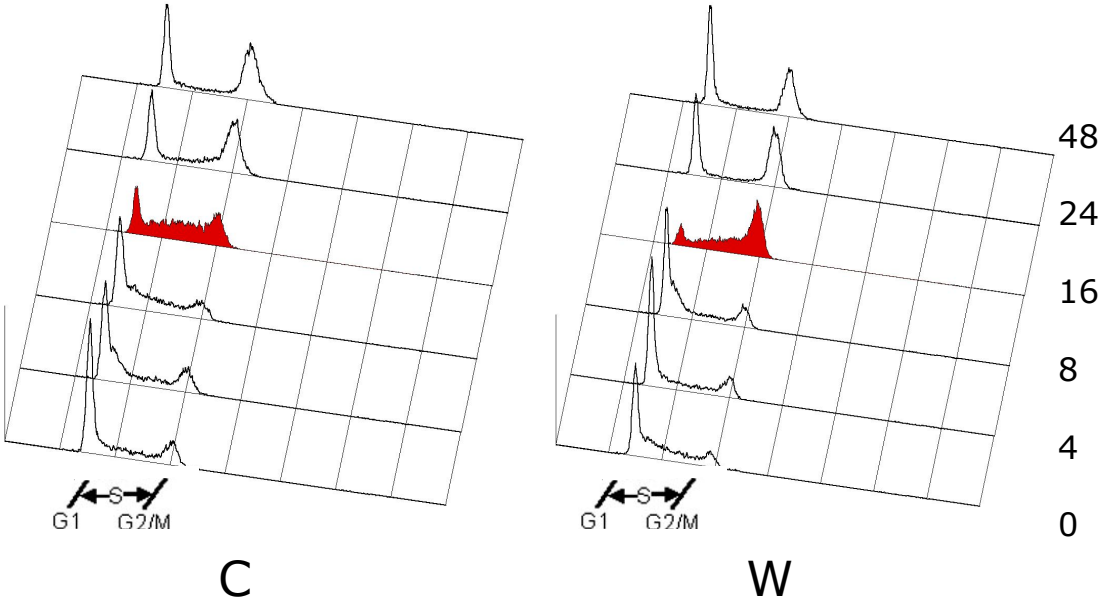
The result of this research study supports the latter hypothesis that WRN protein has cancer suppressor properties. The two lines with WRN gene knocked down grew at least 3-fold faster than the two lines with normal WRN expression. Considering that the WRN protein acts as intermediates in DNA replication, telomere maintenance and DNA damage response, loss of WRN function may trigger chromosomal aberration that is relevant to cancer.

Another more interesting result of this research study is that Nbs1 could be significantly knockdown in WRN cells whereas a complete knockdown of Nbs1 in WRN control cells was somehow lethal. Only NC cells with Nbs1 partially knocked down could be viable and thus obtained in this study. In a similar study, it was reported that knockdown of Nbs1 inhibited ATL mechanism in human fibrosarcoma cell line (Zhong *et al.*, 2007). It was also reported in this paper that incomplete knockdown of Nbs1 permitted partial ALT activity which could be used to explain

why only NC cells with incomplete Nbs1 knockdown were obtained. However, why Nbs1 was nearly completely knocked down in NW cells remains a question. Since enzymatic activities of WRN are needed to suppress telomeric-circle formation in normal cells which express telomerase reverse transcriptase (Li *et al.*, 2008). Loss of WRN function might remediate the ATL mechanism in pathways different from Nbs1 pathway so that ALT activity was still retained in NW cells even while Nbs1 was completely knocked down. Further research is needed to study the different pathways of WRN and Nbs1's correlation with ALT mechanism.

