UNIVERSITY OF CALIFORNIA, SAN DIEGO

Human Schlafen 11: Biology and Molecular Mechanisms

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in

Biology

by

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The Dissertation of Elaine Yi Kao is approved, and it is acceptable

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University of California, San Diego

2015

DEDICATION

For my amazing parents, Daniel and Lavinia Kao.

Thank you for teaching me the value of hard work, determination, and responsibility. Without your visions and sacrifices, I would not have had the opportunity to pursue my dreams.

For my loving husband, James Heatherington.

Thank you for loving me unconditionally. Your unwavering love and support allowed me to accomplish everything I wanted and more. I think I made the right choice when I married you!

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LIST OF ABBREVIATIONS

Interferon (IFN)

- Interferon stimulated genes (ISGs)

Schlafen (Slfn)

- Murine Schlafen (muSlfn)
- Human Schlafen (huSlfn)
- Viral Schlafen (vSlfn)
- ATPases Associated with various cellular Activities (AAA)
- Orthopoxviruses (OPVs)

DNA Damage Response (DDR)

- DNA Damaging Agents (DDAs)
- Double-Strand Breaks (DSBs)
- Ataxia-Telangiectasia Mutated (ATM)
- Ataxia-Telangiectasia Mutated and RAD3-Related (ATR)
- Camptothecin (CPT)
- Topoisomerase I and II (TOPI and TOPII)
- Transfer RNA (tRNA)

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Chapter 2, in full is still currently under research by Kao, Elaine. Elaine Yi Kao was the primary researcher of this material. I would like to thank Dr. Manqing Li for his helpful discussions, and unwavering support.

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ABSTRACT OF THE DISSERTATION

Human Schlafen 11: Biology and Molecular Mechanisms

by

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The *Schlafen* family of proteins have relatively unknown molecular mechanisms and biological functions. Here we study the novel functions of one member in the human Slfn family, huSlfn11, and its role in regulating the DNA damage response (DDR) as well as its ability to stabilize Z-DNA. We found that huSlfn11-expressing cancer cells treated with camptothecin (CPT) display selective inhibition of ATR (ataxia-telangiectasia mutated and RAD3-related)

protein translation efficiency. This selective regulation by huSlfn11 is based on the codon usage of ATR. We have also demonstrated that cancer cells deficient in huSlfn11 expression can be completely re-sensitized to CPT by knocking down the expression of ATR, and moderately re-sensitizied to CPT with the use of ATR inhibitors. Additionally, we have shown for the first time, that huSlfn11N, the C-terminal truncation of full length huSlfn11 that includes the ATPase AAA domain, can stabilize Z-DNA conformation, possibly as a novel antiviral mechanism.

INTRODUCTION

The mechanism by which interferons (IFNs) activate gene expression has been the target of intense research efforts for many years. Binding of type I IFNs, IFNα/β, to their specific cell surface receptors IFNAR1 and IFNAR2 leads to the tyrosine phosphorylation and activation of several members of the Signal Transducer and Activator of Transcription (STAT) family of transcription factors [1]. Among those, STAT1, STAT2, and IRF9 cooperatively form the DNA binding protein complex ISGF3, which is required for upregulation of interferon stimulated genes (ISGs) through an Interferon Stimulated Response Element (ISRE). Numerous ISGs have antiproliferative or pro-apoptotic activity and represent factors that are well known to contribute to alterations in cell cycle progression in response to IFNα/β. Additionally, numerous ISGs also represent components of the antiviral defense such as the 2'-5' oligoadenylate synthetase [2] and the dsRNA activated protein kinase (PKR) [3], cell surface proteins such as ICAM [4] or the MHC class I and II molecules [5]; however, many other ISRE containing genes are of unknown or not well understood function.

Among the ISGs of largely undefined function are the *Schlafen* (Slfn) gene family members. Slfns were first discovered in mice and were found to be located in a cluster on murine chromosome 11. The murine Slfns (muSlfns) encompass a family of proteins that includes at least 10 distinct members. Slfns can be categorized into three distinct groups based on their length of the C terminus: short forms (muSlfn1 and muSlfn2), intermediate forms (muSlfn3 and muSlfn4), and long forms (muSlfn5, 8, 9, and 10). Humans also express Slfns (huSlfns),

and they are located in a cluster on chromosome 17. However, humans lack homologs to the short muSlfn1 and muSlfn2 isoforms. All Slfn proteins share a conserved NH2-terminal region that contains a putative divergent AAA (ATPases Associated with various cellular Activities) domain, whereas long form Slfns possess additional short motifs that resemble domains found in members of the DNA/RNA helicase superfamily.

Genomic and phylogenetic studies demonstrated that the Slfn family is widely distributed in mammals and are found in clusters with close physical proximity to each other on the chromosome of all species that were studied. Additionally, the Slfn genes encode proteins that display 51-96% similarity over a common shared region. All of these suggest that the evolution of this gene family has been shaped by multiple gene duplication events, possibly through unequal crossing-over or non-homologous mechanisms [6]. Further studies of this gene family indicated that the Slfn genes have been evolving under positive selection. Interestingly, the only non-mammalian organism that encodes a Slfn-like protein are all orthopoxviruses (OPVs). Phylogenetic analyses of these viral Slfns (vSlfns) showed that they are similar in length to the murine short isoforms and were most likely acquired through horizontal transfer from rodents and subsequently diverged. Unlike the mammalian Slfn genes, which underwent positive selection, vSlfns most likely evolved under purifying selection, indicating their importance in OPVs [7].

The majority of publications on Slfn proteins have been focused on muSlfns. The muSlfn genes were found to be differentially regulated during

thymocyte maturation and are preferentially expressed in lymphoid tissues such as thymus, lymph nodes, and spleen [8]. The expression of muSlfn1 and muSlfn2 are upregulated during the double positive (DP) to single positive (SP) transition of thymocyte development, whereas the expression of muSlfn4 and muSlfn7 are decreased. Upon T cell activation, the expression of muSlfn1 and muSlfn2 are downregulated, whereas the expression of muSlfn3, 4, 6, 7, and 9 are upregulated [8, 9]. In vivo studies showed that ectopic expression of muSlfn1 and muSlfn 8 results in significant reduction in thymic cellularity along with impaired T cell development and proliferation upon activation [8, 10]. Similarly, transgenic mice that overexpress muSlfn4 displayed a reduction in monocytes and recruited inflammatory macrophages [11]. Together, these findings indicated that muSlfn1, 4, and 8 play important roles during maturation and differentiation in lymphoid and myeloid lineages. The use of transgenic mice have helped to elucidate the role of individual Slfn genes; however, muSlfn1 knockout mice showed no significant phenotype, suggesting potential functional redundancy or compensatory mechanisms among the muSlfn family members [8].

Previous findings demonstrated that muSlfn genes are involved in the proliferation of immune cells, and several studies have reported on the role of Slfns in cell cycle progression and growth. MuSlfn1 expression was shown to cause cell cycle arrest at the G_1 phase in murine fibroblasts and T cells by inhibiting the induction of cyclin D1 through the downregulation of PDGF receptor expression and preventing cyclin D1 transcription [8, 12, 13]. This also suggested that muSlfn1 translocates from the cytoplasm into the nucleus. Additionally,

mouse fibroblasts transfected with expression vectors encoding muSlfn1, 2, and 3 genes resulted in significant reduction in cell proliferation and colony formation [8, 10, 12]. Further studies on muSlfn2 showed that upon its induction by Type I IFNs, muSlfn2 negatively regulates the growth and metastasis of malignant cells because siRNA-mediated knockdown of muSlfn2 results in increased proliferation and anchorage-independent growth in soft agar [14]. Despite all the published findings linking muSlfn1 and 2 overexpression to the inhibition of cell proliferation, *Zhao et al.* failed to observe muSlfn1 and 2 inhibit cell proliferation, cause G1 arrest, or reduce cyclin D1 expression in mouse fibroblasts and various myeloid progenitor cell lines [15]. Unlike the muSlfns described above, expression of muSlfn5, 8, 9, and 10 in mouse fibroblasts did not show any antiproliferative effects [10]. All together, the role of muSlfns in the regulation of cell proliferation continues to be an open area of research.

As described previously, numerous interferon-inducible genes are components of antiviral defense. As an ISG, muSlfns may have important functions in response to viral infections. The expression levels of muSlfns were found to be elevated after infection with intracellular pathogens Brucella [16] and Listeria [10]. Additionally, a loss-of-function muSlfn2 mutation (elektra) showed reduced number of T cells and Ly6C+ inflammatory monocytes, resulting in enhanced susceptibility to bacterial and viral infection [17]. MuSlfn4 expression in bone marrow-derived macrophages was induced by Toll-like Receptor (TLR) 4 agonist lipopolysaccharide (LPS) as well as Poly(I:C), a TLR3 agonist. In addition to differential expression of muSlfn genes in the development and activation of

immune cells as well as infections, inflammation can also modulate the expression of muSlfns. Analysis of collage induced arthritis (CIA) mouse model of rheumatoid arthritis showed increased levels of muSlfn1, 2, and 4 in the joints [10, 11].

The short forms of muSlfns have been implicated in having important functions upon viral and bacterial infections. OPVs encode vSlfns that have sequences closely related to the short muSlfn isoforms, yet only a few members in the poxvirus family (monkeypox, cowpox, mousepox, taterapox, and camelpox) retain intact open reading frame (ORF) that codes for functional protein [7]. In order to understand the function of vSlfn upon viral infection, recombinant vaccinia and variola viruses containing the intact ORF of camelpox (CMLV) vSfln were generated. Expression of vSlfn did not affect vaccinia replication or plaque morphology [10], and in vivo mouse model of intradermal infection did not result in a difference in the size of lesions caused by vaccinia [18]. However, viral titers in infected lungs showed a greater recruitment of lymphocytes but less activation, suggesting vSlfn is a virulence factor that affects the host immune response to infection [18]. Additionally, the N-terminal sequences of vSlfn seem to have an important function because infection with vSlfn containing an N-terminal HA epitope, but not at the C terminus, resulted in reduced body weight and health that is comparable to control virus infection. All together, with the phylogenetic studies that showed that vSlfns underwent purifying selection throughout evolution, suggest vSlfns in OPVs have been retained to counteract antiviral activities from the host Slfn genes.

In recent years, more focus has been shifted to investigate the molecular mechanism and biological functions behind the huSlfn family members. Studies published by *Li et al.* demonstrated for the first time that human *Schlafen* 11 (huSlfn11) is a novel and potent IFN-inducible restriction factor against the retrovirus HIV [19]. Expression of huSlfn11 in HEK293 (human embryonic kidney) and CEM (human T cell lymphoblast) cells attenuated the production of HIV, but had no effect on early steps of the infection cycle encompassing entry, reverse transcription, integration, or production and nuclear export of viral RNA. Furthermore, late stage budding or release of the viral particles was not inhibited. It was also reported that this activity was contained in the NH2-terminal, AAA domain-containing region of the huSlfn11 protein (huSlfn11N, aa 1-579), whereas no effect was observed with the isolated COOH-terminal region (huSlfn11C, aa 523-901).

Viral genomes are known to display bias nucleotide compositions that are quite different from human genes [20-26]. In particular, the *gag* and *pol* sequences of wild-isolate strains of HIV-1 are characterized by low GC content and suboptimal codon usage when compared to the host cell preference [22, 27-30]. Mammalian expression vectors containing the ORF of HIV *gag* with either the original viral codon bias (Gag^{vir}) or with synonymous codon usage optimized for expression in human cells (Gag^{opt}), clearly showed that huSlfn11 strongly affected the expression of Gag^{vir} but not Gag^{opt} [19]. HuSlfn11 was found to selectively inhibit the synthesis of virally encoded proteins on the basis of codon usage discrimination.

Previous reports showed changes in the cellular tRNA levels after HIV infection [31]. Following this observation, *Li et al.* performed electrophoretic mobility shift assay (EMSA) demonstrating that huSlfn11N can bind to 32P-labeled human tRNAs (transfer RNAs) in a dose-dependent manner. Furthermore, tRNA arrays hybridized with isolated tRNAs from the shifted band of EMSA did not show an enrichment of particular tRNAs, indicating that huSlfn11N binds tRNAs non-discriminately [19]. All together, the work published by *Li et al.* has described for the first time a molecular mechanism for any member of the huSlfn family.

In addition to being an executioner of an anti-retroviral innate immune response, recently published reports illustrated the role of huSlfns in regulating cancer malignancies [32-37]. Two published reports demonstrated a strong correlation between huSlfn11 expression and sensitivity of tumor cells to DNA damaging agents (DDAs) such as topoisomerase I and II inhibitors (TOPI and TOPII), DNA synthesis inhibitors, and alkylating agents [32, 33]. Furthermore, cell cycle analysis of cancer cells treated with TOPI showed arrest at the early S phase, and these S phase arrested cells underwent apoptosis only when huSlfn11 is expressed [32].

Other members of the huSlfn family were also implicated in modulating cancer cell differentiation and invasion. HuSlfn5 expression can be induced by IFNα treatment, resulting in decreased anchorage-independent growth, motility, and invasiveness in both malignant melanoma cells and renal cell carcinoma cells [36, 37]. Additionally, huSlfn12 expression results in a less aggressive

phenotype for prostate cancer by regulating differentiation in prostate epithelial cells [34].

The mammalian Slfn genes have been shown to regulate a wide variety of cellular functions including immune cell development and function, cell cycle progression, antiviral innate immune response, as well as cancer cell malignancy. However, the molecular mechanisms and biological functions of other Slfns, including vSlfn, have yet to be fully elucidated. The work included in this dissertation hope to shed some light on the importance of huSlfn11 in the treatment against cancer, as well as provide a novel biological significance to alternative DNA conformation induced by huSlfn11.

CHAPTER 1: Human Schlafen 11 and DNA Damage Response Regulation

Background on DNA Damage Response (DDR)

DNA damage can originate from numerous sources including endogenous causes such as replication errors and oxygen radicals derived from normal cell metabolism, or exogenous causes from the environment such as reactive chemicals and radiations. These damaging agents induce different types of DNA lesions that can result in extreme cytotoxicity if not repaired correctly, or malignant transformation from malfunctioning proteins. The desire to maintain genome stability leads to the basic cellular response of DNA damage repair. However, programmed cell death, or apoptosis, might be the preferable response if the type and amount of DNA damage overwhelms the cellular survival response machinery.

DNA damage response (DDR) is a complex but precise collection of signaling pathways that is initiated when cellular repair machineries are activated in response to DNA damage. Different types of DNA lesions activate different DNA repair mechanisms and induce changes in gene-expression profiles, protein synthesis, trafficking, and degradation [38]. The most severe type of DNA lesions, double-strand breaks (DSBs), results in immediate activation of cell cycle checkpoints at the G1-S and G2-M boundaries as well as in the S phase in order to delay cell cycle progression or modulate DNA replication [39, 40]. Elements of the DDR pathways include sensory protein that detect DNA damage, and effector proteins that are responsible for subsequent cell fate decisions.

Key components of DDR include the ATM-Chk2 and ATR-Chk1 pathways.

ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia mutated and RAD3-related) are large kinases belonging to a family of conserved proteins called PIKK (phosphatidylinositol 3-kinase-like protein kinases) because they contain sequences similar to the PI3K lipid kinases family. Both possess serine/threonine kinase activities that phosphorylate only protein substrates, including their downstream checkpoint effector kinases Chk2 and Chk1 [41, 42]. ATM is primarily activated by radiation and genotoxin-induced DSBs through recruitment with the MRN sensor complex (Mre11, Rad50, Nbs1), whereas ATR is activated mainly by DNA damage caused by replication stress through recruitment and association with its partner protein, ATRIP (ATR-interacting protein) [41, 43-47]. One of the earliest events occurring at DNA damage sites is the ATM- and ATR-dependent phosphorylation of H2AX upon DSB induction and replicative stress, respectively [48-50]. Repeated cycles of H2AX phosphorylation allow for the rapid and powerful amplification of damage signal resulting in recruitment of repair proteins.

Mutations in ATM result in the human genetic disorder ataxia-telangiectasia (A-T) that belongs in a group of human diseases known as genomic instability syndrome [51]. Patients with A-T have predisposition to cancer and show extreme sensitivity to ionizing radiation and DSB-inducing agents, in addition to displaying cerebellar degeneration [52]. This disorder combines hallmarks of a defective DNA damage response, and cultured cells from A-T patients are defective in DSBs response [53]. ATM exists as a homodimer in its inactive form in the nucleus, but upon activation by DSBs

becomes partially activated monomers through autophosphorylation at numerous serine sites [54, 55]. The monomers are then recruited to the site of DSBs by their interaction with the MRN complex to become fully activated [56, 57]. Activated ATM phosphorylates multiple substrates including Chk2, which undergoes transient homodimerization to allow for intermolecular activation loop autophosphorylation to become fully activated [58, 59]. Activated Chk2 then dissociates into monomers from the DNA damage site and disperse throughout the nucleus to act on substrates involved in cell cycle checkpoints and apoptosis [60]. Known targets of Chk2 include p53, Cdc25 phosphatases, BRCA1, and E2F1 transcription factor [45, 60-63].

In contrast, ATR is an essential gene in replicating cells because it is activated by replication stress in every S phase, and homozygous knockout mice are embryonic lethal [47, 64, 65]. Besides being activated by single-stranded DNA (ssDNA) resulting from replication stress and nucleotide excision repair (NER), ATR can also be activated by DSBs that produce ssDNA upon nuclease-mediated end resection [66]. SsDNA is coated by trimeric Replication Protein A (RPA), which recruits ATR and its interacting partner ATRIP to the site of DNA damage. This occurs through direct interaction between a RPA subunit and ATRIP α-helix in order to promote ATR accumulation [47, 67, 68]. Activation of ATR then requires the co-localization of Rad9-Rad11-Hus1 checkpoint clamp complex (9-1-1) to the 5’ junction in order to recruit the ATR activator topoisomerase-binding protein-1 (TOPBP1) [69, 70]. Activation of ATR is finally achieved upon binding of TOPBP1 activation domain with both ATRIP and the

PIKK regulatory domain (PRD) on ATR [71].

Activated ATR coordinates cell cycle checkpoints, replication fork stability, and origin firing. Chk1 is the most well-studied substrate of ATR, and its activation requires phosphorylation by ATR at serine 317 and 345 through the mediation of Claspin and replication-fork-associated complex including Tim (Timeless) and Tipin (Tim-interacting protein) [72-76]. Important Chk1 substrates include Cdc25 phosphatases that control cell cycle transitions, Cdk2-cyclin E kinases that reduce DNA replication by inhibiting origin firing, and Rad51 and BRCA2 involved in recombination [77, 78].

The ATM-Chk2 and ATR-Chk1 pathways do not only act in parallel but are interdependent on each other. Both kinases are activated by DSBs, but ATM is activated rapidly regardless of the cell cycle, whereas ATR is activated more slowly and mainly during S and G2 phase [79]. Furthermore, several publications have demonstrated the importance of ATM in ATR activation. ATM phosphorylates TOPBP1 upon DSBs, resulting in a more efficient activator of ATR [80]. Studies also showed ATM and Mre11 co-localization to sites of ionizing radiation-induced DSBs to generate RPA-coated ssDNA that then recruits ATR [81, 82]. Conversely, there are also numerous studies demonstrating ATR-dependent ATM activation in response to UV treatment and stalled replication fork. ATR is shown to function upstream of ATM by phosphorylating serine 1981, the ATM autophosphorylation site, resulting in Chk2 activation to regulate G2-M checkpoint arrest [83]. The relationship between ATM and ATR signaling pathways is complex and suggests more novel interactions between these two

kinases and their substrates will continue to be elucidated in future studies.

Introduction

The Schlafen (Slfn) family of genes encodes proteins found only in mammalian organisms. Their roles in regulating cancer malignancies have only recently been discovered [34, 36, 37]. Studies using the NCI-60 Antitumor Cell Line Panel and the Cancer Cell Line Encyclopedia (CCLE) aimed to determine gene-expression-based predictors of anticancer drug sensitivity [32, 33]. Topoisomerase I is a popular target for cancer drug therapy because it is an essential enzyme during DNA replication, transcription, and chromatin remodeling [84, 85]. Camptothecin (CPT) and its derivatives, irinotecan and topotecan, are specific TOPI inhibitors that take advantage of highly proliferating cancer cells to create replication fork collisions, resulting in replication-mediated DSBs that can lead to programmed cell death [86, 87].

SiRNA-mediated silencing of huSlfn11 in various human cancer cell lines resulted in a reduction of sensitivity to DNA damaging agents (DDAs) such as topoisomerase I and II inhibitors (TOPI and TOPII), DNA synthesis inhibitors, and alkylating agents, which are all commonly used clinical treatments for cancers. Additionally, cell cycle analysis of cancer cells showed CPT-treated cells accumulating in early S phase. These S phase arrested cells undergo apoptosis when huSlfn11 is expressed, providing evidence that huSlfn11 is causally associated with the activity of DDAs in human cancer cells by playing a role in cell cycle arrest. These studies demonstrated a significant positive correlation between huSlfn11 expression and sensitivity to DDAs [32, 33].

Recently, we published on the role of human huSlfn11 as a novel antiviral

factor that acts at the late stage of virus production by selectively inhibiting viral protein synthesis in HIV-infected cells through codon-bias discrimination [19]. It was previously reported that cellular tRNA levels change after HIV infection [31]. We showed that huSlfn11 interacts directly with tRNA in HIV-infected cells, thereby counteracting HIV-induced changes in tRNA composition initiated to promote viral protein synthesis. Various reports have illustrated that retroviruses such as HIV, induce DSBs during the host genome integration process [88, 89]. This HIV-induced DNA damage triggers the cellular DNA damage response, as HIV wants to maintain cell propagation in order to exploit the infected cells to express viral genes and to release viral progeny.

The trigger of DNA damage response (DDR) results in the activation of ATM-CHK2 and ATR-CHK1 kinase signaling cascades. ATM is activated upon radiation and genotoxins-induced DSBs, whereas ATR is mainly activated by DNA damage resulting from replication stress [44, 46]. Activated ATM and ATR selectively phosphorylate their downstream effector kinases, CHK2 and CHK1, respectively. The resulting effect of these two signaling cascades is the activation of substrates important in the regulation of origin firing, DNA repair, cell cycle arrest, and cell death [43, 44, 46].

Because huSlfn11 has been implicated to confer sensitivity to DDAs in cancer cells, we aim to investigate the mechanism of how huSlfn11 regulates camptothecin-induced DNA damage response by focusing on the ATM and ATR signaling cascades. Furthermore, we explore potential therapeutic treatments for cancer cells deficient in huSlfn11 expression by using kinase inhibitors and

siRNA knockdowns targeting components of DNA damage response.

Materials and Methods

Cell lines, chemicals, and drugs

HEK293 and COLO 375 FG stable cell lines were generated as described in Li et al. HEK293, FG, and MIA PaCa-2 cell lines were maintained in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2mM L-Glutamine, MEM Non-essential Amino Acid, 1mM Sodium Pyruvate, and 55uM 2-Mercaptoethanol. Camptothecin (EMD Millipore) was prepared at 10mM in DMSO. The ATR inhibitors, VE-821 and VE-822 (Selleckchem) were prepared at 25mM and 10mM, respectively, in DMSO. The CHK1 inhibitors, CHIR-124 and LY2603618 (Selleckchem), were both prepared at 10mM in DMSO. For cell-based work, compounds were diluted to give a final DMSO concentration of 0.01% for all compounds and 1% for LY2603618.

MTS cell viability assay

Cells were seeded at 40% confluency in 96-well tissue culture plates 24 hours prior to drug treatment (in high glucose, phenol red-free DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2mM L-Glutamine, MEM Non-essential Amino Acid, 1mM Sodium Pyruvate, and 55uM 2-Mercaptoethanol). After 48hr treatment, MTS reagent was added following manufacture protocol (Promega G3581). Plates were analyzed at 490nm after incubation at 37°C for 2-4 hours.

Whole cell lysis and Western blotting

Cells were lysed in 1X Cell Lysis Buffer (Cell Signaling Technology)

supplemented with Phosphatase Inhibitor Cocktail Set I, Phosphatase Inhibitor Cocktail Set II, and Protease Inhibitor Cocktail Set III (Merck Millipore). NuPAGE 4X LDS Sample Buffer (Life Technologies) was added to the lysates then samples were resolved by 4-12% SDS-PAGE (Life Technologies) and transferred onto Amersham Hybond-LFP PVDF membranes (GE Healthcare). Antibodies against ATR and human Schlafen 11 were obtained from Santa Cruz Biotechnology. Antibodies against ATM, DNA-PK, Mre11, Rad50, and GAPDH were obtained from Cell Signaling Technology. After incubation with HRP-conjugated secondary antibodies, signals were detected with Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare).

Quantitative PCR and Primers

Quantitative PCR was performed using Qiagen FastLane Cell RT-PCR kit. QPCR was performed on Applied Biosystems StepOnePlus Real-Time PCR System using Power SYBR Green PCR Master Mix. Relative RNA level were calculated after normalization to GAPDH.

Polysome profiling by sucrose gradient

COLO 375 FG cells were seeded 24 hours prior to 6-hour DMSO or 40nM CPT treatment. After treatment, cells were incubated with 100ug/mL of cycloheximide (CHX) for 3 minutes at 37°C then washed with PBS supplemented with 100ug/mL CHX. Cells were then harvested and lysed in polysome extraction buffer (0.5% Triton, 10mM Tris pH7.4, 15mM MgCl2, 150mM NaCl, RNase inhibitor, 100ul/ml CHX) before layered onto previously prepared linear sucrose density gradients (10-50%). Ultracentrifugation was performed at 35,000 r.p.m for

2.5 hours. Fractions were then collected using ISCO Gradient Former (Model 160). Total RNA from each fraction was extracted using Trizol, followed by RT to synthesize cDNA. Quantitative RT-PCR was performed using primers against ATR, ATM, GAPDH, β-actin, and 18S rRNA.

35S- protein labeling and immunoprecipitation

HEK293 and COLO 375 FG stable cell lines were treated with DMOS or 40nM CPT. Cultures were starved in methionine and cysteine-free DMEM for 30min at 37°C then labeled methionine and cysteine-free DMEM supplemented with 250uCi of 35S-methionine and cysteine (PerkinElmer EasyTag EXPRESS [^{35}S]-protein labeling mix, 11mCi/ml) for 30 minutes at 37°C. After labeling, cells were washed with room temperature PBS then harvested with trypsin then lysed following the whole cell lysis protocol above. Cellular debris was pelleted by centrifugation, and the resulting 35S-labeled lysates were incubated with anti-ATR antibody (Santa Cruz Biotechnology) or anti-GAPDH antibody (Abcam ab110305) at 4°C for 2 hours. Antibody-antigen complexes were precipitated with Dynabeads ProteinG (Life Technologies), and samples were analyzed following Western blotting method above. The membrane was dried then exposed to Bio-Rad screen for 48hr, and the auto-rad was developed using the Typhoon phosphoimager.

siRNA knock-down

Reverse transfection was performed with cells at day 0 using RNAi Max reagent (Life Technologies) then seeded in 96-well plates. Cell were harvested and lysed 5 day later to analyze for gene knockdown. For siRNA MTS cell

viability assays, cells were treated with camptothecin 72 hours post-reverse transfection, followed by 48-hour treatment with CPT before following MTS cell viability assay protocol above.

Results

HuSlfn11 was demonstrated to selectively inhibit the translation of HIV viral proteins [19], and we are interested in investigating whether huSlfn11 also affects the translation of genes in the DDR pathways. First, to confirm the reported observation that huSlfn11 expression confers sensitivity to the DNA damaging agent, camptothecin (CPT) [32, 33], we performed MTS cell viability assay with increasing concentrations of CPT treatment in two different cell lines. Using the previously established HEK293 control and huSlfn11-KD cell lines [19], as well as a matching set in the pancreatic cancer cell line, COLO 375 FG, we observed significant decrease in the percentage of cell viability upon CPT treatment in both HEK293 and FG control-KD cell lines when compared to their DMSO controls (Fig. 1-1 A, B). We also observed significant tolerance and prolong cell survival for CPT in huSlfn11-KD cell lines in both HEK293 and FG. Additionally, we tested other DDAs that were reported to also confer sensitivity in huSlfn11-expressing cell (Mitoxantron, Gemcitabine, and Chlorambucil), as well as one DDA (Taxol) that did not (Fig. 1-1 C-E). MTS assay results showed that huSlfn11 expression showed similar effect with these DDAs as with to CPT, indicating that the observed phenotype was not limited to CPT in our stable cell line system.

