12-19-2017

DNA Base Excision Repair and Double Strand Break Repair in Human Fibroblast

Mingyang Li
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DNA BASE EXCISION REPAIR AND DOUBLE STRAND BREAK REPAIR IN HUMAN FIBROBLAST

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical and Veterinary Medical Sciences through The Department of Comparative Biomedical Sciences

by

Mingyang Li
B.S., Shenyang Pharmaceutical University, 2008
May 2018
ACKNOWLEDGEMENTS

In the first place, I would like to express the deepest appreciation to my main Professor Dr. Shisheng Li, who has strong motivation and high dedication for scientific research. He guides me to explore the new findings by using new technologies and methods. He is always striving to make me be a good independent scientist in every aspect of laboratory research, with persistent and careful guidance.

I also would like to thank my committee members, Dr. Masami Yoshimura, Dr. Shaomian Yao and Dr. Alison McFarland for their helpful discussion and suggestions for my project.

Furthermore, I am grateful to all of the laboratory members, Dr. Wentao Li, Dr. Kathiresan Selvam, Mr. Tengyu Ko and Mr. Sheikh Arafatur Rahman, who helped me throughout my scientific research, from skills of scientific methods to the troubleshooting of the problems during the experiments. Beside of them, I also want to appreciate all of the suggestions from the faculty, and all of the help of administration and staff of the department.

Finally, I would like to say thank you to my parents who give me endless love and support throughout my life, especially during this period of pursuing Ph.D. degree.
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LIST OF ABBREVIATIONS

3MeA ................................................................. \( N^3 \)-methyladenine
4-OHT ............................................................... 4-Hydroxytamoxifen
7MeG ................................................................. \( N^7 \)-methylguanine
8-oxoG ............................................................... 7, 8-dihydro-8-oxoguanine
ALS4 .............................................................. amyotrophic lateral sclerosis 4
AOA2 .............................................................. ataxia-ocular apraxia 2
AP ................................................................. apurinic/apyrimidinic
APE1 ............................................................ AP endonuclease 1
ATM .............................................................. ataxia telangiectasia mutant
ATP .............................................................. adenosine triphosphate
ATR .............................................................. ataxia telangiectasia and rad3-related
BER .............................................................. base excision repair
Cas9 ............................................................... CRISPR associated protein 9
CDK .............................................................. cyclin-dependent kinase
CRISPR ........................................................ clustered regularly interspaced short palindromic repeats
DMS .............................................................. dimethyl sulfate
dsDNA ........................................................... double-stranded DNA
dRP ............................................................... deoxyribose phosphate
DDR .............................................................. DNA damage response
DSB .............................................................. double-strand break
FAM .............................................................. fluorescein amidite
H\(_2\)O\(_2\) ............................................................ hydrogen peroxide
hAAG ........................................................... human alkyladenine glycosylase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>IR</td>
<td>ionizing radiation</td>
</tr>
<tr>
<td>K/O</td>
<td>knockout</td>
</tr>
<tr>
<td>LAF-Seq</td>
<td>lesion-adjointing fragment sequencing</td>
</tr>
<tr>
<td>LM-PCR</td>
<td>ligation-mediated PCR</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpurine</td>
</tr>
<tr>
<td>NTS</td>
<td>nontranscribed strand</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-oxoguanine glycosylase</td>
</tr>
<tr>
<td>OTX</td>
<td>O-(Tetrahydro-2H-pyr an-2-yl)hydroxylamine</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pol β</td>
<td>polymerase beta</td>
</tr>
<tr>
<td>Pol λ</td>
<td>polymerase lambda</td>
</tr>
<tr>
<td>Pol μ</td>
<td>polymerase mu</td>
</tr>
<tr>
<td>RC-A</td>
<td>reverse complement of adapter A</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DNA</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S_N2</td>
<td>substitution nucleophilic bi-molecular</td>
</tr>
<tr>
<td>snRNAs</td>
<td>small nuclear RNAs</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TS</td>
<td>transcribed strand</td>
</tr>
</tbody>
</table>
UV…………………………………………………………………………………….. ultraviolet
WT…………………………………………………………………………………….. wild type
XRCC4…………………………………………………………………………… X-ray cross-complementing protein 4
XR-seq……………………………………………………………………………… excision repair sequencing
ABSTRACT

In eukaryotes, DNA repair mechanisms detect and repair damaged DNA. DNA damage is primarily caused by a variety of exogenous and endogenous sources. Several types of damage to DNA are repaired by different kinds of DNA repair pathways. This dissertation focused on repair of N-methylpurines (NMPs) and double-strand breaks (DSBs) in human fibroblasts.

NMPs, including $N^7$-methylguanine (7MeG) and $N^3$-methyladenine (3MeA), can be induced by environmental methylating agents (e.g. the soil fumigant methyl bromide), chemotherapeutics (e.g. nitrogen mustards), and natural cellular methyl donors like S-adenosylmethionine. In human cells, NMPs are repaired by the multi-step base excision repair pathway initiated by human alkyladenine glycosylase (hAAG). Repair of NMPs has been shown to be affected by DNA sequence contexts. However, the nature of the sequence contexts has been poorly understood. We developed a sensitive method, LAF-Seq (Lesion-Adjoining Fragment Sequencing), which allows nucleotide-resolution digital mapping of DNA damage and repair in multiple genomic fragments of interest in human cells. We also developed a strategy that allows accurate measurement of the excision kinetics of NMP bases in vitro. We demonstrate that 3MeAs are induced to a much lower level by the Sn2 methylating agent dimethyl sulfate (DMS) and repaired much faster than 7MeGs in human fibroblasts. Induction of 7MeGs by DMS is affected by nearest-neighbor nucleotides, being enhanced at sites neighbored by a G or T on the 3’ side, but impaired at sites neighbored by a G on the 5’ side. Repair of 7MeGs is also affected by nearest-neighbor nucleotides, being slow if the lesions are between purines, especially Gs, and fast if the lesions are between pyrimidines, especially Ts. Excision of 7MeG bases from the DNA backbone by hAAG in vitro is similarly affected by nearest-neighbor
nucleotides, suggesting that the effect of nearest-neighbor nucleotides on repair of 7MeGs in the cells is primarily achieved by modulating the initial step of the base excision repair process.

DSBs can be induced by hydrogen peroxide (H₂O₂), endonuclease I-PpoI and ionizing radiation. Senataxin is a putative RNA/DNA helicase. We demonstrate that senataxin facilitates repair of DSBs and modulates the activation of DNA damage response pathway of ATM-Chk2 and ATR-Chk1 upon DSB damage.
CHAPTER 1
LITERATURE REVIEW DNA BASE EXCISION REPAIR AND DOUBLE-STRAND BREAK REPAIR IN MAMMALIAN CELLS

1.1 Introduction

DNA, genetic material inside cells of all organisms, is an informational chemical component which must maintain the high degree of fidelity in order to replicate itself. This macromolecule is constantly under the attack of various endogenous and exogenous sources, resulting in alterations of the molecular structure of this genetic material. Exogenous sources are a few physical agents (UV, ionizing radiation) and a variety of chemical compounds such as dimethyl sulfide. DNA damage caused by endogenous factors includes mainly oxidative damage induced by free radical species, depurinations, depyrimidinations, replication errors, deamination, and single- or double-strand breaks (Friedberg et al., 2006).

DNA repair is a series of processes that recognize and correct any damage to DNA molecules. Different types of DNA damage are recognized and repaired by their corresponding repair pathways. The main DNA repair pathways in human cells include base excision repair, nucleotide excision repair, mismatch repair, homologous recombination, and non-homologous end joining. Defects in DNA repair result in the accumulation of DNA damage, which could lead to premature aging disorders (Coppede & Migliore, 2010) and increased risk of cancer (Romero-Laorden & Castro, 2017).

1.2 DNA Base Excision Repair

Small base lesions such as deamination events, oxidative damage and methylation to DNA molecules cannot distort DNA helix structure significantly. Most of the damage is caused by the spontaneous degeneration of DNA (Lindahl, 1993), although similar damage could also be
induced by exogenous chemicals. Such damage is corrected by base excision repair (BER). The finding of the role of Escherichia coli uracil-DNA glycosylase in 1974 by the Nobel Prize winner Tomas Lindahl sparked the study of BER mechanism (Lindahl, 1974).

Lindahl found the first step of BER, during which the modified base was excised from DNA backbone by a DNA glycosylase, leaving an abasic site (apurinic or apyrimidinic site, AP site) (Figure 1-1). Depending on the type of modifications on base, one of at least 11 distinct DNA glycosylases initiates the BER pathway (Krokan & Bjoras, 2013). In order to locate and recognize the specific base damage, DNA glycosylases gently pinch the DNA while scanning it, with the result that the DNA bends sharply at the positions of instability due to mismatching. This localized DNA distortion, combined with additional pushing by the enzyme, facilitates the damaged base to flip out of the DNA double helix and enter the binding site of the enzyme. If the damaged base fits well in the binding site pocket, it remains inside long enough, leading to its bond to DNA backbone to be cleaved by the enzyme. This event, named “base flipping”, is an essential step in the enzymatic activity of all DNA glycosylases (Kim & Wilson, 2012).
Figure 1-1. Schematic showing the base excision repair pathway. Adapted from Leyns and Gonzalez, 2012.

After base removal by a DNA glycosylase, an AP endonuclease creates a nick on the DNA backbone immediately 5’ to the abasic site, thus leaving a 3’-OH terminus and a non-conventional 5’-deoxyribose phosphate (dRP). APE1, which is the major AP endonuclease in mammalian cells, accounts for more than 95% of the total AP site nick incision activities
(Demple & Sung, 2005). APE1 is essential for the survival of mice, as a mutant of this gene in mice is lethal (Friedberg & Meira, 2006).

Further processing needs either short-patch (a single nucleotide cap is filled) or long patch (a gap of 2-10 nucleotides is filled) to finish the BER. Short-patch repair is generally considered the dominant pathway in proliferating and non-proliferating cells, while long-patch repair is used mainly in proliferating cells especially during S phase of cell cycle. The choice of these two pathways also depends on the type of glycosylase used in the first step (Fortini, Parlanti, Sidorkina, Laval, & Dogliotti, 1999). In short-patch repair, Pol β incorporates one correct nucleotide and converts 5’-dRP to ligatable 5’-phosphate required for ligation through its phosphodiesterase activity (Beard & Wilson, 2006). Lig III seals the nick in the presence of XRCC1 (X-ray cross-complementing protein 1) which interacts with Lig III to enhance stabilization and ligation. However, 5’-dRP cannot be removed by Pol β, if it has been oxidized for instance (Sung & Demple, 2006). Then long-patch repair can displace the 5’-dRP-containing strand by gap filling of 2-10 nucleotides and remove the 5’-dRP by flap endonuclease 1 (FEN1). Lig I, instead of Lig III, is used for the final ligation. PCNA is an essential partner protein of FEN1 and Lig I to stimulate their activities (Strzalka & Ziemienowicz, 2011).

1.3 Double-Strand Break Repair

Double-strand breaks (DSBs) are one of the most hazardous types of DNA damage. Inefficient repair of DSBs can lead to cell death, chromosomal aberrations associated with potential cancers such as breast and ovarian cancers, and genomic instability syndromes (Khanna & Jackson, 2001; van Gent, Hoeijmakers, & Kanaar, 2001). Therefore, repair of DSBs is
important for cell survival and maintaining genome integrity. DSBs can be introduced by a variety of sources, including endogenous factors such as reactive oxygen species created by biological metabolism system, and exogenous factors such as ionizing radiation and chemotherapeutic agents (Hoeijmakers, 2001). Repair of DSBs is mainly through two kinds of pathways, which are homologous recombination (HR) and non-homologous end joining (NHEJ). The main differences of these two pathways are: 1. Fidelity of the repaired product, meaning that HR produces error-free repaired DNA sequence, by utilizing the homologous sequence region of the undamaged sister chromatid as a template for DNA synthesis. In contrast, NHEJ normally is error-prone, by direct ligation of the broken ends. 2. Timing, meaning that HR is restricted to occur during late-S and G2 cell cycle phase due to high CDK activity (Johnson & Shapiro, 2010), however, NHEJ predominantly exists in all phases of cell cycle.

1.3.1 Homologous recombination (HR)

During S and G2 phases, DSBs are repaired by HR, promoted by the activity of cyclin-dependent kinase 2 (CDK2). MRE11/RAD50/NBS1 complex (MRN) quickly binds to the sites of DSBs (Figure 1-2) via its globular domain, in which MRE11 and NBS1 interact with Walker A and Walker B motifs of RAD50 (Hopfner et al., 2001), holding two DNA ends to each other (Stracker & Petrini, 2011). After binding to DSBs ends, the serine-threonine protein kinase ATM (ataxia telangiectasia mutated) is recruited by MRN complex (Uziel et al., 2003). ATM is activated by autophosphorylating its serine 1981 site (So, Davis, & Chen, 2009) and then signals its cascade by phosphorylating a group of downstream protein substrates including BRCA1 (breast cancer 1), Chk2 (checkpoint kinase 2) and p53, resulting in cell cycle checkpoint, activation of DNA repair, and potential programmed cell death. Mutations in ATM cause ataxia telangiectasia, a neurodegenerative disease with poor coordination, showing high sensitivity to
ionizing radiation and preventing repair of DSBs (Savitsky et al., 1995). MRE11 mutations also cause ataxia telangiectasia-like disorder (Stewart et al., 1999).