4.2 WRN and Nbs1 participate in S-phase checkpoint

4.2.1 CPT treatment



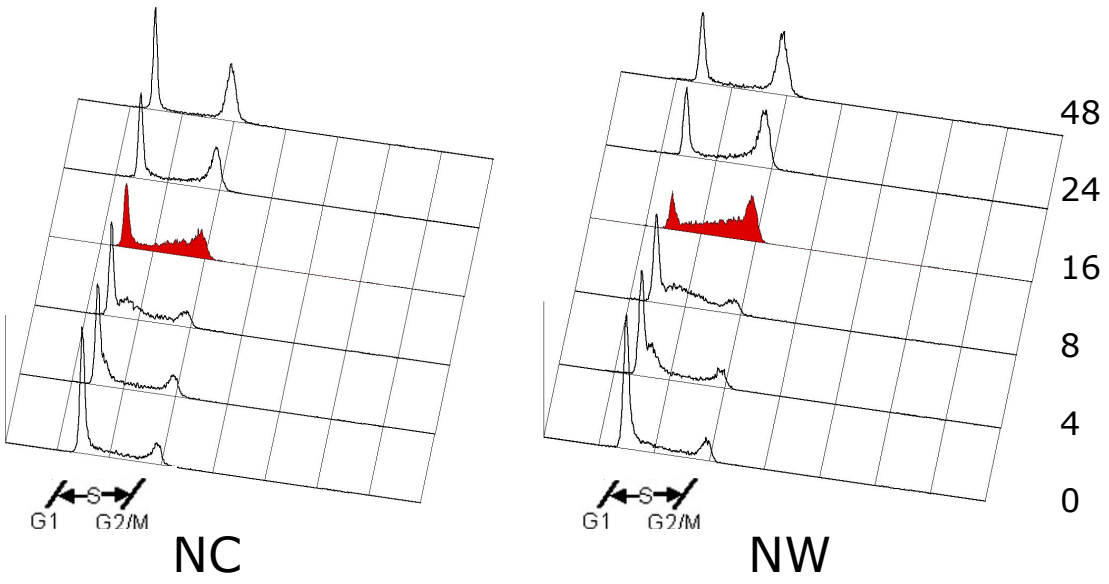


Figure 4.1 Role of WRN and Nbs1 in S-phase checkpoint. The four cell lines were treated with CPT (1 μM, 3 h). Cells were harvested at the indicated time points and analyzed by flow cytometry (n=1). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

W and C cells displayed the same cell cycle profiles as were described in Cheng *et al.*'s paper (Cheng *et al.*, 2008). These date confirms that WRN plays a role in the S-phase checkpoint induced by replication dependent DSBs. The fact that NC progresses into G2/M phase faster than C cells also confirms that notion that the MRN complex and ATM are interdependent in recognizing and signaling DNA DSBs (Berkovich *et al.* 2007). However, NW cells enter G2/M phase much slower than W cells and are at a similar speed as NC cells may indicate that Nbs1, compared to WRN, is a more critical protein in sensing DSBs and plays a role as a bottle neck. Moreover, this data also seem to show that ATM activation by WRN is not dependent

on Nbs1 because a complete depletion of Nbs1 increases ATM activation instead of decreasing it.