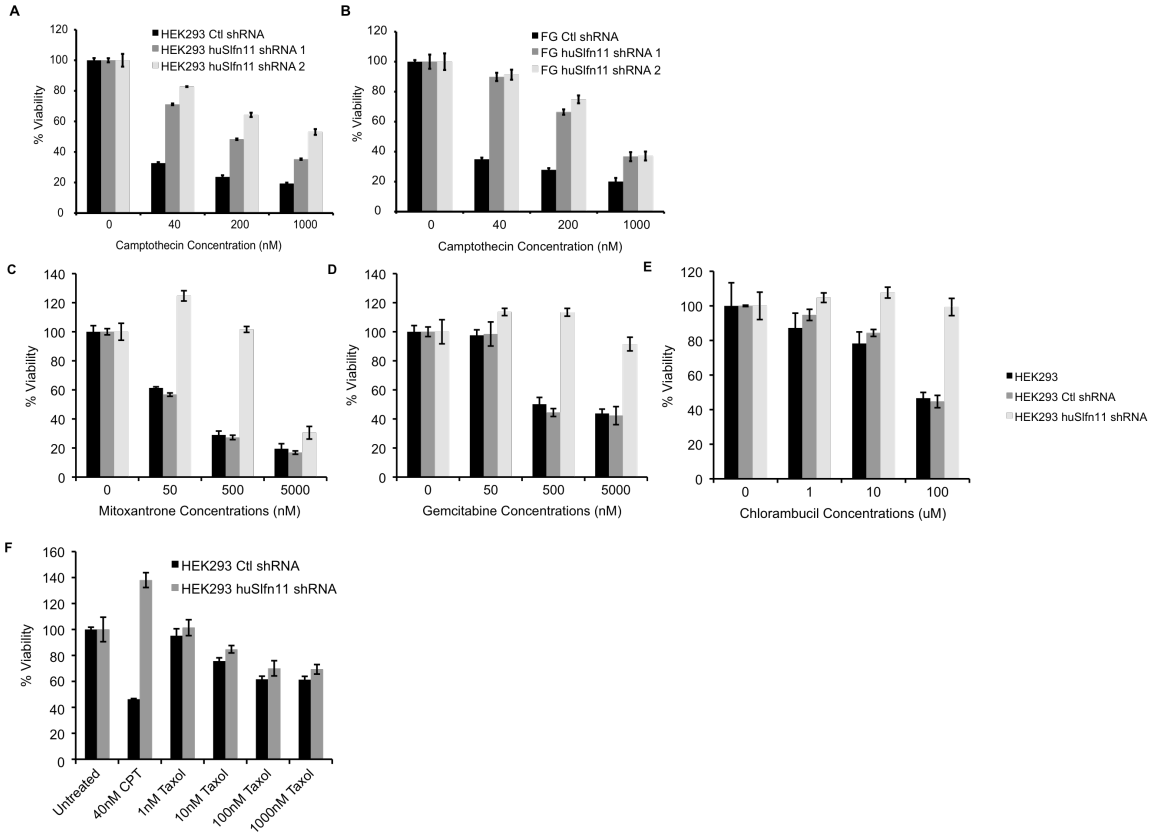

Figure 1-1. Human Schlafen 11 expression confers sensitivity to different DNA damaging agents. HEK293 and FG control and huSlfn11 knockdown cell lines were treated with different concentrations of DDAs for 48 hours, followed by MTS assay to measure cell viability. (**A**) CPT treatment in HEK293 stable cell lines. (**B**) CPT treatment in FG stable cell lines. (**C**) Mitoxantrone, TOPII inhibitor. (**D**) Gemcitabine, DNA synthesis inhibitor. (**E**) Chlorambucil, alkylating agents. (**F**) Taxol, tublin active antimitotic agent.

The presence of CPT in replicating cells results in slower re-ligation of DNA during DNA replication and transcription, which if left unrepaired, will lead to an accumulation of DNA double-strand breaks that is detrimental to the cell [90]. The MTS cell viability assays demonstrated that expression of huSlfn11 results in reduced cell survival upon CPT-induced DNA damage, indicating an impaired DDR. This prompted us to investigate the protein expression of important DDR genes in the context of huSlfn11 expression and CPT-induced DNA damage. Control and huSlfn11-KD cells from both HEK293 and FG stable cell lines were

treated with DMSO or 40nM of CPT for 24 and 48 hours, followed by Western blotting to analyze the expression levels of various DDR proteins. We noted that CPT treatment resulted in the downregulation of ATR expression in a huSlfn11-dependent manner when compared to control conditions (Fig. 1-2), and this expression pattern can be observed in both HEK293 and FG stable cell lines. Surprisingly, the expression of RAD50 is upregulated upon CPT treatment in a huSlfn11-independent manner, indicating that the observed phenotype in ATR protein expression is not the result of global protein synthesis inhibition. We also analyzed the expression levels of other genes in the DDR pathways, such as ATM, DNA-PK, and a component of the MRN complex, Mre11, but these genes did not clearly exhibit the same expression patterns and consistency in both cell lines as compared to ATR.

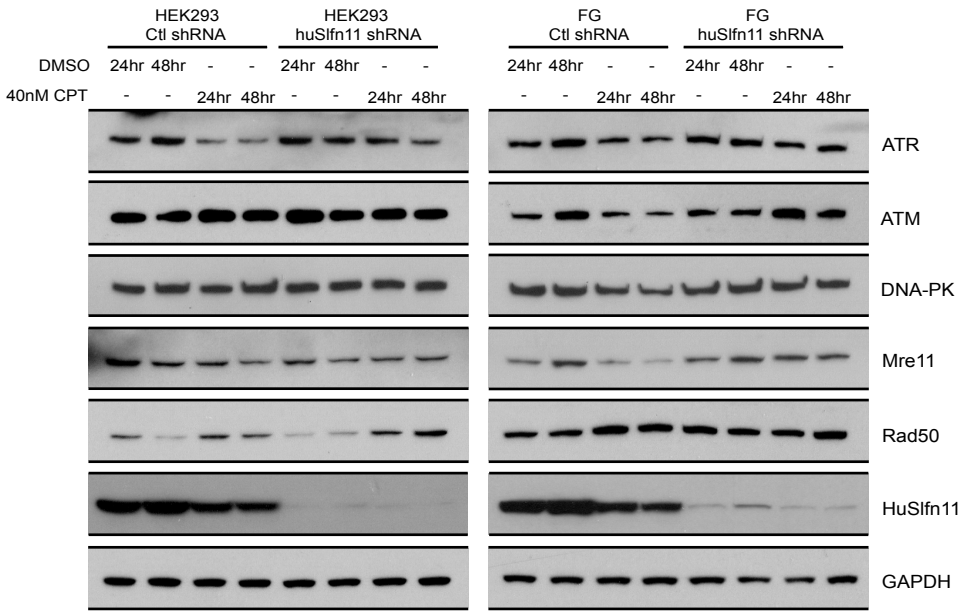

Figure 1-2. CPT treatment results in the downregulation of ATR protein expression in a huSlfn11-dependent manner. HEK293 and FG stable cell lines were treated with DMSO or 40nM CPT for 24 hours and 48 hours. Protein levels of various DDR genes were analyzed using Western blotting.

Next, to determine whether the observed decrease in ATR protein expression level in huSlfn11-expressing cells treated with CPT can be attributed to changes on the transcription level, we conducted quantitative RT-PCR to measure the mRNA levels of proteins we studied in Figure 1-2. Surprisingly, ATR mRNA levels were increased in huSlfn11-expressing cells treated with CPT when compared to DMSO treated cells (Fig. 1-3 A, B). This suggests the cell is compensating for the decrease in ATR protein expression level by increasing ATR mRNA levels. Conversely, in huSlfn11-KD cells ATR protein levels are not downregulated and consequently we did not observe an upregulation in ATR mRNA level. The mRNA levels of ATM and Mre11 were also analyzed and found to display similar QPCR results as compared to ATR (Fig. 1-3 C-F). Together, all of these results demonstrate that huSlfn11 expression negatively affects the viability of cells treated with CPT by selectively inhibiting the protein expression of DDR proteins in a huSlfn11-dependent manner. Because of the importance of ATR as a major signal transducer upon CPT-induced DNA damage, and the clear selective inhibition of ATR protein expression in huSlfn11-expressing cells treated with CPT in both stable cell lines, we decided to focus on ATR and its potential regulation by huSlfn11 for the remainder of this study.

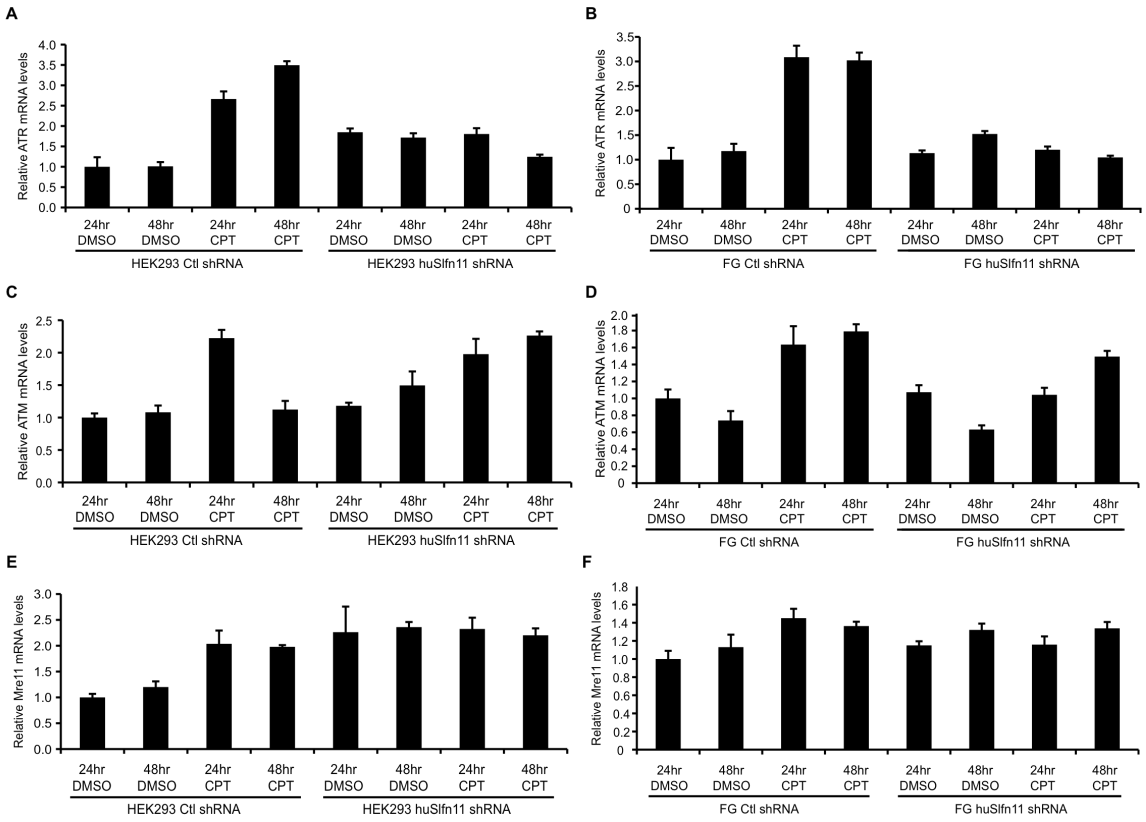

Figure 1-3. CPT treatment of HEK293 and FG huSlfn11-expressing cells resulted in compensatory increase of mRNA levels in response to reduced protein expression. Quantitative RT-PCR in HEK293 and FG stable cell lines measured the mRNA levels of ATR (**A** and **B**), ATM (**C** and **D**), and Mre11 (**E** and **F**). The error bars represent triplicates in an experiment.

Despite an increase in ATR mRNA levels in huSlfn11-expressing cells treated with CPT, ATR protein expression is reduced. To investigate whether ATR is regulated at the level of protein translation, we performed polysome fractionation by sucrose density gradient centrifugation. The separated fractions were then subjected to RNA purification followed by quantification using RT-PCR in order to generate a polysome profile for each gene. FG control and huSlfn11-KD cells were treated with DMSO or 40nM of CPT for 6 hours prior to cycloheximide (CHX) addition in the growth media to prevent ribosome runoffs on the mRNAs. High-speed centrifugation was then used to separate the mRNAs by

their densities to allow for the isolation and for the quantification of free ribosomal subunits, monosomes, and polysomes (translating ribosomes). MRNAs with multiple ribosomes attached have higher densities and were separated into later fractions. These heavier mRNAs, or polysomes, equate to more mRNA translation because more ribosomes are residing on the mRNA at a given time. ATR in FG control-KD cells treated with CPT displayed a decrease in the proportion of polysomes, or a shift to lighter fractions, when compared to the DMSO control (Fig. 1-4 A, left). One possible explanation for this might be lower translation elongation rate on ATR mRNAs in huSlfn11-expressing cells when treated with CPT, resulting in subsequent reduction in ATR protein translation efficiency and protein expression [91]. In support of the Western blotting results, the polysome profiles of FG huSlfn11-KD cells did not show significant differences between the DMSO and CPT-treated conditions (Fig. 1-4 A, right). Together, these data demonstrate that huSlfn11 is required for CPT-induced selective inhibition of protein translation in ATR.

To ensure that the observed decrease in ATR protein translation efficiency was not the result of overall protein translation inhibition caused by CPT, we also analyzed the polysome profiles of 18S rRNA. The polysome profiles of 18S rRNA showed perfectly matched profiles in both cell lines and treatment conditions (Fig. 1-4 E). Furthermore, we also analyzed the polysome profiles of ATM, another important kinase in the DDR pathways, and observed similar polysome profile patterns as ATR (Fig. 1-4 B). Notably, the polysome profiles of genes not involved in DDR, GAPDH and β-actin, have almost identical patterns in both cells

lines, regardless of huSlfn11 expression and treatment conditions (Fig. 1-4 C, D). These results provide strong evidence linking huSlfn11 expression with the selective inhibition of ATR protein translation efficiency.

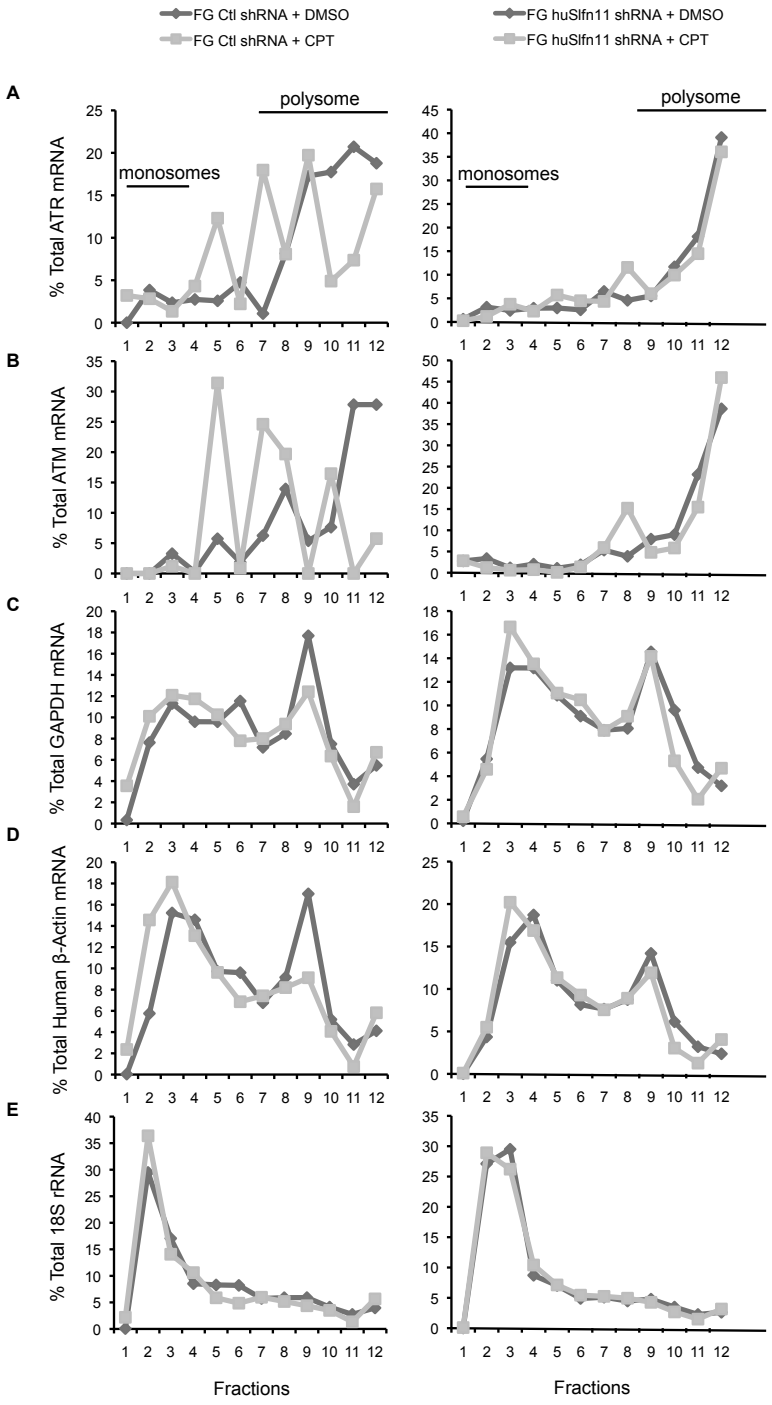

Figure 1-4. Decreased protein translation efficiency of ATR and ATM in huSlfn11-expressing cells treated with CPT. FG control and huSlfn11-KD cells were pre-treated with DMSO or 40nM camptothecin for 6 hours followed by 100ug/mL of cycloheximide treatment. Harvested cells were then centrifuged at high speed through a sucrose density gradient to separate mRNAs with different densities into fractions, followed by total RNA isolation and quantification using RT-PCR. Polysome profiles of ATR (**A**), ATM (**B**), GAPDH (**C**), β-actin (**D**), and 18S rRNA (**E**).