Figure 1-2. Schematic showing HR pathway. Adapted from Hosoya & Miyagawa, 2014; Y. Saito, Fujimoto, & Kobayashi, 2013.
CtIP (CtBP[carboxy-terminal-binding protein]-interacting protein) is permitted to recruit to DSBs through the activated ATM and interacts with NBS1 of MRN complex. CtIP is then phosphorylated by CDK2 during S and G2 phases of cell cycle, driving HR repair of DSBs (Huertas & Jackson, 2009).

The MRN/CtIP complex initiates resection on DNA DSB ends including bulky adducts or covalent changes (Buis et al., 2008) from 5’ to 3’ direction, leaving a 3’ overhang tail. However, for extended 5’-3’ resection to create 3’ overhang strand searching for homology template, it requires the combined functions of a few partnering proteins, either BLM-DNA2-RPA-MRN or EXO1-BLM-RPA-MRN (Nimonkar et al., 2011). In both resection machineries, the helicase BLM (Bloom’s syndrome protein) is needed to promote the endonuclease activity of DNA2 (DNA replication helicase/nuclease 2) or the exonuclease activity of Exo1 (Exonuclease 1). Exo1 itself has a relatively weak 5’-3’ exonuclease activity, therefore partnering with other proteins in the complex is essential for the resection. RPA (replication protein A) is loaded onto the 3’ overhang single-stranded DNA to control the extent and improve polarity, preventing DNA from hybridizing itself to form secondary structures like hairpins and therefore increases 5’-3’ end resection processivity of Exo1 or DNA2 (H. Chen, Lisby, & Symington, 2013). MRE11’s nuclease activity is not required for the resection, but MRN complex is needed for providing the scaffolding domain to interact with Exo1 and DNA2. It is unknown why the resection needs two different machineries, even if both need the combination of MRN, RPA and BLM. The distinct activities of Exo1 and DNA2 may suggest different targets for processing DSB ends.

ATR (ataxia telangiectasia-mutated and Rad3-related) is an essential protein kinase of phosphoinositide (pi) 3-kinase family beside ATM. It is a master checkpoint regulator to protect genome integrity similar as ATM. ATR-ATRIP complex is localized to the RPA-coated ssDNA
strand and activated by autophosphorylation at its threonine 1989 (Liu et al., 2011), which is then recognized by TopBP1 to further facilitate ATR substrate recognition. Chk1 (checkpoint kinase 2) is the downstream substrate of ATR. Upon phosphorylation by ATR, it is triggered to phosphorylate a group of substrate proteins, leading to the activation of DNA damage checkpoint, DNA repair and potential cell death (Patil, Pabla, & Dong, 2013). More evidence emerges for the correlation between DNA damage response (DDR) signaling pathway (ATM-Chk2 and ATR-Chk1) and DNA repair pathway (HR and NHEJ) (Riballo et al., 2004; H. Wang, Wang, Powell, Iliakis, & Wang, 2004). These studies at least suggest that some steps of HR and NHEJ are mediated in ATR or ATM-dependent manners.

After resection finishes, BRCA1 is localized to CtIP of which the serine 327 has been phosphorylated by CDK2, resulting in MRN, CtIP and BRCA1 complex (L. Chen, Nievera, Lee, & Wu, 2008). BRCA2 is then recruited to BRCA1, which in turn binds to PALB2, the nuclear localizer of BRCA2 (Sy, Huen, & Chen, 2009). Mutations in BRCA1, BRCA2 and PALB2 lead to high risk of breast and ovarian cancers (Petrucelli, Daly, & Feldman, 2010). BRCA2 assists RAD51 to be loaded onto the 3’ overhang ssDNA, at the same time displacing RPA. A nucleoprotein filament is constructed by loading around six monomers of RAD51 per one DNA helical turn (Reymer, Frykholm, Morimatsu, Takahashi, & Norden, 2009). The Rad51-coated filament of single-stranded DNA attaches to double-stranded DNA and searches for the homologous sequence fragment. The recombinase Rad51 is the key component for strand exchange in HR. The strand exchange and D-loop formation are also facilitated by BRCA2 and PALB2 (Buisson et al., 2010). Rad51 is then dissociated from dsDNA, the product of DNA strand exchange, via its ATPase activity, facilitated by Rad54 (X. Li et al., 2007). From the 3’
end of invading strand, DNA synthesis is executed by polymerase eta to recover the lost sequence, using the template of the homologous strand (McIlwraith et al., 2005).

For producing cross-over recombinants, the endonucleases MLH1-MLH3 heterodimer binds to holiday junctions, creating single-strand breaks in the double-stranded DNA (Ranjha, Anand, & Cejka, 2014). The resulting nicks can be easily repaired by DNA ligase (Chan & West, 2015).

Table 1-1. Main proteins involved in homologous recombination in mammalian cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Biochemical activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRE11</td>
<td>Nuclease, component of MRN complex</td>
</tr>
<tr>
<td>Nbs1</td>
<td>Nuclear localization, component of MRN complex</td>
</tr>
<tr>
<td>Rad50</td>
<td>ATPase, component of MRN complex</td>
</tr>
<tr>
<td>ATM</td>
<td>Kinase, signaling cascade</td>
</tr>
<tr>
<td>CtIP</td>
<td>DNA binding, promote MRN’s nuclease activity</td>
</tr>
<tr>
<td>RPA</td>
<td>Binding ssDNA, promote resection</td>
</tr>
<tr>
<td>ATR</td>
<td>Kinase, signaling cascade</td>
</tr>
<tr>
<td>Exo1</td>
<td>Exonuclease 5’-3’</td>
</tr>
<tr>
<td>DNA2</td>
<td>Helicase, endonuclease</td>
</tr>
<tr>
<td>BLM</td>
<td>Helicase, facilitate the activity of Exo1 and DNA2</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>PALB2</td>
<td>binding to BRCA1, nuclear localizer of BRCA2</td>
</tr>
<tr>
<td>Rad51</td>
<td>Binding ssDNA, formation of nucleoprotein filament, ATPase</td>
</tr>
<tr>
<td>Pol eta</td>
<td>Polymerase, DNA synthesis</td>
</tr>
<tr>
<td>MLH1-MLH3</td>
<td>Heterodimer, endonucleases, resolution of holiday junctions</td>
</tr>
</tbody>
</table>

1.3.2 Non-homologous end joining (NHEJ)

Repair of DSBs by NHEJ occurs throughout all phases of cell cycle, simply by joining both broken ends together (Figure 1-3). However, broken ends with damaged nucleotides need to be first removed and then ligated back.
The first step of NHEJ is recognition of DSBs by Ku, the heterodimer of Ku70 and Ku80, which each contains a central DNA binding core (Walker, Corpina, & Goldberg, 2001). Due to the structure of the binding core, Ku tethers both of DSB ends together with high affinity and no obvious sequence specificity (Downs & Jackson, 2004). Binding of Ku to the damage site elicits
a group of essential NHEJ repair proteins to be recruited. Once bound, Ku recruits DNA-PKcs (DNA-dependent protein kinase catalytic subunit) at the extreme termini of broken ends, making itself translocate inward from the broken ends (Yoo & Dynan, 1999). Inhibition of the serine-threonine protein kinase DNA-PKcs can enhance radiosensitivity of cells and inhibit DSB repair, which could make DNA-PKcs considered a possible therapeutic target (O'Connor, Martin, & Smith, 2007; Yang et al., 2015). Two DNA-PKcs molecules at terminals interact with each other across DSBs, forming a synaptic complex (DeFazio, Stansel, Griffith, & Chu, 2002), which activates the kinase activity of DNA-PKcs. The DNA-PKcs-Ku-DSB complex tethers the ends of DSBs tightly to prevent nucleolytic degradation by nucleases. The following autophosphorylation of DNA-PKcs produces conformational changes which displace itself from DNA ends (K. Meek, Dang, & Lees-Miller, 2008). This dissociation allows other essential NHEJ proteins to be recruited and process the DNA ends.

After broken DNA ends have been recognized and secured, the following step of NHEJ is the processing of the ends to remove any non-ligatable groups. Depending on the structure of the end groups, different combinations of enzymes are required to remove the corresponding blocking ends, damaged nucleotides, or secondary structures surrounding the break site. NHEJ repairs the DSBs without needing the template strand, and processing of the broken ends may insert or delete some nucleotides, resulting in NHEJ to be an error-prone repair machinery. DNA-PKcs at least partially regulates the step of processing the broken ends and losing some nucleotide sequence (Ding et al., 2003). There are some main enzymes involved in processing the ends, including Artemis, DNA polynucleotide kinase/phosphatase (PNKP), polymerases mu (Pol μ) and lambda (Pol λ). Artemis itself has a 5’-3’ exonuclease activity. Upon forming the complex with DNA-PKcs, Artemis acquires endonuclease activity in the presence of ATP to
cleave ds or ssDNA transitions such as 5’ or 3’ ssDNA overhang, and hairpins (Y. Ma, Pannicke, Schwarz, & Lieber, 2002). Artemis-deficient mice have a similar phenotype to that of DNA-PKcs-deficient ones, including high sensitivity to ionizing radiation, and deficiency in opening hairpin ends (Rooney et al., 2002). Artemis is recruited to DSB sites by DNA-PKcs through phosphorylation (Soubeyrand et al., 2006). However, even though cells lacking Artemis have elevated sensitivity to ionizing radiation, they only have a subtle deficiency in DSB repair, suggesting that Artemis may only repair a small subset of DSBs such as hairpins in vivo (J. Wang et al., 2005). Processing the broken ends may lead to lose nucleotides and create a gap, which needs to be filled in by the X Family of DNA polymerases Pol λ, Pol μ or terminal transferase (Tdt). The choice of usage of different polymerases depends on the length of gap and likely presence of microhomology (Yamtich & Sweasy, 2010). TdT is restricted to express in lymphoid, and its function may only be limited to V(D)J recombination (Nick McElhinny & Ramsden, 2004), whereas Pol λ and Pol μ are expressed in most types of cells and considered to play essential roles in NHEJ (Lieber, 2010). Pol λ and Pol μ interact with Ku and XRCC4 (X-ray cross-complementing protein 4) to be recruited to DSBs (Mueller et al., 2008). The main difference of them in NHEJ is the requirement of DNA template strand. The Pol λ is more dependent on template for gap-filling reactions, whereas Pol μ is relatively less dependent (Nick McElhinny et al., 2005). PNKP has 5’-DNA kinase and 3’-phosphatase activities. It plays an important role to remove non-ligatable end group from DSB ends, converting 5’-OH and 3’-phosphate DNA end groups into the ligatable forms of 5’-phosphate and 3’-OH groups. PNKP interacts with XRCC4, to be recruited to DSB sites (Koch et al., 2004). Knockdown of PNKP in human cells exhibits elevated sensitivity to ionizing radiation and slower repair of radiation-induced DSBs (Rasouli-Nia, Karimi-Busheeri, & Weinfeld, 2004).
After the broken ends have been processed and converted into ligatable ends, ligation needs to be performed as the last step of NHEJ. Ligation is carried out by ligase IV, forming the complex with XRCC4, called X4-L4 complex. Ku recruits this ligation complex to the DSB sites (Nick McElhinny, Snowden, McCarville, & Ramsden, 2000). XRCC4 itself serves as a scaffolding protein to facilitate the recruitment of other NHEJ proteins, without any enzymatic activities. It stabilizes and stimulates the activity of ligase IV (Grawunder et al., 1997). XLF (XRCC-like factor) is also required for NHEJ (Ahnesorg, Smith, & Jackson, 2006). It has a similar structure to XRCC4 and interacts with XRCC4 to form dimers via their head domains (Wu et al., 2011). XLF stimulates the ligation activity of X4-L4 complex (Lu, Pannicke, Schwarz, & Lieber, 2007).

Table 1-2. Main proteins involved in non-homologous end joining in mammalian cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Biochemical activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ku</td>
<td>DSB end binding</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>Artemis</td>
<td>Endonuclease, exonuclease</td>
</tr>
<tr>
<td>Polλ</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>Polμ</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>PNKP</td>
<td>Polynucleotide kinase/phosphatase</td>
</tr>
<tr>
<td>Ligase IV</td>
<td>DNA ligase</td>
</tr>
<tr>
<td>XRCC4</td>
<td>partner of ligase IV</td>
</tr>
<tr>
<td>XLF</td>
<td>DNA binding, stimulates ligation activity</td>
</tr>
</tbody>
</table>

1.4 References


CHAPTER 2
HIGH-RESOLUTION DIGITAL MAPPING OF N-METHYLPURINES IN HUMAN CELLS REVEALS MODULATION OF THEIR INDUCTION AND REPAIR BY NEAREST-NEIGHBOR NUCLEOTIDES

2.1 Introduction

Alkylation damage to DNA can be caused by environmental or chemotherapeutic alkylating agents, or by natural cellular methyl donors such as S-adenosylmethionine (Fu, Calvo, & Samson, 2012). Simple $S_N2$ methylating agents, such as dimethyl sulfate (DMS), produce a variety of damaged bases in DNA, of which $N$-methylpurines (NMPs), including $N^7$-methylguanine (7MeG) and $N^3$-methyladenine (3MeA), constitute ~90% of the lesions (Wyatt & Pittman, 2006). NMPs are repaired by the multi-step base excision repair (BER) pathway. In human cells, the BER process is initiated by human alkyladenine glycosylase (hAAG), which recognizes and excises the methylated purine bases (Fu et al., 2012). The apurinic/apyrimidinic (AP) sites formed are typically recognized by an AP endonuclease that cleaves the DNA on the 5’ side of the AP sites. The repair process concludes following DNA repair synthesis and ligation. BER of NMPs plays an important role in protecting the genome, and at the same time confounds cancer alkylation therapies, by excising the cytotoxic lesions.