4.2.2 HU treatment

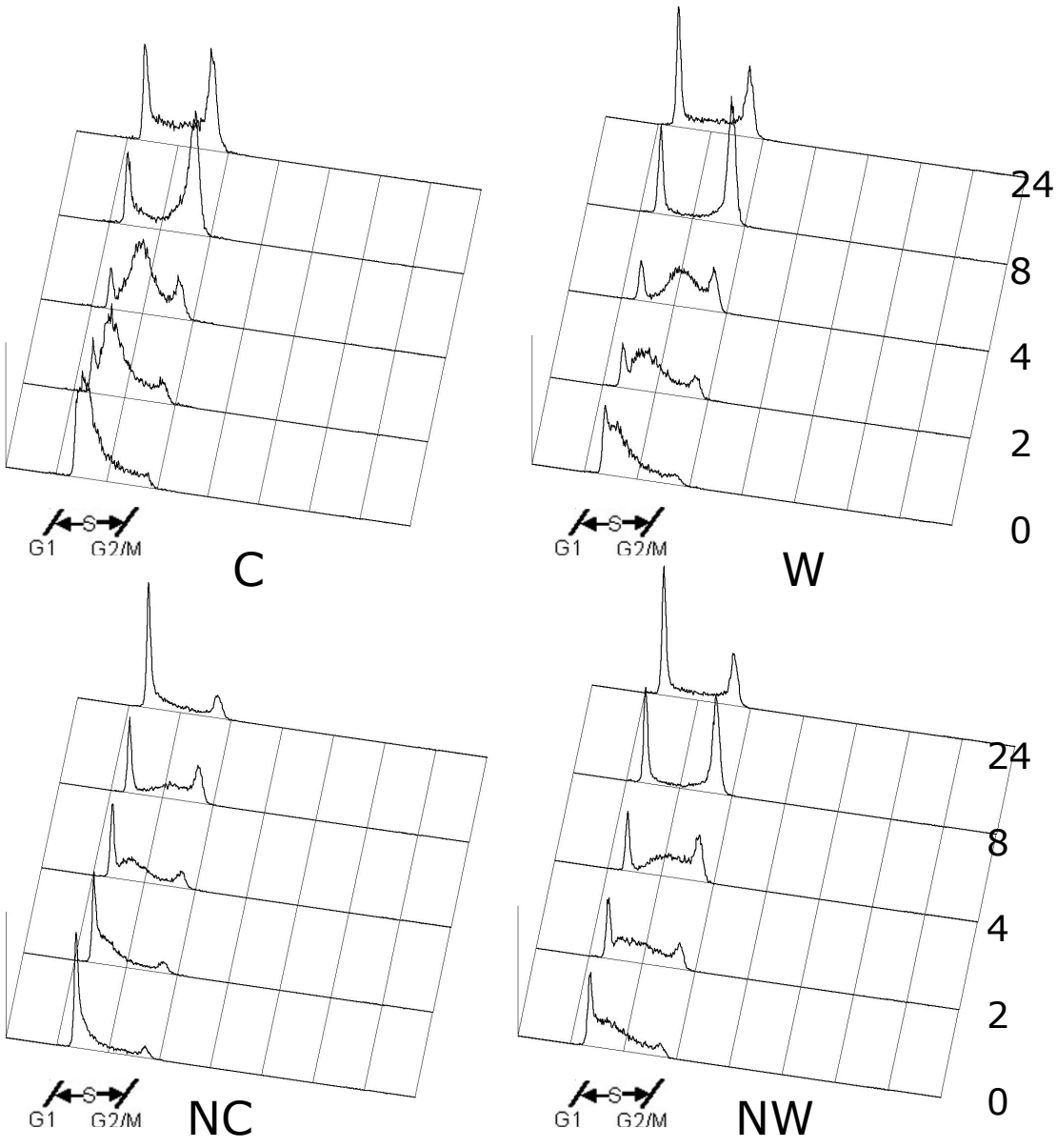


Figure 4.2 Role of WRN and Nbs1 in S-phase checkpoint. The four cell lines were treated with HU (0.5 mM, 24 h). Cells were harvested at the indicated time points and

analyzed by flow cytometry (n=3). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

After HU treatment, W and C cells displayed the same cell cycle profiles as were described in Cheng *et al.*'s paper (Cheng *et al.*, 2008). By 4 h after treatment, more than half of W cells entered G2/M phase while the majority of C cells were still arrested in S phase. Like CPT treatment, this date also confirms that WRN plays a role in the S-phase checkpoint induced by replication dependent DSBs. However, the two Nbs1 knockdown lines NC and NW were still arrested by 4 h after treatment, which, combined with data from CPT treatment, further confirms that that ATM activation by WRN is not dependent on Nbs1 because a complete depletion of Nbs1 increases ATM activations in both lines instead of decreasing them. It could be explained in both treatment that a partial depletion of Nbs1 in NC cells doesn't affect ATM activation substantially. However it is not logical that a large number of NW cells are arrested in S phase because ATM is bound to the MRN complex via Nbs1. With a total completion of Nbs1, it is hard to explain why ATM could still be activated. Another repeat of this experiment is needed.