A decrease in ATR protein translation efficiency suggested that ATR protein stability or ATR protein synthesis might be affected by huSlfn11 expression. To determine if ATR protein stability might be regulated by huSlfn11, we performed 35S-methionine/cystein pulse-chase experiments to track the activity of ATR protein over time. HEK293 and FG stable cell lines were treated with DMSO or 40nM of CPT for 24hrs. Cells were then pulsed with 250uCi of 35S-methionin/cystein labeling mixture (Perkin Elmer) for 30 minutes, followed by chase with cold media for 0, 2, and 4 hours. The cells were lysed then immunoprecipitated for ATR. Figure 1-5A shows significant lower levels of translated ATR protein at 0 hour chase in control-KD cells treated with CPT, indicating that the translation of ATR is immediately affected after treatment with CPT. Additionally, the level of ATR protein does not significantly decrease upon increasing chase time (2 and 4 hours). This demonstrates that ATR itself is an extremely stable protein, but its translation is immediately inhibited with CPT treatment.

Since the decrease in ATR protein translation efficiency is not the result of fast ATR protein turnover rate, we next investigate the protein synthesis of ATR. Using 35S-methionine/cystein-protein labeling experiments, we were able to determine and quantify the effect of huSlfn11 on ATR protein translation. FG control and huSlfn11-KD cells were treated with DMSO or 40nM of CPT for 3 hours and 6 hours prior to immunoprecipitation of ATR and GAPDH. 3 hours post-CPT treatment, we saw significant decrease in ATR protein translation level when compared to GAPDH in control-KD cells, 0.64 to 1.23, respectively (Fig. 1-

5 B). At 6 hours post-CPT treatment, the translation of ATR further decreased in FG control-KD cells treated with CPT, while the translation level of ATR in huSlfn11-KD cells remained high regardless of CPT treatment. We did observe a slight decrease in the GAPDH translation level in control-KD cells treated with CPT for 6 hours (1.23 to 0.69); however, the translation level of GAPDH (0.69) is still significantly higher than that of ATR (0.50). This observed decrease in GAPDH translation level is most likely the result of an accumulation of large amounts of DSBs induced by prolonged CPT treatment. All together, these results demonstrate that upon CPT-induced DNA damage, huSlfn11 selectively inhibits ATR protein synthesis resulting in reduced ATR protein translation, thus the inability of cells to efficiently repair DNA damage which results in eventual cell death.

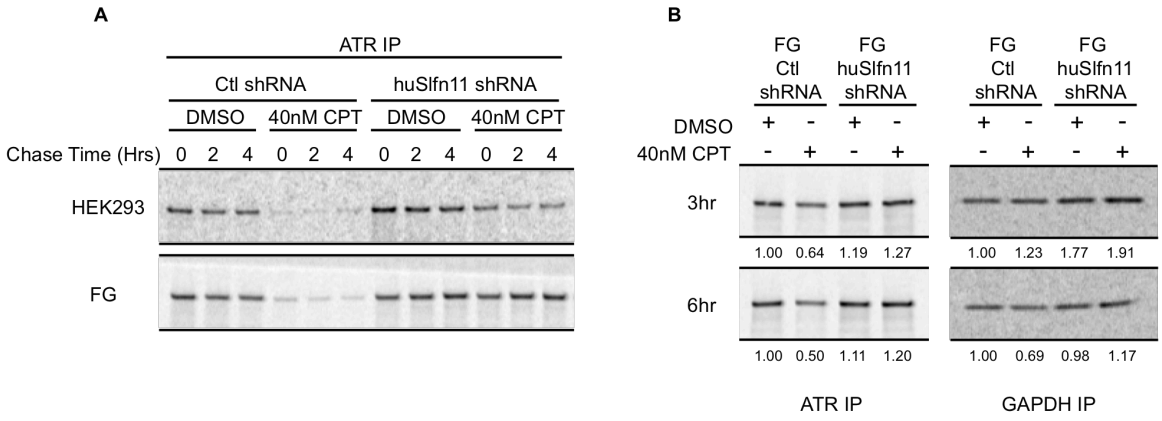

Figure 1-5. Immediate inhibition of ATR protein translation by huSlfn11 in CPT treated cells. (**A**) 35S-methionine/cystein pulse-chase in HEK293 and FG stable knockdown cell lines. Cells were pre-treated with DMSO or CPT for 24 hours prior to labeling and immunoprecipitation. (**B**) 35S-methionin/cystein protein labeling followed by ATR and GAPDH immunoprecipitation. FG stable knockdown cell lines were treated with DMSO or CPT for 3 and 6 hours prior to 35S labeling.

We have thus far provided strong evidence demonstrating that huSlfn11 expression confers sensitivity to the anticancer DNA damaging agent, CPT, by

selectively inhibiting the protein translation of ATR resulting in decreased ATR protein expression and impaired DNA damage response that leads to apoptosis. Therefore, we next wanted to investigate whether we can correct huSlfn11-deficient cancer cells to respond to CPT. ATR inhibitors have been popular candidates for ongoing clinical developments targeting replicating cancer cells because ATR is activated by most cancer chemotherapies [92-94]. To assess whether inhibiting the kinase function of ATR through the use of commercially available ATR inhibitors, VE-821 and VE-822, can completely re-sensitize huSlfn11-KD cells to CPT, we performed MTS cell viability assays on FG control and huSlfn11-KD cell lines. Cells were pre-treated with either ATR inhibitor for 1 hour, followed by treatment with 40nM of CPT for 48 hours. The results from the MTS cell viability assays showed mild to moderate re-sensitization of huSlfn11-KD cells to CPT (Fig. 1-6 C, D).

Because inhibiting the kinase function of ATR did not achieve the complete CPT re-sensitization we were hoping, we next investigated whether the non-kinase function of ATR might be more important in this context. To further follow up on this, we knocked down the expression of ATR using siRNA. Western blotting results showed successful knockdown of ATR protein expression in FG stable cell lines (Fig. 1-6 B, left). The accompanying ATM siRNA knockdown results showed the specificity of siRNAs used in these experiments (Fig. 1-6 B, right). Next, we analyzed the effect of ATR siRNA on the cell viability of FG huSlfn11-KD cells treated to CPT. The results showed complete re-sensitization of huSlfn11-KD cells to CPT, down to the same level as huSlfn11-expressing

cells (Fig. 1-6 A). By contrast, the siRNA knockdown of other genes in the DDR pathways (ATM, Mre11, Nbs1, Rad50) only mildly re-sensitized huSlfn11-KD cells to CPT. Parallel experiments were also performed in HEK293 stable cell lines resulting in similar conclusions (Fig. 1-7).

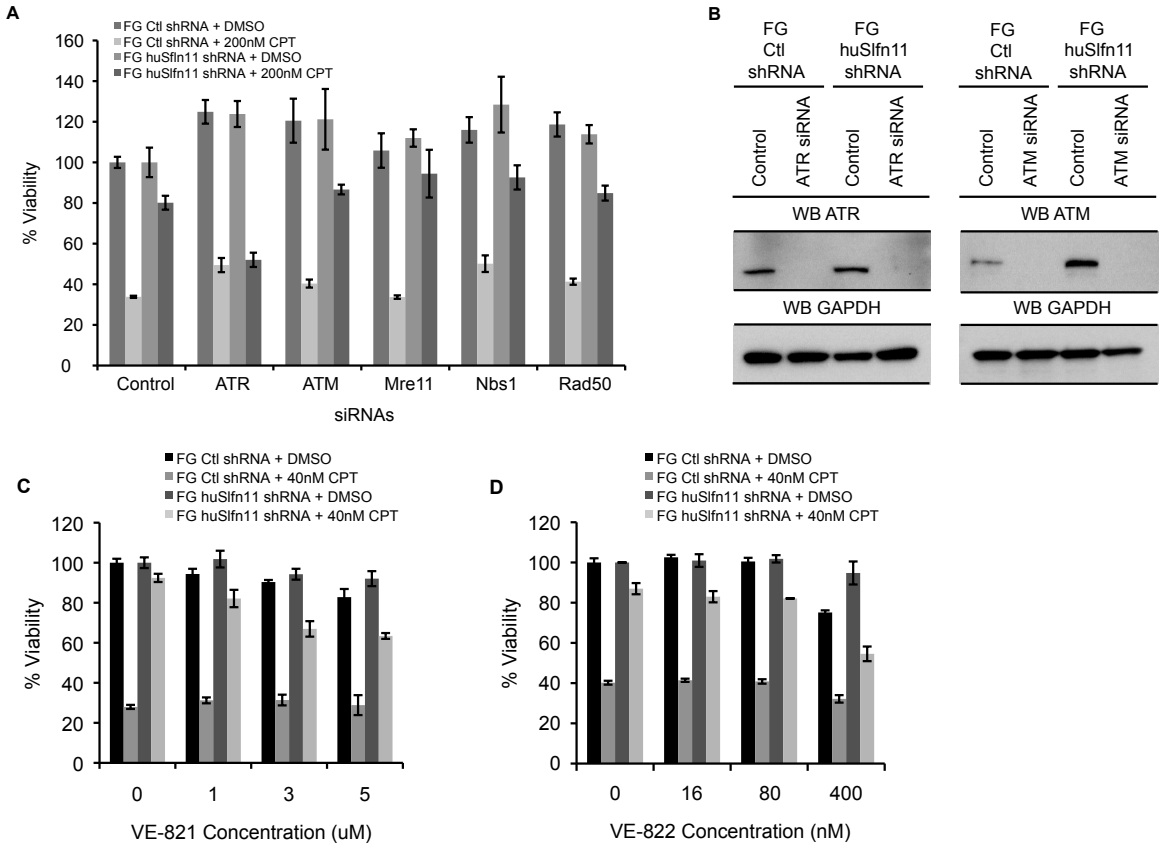

Figure 1-6. ATR siRNA knockdown completely re-sensitized FG huSlfn11-KD cells to CPT. (**A**) FG cell viability was determined by MTS assays 5 days after siRNA reverse transfection and 48 hours after CPT treatment. (**B**) Western blotting results confirmed the specificity of the siRNAs and their effects. MTS assays of cells were pre-treated with ATR inhibitors for 1 hour, followed by 48 hours CPT treatment. FG huSlfn11-KD cells showed only moderate re-sensitization to CPT with ATR inhibitors (**C** VE-821, **D** VE-822).

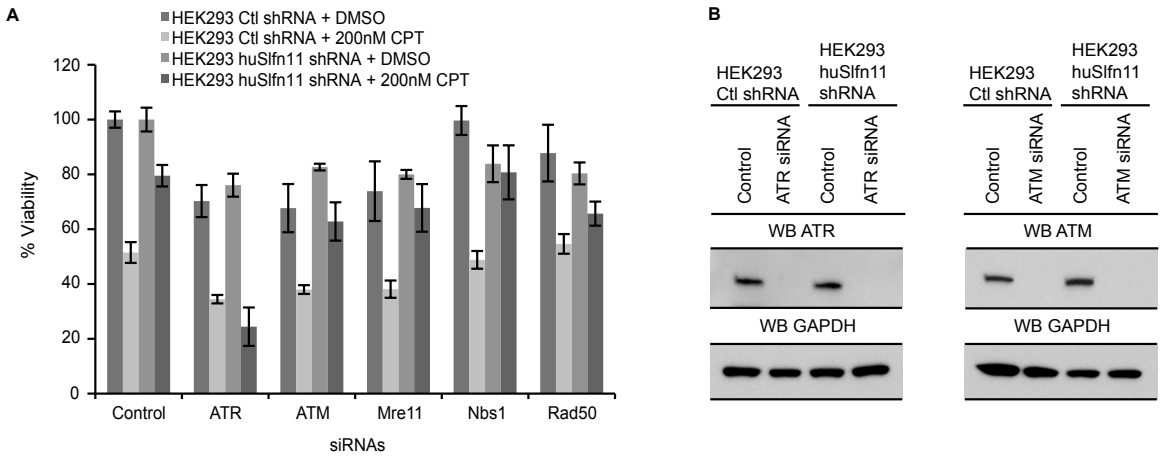

Figure 1-7. ATR siRNA knockdown completely re-sensitized HEK293 huSlfn11-KD cells to CPT. (**A**) MTS assay of ATR siRNA on DMSO- and CPT-treated HEK293 stable knockdown cell lines showed completely re-sensitization of huSlfn11-KD cells to CPT. (**B**) Western blotting results confirmed the knockdown of ATR and ATM by siRNA.

Following the complete re-sensitization of huSlfn11-KD cells to CPT, we next wanted to explore the effect of ATR siRNA on a naturally huSlfn11-deficient cancer cell line. To test this, we used the pancreatic cancer cell line, MIA PaCa-2 (Fig. 1-8 B). Following the same rationale as described previously, we knocked down the expression of ATR using siRNA (Fig. 1-8 C). MTS cell viability assays showed significant re-sensitization of MIA PaCa-2 cells to CPT upon ATR knockdown when compared to DMSO control (Fig. 1-8 A). However, unlike FG huSlfn11-KD cells, treatment of MIA PaCa-2 with the ATR inhibitor, VE-822, resulted in similar level of CPT re-sensitization as the ATR siRNA (Fig. 1-8 D).

By knocking down and inhibiting ATR, we demonstrated complete re-sensitization of MIA PaCa-2 cells to CPT. Numerous reports have looked into other important kinases in the DDR pathways as a target for increasing the efficiency of anticancer treatment. In particular Chk1 because it is immediate downstream of ATR. Using two different Chk1 inhibitors, CHIR-124 and

LY2603618, we saw significantly stronger re-sensitization of MIA PaCa-2 cells to CPT as compared to VE-822 (Fig. 1-8 E, F). These results demonstrate that Chk1 might be a more suitable and effective candidate for kinase inhibitor-targeted cancer therapy for CPT re-sensitization in huSlfn11-deficient cancer cells.