The efficiency of BER can be affected by local DNA sequences (Donigan & Sweasy, 2009). The different efficiencies may have implications for genome stability, as lesions that

persist longer in DNA have a greater chance of leading to mutagenesis. It was demonstrated that the rates of NMP repair in the PGK1 gene of human cells are highly heterogeneous and can be affected by some DNA sequence contexts (Ye, Holmquist, & O'Connor, 1998). However, until now, the exact nature of the sequence contexts that affect the repair of NMPs in human cells has been poorly understood. Also, the excision kinetics of NMP bases have been difficult to measure in vitro because the methylated bases are labile to spontaneous depurination and the AP sites formed after excision of the methylated bases are labile to spontaneous strand cleavage. This difficulty has made it impossible to unambiguously address the question as to whether the heterogeneity of NMP repair is caused by the excision of the methylated bases or by a later step of the BER process.

Multiple methods have been developed to map the formation and repair of DNA adducts in living cells (Henderson, 2005; Vaughan, 2000). Broadly speaking, these methods can be grouped into genome overall level, gene/DNA fragment level and nucleotide level. The nucleotide level methods can be powerful for delineating the modulation of DNA adduct formation and repair by various cellular elements, such as DNA sequence context, chromatin structure, epigenetic modifications and certain cellular processes (e.g. transcription) (S. Li, Waters, & Smerdon, 2000). However, most of the nucleotide-level methods are only sensitive enough for mapping DNA adduct formation and repair in prokaryotes and lower eukaryotes (i.e. organisms with small genomes) or in multi-copy sequences (e.g. those in mitochondria) of mammalian cells. Indeed, until very recently, ligation-mediated PCR (LM-PCR) had been the only method that allowed nucleotide level mapping of DNA lesions in single-copy sequences in mammalian cells (Besaratinia & Pfeifer, 2012). However, the LM-PCR technique is unsuitable for large scale analyses of DNA damage and repair in the cell. Also, the gel bands corresponding to DNA
lesions cannot always be well separated, and the lesions cannot be precisely counted. Very recently, a high-throughput method, called XR-seq (excision repair sequencing), which allows mapping of nucleotide excision repair (NER) of UV photoproducts (cis-syn cyclobutane pyrimidine dimers and 6-4 photoproduct) in human cells was developed (Hu, Adar, Selby, Lieb, & Sancar, 2015). This method can provide a snapshot measurement of UV photoproducts-containing oligonucleotides (~ 30 nucleotides) that are excised during NER. As it relies on immunoprecipitation of the excised oligonucleotides that transiently exist in the cell, the XR-Seq method is not suitable for accurately measuring the kinetics of NER. Also, the XR-Seq method cannot be used for mapping the formation and repair of DNA lesions that are not NER substrates.

Here, we report the development of a sensitive method, LAF-Seq (Lesion-Adjoining Fragment Sequencing), which allows nucleotide-resolution digital mapping of DNA damage and repair in multiple genomic fragments of interest in human cells. This method is suitable for mapping any type of DNA lesions, provided that they can be converted into DNA single- or double-strand breaks after isolation of total genomic DNA. We also developed a strategy that allows accurate measurement of the excision kinetics of NMP bases in vitro. We demonstrate that 3MeAs are induced to a much lower level by DMS and repaired much faster than 7MeGs in human fibroblasts. Induction of 7MeGs is affected by nearest-neighbor nucleotides, being enhanced at sites neighbored by a G or T on the 3’ side, but impaired at sites neighbored by a G on the 5’ side. Repair of 7MeGs is also affected by nearest-neighbor nucleotides, being slow if the lesions are between purines, especially Gs, and fast if the lesions are between pyrimidines, especially Ts. Furthermore, we show that the excision of 7MeG bases by hAAG in vitro is similarly affected by nearest-neighbor nucleotides, suggesting that the effect of nearest-neighbor
nucleotides on repair of 7MeGs in human cells is primarily achieved by modulating the initial step of the BER process, namely the excision of 7MeG bases by hAAG.

2.2 Materials and Methods

2.2.1 Cell culture, DMS treatment and DNA isolation

Telomerase-immortalized human foreskin fibroblast R2F/TERT cells (Rheinwald et al., 2002) were cultured in DMEM/F12 medium supplemented with 15% newborn calf serum and 10 ng/ml of epidermal growth factor. A fraction of the cultured cells was saved as control, and the rest were treated with 0.005% (v/v) DMS for 10 minutes. This treatment induced ~ 1 NMP per 10 kb of genomic DNA and caused no obvious cell killing. The cells were washed twice with phosphate buffered saline (PBS) containing 0.005% (v/v) β-mercaptoethanol, washed twice with PBS, and then replenished with fresh medium and incubated at 37°C. At 0, 6, 12, 24 hours of the incubation, an aliquot of the cells was harvested. Each aliquot (10 million cells) was resuspended in 2 ml of nuclei isolation buffer (10 mM Tris-Cl, 10 mM NaCl, 3 mM MgCl₂, 0.5 % NP-40, pH 7.9) and incubated on ice for 30 minutes. The nuclei were pelleted by centrifugation, resuspended in 10 ml of lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 400 mM NaCl, 1% SDS, 10 μg/ml of proteinase K, pH 8.0) and incubated at 65°C for 12 hours. In addition to releasing DNA, this 12-hour incubation at 65°C caused complete depurination of NMP bases from the DNA backbone. The AP sites formed might be further cleaved by spontaneous β elimination or β and δ eliminations, leaving an unsaturated sugar or a phosphate at the 3’ ends of the cleaved sites. Each of the samples was mixed with 5 ml of 5 M NaCl and incubated on ice overnight. The samples were centrifuged at 12,000 rpm for 30 min at 4°C and the supernatant was collected. The genomic DNA was precipitated with isopropanol.
2.2.2 Construction and sequencing of libraries of DNA fragments of interest

Six µg of genomic DNA of each sample (DMS-treated and untreated) were digested with 12 units of MseI and 3 units of endonuclease IV (New England Biolabs) in 100 µl of NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT, 100 µg/ml BSA, pH 7.9) overnight. MseI digestion releases the three fragments encompassing exons 11 (281 bp) and 15 (264 bp) of the $BRAF$ gene and the coding exon 1 (228 bp) of the $NRAS$ gene. In addition to incising the DNA on the 5’ side of the AP sites formed after spontaneous depurination of NMPs, endonuclease IV also removes unsaturated sugar or phosphate groups from the 3’ ends of the DNA (Demple, Johnson, & Fung, 1986). Each of the samples was added with NaCl to a final concentration of 1 M and a mixture (0.5 pmol each) of biotinylated oligonucleotides (Table 2-1). The samples were heated at 95°C for 5 min and then incubated at 50°C for 30 minutes. Ten µl of streptavidin magnetic beads (Dynabeads M-280 Streptavidin, Life Technologies) were added to each of the samples and incubated at room temperature for 30 minutes. The beads were washed with STES (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 7.5) at room temperature and with STE (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5) at 55°C. To estimate the efficiencies of fishing out the fragments of interest, a small fraction of the beads was resuspended in 25% ammonium hydroxide. The eluted fragments of interest (contained in the supernatants) were vacuum-dried and quantified by real-time PCR (using SYBR Select Master Mix from Life Technologies).
Table 2-1. Oligonucleotides used for construction and sequencing of libraries of DNA fragments of interest. NTS, nontranscribed strand; TS, transcribed strand.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5’ → 3’)</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapter P1</td>
<td>CCTCTCTATGGGCAGTCGGTGAT</td>
<td>PCR amplification and sequencing of fragment libraries.</td>
</tr>
<tr>
<td>Adapter A</td>
<td>CCATCTCATCCCTGCGTGTC</td>
<td>PCR amplification and sequencing of fragment libraries.</td>
</tr>
<tr>
<td>RC-A1</td>
<td>Phosphate-ATCGTTACCTTAGTGAGTCGGAGACACGCA GGATGAGATGG-inverted T</td>
<td>Reverse Complement of Adapter A with barcode 1</td>
</tr>
<tr>
<td>RC-A2</td>
<td>Phosphate-ATCGTTCTCTCTTAGTGAGTCGGAGACACGCA GGATGAGATGG-inverted T</td>
<td>Reverse Complement of Adapter A with barcode 2</td>
</tr>
<tr>
<td>RC-A3</td>
<td>Phosphate-ATCGGATCTCTCTCTTAGTGAGTCGGAGACACGCA GGATGAGATGG-inverted T</td>
<td>Reverse Complement of Adapter A with barcode 3</td>
</tr>
<tr>
<td>RC-A4</td>
<td>Phosphate-ATCGATCTTGGTACTGAGTCGGAGACACGCA GGATGAGATGG-inverted T</td>
<td>Reverse Complement of Adapter A with barcode 4</td>
</tr>
<tr>
<td>RC-A5</td>
<td>Phosphate-ATCGTTCTCTCTCTTAGTGAGTCGGAGACACGCA GGATGAGATGG-inverted T</td>
<td>Reverse Complement of Adapter A with barcode 5</td>
</tr>
<tr>
<td>BRAF11-1</td>
<td>AGGGATACAGGAAGATCCCCCTTAATCACCGACTGCATAGAGAGG-biotin</td>
<td>Fishing out the NTS of BRAF exon 11; assisting ligation of adapter P1.</td>
</tr>
<tr>
<td>BRAF11-2</td>
<td>GTGACATTGTGACAAGTCATTTAATGAGATATGTATGCTAATACCATAGAGAGG-biotin</td>
<td>Fishing out the TS of BRAF exon 11; assisting ligation of adapter P1.</td>
</tr>
<tr>
<td>BRAF15-1</td>
<td>GAGTTTAGGTAAGATCTAATTTCCTCTATAATTCTGTAATATAATATATCTTTTAATCACCAGACTGCATAGAGAGG-biotin</td>
<td>Fishing out the NTS of BRAF exon 15; assisting ligation of adapter P1.</td>
</tr>
<tr>
<td>BRAF15-2</td>
<td>GGTTAGATGGGCTATTATTTTCTACTGATATTACCGACTGCCCATAGAGAGG-biotin</td>
<td>Fishing out the TS of BRAF exon 15; assisting ligation of adapter P1.</td>
</tr>
<tr>
<td>NRAS1-1</td>
<td>CGTTGAGAATTTGGAGAGCATATGTTTCTATCATTCTGTAATTATATATGAGAGGAGAGG-biotin</td>
<td>Fishing out NTS of NRAS coding exon 1; assisting ligation of adapter P1.</td>
</tr>
<tr>
<td>NRAS1-2</td>
<td>TGCTGGGATGACACTCTCTCTATGATCTGTAATTATATATGAGAGGAGAGG-biotin</td>
<td>Fishing out the TS of NRAS coding exon 1; assisting ligation of adapter P1.</td>
</tr>
</tbody>
</table>

To ligate adapter RC-Ai (Table 2-1) to the 3’ ends of the fragments of interest, the beads were resuspended in 10 µl of CircLigase buffer (50 mM MOPS, pH 7.5, 20% PEG 8000, 10 mM KCl, 5 mM MgCl₂, 0.5 M betaine, 20 µM ATP, 2.5 mM MnCl₂, 200 µg/ml BSA, 1 mM DTT), which we have extensively optimized to allow efficient ligation of single-stranded DNA molecules by CircLigase (Epicentre) (Blondal et al., 2005). Two hundred pmol of RC-Ai and 10 units of CircLigase were added to each of the samples and incubated at 55°C for 1 hour. The beads were washed with STES at room temperature, binding and washing buffer with SDS (10
mM Tris, 1 M NaCl, 1 mM EDTA, 0.5% SDS, pH8.0) at 55°C and Tris-EDTA buffer (pH 8.0) at room temperature. The RC-Ai-ligated fragments were eluted from the beads by using 25% ammonium hydroxide and vacuum-dried.