4.2.3 Aph treatment

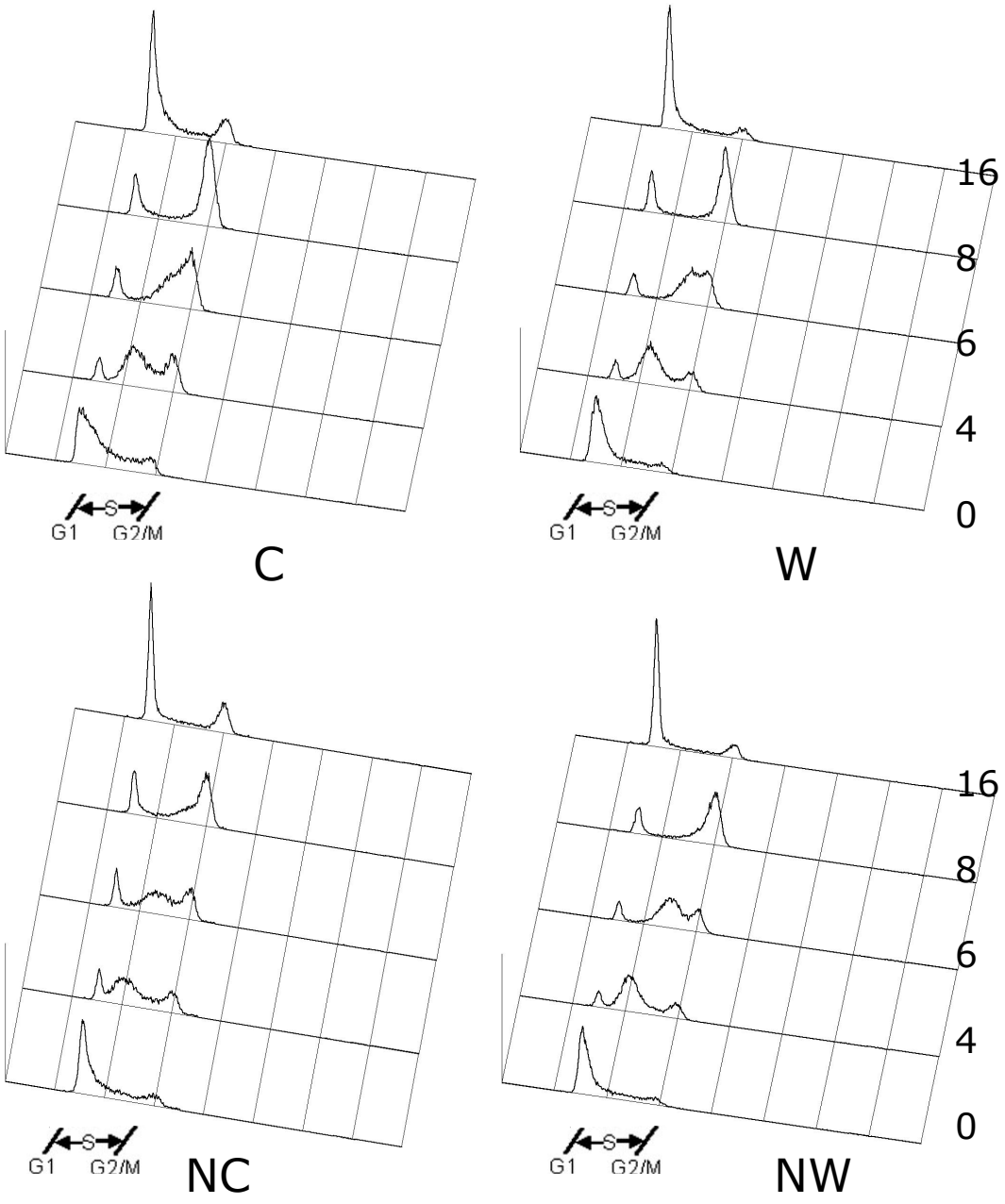


Figure 4.3 Role of WRN and Nbs1 in S-phase checkpoint. The four cell lines were treated with Aph (1 μg/ ml, 24 h). Cells were harvested at the indicated time points and analyzed by flow cytometry (n=3). NC: Nbs1/WRN control; NW: Nbs1/WRN; C: WRN control; W: WRN.