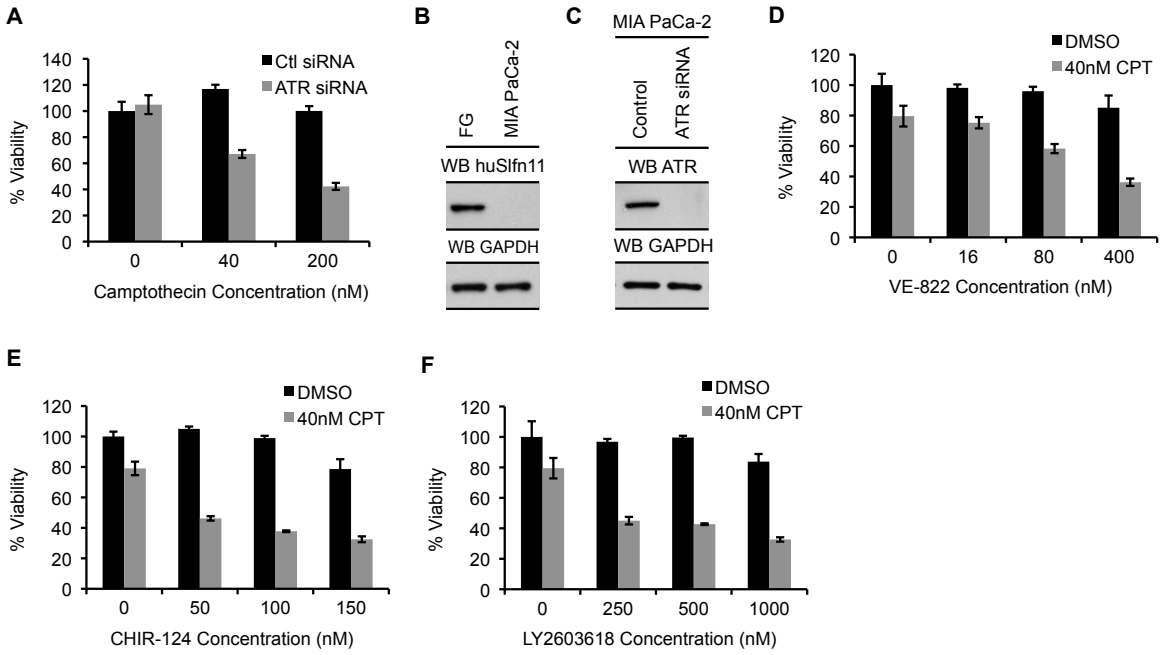

Figure 1-8. MIA PaCa-2 can be re-sensitized to CPT by ATR siRNA, ATR and Chk1 inhibitors. (**A**) MTS cell viability assay showed CPT re-sensitization by ATR siRNA. Western blotting results showing huSlfn11 (**B**) and ATR expression (**C**). Cells were pre-treated with inhibitors for 1 hour followed by CPT treatment for 48 hours prior to MTS assay. MTS cell viability assays showed re-sensitization to CPT by ATR inhibitor (**D**), and Chk1 inhibitors (**E** and **F**).

It has been reported that codon bias can be a way to regulate gene expression through controlling protein translation [95]. Additionally, previous study on the role of huSlfn11 in selectively inhibiting viral proteins synthesis based on their bias codon usage [19] prompted us to investigate whether huSlfn11 is acting through the same mechanism with ATR in response to CPT-

induced DNA damage. Using an algorithm developed by GenScript, we analyzed the codon usage frequency along the ATR coding sequence. ATR displays very strong bias towards rare codons, even more than the HIV *gag* gene (Fig. 1-9 A, B). Furthermore, it has been reported that longer genes, such as ATR, have stronger codon bias. This might be attributed to more strict regulation of larger proteins in order to prevent wasting of resources for mistranslation [96]. Analysis of codon frequency usage on genes not regulated by huSlfn11, such as GAPDH and β-actin, showed they are more prone to use optimized codons (Fig. 1-9 C, D).

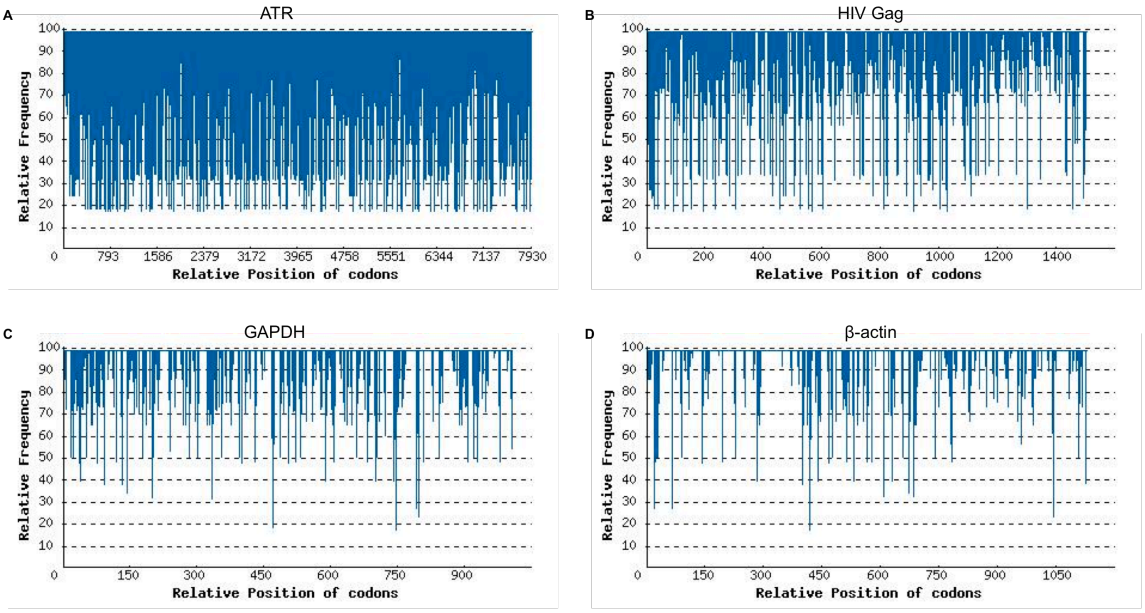

Figure 1-9. Codon usage frequency of ATR showed tendency to use rare codons. X-axis shows the relative position of codons along the coding sequence of each gene. Y-axis shows the relative frequency, or the frequency of codon used at each position normalized to the frequency of synonamous codons used most frequently in the human genome (set as 100). The more rare the codon, the less frequent it is used and the lower the relative frequency. (**A**) The codon usage frequency of ATR displays strong bias towards rare codons. (**B**) Codon usage frequency of HIV *gag* gene. Codon usage frequency of GAPDH (**C**) and β-actin (**D**) showed these genes utilize more frequently used codons.

Discussion

It is of strong interest in the clinics to develop efficient and accurate biomarkers and treatments to combat the large variety of human cancers. The role of huSlfn11 in conferring cancer cell sensitivity to DNA damaging agents, such as camptothecin, has previously been described and further confirmed in this study [32, 33]. In the current study, we demonstrated for the first time that huSlfn11 selectively inhibits the protein translation of ATR, an important signal transducer in the DNA damage response pathways. Through this selective protein translation inhibition of ATR, cancer cells deficient in huSlfn11 expression have prolonged cell survival upon CPT treatment (Fig. 1-1). Polysome profiles of ATR and ATM, kinases that are essential in the DDR pathways, revealed significant decrease in the protein translation efficiency of FG control-KD cells compared to huSlfn11-KD cells treated with CPT (Fig. 1-4 A, B). However, GAPDH and β-actin, genes not involved in DDR, showed no significant difference in their polysome profiles, regardless of huSlfn11 expression and CPT treatment (Fig. 1-4 C, D).

This difference in protein translation was further demonstrated to be the result of huSlfn11-mediated decrease in ATR protein synthesis, rather than an effect on ATR protein stability (Fig. 1-5). Optimal protein expression is determined by high translation initiation and elongation rate [91]. Faster translation elongation can occur by using more frequently used codons, which are recognized by abundant tRNAs [97]. As shown above, huSlfn11-expressing cells treated with CPT displayed ATR polysome profile with an increase in lighter

fractions, indicating less ribosomes attached to ATR mRNAs and subsequent decrease in protein translation efficiency (Fig. 1-4 A, left). Together with the result of the ATR codon usage frequency, which shows stronger bias towards rare codons, suggest that the observed decrease in ATR protein translation efficiency might be attributed to the use of rare codons with less abundant cognate tRNAs, resulting in reduced protein translation elongation rate [98, 99]. Furthermore, huSlfn11 has been shown to interact non-discriminately with tRNAs and potentially affect their expression, maturation, or charging [19]. DDA-treated yeast has also been shown to accumulate unspliced tRNAs in the nucleus and to upregulate specific tRNA-modifying enzymes to enhance codon-specific translation elongation [100, 101]. All together, these suggest that huSlfn11 might be regulating ATR protein translation upon CPT-induced DNA damage by modifying or sequestering tRNAs to interfere with their availability and capability to bind these rare codons.

We observed a slight decrease in the protein translation of GAPDH in FG control-KD cells treated with CPT upon increasing CPT exposure time (Fig. 1-5 B). This slight decrease in GAPDH protein translation was probably the result of an increase in DNA damage accumulated upon increased CPT treatment time, from 3 hours to 6 hours. Upon severe DNA damage, cells most likely devote majority of their resources into machineries important for DNA repair and cell cycle checkpoints [90], so protein translation of such ubiquitous protein as GAPDH is probably not top priority. Additionally, as cells are exposed to CPT for longer time periods, they are now entering into early stages of apoptosis, and we

can observe evidence of cell death in the cultures of these CPT-treated cells (data not shown).

Upon elucidating the mechanism of huSlfn11-mediated selective ATR protein translation inhibition, we investigated potential therapeutic treatments targeting cancer cells that do not express huSlfn11, therefore unresponsive to CPT as an anticancer treatment. Following previous studies that investigated ATR inhibitors as a way to improve the efficiency of cancer therapy [92-94], we demonstrated that ATR inhibitors are limited at re-sensitizing huSlfn11-deficient cancer cells to CPT. In our study, we saw complete re-sensitization of MIA PaCa-2 cancer cells to CPT when used in combination with ATR inhibitor (Fig. 1-8 D), but we were unable to achieve the same degree of CPT re-sensitization in FG huSlfn11-KD cells (Fig. 1-6 C, D). Interestingly, we were able to completely re-sensitize both FG huSlfn11-KD cells and MIA PaCa-2 to CPT when we ablated ATR expression using siRNA (Fig. 1-6 A and Fig. 1-8 A). These results highlighted the multi-faceted role of ATR within the cell. ATR is a very large protein, and the functions of many of its domains are still unknown. Besides serving as an important kinase in the DNA damage response pathways, ATR has been implicated to function as a scaffolding protein, possibly through its understudied amino terminal domain [41]. In this regard, we conclude that kinase inhibitors are not potent enough to modulate all functions of ATR and bring about complete re-sensitization of huSlfn11-deficient cancer cells to CPT.

We also provided strong evidence illustrating the effectiveness of Chk1 inhibitors in re-sensitizing certain huSlfn11-deficient cancer cells to CPT (Fig. 1-8

E, F). This demonstrates that perhaps targeting a kinase that only belongs in the DDR pathways, rather than a multi-functional kinase such as ATR, might be a more effective way to improve cancer therapy. All human cancers are full of extremely variable mutations and drug sensitivities, but we have presented a novel approach to an effective targeted cancer therapy by identifying huSlfn11 as a regulator of genes essential in the DNA damage response pathways.

One unanswered question centers on the signaling pathway that activates huSlfn11. Our previous discovery that huSlfn11 is a executioner of an anti-retroviral innate immune response was completely novel and unique, and described for the first time a molecular mechanism for any member of this family. In the current study, we provided evidence implicating huSlfn11 in cell fate decisions in response to DDAs, indicating that huSlfn11 fulfills another vital cellular function. One key question is whether these two cellular functions are independent of each other, or whether a common mechanism can be identified that controls both retroviral replication and DNA damage responses. We can eliminate the possibility of CPT directly activating huSlfn11 because huSlfn11-expressing cancer cells also showed the same sensitivity when treated with other DDAs (Mitotoxantrone, Chlormabucil, and Gemcitabine). Based on preliminary results from currently ongoing research in the lab, our hypothetical model suggests DNA damage might indirectly activate huSlfn11 by first activating a phosphatase, which in turn de-phosphorylates huSlfn11 at specific serine sites in order to activate its function. The selective translation inhibition of retroviral proteins based on codon usage by huSlfn11 might simply be the result of

huSlfn11 activation induced by DNA damage that occurs during HIV viral DNA integration into the host genome.

Additionally, another unanswered question centers on how huSlfn11 might interact with tRNA to regulate protein translation efficiency based on codon usage. Our previous study on HIV showed that huSlfn11N binds non-discriminately to tRNAs; however, the full length huSlfn11 protein might have specificity for certain tRNAs. Alternatively, huSlfn11 might not bind tRNAs at all but rather interact with tRNA synthetase to interfere with the charging of specific cognate amino acid to its compatible cognate tRNAs. Unpublished data from our collaborator also showed that huSlfn11 can form hexamers through interactions at the AAA domains. It is possible that the formation of hexamer is required for complete huSlfn11 function. Lastly, a recent study in reticulocytes showed that huSlfn14 can bind directly to ribosomes and is an endoribonuclease that is capable of cleaving RNAs [102]. It is feasible that huSlfn11 could have the same endoribonuclease activity and functions to degrade tRNAs, thus limiting the availability of rare codons even more.

Acknowledgements

Chapter 1, in full is currently being prepared for submission for publication by Kao, Elaine. Elaine Yi Kao was the primary author and researcher of this material. I would like to thank co-authors Dr. Manqing Li, Xia Gao, Marcos Arribas-Layton, and Dr. Jean Y. J. Wang for their hard work, helpful discussions, and unwavering support.

CHAPTER 2: Human Schlafen 11 and Z-DNA Formation

Background on Z-DNA

DNA is capable of assuming many different conformations. The more familiar right-handed B-DNA configuration has been widely studied. However, B-DNA can change its shape dramatically to the less well-known left-handed Z-DNA conformation. This conformation has been observed through X-ray diffraction of DNA fibers when water and ion content varied [103]. Initial reports on Z-DNA focused on the in-vitro structural and chemical formation of this DNA configuration. Optical studies demonstrated that polymer with alternating deoxyguanosine and deoxycytidine residues undergoes a salt-dependent and reversible conformational transition [104]. This transition involves “flipping” the base pairs upside-down, resulting in inverted deoxyguanosines to the syn conformation while keeping deoxycytidines in the usual anti conformation. This results in the disappearance of the major groove and the backbone now follows a zigzag pattern. This reorganization results in phosphate groups becoming closer to each other than in B-DNA. The Z conformation was characterized by an inverted UV circular dichroic spectrum compared to the B conformation [104], and further confirmed by the Raman spectra of crystalline Z-DNA and high salt polynucleotides solution [105].