To ligate adapter P1 to the 5’ ends of the fragments of interest, the DNA samples were dissolved in 50 µl of T7 DNA ligation buffer (66 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 7.5% PEG 6000, pH7.6) containing a mixture (0.25 pmol each) of the biotinylated oligonucleotides and 20 pmol of adapter P1 (Table 2-1). The samples were heated at 95°C for 5 min, 50°C for 30 minutes and then cooled to room temperature. The samples were added with 3 units of T7 DNA ligase (Molecular Cloning Laboratories) and incubated at room temperature for 20 minutes. Each of the samples was added with 50 µl of 2x binding and washing buffer (20 mM Tris, 2 M NaCl, 2 mM EDTA, pH8.0) and 10 µl of streptavidin magnetic beads and incubated at room temperature for 30 minutes. The beads were washed with STES, Tris-EDTA buffer (pH 8.0) and H₂O at room temperature. The adapter-ligated fragments were eluted from the beads by using 25% ammonium hydroxide and vacuum-dried. The efficiencies of adapters RC-Ai and P1 ligations were estimated by real time PCR using a small fraction of the eluted fragments as templates.

The libraries of DNA fragments were amplified by 10 – 14 cycles of PCR, using Herculase II Fusion DNA polymerase (Agilent Technologies) and adapters A and P1 (Table 2-1) as primers. The qualities and quantities of the libraries were analyzed by using a Bioanalyzer (Agilent). The barcoded libraries were then pooled, and the sequencing templates were prepared on Ion Sphere particles by using the Ion OneTouch 2 System. The templates were loaded onto Ion 318 chips and sequenced from the RC-Ai-ligated ends (by “reading” the complementary strand in the 5’ to 3’ direction) on an Ion Torrent personal genome machine.
2.2.3 Analysis of sequencing data

The sequencing reads were sorted according to their barcodes by using the Torrent Suite software. After being trimmed of barcode sequences, the reads were aligned to the sequences of the fragments of interest by using Bowtie 2 in Galaxy (https://usegalaxy.org). As the NMPs were induced to a relatively low level (~ 1 NMP per 10 kb of DNA), the majority (≥ 97%) of the reads correspond to the complementary strands of the full-length (undamaged) fragments of interest. A small fraction of the reads (≤ 3%) corresponds to the complementary strands of the fragments of interest adjoining the NMP sites. The total numbers of reads from the DMS-treated samples were normalized to those from the control (not treated with DMS) samples. The 5’ ends of the reads that align to the internal sites of the fragments of interest would correspond to the NMPs induced or remaining (at different times of repair) at sites on the complementary strands. The reads whose 5’ ends align to the same sites of the fragments of interest were counted. To remove background signals, the read counts aligned to different sites of the fragments of interest from the DMS-treated samples were deducted by the corresponding counts from the control samples. Only those sites with > 10 counts of lesions at 0 hour of repair were analyzed for NMP repair.

2.2.4 Excision of 7MeGs between different nucleotides by hAAG in vitro

Eighty pmol of each of the substrates (Figure 2-9A) was treated with 0.6% (v/v) DMS in a total volume of 100 µl of Tris-EDTA buffer (pH 8.0) at room temperature for 15 min, which induced ~ 0.05 7MeG per FAM labeled fragment of the substrates. The DMS treated substrates were purified and dissolved in 80 µl of ThermoPol buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 100 µg/ml BSA, 0.1% Triton X-100, pH 8.8 at 25°C). Two pmol of purified hAAG (New England Biolabs) were added to each of the samples and incubated at 37°C. At different times of the incubation, an aliquot of 10 µl (10 pmol of DNA)
was taken, and the DNA was purified. Each of the aliquots was then treated with 100 mM O-(Tetrahydro-2H-pyr-an-2-yl)hydroxylamine (OTX) (Sigma) in a total volume of 25 µl STE at 37°C for 1 hour. Each of the aliquots (10 pmol of DNA) was then treated with 1.2 pmol of hAAG and an excess amount of APE1 (New England Biolabs) at 37°C for 3 hours to excise all the remaining NMPs and completely cleave the DNA at the resulting unprotected AP sites.

To analyze the samples on a Genetic Analyzer (3130xl, Applied Biosystems), 0.1 pmol of each of the samples was loaded into an 80-cm capillary column filled with POP-6 polymer. The FSA binary files generated were converted to text files by using BatchExtract (ftp://ftp.ncbi.nih.gov/pub/forensics/BATCHEXTRACT). The fluorescent signals were then analyzed by using Microsoft Excel.

2.3 Results

2.3.1 Method development

We reasoned that DNA lesions at specific sites of the genome could be digitally mapped if single-stranded DNA fragments adjoining the lesions could be fished out, sequenced and counted. Exons 11 and 15 of the BRAF gene and the coding exon 1 of the NRAS gene contain frequent mutations in cutaneous melanoma and other human cancers (Hocker & Tsao, 2007). We chose to develop the method by mapping NMP induction and repair in both strands of genomic DNA fragments encompassing these exons.

Human fibroblasts were treated with DMS to induce a low level of NMPs in the genomic DNA (~ 1 NMP per 10 kb of DNA). Following different times of repair incubation, aliquots were taken and total genomic DNA was isolated. DNA samples from the DMS-treated cells at different times of repair and from the untreated cells was separately cut with a restriction enzyme to release the fragments of interest and cleaved at the NMP sites (Figure 2-1). The fragments of
interest were simultaneously fished out from each of the samples by using excess copies of a mixture of biotinylated oligonucleotides that are complementary to the 5’ end region of the fragments of interest (Table 2-1). The efficiency of fishing-out the genomic fragments of interest from the total genomic DNA was around 70% (Figure 2-2). To allow sequencing by the Ion Torrent personal genome machine, adapter RC-Ai (Reverse Complement of adapter Ai, “i” stands for a barcode number) and adapter P1 were ligated to the 3’ and 5’ ends of the fragments of interest, respectively. Each of the samples was ligated with specifically barcoded RC-Ai. Under our optimized conditions, over 90% of the fished-out fragments of interest were ligated to adapters RC-Ai and P1 (Figure 2-3). The adapter-ligated fragments with different barcodes were pooled and sequenced simultaneously from the adapter RC-Ai ligated ends (by “reading” the complementary strand in the 5’ to 3’ direction) on Ion Torrent 318 chips, which typically generate 5 – 6 million high quality sequencing reads each run (Figure 2-4). Most of the sequencing reads can be aligned to the reference sequences of the fragments of interest (94 and 99% for the two sequencing runs, respectively). The low read counts aligned to specific sites of the fragments of interest from the control (untreated with DMS) samples reflect background signals, which were deducted from the corresponding sites from the DMS-treated samples. NMPs induced (at 0 hour of repair) or remaining (at different times of repair) at specific sites of the fragments of interest were then counted by tallying the sequencing reads whose ends align to the respective damaged sites.
Figure 2-1. Construction of a sequencing library of DNA fragments that are full-length (undamaged) or adjoining the lesion sites. Red circles represent lesions induced initially or remaining after different times of repair. Dashed line box indicates a fragment that can be released by restriction digestion. Gray circles marked with B denote the biotin group. The 3’ (pink) and 5’ (black) regions of the biotinylated oligonucleotides are complementary to adapter P1 and the 5’ end region of fragments of interest, respectively. Adapter RC-Ai (“i” stands for a barcode number) is an oligonucleotide whose 5’ end is phosphorylated and 3’ end blocked by an inverted dT. The 5’ region (cyan) of RC-Ai is a barcode sequence (i). The 3’ region (brown) of RC-Ai is complementary to adapter A. See Table 2-1 for sequences of the oligonucleotides.
Figure 2-2. Estimation of efficiencies of fishing out the fragment of interest. A and B, schematic representation of the experiment for real-time PCR using restricted genomic DNA (A) versus fished-out template (B). Fa and Fb are PCR primers. Oligo, oligonucleotides. C, examples of real-time PCR results. Amplifications of the fragment encompassing exon 15 of the BRAF gene are shown. The templates used for each of the repeats were 100 ng of the restricted genomic DNA or the fragment fished out from the equivalent of 100 ng of the restricted genomic DNA. D, gel showing products of the real-time PCR reactions (after 40 cycles) shown in C. M, DNA size maker.
Figure 2-3. Estimation of ligation efficiencies of adapters RC-Ai and P1 to the fished-out fragments of interest. A, schematic showing real-time PCR of the ligated fragment with the indicated primer pairs. B, examples of real-time PCR results. Shown here are amplifications of the fragment encompassing exon 15 of the *BRAF* gene ligated to adapters RC-A1 and P1. The templates used for each of the repeats were fragments fished out from the equivalent of 100 ng of the restricted genomic DNA. C, gel showing products of the real-time PCR reactions (after 40 cycles) shown in B. M, DNA size marker.

<table>
<thead>
<tr>
<th>Ligation</th>
<th>Primer pair</th>
<th>PCR length (bp)</th>
<th>Repeat</th>
<th>Ct value</th>
<th>Calculated number of starting template molecules</th>
<th>Ligation efficiency</th>
<th>Mean ligation efficiency (±SD)</th>
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<tr>
<td>RC-A1</td>
<td>A-Fa</td>
<td>270</td>
<td>1</td>
<td>22.75</td>
<td>24807</td>
<td>0.930</td>
<td>0.932 (±0.017)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>22.89</td>
<td>22440</td>
<td>0.917</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>22.92</td>
<td>21963</td>
<td>0.950</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>P1-Fb</td>
<td>273</td>
<td>1</td>
<td>23.95</td>
<td>26224</td>
<td>0.984</td>
<td>0.964 (±0.024)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>24.15</td>
<td>22920</td>
<td>0.936</td>
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<tr>
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<td>3</td>
<td>24.18</td>
<td>22462</td>
<td>0.972</td>
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</tr>
<tr>
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<td>23.46</td>
<td>26662</td>
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<td>3</td>
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<td>23119</td>
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</tr>
</tbody>
</table>
Figure 2-4. Summary of sequencing runs and alignments. A and B, statistics of one sequencing run and alignment of the sequencing reads to DNA fragments of interest. C and D, statistics of another sequencing run and alignment of the sequencing reads to DNA fragments of interest. ISP, Ion Sphere Particle (on which the sequencing templates were prepared).
2.3.2 Induction and repair of NMPs in human cells

Lesion counts at different sites of the fragments analyzed are shown in Figure 2-5, 2-6 and 2-7. As can be seen, most (~ 90%) of the NMPs induced by DMS were 7MeGs and a small fraction (~ 10%) were 3MeAs. For five out of six strands of the three fragments analyzed, NMPs that can be detected are located in sites that are $\geq 2 – 9$ nucleotides from the regions that are complementary to the biotinylated oligonucleotides used to fish out the fragments (Figure 2-1). This is consistent with the fact that CircLigase we used to ligate adapter RC-Ai to the 3’ end of the fragments of interest can only ligate single-stranded but not double-stranded DNA (Blondal et al., 2005). However, we detected 7MeGs at a site of the region that is complementary to BRAF11-2 (Table 2-1). This site is complementary to the 5th nucleotide (from the 5’ end) of BRAF11-2 and is located in the transcribed strand of the fragment encompassing exon 11 of the BRAF gene (Figure 2-5, see the “g” site that is the second nucleotide from the 5’ end of the transcribed strand). Presumably, the fragment adjoining 7MeG at this site was ligated to adapter RC-Ai due to “breathing” of double-stranded DNA. The “breathing” at 55°C (the temperature we used for ligation of RC-Ai) might be frequent but did not cause detachment of the fragment from the biotinylated BRAF11-2.
Figure 2-5. Lesion counts in the genomic DNA fragment encompassing exon 11 of the *BRAF* gene in human fibroblasts. Bottom and top panels show the transcribed and nontranscribed strands, respectively. Bars in the panels denote lesion counts per $10^5$ DNA molecules at the indicated sites at 0 (blue), 6 (red), 12 (cyan), and 24 (violet) h of repair incubation. Nucleotide positions are numbered from “A” in the start codon (ATG) of the DNA sequences present in the mature mRNA. Arrows indicate sites that are frequently mutated ($\geq 10$ recurrence in human cancers; based on the COSMIC). Nucleotides underneath the arrows indicate common nucleotide changes at the mutation sites.
Figure 2-6. Lesion counts in the genomic DNA fragment encompassing exon 15 of the \textit{BRAF} gene in human fibroblasts. Bottom and top panels show the transcribed and nontranscribed strands, respectively. Bars in the panels denote lesion counts per $10^5$ DNA molecules at the indicated sites at 0 (blue), 6 (red), 12 (cyan), and 24 (violet) h of repair incubation. Nucleotide positions are numbered from “A” in the start codon (ATG) of the DNA sequences present in the mature mRNA. Arrows indicate sites that are frequently mutated ($\geq 10$ recurrence in human cancers; based on the COSMIC). Nucleotides underneath the arrows indicate common nucleotide changes at the mutation sites.
Figure 2-7. Lesion counts in the genomic DNA fragment encompassing coding exon 1 of the NRAS gene in human fibroblasts. Bottom and top panels show the transcribed and nontranscribed strands, respectively. Bars in the panels denote lesion counts per 10^5 DNA molecules at the indicated sites at 0 (blue), 6 (red), 12 (cyan), and 24 (violet) h of repair incubation. Nucleotide positions are numbered from “A” in the start codon (ATG) of the DNA sequences present in the mature mRNA. Arrows indicate sites that are frequently mutated (≥ 10 recurrence in human cancers; based on the COSMIC). Nucleotides underneath the arrows indicate common nucleotide changes at the mutation sites.
In addition to inducing the predominant NMPs, DMS induces a small fraction (0.3%) of $O^6$-methylguanine, which is stable (Wyatt & Pittman, 2006) and cannot be converted to a strand break and detected by the LAF-Seq method. DMS also induces ~10 other minor adducts (e.g. $N^\prime$-methyladenine, $N^7$-methyladenine, $N^3$-methylguanine and $O^2$-methylcytosine), some of which are heat-labile (Wyatt & Pittman, 2006) and may be detected by the LAF-Seq method. However, as together they combined comprise < 5% of the total lesions induced by DMS (Wyatt & Pittman, 2006), the levels of the minor adducts were expected to be marginal when compared with NMPs we detected here.