After Aph treatment, W and C cells displayed the same cell cycle kinetics as were described in Cheng *et al.*'s paper (Cheng *et al.,* 2008) showing that WRN only participates in S phase checkpoint induced by replication-dependent DSBs. However, the two Nbs1 knockdown lines NC and NW were still arrested by 6 h after treatment, which, considering the treatment of Aph as a DNA polymerase inhibition, Nbs1 may participate other cell cycle checkpoints induced by Aph stress which is different from DSBs. There is evidence showing that it is Mre11 binding domain instead of FHA/BRCT domains of Nbs1 taking part in the efficient induction of the intra-S checkpoint (Stewart *et al.,* 2003; Goldberg *et al.,* 2003). G1 and G2 checkpoints may also involve Nbs1 (Kobayashi *et al.,* 2004). However reports are controversial and the mechanism behind them remains unsolved, more investigation needs to be done.

4.3 Conclusion and prospect

Previous study showed that WRN protein was involved in ATM pathway activation when cells are exposed to DSBs induced by replication fork collapse. Because the Mre11-Rad50-Nbs1 (MRN) complex, a sensor of DSBs, is known to interact with WRN and ATM via Nbs1 subunit, we are interested in whether the MRN complex mediates the WRN-dependent ATM pathway activation. In this study, we employed short-hairpin RNA to generate WRN- and Nbs1-deficient U-2 OS cells and treat cells with clastogens which induce collapsed replication forks, thus provided primary proof for whether WRN facilitates ATM activation via MRN complex. We also performed

cell survival assay on all cell lines, hoping to get auxiliary information on the function of these two target proteins. Upon analyzing our results, we concluded that:

1. The result of this research study supports the hypothesis that WRN protein has cancer suppressor properties by affecting ATL mechanism. Nbs1, on the other hand, also affects the ATL mechanism in a way that is different from WRN pathways.

2. Our data suggest that ATM activation by WRN is not dependent on Nbs1 although ATM interacts with both WRN and the WRN complex.

Our ultimate task is to understand aging as well as the relationship between cell cycle checkpoints and genomic stability by studying protein-protein interaction. Thorough understanding on the novel WRN-dependent ATM activation will shed light on some unknown cellular network. To achieve this goal, there are works left to be done in future.

Zhong *et al.*, used a scrambled control shRNA sequence to make a control pCIpur vector (Zhong *et al.*, 2007). As a result, both Nbs1 deficient and control cell line all grew under puromycin condition. We did not obtain the control plasmid so the W and C cell lines we used as control were cultured only under hygromycin B without puromycin pressure. Meanwhile the NC and NW cell lines were under the pressure of both hygromycin B and puromycin, which makes the data not persuasive enough. It is

better to repeat all the experiments using a control plasmid so that all four cell lines are cultured under the same condition.

FACS data only provide indirect information on ATM activation. ATM autophosphorylation on Ser-1981 needs to be quantified by Western Blotting using the four cell lines after treatment according to 2.2.7. Downstream ATM pathway should also been analyzed to further support ATM activation. ATM substrates like SMC1, BRCA1 and CHK1 could be tested by Western Blotting on autophosphorylation. Besides Western Blotting assay, immunostaining could also be used to confirm ATM phosphorylation according to the protocol described in Cheng *et al.*'s 2008 paper.

Protein colocalization could be tried either under stress or without stress by performing coimmunofluorescence. The data could also be used to know at which cell cycle phase when proteins interact with each other.

In order to confirm that DSB formation exist in all four cell lines. Assays on γH2AX formation also need to be performed to show whether DSB formation are inhibited by gene expression knockdown.