Z-DNA is favored in nucleotide sequences with alternating purines and pyrimidines, especially alternating GC sequences. It can be generated from synthetic polynucleotides under high salt concentration (above 2.5M NaCl or 0.7M $MgCl_2$) [104], in addition to numerous chemical and environmental

modifications that were shown to influence the B/Z-DNA equilibrium. Naturally occurring polyamines such as spermine and spermidine [106], bromination [107], methylation on cytosine [108], and negative supercoiling [109] can all stabilize the Z conformation. Z-DNA conformation is less stable in physiological conditions and resides in a high-energy state due to the close proximity of negatively charged phosphates on opposite strands resulting in electrostatic repulsion. In-vitro high salt conditions reduce this repulsion and stabilize the Z conformation [110]. All these indicate that the sequence of the base pairs is important in determining the energy that is required to transition from B- to Z-DNA.

The discovery that Z-DNA can form under physiological salt conditions upon methylation and be stabilized by negative supercoiling suggested that Z-DNA might have biological functions. Unlike B-DNA, Z-DNA is extremely immunogenic which has not only allowed for the production of highly specific antibodies for research purposes [111], but has also given rise to anti-Z-DNA antibodies in autoimmune disease such as lupus erythematosis [112]. Z-DNA antibodies identified Z-DNA presence in *Drosophila* polytene chromosomes [113] and ciliated protozoa macronucleus [114]. Both structures are associated with high levels of transcriptional activity, thus indirectly links Z-DNA presence with transcription. In-vitro studies with *E. coli* RNA polymerase demonstrated that negative torsional strain is generated behind a moving polymerase resulting in Z-DNA stabilization [115, 116]. Additional support that links Z-DNA with transcription showed that there are high concentrations of Z-DNA forming sequences near transcription start sites [117]. However, the most convincing

evidence that solidified a direct correlation between transcription and Z-DNA conformation came from experiments using metabolically active permeabilized mammalian nuclei with nearly in-vivo rate of DNA replication and transcription [118]. The levels of Z-DNA was shown to be regulated by torsional strain and increased upon an increase in transcriptional activity [119]. Additionally, DNA unwrapped from nucleosomes have been found to be in the Z conformation and unable to reform into nucleosomes. This allows for the accumulation of transcription factors, and implicates Z-DNA as a trigger for transcription initiation [120, 121].

In opposition to the studies described above, Z-DNA-forming sequence was shown to partially block transcription by T7 RNA polymerase in-vitro. This effect was increased with negative supercoiling of this sequence but disappeared when the sequence was disrupted [122]. Z-DNA-forming sequences was also demonstrated to generate high levels of genetic instability by inducing DSBs that result in large-scale deletions in mammalian cells. The locations of these Z-DNA-induced DSBs are consistent with chromosomal breakpoints found in human tumors [123]. All together, Z-DNA-forming sequences are suggested to be causative factors for translocation-related diseases such as leukemias and lymphomas.

In order to establish the presence of Z-DNA and its functions in-vivo, numerous studies were conducted to identify Z-DNA binding proteins. The discovery of a Z-DNA-binding and RNA-editing enzyme, ADAR1, ended widespread skepticism that Z-DNA is not associated with any biological functions

[124]. ADAR1 edits double-stranded segments in pre-mRNA by deaminating adenosine to inosine, which is then interpreted as a guanine by ribosomes. The N-terminus of ADAR1 contains the Z_α domain that binds to Z-DNA through recognition of the syn-conformation of guanine [125, 126]. This domain can also stabilize Z conformation and B-Z junctions in-vitro [127]. In addition to binding Z-DNA, human ADAR1 Z_α domain can also bind to Z-RNA formed from dsRNA [128], demonstrating that recognition and binding by ADAR1 is structure-specific and not sequence-specific.

Besides ADAR1, other Z-DNA binding proteins have also been identified. DLM-1 (or ZBP1/DAI) is upregulated by IFNγ in tissues near tumors and contains a Z_α domain with sequence similar to that of ADAR1 Z_α domain [129]. PZ proteins isolated from the DNA tumor virus SV40 were found to bind to alternating purine-pyrimidine sequences that could form Z-DNA upon negative supercoiling caused by DNA unwinding. This sequence is located in the control region of the virus, which contains the early and late promoters that are responsible for replication and transcription [130, 131]. Additionally, poxviruses express the Z-DNA binding protein E3L that is exported into the nucleus upon infection and is necessary for its pathogenicity [132]. To explore the connection between Z-DNA binding activities and viral pathogenicity, chimeric viruses were created by replacing the Z-DNA binding domain of E3L with the Z_α domain from either ADAR1 or DLM-1. The chimeric viruses were shown to be as pathogenic as wildtype virus. Further investigation demonstrated that weakening the Z-binding activity by mutations or deletion resulted in reduced viral pathogenicity

[133].

Z-DNA has been a mystery in the field of biology for over three decades. This has mainly been attributed to the transient nature of the Z-DNA conformation. Additionally, its biological activity has also been difficult to study in-vivo because it is induced only upon Z-DNA formation then quickly disappears. The discovery of Z-DNA binding proteins has shed some light on the potential biological functions of Z-DNA. Future studies will no doubt reveal the still unknown properties and biological functions of this left-handed DNA double helix.

Introduction

Immediately after its discovery, Z-DNA generated a lot of interest among a diverse group of scientists both in biology and physical chemistry. Several laboratories determined structural features of Z-DNA sequences, which provided detailed information about the Z conformation as well as B/Z-DNA transition regions. Many studies described the in-vitro formation of Z-DNA including DNA methylation [108] or bromination [107], or interaction with positively charged molecules such as spermine and spermidine that can stabilize Z conformation [106]. Alternatively, high ionic strength (> 4M NaCl) can promote conversion of B-DNA into the Z-configuration in supercoiled plasmid DNA [104, 109]. Unfortunately, the ionic conditions that were suitable for stabilizing Z-DNA in in-vitro experiments were very different from those present in a cell.

Although some studies supported a functional role for Z-DNA in transcription, the results were unexplainably variable among different genes. More recently information about the potential biological roles of Z-DNA emerged. Results obtained from the DNA tumor virus SV40 suggest that Z-DNA may play a role as a transcriptional regulatory element [131]. Other evidence for a biological function of Z-DNA came from the discovery that DNA behind a moving RNA polymerase is unwound and forced into negative torsional strain, which leads to Z-DNA formation at the ends of actively transcribed genes [116]. Sequences with Z-DNA forming potential were also found near transcription start sites, suggesting that these elements might have a functional role in the regulation of gene expression [117]. Additionally, the discovery of Z-DNA binding proteins,

human ADAR1 and poxvirus E3L, suggested that Z-DNA might play a role in the immune response. Currently, only a single lab is actively investigating Z-DNA, specially the role of B-DNA to Z-DNA transition points as areas of high susceptibility to DSBs [134].

Although Z-DNA (and RNA) were discovered decades ago, current investigation of their physiological role is almost non-existing. Recent published data from our lab defined a biological function of huSlfn11 protein in attenuating the production of retroviruses including HIV. In the course of our studies we found that the N-terminal region (contains the AAA domain) of huSlfn11 (huSlfn11N) binds tRNA and selectively inhibits viral protein synthesis based on codon usage [19]. Unpublished data from our lab demonstrated another member of the Slfn family, muSlfn2, is able to bind to all forms of nucleic acids (ss- and ds-DNA and RNA). Further studies revealed that muSlfn2 could introduce changes into plasmid DNA that affects its mobility on agarose gels in a Mg^{2+}-dependent manner. These migration differences were abolished by linearization of the plasmid. We explored numerous possibilities to explain this shift in DNA mobility such as nicking, coiling, rearrangement into concatamers, and the somewhat remote possibility of Z-DNA formation.

Because muSlfn2 is similar in length and sequence to huSlfn11N, and we already understand the anti-viral function of huSlfn11, we decided to investigate the potential of huSlfn11N to generate or stabilize Z-DNA. We hypothesized that Z-DNA not only fulfills regulatory functions by controlling transcriptional events, but that Z-DNA also contributes to both innate and adaptive immune responses.

This hypothesis is rooted in the fact that all known Z-DNA binding proteins are either of viral origin (E3L), or those of mammalian derivation are induced by IFN (DAI). In addition, poxviruses also encode vSlfn that is important for its virulence, further suggesting that poxviruses evolved a countermeasure against host anti-viral defense. We hope to provide new insights into the host-pathogen interactions involving Slfn proteins, in addition to create a better understanding of the biological significance of left-handed nucleic acid conformations.

Materials and Methods

Cloning and expression

The huSlfn11N protein was cloned in pET101 vector that includes a C-terminal V5-6xHis tag (Life Technologies) and expressed in *E. coli* DX competent cells. Cells were grown at 37°C until log phase (OD_{600} ~ 0.5-0.7) then transferred to 25°C for induction of protein expression overnight (20-24 hours). Protein expression was induced with 0.1mM of IPTG and 1mg/mL of L-Arabinose.

Protein purification with FPLC

Isolated cell pellets were resuspended in 20mL of binding buffer containing 20mM sodium phosphate, 0.5M NaCl, 10mM imidazole at pH7.4, 1 tablet of EDTA-free protease inhibitor (Roche), and incubated for 30 minutes on ice with 20mg of lysozyme (USB) followed by sonnication (8X 10 seconds, on ice). 10ug/mL RNaseA and 5ug/mL DNaseI (Roche) were added to the lysates then incubated on ice for 15 minutes. All samples were clarified by centrifugation at 10,000 R.P.M for 30 minutes followed by filtration through 0.45um and 0.22um filters (Millipore).

The standard purification protocol includes the following chromatographic steps:

(1) FPLC (fast protein liquid chromatography) using a 1-mL HisTrap FF affinity column (GE Healthcare) charged with Ni. Proteins were eluted with a linear gradient of imidazole (up to 250mM) in the same buffer. Each fraction was monitored at 280nm, collected at a volume of 1mL.

(2) Verification of protein expression in eluted fractions using Coomassie

staining.

(3) Buffer-exchange chromatography on desalting spin column with 40mM Tris pH7 (Thermo Scientific).

Dot blot assay and Z-DNA detection

For each dot blot reaction, 250ng of purified protein was mixed with 100ng of plasmid DNA and 1mM DTT on ice. Nylon membranes were washed 1X with 20mM Tris pH7 then samples were spotted. Membrane was dried at room temperature then blocked with 5% milk diluted in TBS-Tween 20 for 1 hour. Z-22 anti-Z-DNA antibody was added at 1:10,000 diluted in 5% BSA for 1.5 hour at room temperature. Membrane was washed 3X with TBST before incubation with mouse fluorescent secondary antibody (APC) for 1 hour at room temperature followed by 3X washing then Z-DNA signal was detected using phosphoimager.

Results

During the course of our investigations into the general nucleic acid binding properties of huSlfn11, we explored the possibility that huSlfn11N might generate or stabilize Z-DNA formation. After obtaining an aliquot of a mouse monoclonal anti-Z-DNA antibody (clone Z-22) from Dr. David Stollar that was extensively used in the 1980s and 90s [111], we verified by dot blot assay that FPLC-purified huSlfn11N protein promoted Z-DNA formation in pUC18 plasmid (Fig. 2-1). To confirm what we observe with pUC18 was not an artifact of the in-vitro conditions or the anti-Z-DNA antibody, we performed dot blot assay with DNA plasmids of varying sizes along with a no protein control (Buffer), a negative control protein (GFP), and huSlfn11N (Fig. 2-2 A). All plasmids tested were able to generate Z-DNA with purified huSlfn11N protein but not with buffer and GFP. We also quantified the relative Z-DNA signal in each condition and confirmed that Z-DNA signal was significantly higher in the presence of huSlfn11N (Fig. 2-2 B). Interestingly, we noted that plasmids alone (Buffer) or with GFP seem to contain low basal levels of Z-DNA signal (Fig. 2-2 A, lane 1 and 2). This could be attributed to the DNA purification kits used by our lab that yield highly supercoiled purified DNA.

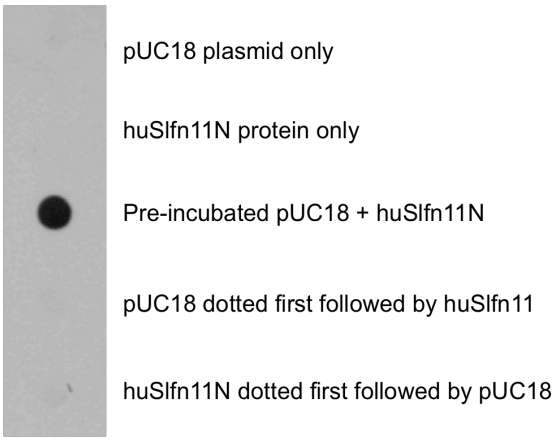

Figure 2-1. Human Schlafen11N promoted Z-DNA formation in pUC18. Pre-incubation of pUC18 with FPLC purified huSlfn11N protein resulted in Z-DNA detection with Z-22 antibody at 1:10,000 dilution. Plasmid or huSlfn11N alone does not generate Z-DNA. Sequential dotting of pUC18 and huSlfn11N also does not generate Z-DNA.

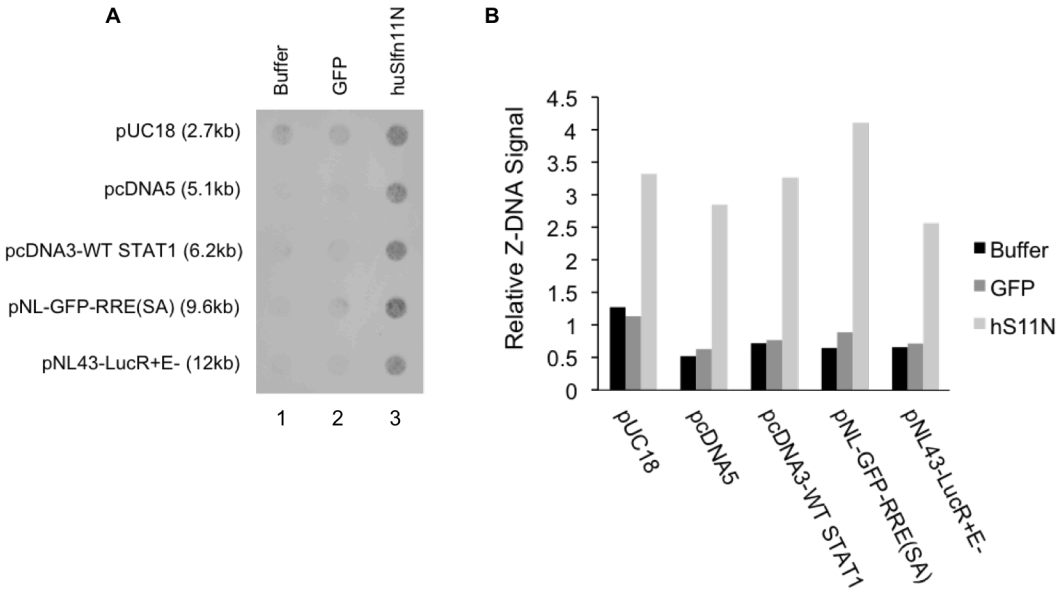

Figure 2-2. Human Schlafen11N promoted Z-DNA formation in DNA plasmids of varying sizes. (**A**) Dot blot assay of buffer (no protein control), GFP (negative control protein), or huSlfn11N with plasmids of varying sizes confirmed Z-DNA detection is not an artifact of in-vitro conditions or Z-22 antibody. (**B**) Quantification of relative Z-DNA signal.