The induction levels and repair rates of NMPs were highly heterogeneous. However, the heterogeneity did not seem to correlate with the hotspots of carcinogenic mutations in these fragments documented in the Catalogue of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic) (Figure 2-5, 2-6 and 2-7), suggesting that NMPs may not particularly cause more mutations at these hotspots. According to data deposited in the UCSC Genome Browser (https://genome.ucsc.edu), some weakly positioned nucleosomes are present in the fragments we analyzed. The repair rates did not seem to significantly correlate with these weakly positioned nucleosomes (not shown).

7MeGs were induced to higher levels at sites of aGg, aGt, tGg and tGt, but to lower levels at sites of gGa, gGc, gGg and gGt (Gs are the sites of 7MeG formation, and lowercase letters denote nearest-neighbor nucleotides) (Figure 2-8A, compare 0 h lesion levels at the different sites). It appears that 7MeG induction was enhanced at sites neighbored by a G or T on the 3’ side, but impaired at sites neighbored by a G on the 5’ side. In G tracts (with ≥ 2 continuous Gs), 7MeG induction tended to be high at the 5’-most G site but low at the other G sites (Figure 2-5, 2-6, 2-7 and 2-8A).
The 7MeG counts at some sites, especially those between purines (As or Gs), were higher after certain times of repair than those at 0 hour of repair (Figure 2-5, 2-6, 2-7, 2-8A and B), presumably reflecting certain levels of continued induction of the lesions after DMS was removed from the medium and slow repair at these sites. Indeed, repair of 7MeGs appeared to be significantly affected by nearest-neighbor nucleotides, being slow for the lesions between purines, especially Gs, and fast for the lesions between pyrimidines, especially Ts (Figure 2-8A, B, C and D). 7MeGs neighbored by a pyrimidine on one side and a purine on the other were repaired at intermediate rates. In contrast, the second- or third-nearest nucleotides did not seem to significantly affect the rate of 7MeG repair (data not shown). The average repair rate of all 3MeAs in these fragments was much faster than that of 7MeGs (Figure 2-8E). Indeed, the average half-life of 3MeAs was ~ 7 hours, whereas that of 7MeGs was ~ 22 hours.
Figure 2-8. NMPs remaining at different times of repair. A, 7MeG counts (means ± standard deviation) at sites with different nearest-neighbor nucleotides. Gs indicate 7MeG sites, and lowercase letters denote nearest-neighbor nucleotides. The levels of 7MeG at the cGt site are not shown because <10 7MeGs were induced at the sole cGt site in all the three fragments analyzed. No error bars are shown for the cGc site as >10 7MeGs were induced at only one of three cGc sites in all the three fragments analyzed. The levels of 7MeG induced (0 h of repair) at aGg, aGt, tGg, and tGt sites were significantly higher than those at gGa, gGc, gGg, and gGt sites (p < 0.01, Student’s t test). B, the percentage of 7MeGs remaining (mean) at sites with different nearest-neighbor nucleotides. The values at sites of fast repair (tGt and tGc) are significantly different from those at sites of slow repair (aGg, gGg, gGa, aGa) at all the time points of repair (p < 0.05, Student’s t test). C, 7MeG counts (mean ± standard deviation) at sites neighbored by purines on both sides (rGr), by a purine on one side and a pyrimidine on the other (rGy/yGr), and by pyrimidines on both sides (yGy). D, the percentage of 7MeGs remaining (mean) at sites of rGr, rGy/yGr, and yGy. The value at each of the repair time points is significantly different from one (figure caption continued)
context of the neighbor nucleotides to any of the other two (p < 0.01, Student’s t test). E, the percentage of 3MeAs and 7MeGs remaining (mean). The percentage of 3MeAs remaining is significantly different from the percentage of 7MeGs remaining at each of the repair time-points (p < 0.01, Student’s t test).

2.3.3 Excision of 7MeGs between different nucleotides by purified hAAG in vitro

The rates of 7MeG repair we measured in the cells reflect the speeds of the whole BER process. We wondered whether the heterogeneity of 7MeG repair in the cells was caused by the initial step (i.e. the excision of 7MeG bases) of the BER process. The excision kinetics of 7MeG bases by purified hAAG had been difficult to measure because 7MeG bases are labile to spontaneous depurination and the AP sites formed after excision of the 7MeG bases are labile to spontaneous strand cleavage, especially at an elevated temperature. We therefore developed a new strategy for the measurement (Figure 2-9). All the DNA substrates we used were identical except for the 2 nucleotides neighboring the G in the middle of the 5’-FAM-labeled strand (Figure 2-9A). The substrates were treated with DMS to induce ~ 0.05 7MeG per FAM-labeled fragment of the substrates. The inductions of 7MeGs by DMS are largely random events, although they may be modulated by certain DNA sequences. At an induction level of 0.05 7MeG/fragment, the chance that the fragment molecules may contain > 1 lesion is very low (0.12%). The DMS-treated substrates were incubated with a limited amount of hAAG. At different times of the incubation, aliquots were taken and the AP sites formed after excision of the damaged bases were stabilized by treatment with OTX. AP sites stabilized by OTX are resistant to cleavage by heat and the human AP endonuclease APE1 (Luke & Nakamura, 2012; Rosa, Fortini, Karran, Bignami, & Dogliotti, 1991). The DNA was then completely cleaved at the remaining 7MeG sites by extended incubation with excess amounts of hAAG and APE1. The DNA fragments were analyzed by capillary electrophoresis on a 3130xl Genetic Analyzer, which
is commonly used for routine DNA sequencing. We found that, when compared with DNA sequencing gels, the capillary electrophoresis has a much higher resolution in separating bands of DNA fragments, and allows much more sensitive detection (the limit is ~ 0.1 fmol or $6 \times 10^7$ of FAM labeled molecules for detecting a band) and accurate quantification of the bands.

Figure 2-9. Experimental design for measuring the excision of 7MeG bases by hAAG in vitro. A, DNA substrates. 7MeGs formed at the underlined Gs neighbored by G (gGg), A (aGa), C (cGc), or T (tGt) in the FAM-labeled strand were intended for analysis. B, schematic outlining the process of the analysis. Open circles denote 7MeGs formed in the FAM-labeled strand. Open triangles indicate OTX-reacted AP sites formed after excision of the 7MeG bases.
As can be seen in Figure 2-10, 7MeG bases between purines, especially Gs, were excised much slower than those between pyrimidines, especially Ts. The trend of nearest-neighbor nucleotide effect on excision of 7MeG bases by hAAG in vitro resembles that on 7MeG repair in human cells. The overall excision rates of 7MeG bases in vitro under our conditions were much faster than those of 7MeG repair in the cells, presumably because 1) a relatively low level of hAAG existed in the cell and/or 2) the rates of 7MeG repair we measured in the cell reflected the whole process of BER, rather than just the excision of the damaged bases. The scattered distribution of 7MeGs and the dynamics of DNA structure in the genome might also contribute to the slow repair of 7MeGs in the cell. These results support the idea that the effect of nearest-neighbor nucleotides on repair of 7MeGs in human cells is primarily achieved by modulating the initial step of the repair process, namely the excision of the damaged bases by hAAG.
Figure 2-10. Measurement of the excision of 7MeG bases by hAAG in vitro. A–D, overlays of FAM signals in capillary columns loaded with samples that had been treated with a limited amount of hAAG for the indicated lengths (in min) of time. FAM-labeled DNA substrates (Figure 2-9A) containing 7MeGs neighbored by G (gGg), A (aGa), C (cGc), or T (tGt) were treated with a limited amount of hAAG for different lengths of time. The resulting AP sites were stabilized by treatment with OTX. The DNA substrates were then cleaved at all the remaining 7MeG sites and subjected to capillary electrophoresis. The nucleotide positions of the peaks are indicated at the bottom of each of the panels. The underlined Gs in red indicate the sites of 7MeG bases that were intended for analysis. E, the percentage of 7MeG bases remaining. The values are averages of three measurements. The values are significantly different from one context of the neighbor nucleotides to any of the other three (p < 0.01, Student’s t test).
2.4 Discussion

We developed the LAF-Seq method for high-resolution digital mapping of NMP induction and repair in human cells. Compared to currently available gel-based methods, the LAF-Seq method has several advantages. First, the LAF-Seq method is highly sensitive. We mapped lesions that were induced to a level of ~ 1 per 10 kb of DNA. Second, the LAF-Seq method is much less labor-intensive, especially for simultaneously mapping DNA damage and repair in multiple sequences of the genome. Although we have tested fishing out and mapping three fragments of interest, there is no reason why more fragments cannot be fished out and analyzed simultaneously. Third, the LAF-Seq method can be used to achieve true nucleotide resolution mapping and digital quantification of DNA lesions. This contrasts to gel based methods where bands cannot always be well separated and lesions cannot be precisely counted. Furthermore, the LAF-Seq method should be able to be used for mapping other types of DNA lesions if they can be converted to single- or double-strand breaks after the genomic DNA is isolated from the cells. Many enzymes are available that can specifically incise DNA at the sites of different lesions. For example, the bifunctional DNA glycosylases FPG and endonuclease VIII can excise oxidized purine and pyrimidine bases, respectively, and incise the DNA 3’ (through β-elimination) and 5’ (through δ-elimination) to the resulting AP sites, leaving a phosphate group at the 3’ end (Prakash, Doublie, & Wallace, 2012). On the other hand, T4 endonuclease V can incise DNA at cyclobutane pyrimidine dimers, resulting in an unsaturated sugar at the 3’ end (Dodson, Michaels, & Lloyd, 1994). To make the 3’ ends ligatable to adaptor RC-Ai, the lesion-incised DNA samples can be treated with endonuclease IV, which seems to be able to remove essentially any non-OH groups at the 3’ ends of DNA formed after incision by different damage-specific enzymes (Demple et al., 1986). However, certain fragments cannot be fished out from the
genomic DNA due to lack of appropriate restriction sites. Therefore, although it can be used for simultaneous mapping of DNA lesions in multiple restriction fragments, the LAF-Seq method is not suitable for genome-wide mapping of DNA lesions.

The $N^7$ position of Gs faces the major groove of DNA and has a high electrostatic potential, which is the major reason why this position is preferentially methylated by such $S_N$2 methylating agents as DMS (Wyatt & Pittman, 2006). It was reported that DMS preferentially methylates the 5’ end G in G runs, presumably due to the highest electrostatic potential at the $N^7$ position of the 5’ end G (Cloutier, Drouin, & Castonguay, 1999). Our result that 7MeG induction is high at the 5’-most G site but low at the other G sites in a G tract agrees well with the previous report. However, different from the previous report showing that an A present in a G run did not affect the damage pattern, we found that an A present in a G run will disrupt the pattern (i.e. $N^7$-methylation of a G 3’ to the A is not impaired by the G(s) 5’ to the A (Figure 2-5, 2-6 and 2-7)). We also found that 7MeG induction was enhanced at sites neighbored by a G or T on the 3’ side. The effects of the 3’ neighbor nucleotides on the 7MeG induction may also be due to modulation of the electrostatic potential. It can be predicted that the electrostatic potential at the $N^7$ position of a G can be reinforced by a 3’ G or T (Pullman & Pullman, 1981; Weiner, Langridge, Blaney, Schaefer, & Kollman, 1982).

Each human cell contains thousands of copies of mitochondrial DNA (Miller, Rosenfeldt, Zhang, Linnane, & Nagley, 2003). Using a gel-based method that is only sensitive enough for mapping DNA damage in multi-copy sequences in the human genome, we previously found that repair of 7MeGs in human mitochondria is affected by nearest-neighbor nucleotides, being slow for the lesions between purines and fast for the lesions between pyrimidines (S. Li, 2011). It was found recently that hAAG is present in human mitochondria and associates with mitochondrial
single-stranded DNA binding protein (van Loon & Samson, 2013). Taken together with our results, it appears that excision of 7MeG bases by hAAG may be similarly affected by neighbor nucleotides in the nucleus and mitochondria, although the local environments can be quite different.

The crystal structure of hAAG showed that a damaged base is flipped from the DNA base stack into a sequestered active site pocket where the glycosidic bond between the damaged base and the deoxyribose is hydrolyzed (Lau, Scharer, Samson, Verdi, & Ellenberger, 1998). The stability of base stacking between two adjacent bases follows the order: purine–purine $>>$ purine–pyrimidine $>$ pyrimidine–purine $>$ pyrimidine–pyrimidine, with a 2-kcal/mol free energy spread between the most stable purine-purine and the least stable pyrimidine-pyrimidine base stacks (R. A. Friedman & Honig, 1995). Under the physiological pH, a 7MeG base can be positively charged (protonated) (Ruszczynska et al., 2003). The positive charge may increase base stacking through the cation-$\pi$ interaction (Mahadevi & Sastry, 2013). To date, the free energies of base stacking between 7MeG and normal purines or pyrimidines have not been documented. It is likely that a 7MeG base has more stable stacking with purines than with pyrimidines. The different base stacking stabilities may affect the flipping of the 7MeG base by hAAG, thereby modulating the excision of the damaged bases. Indeed, it has been shown that hAAG is exquisitely sensitive to the structural context of a deoxyinosine lesion (an uncharged substrate of hAAG) and an inverse correlation between duplex stability and catalytic efficiency was observed (Lyons & O'Brien, 2009).