Our data bring question on Nbs1's role on other cell cycle checkpoints. An analysis on DNA synthesis in replicating cells should be able to answer the question. The

kinetics of cell replication under different treatment could be compared to investigate the role of WRN and Nbs1 on other cell cycle checkpoints.

Appendices

Appendix I: Chemicals and reagents

2-Mercaptoethanol	Sigma M3148
2-Propanol	Fisher A451-4
10XTBS	Bio-Rad 170-6435
10XTG	Bio-Rad 161-0771
10XTBS	Bio-Rad 170-6435
10% SDS	Teknova S0184
ampicillin	Sigma A-9518
aphidicolin	Calbiochem 178273
camptothecin	MP Biomedicals IC15973250
DMEM,1X	Cellgro 10-017-CV
DMSO	Sigma D5897
Ethanol (absolute)	Merck 100986
EDTA	Quality Biological 351-027-10
Fetal Bovine Serum	Atlanta S11550
Glycerol	Sigma G-5516
hydroxyurea	MP Biomedicals AAAL01120-03
hygromycin B	Invitrogen A98670309
KCl	Baker 4001-01
KH_2PO_4	Baker 4008-01
LB Agar, Miller	Fisher BP1425-500
LB Broth	Fisher BP1426-500
Methanol	Fisher A452-4
NaCl	Baker 4058-05
$Na_{2\ h}PO_4$	Sigma S-7907
Non-Fat Dry Milk	Bio-Rad 170-6404
NP40	Calbiochem 492016

Penicillin-Streptomycin	Cellgro 30-002-CI
Precision Plus Protein™ Kaleidoscope™ Standards	BIO-RAD 161-0375
Protease inhibitor cocktail tablets	Complete 11697498001
Puromycin	Calbiochem 540222
Tris-HCl	Quality Biological 351-007-101
Trypsin EDTA, 1X	Cellgro 25-052-C
Tween20	Calbiochem 655204

Appendix II: Buffer, solution and gel

5X loading buffer	
50X proteinase inhibitor solution	1 Protease inhibitor cocktail tablet, 1 mL 1XPBS
10X PBS	80 g NaCl, 10 g KCl, 72 g $Na_{2\ h}PO_4$, 12 g KH_2PO_4, 1 L dd H_20
Cell culture medium	500 mL DMEM medium, 50 mL Fetal Bovine Serum, 5 mL Penicillin-Streptomycin
Blocking buffer	5 g Non-Fat Dry Milk, 100 mL TBS-T
LB broth	25 g/L LB powder, autoclave
LB agar plate	40 g/ L LB Agar, Miller, autoclave
Lysis buffer	50 mM Tris-HCl (7.4), 250 mM NaCl, 5 mM EDTA, 0.1% NP40
Protein gel	Criterion™ Precast Gel 345-0033
Running buffer	100 mL 10XTGS, 900 mL ddH_20
TBS-T	100 mL 10XTBS, 900 mL ddH_20, 10 mL 10% Tween20
Transfer buffer	700 mL ddH_20, 100 mL 10XTG,200 mL methanol, 5 mL 20% SDS

Appendix III: Commercial kits

BCA™ Protein Assay Kit	Thermo JI124811
PolyFect® Transfection Reagent	Qiagen 301107
PureYield™ Plasmid Maxiprep System	Promega A2393
SuperSignal® West Pico Chemiluninescent Substrate	Thermo IF113677
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo IF129579

Appendix IV: Equipments and facilities used

Balance	Denver Instrument S-403
Biological Safety Cabinet	Thermo 109578
CO2 incubator	Thermo 3595
FLUOstar OPTIMA	BMG 413-3128
Isotemp Air Bath	Fisher 11-715-1250
Isotemp Water Bath	Fisher 15-462-01
Legen RT centrifuge	Thermo 75004377
Optic Microscope	Motic AE21
PowerPac™ HC	Bio-Rad 043BR18642
Rocker	VWR 12620-906
Roto-Shake Genie	Scientific Industries S1-1100
Vortex-Genie	Scientific Industries 2-401968

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