Next, to determine the kinetics of Z-DNA formation or stabilization by huSlfn11N, we performed dot blot assay with increasing molar ratios of purified huSlfn11N protein to pUC18 plasmid DNA over time at 4°C. There was virtually

no observable kinetics in huSlfn11N-mediated Z-DNA formation or stabilization as Z-DNA signal was detectable immediately (0 minute) in the presence of huSlfn11N protein and is extremely stable once formed (Fig. 2-3 A). This suggests that Z-DNA “formation” by huSlfn11N is not an enzymatic reaction, but rather a binding conformational change in which huSlfn11N binds and stabilizes the Z-DNA structure, resulting in a shift in the B/Z-DNA equilibrium in favor of Z-DNA. We also observed an increase in the level of relative Z-DNA signal corresponding to an increase in the molar ratio of huSlfn11N protein to pUC18 plasmid DNA and reaction time (Fig. 2-3 B). Together, this suggests that more huSlfn11N protein results in more efficient conversion of B-DNA to Z-DNA, possibly as a result of increased stabilization of B-Z junctions and favoring of the Z conformation.

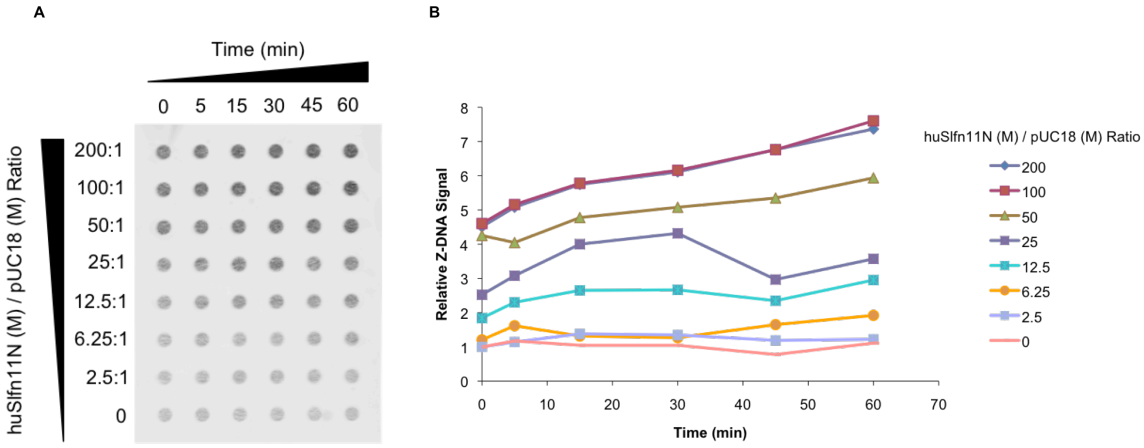

Figure 2-3. Kinetics of Z-DNA generation by huSlfn11N. (**A**) Dot blot assay showed detection of Z-DNA signal over time with increasing molar ratios of huSlfn11N protein to pUC18. (**B**) Quantification of relative Z-DNA signal shown in A.

Previous publications demonstrated that Z-DNA formation is stabilized by negative supercoiling [109]. Consistent with existing literature, plasmid DNA pre-

treated with GyraseB, which negatively supercoils closed circular dsDNA, showed Z-DNA conformation as detected by the Z-22 antibody (Fig. 2-4 A, 1E). The level of detectable Z-DNA signal in Gyrase-treated plasmid DNA was significantly higher in the presence of huSlfn11N protein (Fig. 2-4 A, 3E; Fig. 2-4 B) compared to the no protein control (Buffer) and the negative control (GFP) (Fig. 2-4 A, 1E and 2E). Alternatively, Z-DNA conformation was abolished when DNA was pre-treated with Topoisomerase, which relaxes supercoiling (Fig. 2-4 A, 1F and 2F). It appears that even in the presence of huSlfn11N, relaxed plasmid DNA cannot generate detectable levels of Z-DNA (Fig 2-4 A, 3F). As noted previously, untreated plasmid alone or with control protein GFP contained low levels of detectable Z-DNA (Fig. 2-4 A, 1B and 2B). Interestingly, plasmids linearized with restriction enzymes, BamHI and ScaI, also possessed low levels of detectable Z-DNA signal alone or with GFP (Fig. 2-4 A, 1C and D, 2C and D; Fig. 2-4 B). This is most likely the result of anti-Z-DNA antibody stabilizing Z-DNA in linear plasmids [135]. It appears that the Z-DNA conformation is quite easy to detect; however, dot blot results and Z-DNA signal quantification showed that negative superociling is better at stabilizing Z-DNA than anti-Z-DNA antibody. Regardless of basal Z-DNA levels and plasmid pre-treatments, the relative Z-DNA signal was consistently higher with purified huSlfn11N protein for all plasmid conditions (except for no plasmid control and topoisomerase-treated pUC18), and the signal was strongest with Gyrase-treated plasmid DNA (Fig. 2-4 A, lane 3 and Fig. 2-4 B).

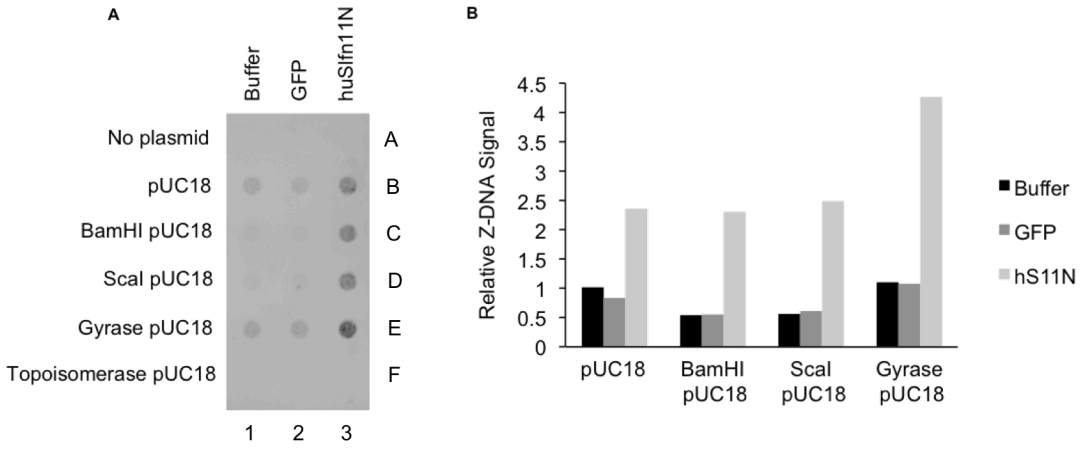

Figure 2-4. Negative supercoiling significantly enhanced Z-DNA stabilization by huSlfn11N. (**A**) Dot blot assay showed Z-DNA formation by huSlfn11N was enhanced by negatively suprecoiling the plasmid DNA with Gyrase, but abolished when relaxed with Topoisomerase. (**B**) Quantification of relative Z-DNA signal in all conditions listed with the exception of no plasmid control and Topoisomerase-treated plasmid DNA (signals were not detectable).

Next, we wanted to further characterize the conditions that are permissive for huSlfn11N-mediated Z-DNA stabilization. To do this, we first investigated the effect of temperature changes on Z-DNA formation. Plasmid alone or with GFP or huSlfn11N purified proteins were pre-mixed together followed by incubation at 4°C, 30°C, or 55°C for various lengths of time (Fig. 2-5 A). Results from dot blot assay as well as relative Z-DNA signal quantification showed that Z-DNA forms robustly with huSlfn11N protein at all the temperatures listed and is very stable once formed (Fig. 2-5 A, B). We also noted an increase in Z-DNA signal with rising temperature (Fig. 2-5 A, lane 3), which indicates a temperature-driven right-handed helix to left-handed helix transition that is consistent with previous reports [136]. Additional experiments that varied the temperatures and lengths of incubation time for each reaction showed that huSlfn11N-mediated Z-DNA conformation is very stable once formed (Fig. 2-5 C, lane 2; Fig. 2-5 D). Notably,

when huSlfn11N was denatured at high temperature (55°C), less Z-DNA was detected when compared with GFP (Fig. 2-5 C, row C). As suggested previously, these results further support the hypothesis that Z-DNA stabilization by huSlfn11N is a binding conformational change and not an enzymatic reaction.

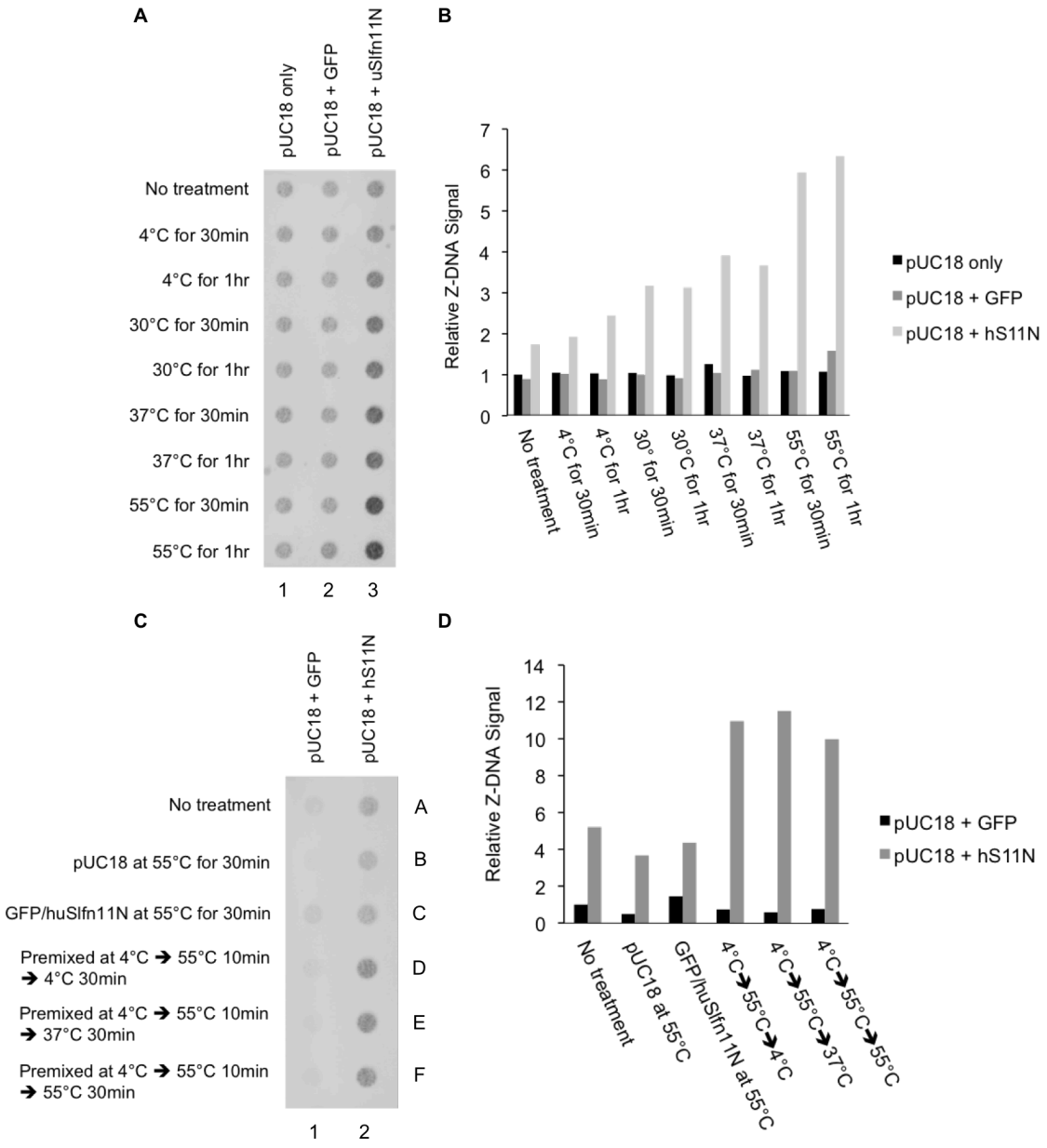

Figure 2-5. Changes in temperature and incubation time does not affect huSlfn11N-mediated Z-DNA stabilization. (**A**) Dot blot assay showed plasmid DNA formed Z-DNA more readily with huSlfn11N in all temperatures and reaction incubation times tested. (**B**) Relative Z-DNA signal quantification of A. (**C**) Effects of different temperature combinations on huSlfn11N-mediated Z-DNA stabilization. (**D**) Z-DNA signal quantification of C.

Metal ions are known to play important roles in biological activity and structural stabilization of DNA so we next investigated the effect of metal ions on Z-DNA stabilization by huSlfn11N. Dot blot assay results showed that huSlfn11N-

mediated Z-DNA stabilization could occur in the presence of most ions with the exception of $ZnCl_2$ (Fig. 2-6 A). Zinc is an abundant trace element in the cell, with 30-40% of total zinc ions located in the nucleus [137]. Recent studies showed that minimal Zn^{2+} concentrations can block annealing of GC-rich DNA sequences, resulting in de-stabilized DNA [138]. We also noted that the presence of Mn^{2+} ions resulted in a moderate decrease in huSlfn11N-mediated Z-DNA signal. Mn^{2+} ions are known to interact with DNA at the N7 position of purines, especially guanine. Binding of Mn^{2+} ion has been reported to induce perturbation of G-C base-pairing, which might result in de-stabilization of the Z-DNA conformation [139]. The presence of DNA intercalating agent, ethidium bromide, also had no effect on huSlfn11N-mediated Z-DNA formation. The relative Z-DNA signal quantification also reflects the same conclusion (Fig. 2-6 B). It has been known that high ionic strength can promote conversion of B-DNA into the Z-configuration in supercoiled plasmid DNA [104]. This observation was verified with dot blot assay showing pUC18 plasmid alone progressively transitioned into more Z-conformation upon increasing NaCl concentration (Fig. 2-6 C, top row). Unfortunately, the ionic conditions that were suitable for stabilizing Z-DNA in in-vitro experiments were very different from those present in cell. However, we found that Z-DNA stabilization by huSlfn11N was most efficient at physiological salt concentrations (Fig. 2-6 C, bottom tow), which represents an important step forward in the study on the functional role of Z-DNA as well as another novel function of the Slfn protein family.