The overall repair of NMPs in the yeast Saccharomyces cerevisiae (S. Li & Smerdon, 1999, 2002) appears to be much faster than that in the human cells we reported here. Interestingly, however, repair of 7MeGs in the yeast has also been found to be affected by
nearest-neighbor nucleotides, being slow if they are between purines and fast if they are between pyrimidines (S. Li & Smerdon, 1999, 2002). Repair of NMPs in the yeast is solely initiated by Mag1, the homolog of hAAG (S. Li & Smerdon, 2002). The crystal structure of the yeast Mag1 complexed with its substrate has not been reported. The nearest-neighbor nucleotides appear to affect hAAG and Mag1 similarly in excising the damaged bases.

Similar to hAAG, most DNA glycosylases have converged on a single mechanistic solution for damaged base recognition and excision: flip of the damaged base from the DNA base stack into a sequestered active site pocket (J. I. Friedman & Stivers, 2010). The activities of some but not all glycosylases have been shown to be modulated to some extent by DNA sequence context [for a review, see (Donigan & Sweasy, 2009)]. Interestingly, altering either the global DNA sequence or the 5’-flanking base pair failed to influence the excision of 7,8-dihydro-8-oxoguanine (8-oxoG) by human 8-oxoguanine glycosylase (OGG1) in vitro (Sassa, Beard, Prasad, & Wilson, 2012). However, an 8-oxoG located in the CAGGGC[8-oxoG]GACTG motif is poorly excised by OGG1 (Allgayer, Kitsera, von der Lippen, Epe, & Khobta, 2013). Therefore, the excision of 8-oxoG by OGG1 does not seem to be significantly affected by base-stacking with nearest-neighbor nucleotides. It seems that the activities of alkyladenine glycosylases (e.g. hAAG and Mag1) are more dramatically modulated by nearest-neighbor nucleotides than those of any other DNA glycosylases analyzed so far.

2.5 References


CHAPTER 3
THE ROLE OF SENATAXIN IN DOUBLE-STRAND BREAK REPAIR IN HUMAN FIBROBLAST

3.1 Introduction

DNA double-strand breaks (DSBs), the most detrimental and cytotoxic type of DNA damage, are induced either by exogenous sources such as ionizing radiation and chemotherapeutic drugs including cisplatin and mitomycin C, or endogenous factors, including free radicals caused by metabolic reactions and DNA abnormal structures that result from replication stress (Mehta & Haber, 2014). During many biological processes, DSBs can also occur during V(D)J recombination (Jackson & Jeggo, 1995), meiosis I (de Massy, 2013), and immunoglobulin class switching recombination of B lymphocytes (Jolly, Cook, & Manis, 2008).

DSBs are repaired in two distinct pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). HR leads to a higher fidelity of repair by searching for homology templates, and it occurs in the S and G2 phases of the cell cycle, whereas NHEJ, which is viewed as error-prone, often results in base changes and short deletions or insertions and functions throughout all phases. The divergent step between these two pathways is end resection and is regulated by the cell cycle phase in which the cyclin-dependent kinase (CDK) can activate the exonuclease Exo1 to enforce end resection (Tomimatsu et al., 2014). The choice of the pathways also depends on the competition between BRCA1 and p53-binding protein 1 (53BP1) (Daley & Sung, 2014).

Senataxin was first discovered as a novel protein in the year 2004 when new types of inherited rare neurodegenerative diseases, autosomal recessive ataxia-ocular apraxia 2 (AOA2) (Moreira et al., 2004) and a familial form of amyotrophic lateral sclerosis (ALS4) (Y. Z. Chen et
al., 2004) were identified. These studies identified causative genetic mutations in the gene SETX, which encodes senataxin. SETX received its name from its yeast homolog Sen1, which has been widely studied (X. Chen, Muller, Sundling, & Brow, 2014). Patients with either AOA2 or ALS4 have an early childhood onset of difficulties with muscle control and movements and require lifelong medical services (Anheim et al., 2009; Rabin et al., 1999).

After SETX mutations were identified, it was observed that a common region of the RNA/DNA helicase domain near the C-terminal was shared by both senataxin and Sen1p, and this domain was found to be essential for survival in the yeast Saccharomyces cerevisiae (Doris Ursic, DeMarini, & Culbertson, 1995). Sen1 was originally referred to as a splicing endonuclease of the introns of all 10 families of precursor tRNAs (DeMarini, Winey, Ursic, Webb, & Culbertson, 1992). Further studies revealed that it was required for processing a diverse variety of non-coding RNA classes, including tRNAs, rRNAs, and small nuclear and nucleolar RNAs (Rasmussen & Culbertson, 1998; D. Ursic, Himmel, Gurley, Webb, & Culbertson, 1997). Sen1 interacts with the RNA-binding protein Nrd1 and Nab3 to form the complex NRD which directly interacts with the C terminal domain of RNA Pol II and functions in the transcription termination of non-coding RNAs (Eric J. Steinmetz, Conrad, Brow, & Corden, 2001) and in protein coding mRNA in a fail-safe manner (Rondon, Mischo, Kawauchi, & Proudfoot, 2009). Moreover, it can control the distribution of RNA Pol II genome-wide (E. J. Steinmetz et al., 2006). Sen1 was also found to contribute to protecting genomes from instability, as it was evidenced by recent studies that it facilitates transcription-coupled repair (W. Li, Selvam, Rahman, & Li, 2016) and revolves the R loops resulting from the RNA-DNA hybrid during transcription, preventing transcription-associated recombination (Mischo et al., 2011).
It was hypothesized by researchers that the key functions of Sen1 were likely expected to occur in human senataxin. Several studies aimed to prove this hypothesis; however, it was difficult to extrapolate the well-studied Sen1-related transcription termination process to mammalian systems in which the key complex of NRD in yeast is not well conserved in higher eukaryotes, as there is no human homolog of Nab3 which is required for recruiting Sen1 in the NRD-dependent RNA Pol II transcription termination (Kuehner, Pearson, & Moore, 2011). In addition, it was discovered that senataxin was not involved in the termination of snRNA (Suraweera et al., 2009); however, recent studies have revealed the key retained roles of human senataxin. The SUMOylation of senataxin facilitates its interaction with a subunit of an exosome to target transcription-induced DNA damage (Richard, Feng, & Manley, 2013). The R-loop formed at the G-rich pause sites can be resolved by senataxin with the assistance of exonuclease Xrn2’s transcript degradation, leading to RNA Pol II termination (Skourti-Stathaki, Proudfoot, & Gromak, 2011).

The role of senataxin implicated in DSB repair was suggested by a research study, which detected a higher amount of phosphorylated histone H2AX (γH2AX) foci, the marker of DSBs, in response to hydrogen peroxide (H$_2$O$_2$) in the senataxin mutant cells isolated from AOA2 patients compared with that of control cells (Suraweera et al., 2007). Another study showed that during the meiotic recombination of the spermatocytes of Setx$^{-/-}$ male mice, the initiated DSBs, as indicated by the staining of γH2AX foci, persistently remained unrepai red on the synapsed autosomes at the pachytene stage compared to Setx$^{+/+}$ (Becherel et al., 2013). Until now, no direct tests have proven the effect of senataxin on DSB repair. In this article, evidence is provided that senataxin plays an essential role in DSB repair.
3.2 Materials and Methods

3.2.1 Plasmid construction

For the construction of pLentiCRISPR-LoxP-GFP-SETX-ex3 (Figure 3-1) in the CRISPR/Cas9 knockout, the best sgRNA (single guide RNA) target region of SETX exon3 for avoiding an off-target knockout was chosen by using the CRISPR design tool online (http://crispr.mit.edu/). Plasmid lentiCRISPR v2 (Addgene, #52961) was used as a template, and two PCR products amplified by the primer pairs of lenti-lox-crispr-F1/lenti-lox-crispr-R1 and lenti-lox-crispr-F2/lenti-lox-crispr-R2 (all sequences of the primers used are shown in Table 3-1) were digested with AvrII/XhoI and BamHI/XhoI, respectively, and ligated with the vector backbone of BamHI/AvrII-digested lentiCRISPR v2 to create pLentiCRISPR-LoxP. Next, two PCR products amplified by the primer pair of maxGFP-F/maxGFP-RM with the template of pMax-GFP (Lonza) and by the primer pair of maxGFP-FM and lenti-loxp-crispr-R1 with the template of pLentiCRISPR-LoxP were digested with BamHI/BstEII and BstEII/XhoI, respectively, and ligated with the vector backbone of BamHI/XhoI-digested pLentiCRISPR-LoxP to create pLentiCRISPR-LoxP-GFP. The hybridized oligonucleotides SETX-sgRNA-Ex3A and SETX-sgRNA-Ex3B were then ligated to the vector backbone of Esp3I-digested pLentiCRISPR-LoxP-GFP to create pLentiCRISPR-LoxP-GFP-SETX-ex3. The sequence was confirmed to be correct.
Figure 3-1. Plasmid map of pLentiCRISPR-LoxP-GFP-SETX-ex3.

To construct pLenti-IPpol-BSD (Figure 3-2), the PCR product of the IPpoI cassette amplified by the primer pair of Ippl-1/IppoI-2 (Table 3-1) with the template of pCL20C-ddIPpoI (Addgene, #49053) was digested with AgeI/RsrII and ligated with the vector backbone of AgeI/RsrII-digested pLenti-DBT-BSD to create pLenti-IPpol-BSD. The sequence was confirmed to be correct.
Figure 3-2. Plasmid map of pLenti-I-PpoI-BSD. For the regulation of the expression of I-PpoI, the cassette of I-PpoI contained a destabilization domain (DD, blue) and a hormone-binding domain (HBD, green) upstream of I-PpoI (red). Blasticidin (BSD, black) was used as a selection marker for I-PpoI expressing cells. The whole I-PpoI cassette was inserted into a lentiviral vector.

To construct the PiggyBac transposon vector pPB-SETX-Ex3M (Figure 3-3 B), the mutations on the SETX exon 3 sgRNA target site were introduced to avoid being recognized as well as to avoid a knockout by the CRISPR/Cas9 system. First, two PCR products amplified by the primer pairs of SETX-cDNA1/SETX-cDNA3 and SETX-cDNA2/SETX-cDNA4 (all sequences of the primers used are shown in Table 3-1) with the template of pCR-XL-TOPO-SETX (WT cDNA, Dharmaco) were digested with NotI and ClaI, respectively, and ligated with the vector backbone of NotI/ClaI digested pCR-XL-TOPO-SETX to create pCR-XL-TOPO-SETX-Ex3M. Second, the hybridized oligonucleotides pPB-a1 and pPB-a2 were ligated with the vector backbone of BsrGI-digested pPB-CAG-Klf4-pA-pgk-hph (Addgene, #74906) to create
pPB-a. The cDNA fragment SETX-Ex3M amplified by the primer pair of SETX-c1/SETX-c2 with the template of pCR-XL-TOPO-SETX-Ex3M was then digested with AvrII/NheI and ligated with the vector backbone of AvrII/NheI-digested pPB-a to create pPB-SETX-Ex3M. The sequence was confirmed to be correct.

![Figure 3-3. Plasmid map of pCMV-hyPBase (A) and pPB-SETX-Ex3M (B).](image)

### 3.2.2 Cell lines

Telomerase-immortalized human foreskin fibroblast R2F/TERT cells (Rheinwald et al., 2002) were cultured in DMEM/F12 medium supplemented with 15% newborn calf serum and 10 ng/ml of epidermal growth factor.

The lentivirus of a specific lentiviral construct was produced by the transfection of HEK293T cells by calcium phosphate. After 24 hours, the lentivirus from the supernatant of the HEK293T cells was collected and concentrated by centrifuge.

For the generation of a stable SETX knockout cell line in R2F/TERT via CRISPR/Cas9, R2F/TERT cells were transduced with the lentiviral construct of pLentiCRISPR-LoxP-GFP-
SETX-ex3. After 5 days of transduction, cells were trypsinized, and one single cell expressing GFP was deposited into each well of 384-well plates. P1F/TERT cells were also added as feeder cells to facilitate the growth of a single SETX knockout cell. When confluence was reached, the colonies of SETX-knockout R2F/TERT with a puromycin selection marker were selected by puromycin, and all feeder cells P1F/TERT without a puromycin selection marker were removed. The puromycin-resistant cell clones were further confirmed to contain GFP under a fluorescence microscope. Genomic DNA was then isolated from a set of clones. Each clone’s SETX exon 3 region surrounding the sgRNA target site was PCR amplified individually with SETX3-F1 and SETX3-R1 (Table 3-1, Figure 3-4), and the two resolved bands (two alleles with different types of deletion or insertion) of each PCR product from each clone by agarose gel were sequenced by SETX3-F1. Two cell clones with deletion or/and insertion around the SETX exon 3 region were recognized to be correct knockouts by creating frameshifts in both alleles (Figure 3-4).

![Diagram](image)

Figure 3-4. Schematic showing the deletion or insertion of both alleles in two clones of SETX knockout cells (K/O c1 and K/O c2). The number represents the amount of nucleotides deleted or inserted.
Table 3-1. Primers sequence.

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<th>Name</th>
<th>Sequences (5’ → 3’)</th>
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<tr>
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</tr>
<tr>
<td>SETX3-R1</td>
<td>TGAAGAAGTCAAGAGAAGGAGTCTTCTTCTGAG</td>
</tr>
<tr>
<td>28s-1</td>
<td>CCTCTCTATAGGGCGATGCTGGAAGGTAGCCAAATGCGATGCTGACAGCGATGCTGCTGCTGCTGTC</td>
</tr>
<tr>
<td>28s-2</td>
<td>CCTCTCTATAGGGCGATGCTGGAAGGTAGCCAAATGCGATGCTGCTGCTGCTGTC</td>
</tr>
<tr>
<td>28s-3</td>
<td>TCACCGGTCAGTGAAAAACG</td>
</tr>
</tbody>
</table>

To knockout GFP cassettes of the SETX knockout cell line for use in flow cytometry to detect apoptotic cells, 30% confluent SETX K/O cells containing GFP were transduced with an adeno-associated virus type 2 (AAV2)-based vector construct of pLM-CMV-R-Cre (Addgene, #27546) for one week. After 1 week, almost all of the transduced cells lost GFP under a fluorescence microscope. In the K/O cells transduced with lentiviral vector pLentiCRISPR-LoxP-GFP-SETX-ex3, during reverse transcription of the viral RNA, the U3 region of the
lentiviral 3’ LTR was copied to form the corresponding region of the 5’ LTR. After integration into the host genome, both of 5’ and 3’ LTR contained one LoxP site. The expression of Cre recombinase mediated the recombination of these two LoxP sites, resulting in the deletion of the Cas9-GFP cassette.

For the generation of I-Ppol-expressing cell lines, WT and SETX knockout cells were transduced with the lentiviral construct of pLenti-I-Ppol-BSD (Figure 3-2). After 4 days of transduction, cells expressing I-Ppol-BSD cassettes were selected using 10 μg/ml blasticidin for 1 week.

For the complementation of SETX knockout cells by introducing a wild-type senataxin, PiggyBac transposon vector pPB-SETX-Ex3M and pCMV-hyPBase (Wellcome Trust Sanger Institute, Figure 3-3 A) expressing transposase were transfected into cells with Lipofectamine 3000 (ThermoFisher) according to the manufacturer’s protocol. After 4 days, cells expressing SETX cassettes were selected using 20 μg/ml hygromycin for 1 week.

3.2.3 Test of hydrogen peroxide sensitivity

Cells were treated with 1mM hydrogen peroxide (H₂O₂, Sigma). At 4 and 8 hrs, cells were washed with PBS, trypsinized and resuspended with fresh medium. After a brief spin, the supernatant was removed, and cells were washed again with cold PBS. After another brief spin, supernatant was removed, and cells were stained with Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (ThermoFisher) according to the manufacturer’s protocol. For apoptotic cells, phosphatidyl serine was translocated from the inner to the outer leaflet of the plasma membrane. Annexin V conjugated with Alexa Fluor® 488 can identify apoptotic cells through binding to phosphatidyl serine exposed on the outer leaflet of the plasma membrane. Flow cytometry was performed to detect the percentage of apoptotic cells.
For the analysis of genomic DNA, cells were treated with hydrogen peroxide at different concentrations of 0.5 mM, 2 mM and 8 mM for 1 hour. Cells were then washed with PBS, replenished with fresh medium and incubated at 37 °C. After 24 hours, genomic DNA was isolated from cells, treated with Fpg and Endonuclease VIII (NEB) in NEBuffer 3.1, and resolved on alkaline agarose gel electrophoresis.

3.2.4 Repair analysis of I-PpoI-induced double-strand breaks

The endonuclease I-PpoI recognizes a specific sequence and creates double-strand breaks in the genome. The I-PpoI cassette of I-PpoI-expressing cells contains a destabilization domain where binding of the ligand Shield-1 (Cheminpharma) can protect I-PpoI from proteasomal degradation. It also includes a hormone-binding domain where the binding of 4-Hydroxytamoxifen (4-OHT, Sigma) can translocate I-PpoI to the nucleus, leading to the initiation of the induction of double-strand breaks created by I-PpoI. Cells were treated with 1mM Shield-1 and 2 mM 4-OHT for 5 hours and then washed with PBS and replenished with fresh medium for incubation at 37 °C. At 3, 6, 12 and 24 hours, genomic DNA was isolated. The repair of the I-PpoI-induced double-strand breaks in 28S of ribosomal DNA (rDNA) was measured by the novel method presented in Figure 3-5. The oligonucleotide 28s-1 (all sequences of the primers used are shown in Table 3-1) was designed to have a 3’ blocking (purple dot) and to contain a unique sequence (red line) different from genomic DNA sequence at the 5’ end followed by a complementary sequence (green line) of 28S beginning from the DSB site. Genomic DNA was denatured, annealed with 28s-1, and extended; however, only the 28S fragments, which were cut at the specific DSB site by I-PpoI, were able to perform extension. The resulting reaction mixture was used for quantitative real-time PCR with the primers of 28s-2
The signal of qPCR originated from all the fragments cut specifically at the 28S DSB site.

![Diagram](image)

Figure 3-5. Schematic showing the strategy used to measure I-Ppol-induced double-strand breaks in the 28S region by qPCR. The oligonucleotide 28s-1, from 5'-3', constitutes a segment (red line) of a unique sequence different from the genomic DNA sequence followed by a segment (green line) of a sequence that is complementary to the 28S bottom strand beginning from the double-strand break site and a 3' blocking (purple dot). After annealing of 28s-1, the bottom strand fragment was extended from the double-strand break site to the end (blue dashed line) using the template (red line) of 28s-1. The extended product was used as a template for qPCR with the primer pair of 28s-2 and 28s-3. The sequence of 28s-2 is complementary to the extension segment (blue solid line).

### 3.2.5 Ionizing radiation

Cells attached in 6-well plates were treated with gamma radiation from the radioactive isotope radioisotope cobalt-60 at the dose rate of 2.9 Gray/min for 7 minutes. After radiation, cells were incubated at 37 °C. At 1.5 and 3 hours, the cells were harvested, and the whole cell extracts were prepared for western blot.
3.2.6 Western blot

The whole cell extracts were prepared as follows. Cells from each well of 6-well plates after treatment were washed, collected, and centrifuged at the bottom of a 1.5 ml Eppendorf tube. The supernatant was removed. Each sample (cells from one well of 6-well plates) was vortexed with 100 μl of phenol solution (equilibrated with 10mM Tris HCl, pH 8.0, with 1mM EDTA, Sigma) and 5 μl 2-mercaptoethanol (Fisher) for 15 minutes. Then, 1.2 ml of methanol containing 0.1 M ammonium acetate was added and mixed well with each sample. After 30 minutes of 14,000 rpm centrifuge at 4 °C, the supernatant was removed from the protein pellet. The pellet was washed with 1ml methanol with 0.1 M ammonium acetate once and then with 1ml of 80% cold acetone twice. After removal of any residual supernatant, each pellet was air-dried, dissolved with 200 μl of 2 × SDS gel loading buffer (120 mM Tris-Cl, 4% SDS, 0.2% bromophenol blue, 20% glycerol, pH 6.8) containing 5% 2-mercaptoethanol, and boiled for 5 minutes. The protein concentration was measured by Qubit Protein Assay Kit (ThermoFisher). 10 μg of protein extract were resolved on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Senataxin (Novus), p-ATM S1981(Cell Signaling), ATM (Santa Cruz), p-ATR T1989 (GeneTex), ATR (Santa Cruz), p-Chk2 T68 (Cell Signaling), Chk2 (Cell Signaling), p-Chk1 S345 (GeneTex), Chk1 (Santa Cruz), p-p53 S15 (Cell Signaling), p53 (Cell Signaling), and GAPDH (Santa Cruz) were used as primary antibodies in TBST containing 1% fish gelatin (Sigma). The HRP-coupled goat anti-mouse and anti-rabbit IgG second antibodies (ThermoFisher) were used accordingly. Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific), and the protein bands were detected using
the ChemiDocTM XRS+ System (Bio-Rad). The relative band intensity was quantified by ImageJ.

3.3 Results

3.3.1 The SETX knockout cell clone was confirmed by sequencing and western blot

The SETX knockout cell clone was confirmed to be correct. The sequence result showed that two alleles in the SETX gene were both disrupted (Figure 3-4).

Western blot was performed using an anti-senataxin primary antibody to confirm the disruption of SETX gene expression. The knockout cell clone completely abolished the expression of senataxin (300 kDa protein) compared with WT cells (Figure 3-6).

![Western blot](image)

Figure 3-6. Western blot of the expression level of senataxin in WT and K/O cells. Anti-Rpb1 was used as a loading control of the samples. 10 μg whole cell extract was loaded for each lane.

3.3.2 Deficiency of senataxin dramatically improves resistance to H₂O₂

To measure the sensitivity of WT and K/O cells to H₂O₂, cells were treated with 1mM H₂O₂ continuously for 4 and 8 hours. The apoptotic cells were identified by Annexin V conjugated with Alexa Fluor 488 and counted by flow cytometry. At 4 and 8 hours, the apoptotic cells of WT accounted for a total of 31.00% (±2.27%) and 52.48% (±1.84%), respectively, whereas senataxin-deficient cells presented a significant resistant phenotype to H₂O₂ with a
relatively small fraction of apoptotic cells: 7.84% (±0.29%) at 4 hours, and 7.27% (±0.62%) at 8 hours (Figure 3-7), indicating almost no increasing number of apoptotic cells between 4 and 8 hours. A longer treatment time for H₂O₂ was tested for 24 hours, and WT cells continued to contain an increasingly large number of apoptotic cells; however, knockout cells persistently showed a dramatic resistance phenomenon (results not shown).

Figure 3-7. Plot showing H₂O₂ sensitivity of WT and K/O cells by flow cytometry. Senataxin K/O cells are significantly resistant to 1mM H₂O₂ after 4 and 8-hour treatments compared to WT cells (P < 0.01, Student’s t-test).

To assess the characteristics of nuclear DNA from H₂O₂-treated cells, total genomic DNA was isolated from the cells (including floated dead, dying and live cells in each sample) treated with 0.5, 2 and 8 mM H₂O₂. WT cells showed a higher level of DNA smearing compared to K/O
cells in both Fpg/EndoVIII treated and untreated conditions, as shown by alkaline gel electrophoresis (Figure 3-8). This suggested that far more apoptotic cells existed in WT cells, contributing to more DNA degradation triggered by apoptosis, which is in agreement with the quantification of apoptotic cells by flow cytometry (Figure 3-7).

![Figure 3-8](image)

Figure 3-8. Alkaline gel showing the smears of genomic DNA from cells that were treated with different concentrations of H$_2$O$_2$ for 1 hour and then repaired after 24 hours. The denatured genomic DNA of K/O cells shows a significantly low level of smears compared to that of WT cells.

3.3.3 Disruption of senataxin enables the DNA damage response (DDR) pathway to remain activated for a longer period of time

To determine whether senataxin plays a role in the DDR signaling pathways ATM–Chk2 and ATR–Chk1 in response to strand breaks induced by H$_2$O$_2$, western blot was performed to detect the activation of these two pathways by probing phosphorylated ATM (serine 1981), Chk2 (threonine 68), ATR (threonine 1989), and Chk1 (serine 345). In senataxin knockout cells, p-ATM peaked immediately at 1 hour, and was reduced to a specific level, which was sustained
during the range of 2 and 8 hours, whereas it was completely diminished to the basal level in WT cells (Figure 3-9). It was also observed that p-Chk2 of K/O was retained for at least 3 hours, but it almost disappeared at 2 hours for WT. p-ATR was elicited at 3 hours in K/O; however, it was delayed to 8 hours for WT. A similar trend was observed in phosphorylated Chk1 in which WT’s p-Chk1 was significantly reduced at 6 hours, but K/O’s cells maintained the same activated level at that time and even decreased to a level higher than that of WT at 8 hours. The level of phosphorylated p53 at Serine 15, which represents the accumulation and activation of p53 responsible for inducing apoptosis, was checked. Therefore, the higher level of p-p53 in WT cells compared to K/O cells (Figure 3-9) is in agreement with the result of the apoptosis assay (Figure 3-7).

Figure 3-9. Western blot (A) showing the phosphorylation level of ATM-Chk2 and ATR-Chk1 kinase in the DDR pathway and downstream p53 in senataxin WT and K/O cells treated with 300 μM H₂O₂ within 8 hours. The relative band intensity of western blot was quantified (B). GAPDH was used as a loading control. U denotes untreated controls.