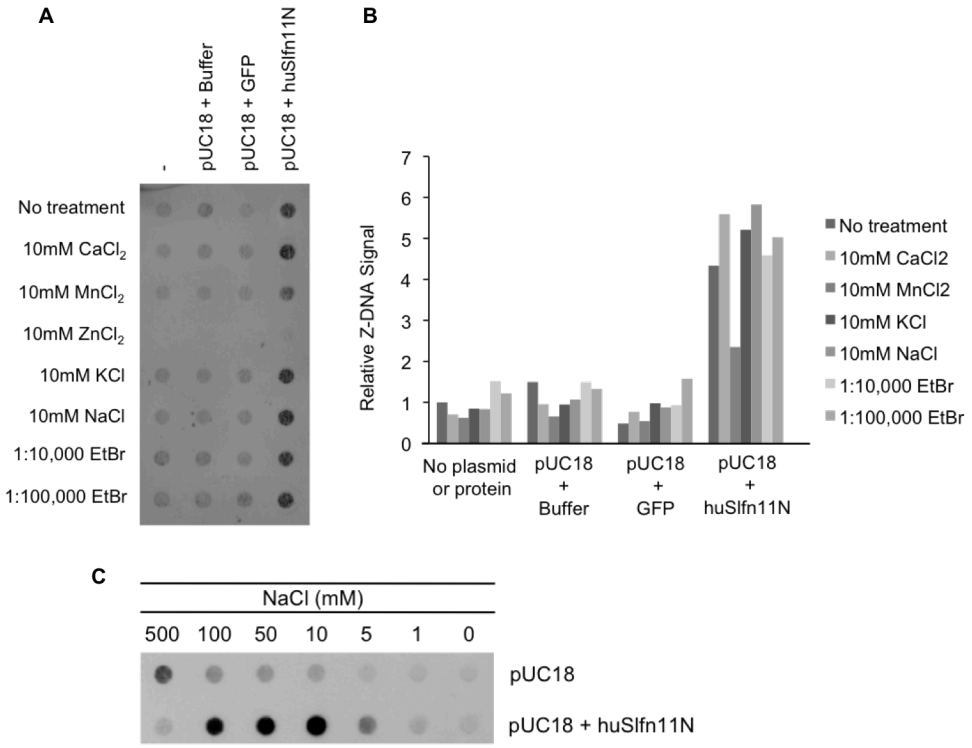

Figure 2-6. Z-DNA stabilization by huSlfn11N occurs most efficiently at physiological salt concentrations. (**A**) Dot blot assay showed most metal ions does not affect huSlfn11N-medaited Z-DNA stabilization. (**B**) Quantification of relative Z-DNA signal as shown in A. (**C**) Z-DNA forms naturally in plasmid DNA as NaCl concentrations increase. HuSlfn11N-mediated Z-DNA stabilization occurs most efficiently at physiological salt concentrations.

Pattern recognition receptors (TLR9) can recognize CpG oligodeoxynucleotides. Strikingly, they contain GC-rich sequences that possess the inherent capability to form Z-DNA and raise the possibility that Z-DNA structures might be a recognizable target for these pathogen receptors. Z-DNA containing regions have been identified in the SV40 genome [131], and Z-DNA elements can negatively impact transcription [122]. We demonstrated that huSlfn11N could enhance Z-DNA in SV40 genomic DNA in-vitro (Fig. 2-7 A, 3D). Gyrase-treated SV40 genomic DNA could also induce Z-DNA formation with huSlfn11N but to a lesser extent than untreated purified SV40 genomic DNA (Fig. 2-7 A, 3F; Fig. 2-7 B). Alternatively, Topoisomerase-treated SV40 genomic DNA

lost all ability to transition to Z-DNA, even with huSlfn11N protein (Fig. 2-7 A, row E). This suggests that the stabilization of Z-DNA in the genomic DNA of invading pathogens or viruses by huSlfn11N might be a trigger of the innate immune response and prevents transcription of viral DNA.

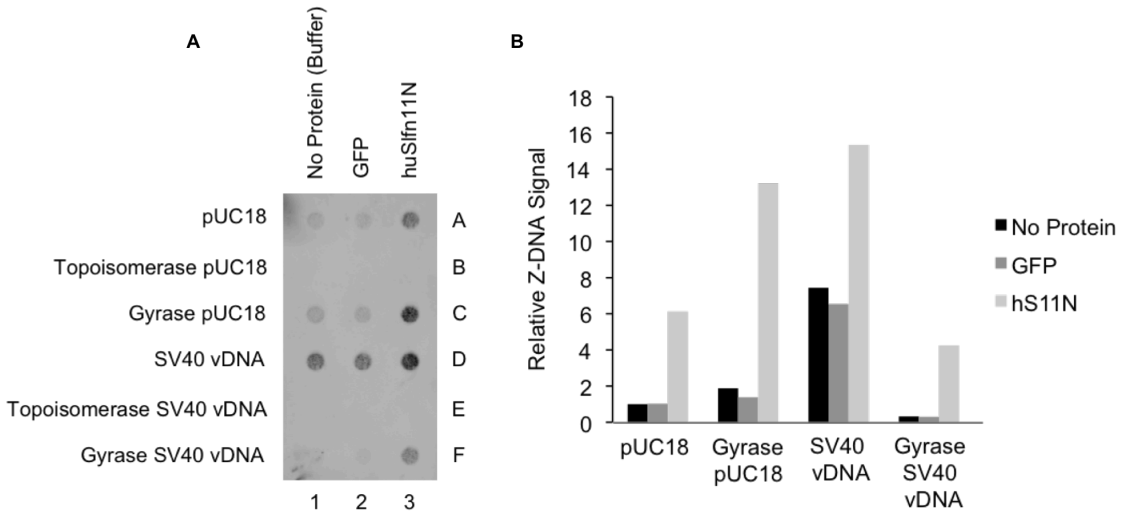

Figure 2-7. HuSlfn11N enhanced Z-DNA in SV40 genomic DNA in-vitro. A SV40 genomic DNA can form Z-DNA with huSlfn11N. **B** Quantification of Z-DNA signal as seen in A.

Discussion

Z-DNA has been a mystery in the field of biology for many decades. In this study, we have demonstrated for the first time, the ability of huSlfn11N protein to stabilize Z-DNA conformation. We also characterized the different conditions that are permissive for huSlfn11N-mediated Z-DNA stabilization. With the use of an anti-Z-DNA antibody (Z-22), we were able to detect and quantify relative Z-DNA signal. We found that huSlfn11N can mediate Z-DNA stabilization in plasmid DNA of all sizes (Fig. 2-2). Kinetics and temperature studies showed that Z-DNA could form immediately upon huSlfn11N protein presence at all temperatures tested (4°C, 37°C, and 55°C) and were stable once formed (Fig. 2-3 and 2-5). The lack of observable kinetics and sustained stability of the Z-DNA conformation mediated by huSlfn11N also suggest that this interaction is not an enzymatic reaction but rather a binding stabilization that shifts the B/Z-DNA equilibrium in favor of Z-DNA.

In agreement with previous publications, we found that negative supercoiling of plasmid DNA using GyraseB can stabilize Z-DNA conformation [109]. The level of detectable Z-DNA signal was also higher in Gyrase-treated pUC18 in the presence of huSlfn11N as compared to no protein control (Buffer) and GFP (Fig. 2-4). Z-DNA formation with huSlfn11N could also occur in the presence of most metal ions (Fig. 2-6). Furthermore, we found that huSlfn11N-mediated Z-DNA stabilization occurs most efficiently at physiological salt concentrations, which will allow us to further study the functional role of Z-DNA together with the Slfn protein family.

It is clear that huSlfn11N suffices to increase the amounts of Z-DNA. The N-terminal region of huSlfn11N contains the ATP-binding AAA domain and includes a number of highly invariant residues that align with AAA sequences found in other proteins that affect function. Additionally, the Z-DNA binding domains in all other known Z-DNA binding proteins (ADAR1 and DAI) are located in the N-terminus. It will be interesting to study whether these residues might be important in huSlfn11 recognition of Z-DNA. Additionally, most of our studies used huSlfn11N, and all long Slfns harbor domain that is conserved among them and weakly resembles that of bacterial RNA/DNA helicases. It is feasible that while the N-terminal region of huSlfn11 is a Z-DNA binding domain without enzymatic activity that only shift the equilibrium towards Z-DNA, but the C-terminal domain might act on the bound Z-DNA to either enhance negative supercoiling to further Z-DNA formation or relieve tension caused by supercoiling in order to prevent DSBs.

In the current study, we also demonstrated that huSlfn11N can enhance Z-DNA in SV40 genomic DNA in-vitro (Fig. 2-7). Z-DNA containing regions have been identified in the control region of SV40, which is responsible for viral replication and transcription [131]. Z-DNA elements have also been shown to negatively impact transcription [115, 122]. All together, this suggests that Z-DNA structures might be a signal to the cellular immune system against invading pathogens. In support of this, recent reports have shown that the cytoplasmic DNA sensor DAI is required for innate immune response activation (including IRF3) during HCMV infection [140, 141]. DAI has been identified as a Z-DNA

binding protein through its Z_α domain in the N-terminus [129]. As huSlfn11N is also capable of creating Z-DNA under physiological conditions, it will be interesting to study whether generation of Z-DNA within the genome of invading pathogens allows for the effective recognition of the foreign DNA by the sensor DAI.

As indicated earlier, the only non-mammalian organism that encode Slfn-like proteins are all members of the poxvirus family [7]. Intriguingly, all poxviruses also harbor a gene for E3L, which is another one of the few known Z-DNA binding proteins [132]. Together, we hypothesize that a connection exists that links Slfn, DAI, and E3L together functionally to support and/or fight against the virulence of these pathogens (Fig. 2-8). Our hypothetical model suggests that Slfn stabilizes Z-DNA conformation in viral DNA to allow recognition by DAI either directly or indirectly, resulting in downstream IRF3 activation to fight against the viral infection. Additionally, Z-DNA conformation might also be maintained by Slfn proteins to allow for the formation of DSBs in viral genomic DNA [142]. Z-DNA stabilization by Slfn will also result in blocking transcription and replication [122], thus further obstructing viruses from propagating within the cell. Alternatively, poxviruses have evolved to encode not only vSlfn but also a Z-DNA binding protein (E3L), most likely to counteract the host anti-viral factors.

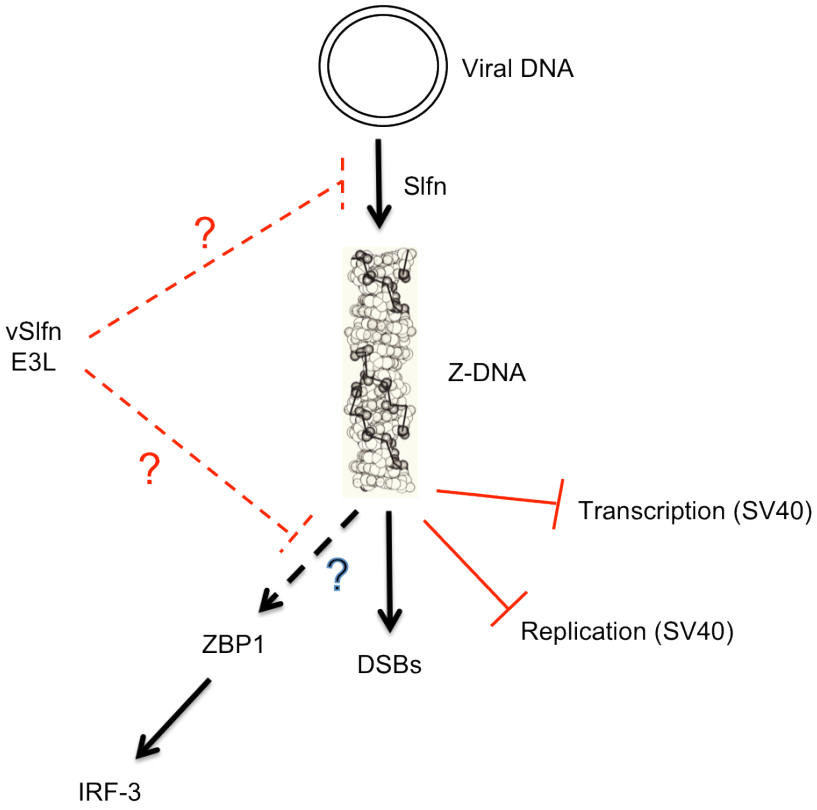

Figure 2-8. Hypothetical model on the biological significance of Slfn-mediated Z-DNA stabilization. Slfn stabilizes Z-DNA conformation in viral DNA thus allowing for DAI recognition and activation of IRF3 anti-viral signaling pathways. Z-DNA stabilization by Slfn could also permit the formation of DSBs as well as block in transcription and replication of the viral genome.

Acknowledgements

Chapter 2, in full is still currently under research by Kao, Elaine. Elaine Yi Kao was the primary researcher of this material. I would like to thank Dr. Manqing Li for his helpful discussions and unwavering support.

Conclusion

Schlafens are a family of fairly unknown proteins whose biological functions and significances have only been described in the past decade. Initial studies have mainly been focused on muSlfns and their roles in thymocyte development and cell cycle progression [8, 10]. The expression of short form muSlfns has been shown to be differentially regulated upon viral and bacterial infections [16, 17]. Furthermore, all members of the poxvirus family encode a vSlfn, thus suggesting the importance of mammlian Slfns in the innate immune response.

In recent years, novel discoveries have been made on the biological roles of human Slfns. HuSlfn11 was found to be a novel antiviral factor that can attenuate the production of retroviruses including HIV by specifically inhibiting the synthesis of viral proteins based on their codon usage [19]. Further investigation revealed that huSlfn11 binds directly to tRNAs. Additionally, huSlfn11 expression was also illustrated to have a strong correlation with sensitivity to DNA damaging agents [32, 33]. The work presented in this dissertation describes the findings on huSlfn11 and DNA damage response.

Cancer cells that express huSlfn11 are highly sensitive to the TOPI inhibitor, CPT, but cells deficient in huSlfn11 expression are resistant to CPT and have prolonged cell survival upon treatment. We found that the expression of huSlfn11 selectively decreased the protein translation efficiency of ATR, an essential signal transducer in the DNA damage response pathways. The codon usage frequency analysis of ATR displayed very strong bias towards rare

codons, indicating reduced protein translation elongation rate [98, 99].
This reduction in ATR protein translation efficiency results in lower ATR protein expression leading to impaired DNA damage response and sensitivity to CPT-induced DNA damage that leads to DSBs and eventually apoptosis. Alternatively, ablating the expression of ATR using siRNA results in complete re-sensitization of huSlfn11-deficient cancer cells to CPT. ATR inhibitors were also capable of moderately re-sensitizing huSlfn11-deficient cancer cells to CPT, but the effect was not as significant and complete as ATR siRNA. We propose that huSlfn11 is activated indirectly by DNA damage, possibly through de-phosphorylation by a still unknown phosphatase. This activation results in a still unidentified interaction between huSlfn11 and tRNAs, thus regulating the level of available tRNA for protein translation.

In addition to regulating components of the DDR, huSlfn11 was also found to stabilize the formation of Z-DNA. This huSlfn11N-mediated Z-DNA stabilization can occur at all temperatures and in the presence of most metal ions. The Z-conformation was present in negatively supercoiled plasmid DNA, and even more pronounced when huSlfn11N was present. We proposed that that huSlfn11N-mediated Z-DNA conformation has an innate immune response function. Z-DNA is stabilized by huSlfn in the cell, resulting in inhibition of viral replication and transcription. The maintenance of Z-DNA also allows for the recognition by cytosolic DNA sensor, DAI, which can activate downstream IRF3 signaling to combat the viral infection.

In conclusion, the work presented in this dissertation has contributed significantly to understanding the biological function and molecular mechanism of huSlfn11. However, there is still much we do not understand, such as how huSlfn11 is activated and its association with tRNAs. In addition, other members of the huSlfn family might also play novel or redundant roles in DNA damage response regulation and antiviral defense as well as functions that are still yet to be discovered. We have only just begun to understand the Slfn family of proteins, and there will sure to be more interesting discoveries in the near future.

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