(figure continued)
3.3.4 Deletion of SETX slows down the repair of DSBs

To determine the effect of senataxin implicated in the DSB repair pathway, an improved I-PpoI system (Goldstein, Derheimer, Tait-Mulder, & Kastan, 2013) was utilized in which
endonuclease I-PpoI can recognize a 15-bp unique sequence and can create DSBs under tight control of its expression by its destabilization domain (DD). A novel method based on quantitative PCR (Figure 3-5) was developed to measure the repair of the specific DSBs induced by I-PpoI in the gene segment of 28S of ribosomal DNA (rDNA) within a period of 24 hours. Around 41% of all 28S segments had the specific DSBs in both WT and K/O cells after 5 hours of I-PpoI induction. The repair was then assessed at 3, 6, 12, and 24 hours (Figure 3-10). At 3 and 6 hours, WT cells repaired the DSBs significantly faster than K/O cells (with $P$ values of 0.0036 at 3 hours, and 0.0002 at 6 hours, $P<0.01$, Student’s t-test). At 12 hours, almost all DSBs were repaired in WT cells with a quite small amount of 1.32% ($\pm 0.72\%$) remaining, but in senataxin-deficient cells, 18.50% ($\pm 2.89\%$), which was nearly half of the total induced DSBs (41%), was still unrepaired. At 24 hours, the DSBs in the K/O cells were completely repaired.

Figure 3-10. Plot showing the percentage of double-strand breaks induced by I-PpoI in the WT and K/O I-PpoI-expressing cells within 24 hours of repair.
3.3.5 Senataxin-deficient cells enhance and sustain the signaling of cell cycle checkpoints through ATM and ATR

In both the I-PpoI expressing WT and K/O cell lines, the HA-tagged I-PpoI proteins were expressed at a similar level throughout the course of 24 hours (Figure 3-11). The phosphorylated components critical for the signaling of cell cycle checkpoints in response to DSBs induced by I-PpoI, as shown in Figure 3-11, were examined again. For p-ATR, K/O cells showed a higher level at 12 and especially at 24 hours. A similar trend was observed for p-ATM; thus, there is clearly a much more intense signal in K/O cells at 12 hours. The phosphorylation of Chk2 was highly strengthened, and its level was maintained in K/O cells compared to WT cells throughout the 24-hour period. A similar pattern was observed for p-Chk1. Phosphorylated p53 at Serine 15 was also detected. Interestingly, an enhanced and enduring level of p-p53 was observed, which is the same pattern as the above-mentioned DDR regulators.
Figure 3-11. Western blot (A) showing the phosphorylation level of ATM-Chk2 and ATR-Chk1 kinase in the DDR pathway and downstream p53 in senataxin WT and K/O I-Ppol-expressing cells within 24 hours after I-Ppol was induced for 5 hours by 1mM Shield-1 and 2 mM 4-OHT. The relative band intensity of western blot was quantified (B). U denotes untreated controls.
ATM and ATR activated by a response to the ionizing radiation of 20 Gray were also examined. A rather high level of p-ATM (S1981) and p-ATR (T1989) was observed at 1.5 and 3 hours, respectively, in senataxin K/O cells compared to WT cells (Figure 3-12).

Figure 3-12. Western blot (A) showing the phosphorylation level of ATM and ATR kinase in the DDR pathway in senataxin WT and K/O cells within 3 hours after being treated with 20 Gray ionizing radiation. The relative band intensity of western blot was quantified (B). GAPDH was used as a loading control. U denotes untreated controls.
3.4 Discussion

A previous study showed that the fibroblasts isolated from one AOA2 patient indicated an increased sensitivity to H$_2$O$_2$ (Suraweera et al., 2007). They stated that exons 14–21 of SETX were deleted, which actually resulted in the removal of only one half of senataxin’s helicase domain (X. Chen et al., 2014). They further confirmed the detection of a lower molecular weight of senataxin by western blot. This truncated senataxin might not lose its full function, suggesting that this chosen cell line may not be a completely valid deletion model. In addition, a much lower dosage of 0.05-0.2 mM H$_2$O$_2$ for 30 minutes was used for the treatment, and then after 2-3 weeks, the viability of the cells was measured by counting colony formations. Both double- and single-stand breaks can be induced by H$_2$O$_2$ (Driessens et al., 2009), and a higher dose could contribute to more frequent DSBs (Olson, 1988). The dose used and cell types could be important factors to determine the frequency and types of strand breaks. In our study, cells were treated with 1 mM H$_2$O$_2$, a quite lethal concentration, for a longer time (8 hours). It is proposed that senataxin may be directly or indirectly related to the mediation of the activation and deactivation of cell cycle checkpoints in the DDR pathways of ATM-Chk2 and ATR-Chk1. When encountering an overwhelming extent of DNA damage, senataxin may acutely repress the DDR pathways (Figure 3-9) and compel cells to rapidly undergo apoptosis (Figure 3-7); however, in the absence of senataxin, the DDR pathways continue to be activated and maintain cell cycle arrest, thus dramatically delaying the apoptosis event. The mild treatment of H$_2$O$_2$, as in their study, could cause a relatively low level of DNA damage, in which case senataxin might react with the DDR pathways in a completely different manner. Their long-term monitoring of cell survival was an effective method used to indicate whether the disruption of senataxin
affected the efficiency of repairing $\text{H}_2\text{O}_2$-induced damage. Therefore, the discrepancy in sensitivity to the DNA damaging agent may be a result of dosage, monitoring time, or cell lines.

An innovative technique was developed to detect the site-specific DSBs generated by the endonuclease I-PpoI in this study (Figure 3-5). The updated version of I-PpoI has a feature that highly controls its expression (Goldstein et al., 2013). The binding of the ligand Shield-1 on its tagged destabilization domain stabilizes the entire protein and prevents it from proteasomal degradation (Banaszynski, Chen, Maynard-Smith, Lisa Ooi, & Wandless, 2006). Removal of the ligand will reversibly degrade I-PpoI. The estrogen receptor hormone-binding domain of I-PpoI is another important feature related to the regulation of DSB induction. I-PpoI constitutively remains in the cytoplasm until the treatment of 4-OHT leads to a translocation of the I-PpoI protein from the cytoplasm to the nucleus (Vigo et al., 1999). The expression of I-PpoI in human cells can create cleavage at 200–300 target sites per genome (Mladenova, Mladenov, & Iliakis, 2016). The repair of I-PpoI-induced DSBs in 28S of rDNA was successfully analyzed; however, this technique may need to be further optimized to improve the sensitivity of measuring other target sites (two copies per each gene), as 28S exists in many copies in human cells (300-400 copies organized in tandem repeats) (Henras, Plisson-Chastang, O'Donohue, Chakraborty, & Gleizes, 2015). The findings of a reduced capacity to repair DSBs in the absence of senataxin (Figure 3-10) are consistent with a previous report that visualized the phosphorylated H2AX foci at damage sites for the quantitative assessment of DSBs and showed that far more foci of phosphorylated H2AX still remained in the senataxin mutant cells 8 hours after $\text{H}_2\text{O}_2$ treatment compared to the control, indicating a defect in the repair of DSBs (Suraweera et al., 2007).

Regarding the implication of the involvement of senataxin in the DDR pathway, it is proposed that senataxin may be related to regulating the activation and deactivation of the DDR
components, as shown by the western blot (Figure 3-9, Figure 3-11, Figure 3-12). A loss of senataxin might tend to gradually accumulate the DDR signaling with a weak deactivation activity; however, the presence of senataxin could deactivate the DDR pathways in an efficient manner.

The phosphorylation of p53 on Serine 15, which is caused by both ATM and ATR protein kinases (D. W. Meek, 2009; D. W. Meek & Anderson, 2009), occurs rapidly in response to DNA DSBs and is the primary target of the DDR pathway on the p53 protein (Loughery, Cox, Smith, & Meek, 2014). Ser 15 phosphorylation also elicits a series of other phosphorylation sites in p53, including the phosphorylation of Ser 20, Ser 46, and Thr18, to further activate p53 (Dumaz, Milne, & Meek, 1999; Saito et al., 2002; Saito et al., 2003). Therefore, in this study, Ser 15 phosphorylation was the major focal point for examining the activation of p53. After stimulation, p53 can lead to apoptosis or cell cycle arrest, and the outcome depends on a variety of factors. The genetic background of the cells can be important, and the extent of DNA damage and the level of p53 can also contribute to determining the outcome of life or death (Pucci, Kasten, & Giordano, 2000). As indicated by the experiments (Figure 3-7, Figure 3-9), in the case of suffering from extensive H2O2-induced DNA damage, the wild-type cells were committed to die quickly, which was promoted by a higher level of p-p53 (S15), while cells with the deletion of SETX could undergo cell cycle arrest with the assistance of persistently activated checkpoint kinases Chk1 and Chk2. Interestingly, it was observed that for DSBs induced by I-PpoI, phosphorylated p53 (S15) was elevated in K/O cells, which was most significant at 24 hours, presumably caused by the gradually increasing level of activated ATM and ATR (Figure 3-11); however, we failed to observe any apoptotic cells even after 24 hours, perhaps because this
relatively low level of damage could be sufficiently repaired, and the higher level of p-p53 mainly contributed to cell cycle arrest rather than cell death.

The mechanism that causes the disruption of senataxin to slow down homologous recombination-mediated DSB repair can be inferred from a recent study (Ohle et al., 2016), which revealed that around DSB sites, RNA Pol II is recruited and produces RNA-DNA hybrids to maintain genomic stability. To make RPA loaded onto 3’ ssDNA overhangs and to continue the repair process, RNA-DNA hybrids must be degraded by RNase H enzymes. Senataxin has been found to resolve RNA/DNA hybrids over G-rich pause sites behind elongating Pol II (Skourti-Stathaki et al., 2011). It is likely that senataxin facilitates RNaseH in removing RNA-DNA hybrids around DSBs. Thus, in the absence of senataxin, the progress of loading RPA could become sluggish due to the reduced capability to remove RNA-DNA hybrids, ultimately delaying the progression of HR-mediated DSB repair. On the other hand, the speed and the length of the 5’ strand resection could be controlled by RNA-DNA hybrids by terminating or stalling RNA Pol II transcription. If this is the case, the deficiency of senataxin could affect the process of strand resection. In future work, the speed of resection can be measured as previously described (Chung, Zhu, Papusha, Malkova, & Ira, 2010; L. Ma, Milman, Nambiar, & Smith, 2015).

In conclusion, it was found that senataxin facilitates the repair of DSBs and regulates the activation of the DNA damage response pathway upon DSB damage. The proposed mechanism that causes this phenomenon remains to be further tested.

3.5 References


CHAPTER 4
CONCLUDING REMARKS

4.1 Research Summary

By using human fibroblast as a model organism, this dissertation has studied on characteristics of repairing $N$-methylpurines at different DNA sequence contexts, and the role of senataxin in response and repair of double-strand breaks. The major findings are as follows:

1. 3MeAs are induced to a much lower level by DMS and repaired much faster than 7MeGs in human fibroblasts. Induction of 7MeGs is affected by nearest-neighbor nucleotides, being enhanced at sites neighbored by a G or T on the 3’ side, but impaired at sites neighbored by a G on the 5’ side. Repair of 7MeGs is also affected by nearest-neighbor nucleotides, being slow if the lesions are between purines, especially Gs, and fast if the lesions are between pyrimidines, especially Ts.

2. DSBs are repaired faster in the presence of senataxin, and DNA damage response pathway of ATM-Chk2 and ATR-Chk1 can be mediated by senataxin in the case of DSB induction.

4.2 Future Direction

The yeast homolog of human senataxin, Sen1, is found to play an important role for transcription coupled repair (TCR), and truncation of N-terminal non-essential region of Sen1 significantly reduces TCR at essentially all sites in the RPB2 housekeeping gene (W. Li et al., 2016). It is uncertain that the knockout of human senataxin may slow down TCR in human fibroblast. Southern blot can be performed, as described in the previous study (W. Li et al., 2016), to measure TCR of UVC-induced CPD damages in one housekeeping gene fragment in the human senataxin WT and knockout fibroblast cell lines.
To further confirm the exclusive role of senataxin in DSB repair, the complementation of senataxin knockout cells will be achieved by introducing wild-type senataxin through Piggybac transposon system (Yusa, Zhou, Li, Bradley, & Craig, 2011) which is utilized owing to its benefit of a large cargo capacity of covering 8,000 bp SETX cDNA. The senataxin-complemented cell line is expected to restore the phenotype, the same as wild-type cells.

The mechanism of how knockout of senataxin slows down DSB repair, needs to be further investigated. The rate of resection at DSBs may be decrease in the absence of senataxin. This speed can be measured by the southern blot, as described previously (Chung et al., 2010; L. Ma et al., 2015).

4.3 References


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Last updated January 3, 2018

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VITA

Mingyang Li, a native of Liaoning, China, received his Bachelor of Science degree from Shenyang Pharmaceutical University in Shenyang, China, in July 2008. Thereafter, he worked in a local pharmaceutical company in the same year. As his interest in biomedical sciences grew, he made the decision to enter the doctoral program at Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana, in the fall of 2011. He is currently completing his doctoral degree in Dr. Shisheng Li’s DNA repair laboratory.