

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

The Regulation and Activity of Schlafen11

Permalink

<https://escholarship.org/uc/item/4td11694>

Author

Malone, Dane Michael

Publication Date

2017

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Regulation and Activity of Schlafen11

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biology

by

Dane Michael Malone

Committee in charge:

Professor Michael David, Chair
Professor Dan Donoghue
Professor John Guatelli
Professor Jens Lykke-Andersen
Professor Jean Wang

2017

Copyright

Dane Michael Malone, 2017

All rights reserved

The dissertation of Dane Michael Malone is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2017

TABLE OF CONTENTS

SIGNATURE PAGE.....	iii
TABLE OF CONTENTS.....	iv
LIST OF ABBREVIATIONS	v
LIST OF FIGURES	vii
ACKNOWLEDGEMENTS.....	viii
VITA.....	ix
ABSTRACT OF THE DISSERTATION.....	x
BACKGROUND	1
CHAPTER 1: tRNA CLEAVAGE VIA SLFN11 INHIBITS ATR TRANSLATION TO PROMOTE DNA DAMAGE-INDUCED APOPTOSIS	9
Abstract.....	9
Introduction	10
Materials and Methods.....	12
Results	19
Discussion.....	32
Extended Data	35
Acknowledgements	39
CHAPTER 2: DE-PHOSPHORYLATION OF SLFN11 INDUCES DOWN- REGULATION OF TYPE II tRNA	40
Abstract.....	40
Introduction	41
Materials and Methods.....	43
Results	48
Discussion.....	55
Extended Data	59
Acknowledgements	61
CONCLUDING REMARKS AND REMAINING QUESTIONS.....	62
REFERENCES	65

LIST OF ABBREVIATIONS

Anti-retroviral therapy (ART)

Antigen presenting cell (APC)

Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)

SAM domain and HD domain-containing protein 1 (SAMHD1)

Ataxia telangiectasia and Rad3 related (ATR)

Ataxia telangiectasia mutated (ATM)

ATPase associated with diverse cellular activities (AAA)

Camptothecin (CPT)

Cancer Institute Antitumor Cell Line panel (NCI-60)

Codon adaptation index (CAI)

DNA damage agent (DDA)

DNA damage response (DDR)

Enhanced green fluorescent protein (EGFP)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Human immunodeficiency virus (HIV)

Interferon (IFN)

Interferon regulatory factor (IRF)

Interferon stimulated genes (ISGs)

Melanoma differentiation associated gene 5 (MDA5)

Micro RNA (miRNA)

Phosphatidylinositol-3-kinase-like family (PIKK)

Protein phosphatase catalytic subunit 1 gamma (PPP1CC)

Protein phosphatase catalytic subunit 6 (PPP6)

Retinoic acid-inducible gene I (RIG-I)

Reverse transcriptase (RT)

Schlafen (SLFN)

Schlafen (SLFN11)

Toll-like receptor (TLR)

Topoisomerase 1 (Top1)

Topoisomerase 2 (Top2)

Transfer RNAs (tRNA)

tRNA-derived fragments (tRFs)

(VSV)-G pseudotyped HIV (HIV^{VSV-G})

Vesicular stomatitis virus (VSV)-G pseudotyped HIV (HIV^{VSV-G})

Wild type green fluorescent protein (wtGFP)

LIST OF FIGURES

Figure 1-1. SLFN11 selectively inhibits ATR and ATM translation and promotes cell death upon CPT treatment.....	22
Figure 1-2. Attenuation of ATR expression re-sensitizes SLFN11 deficient cells to CPT-induced apoptosis.....	24
Figure 1-3. SLFN11-mediated reduction of type II tRNAs upon CPT treatment.....	27
Figure 1-4. SLFN11 mediates tRNAs cleavage and inhibits the translation of mRNAs based on codon TTA(Leu) usage.....	31
Extended Data Figure 1-1. SLFN11 selectively inhibits ATR translation and promotes cell death upon CPT treatment in 293 cells.....	35
Extended Data Figure 1-2. Small molecule ATR inhibitors partially re-sensitize SLFN11-deficient cells to CPT-induced cell death.....	36
Extended Data Figure 1-3. SLFN11 mediates the down-regulation of type II tRNAs in 293 SLFN11-KO (CRISPR) cells upon CPT treatment.....	37
Extended Data Figure 1-4. Expression of SLFN11 does not significantly alter mRNA levels of EGFP regardless of codon usage.....	38
Figure 2-1. SLFN11 phospho-mimetics reveal de-phosphorylation activates SLFN11.....	50
Figure 2-2. SLFN11 Alanine mutants down-regulate type II tRNA greater than SLFN11 Aspartic acid mutants.....	52
Figure 2-3. SLFN11 phospho-mutants inhibit specific EGFP Leucine codon bias constructs.....	54
Extended Data Figure 2-1. SLFN11 phospho-mimetics reveal de-phosphorylation activates SLFN11.....	59
Extended Data Figure 2-2. Verification of type I and II tRNA northern blot probes.....	60

ACKNOWLEDGEMENTS

Chapter 1, in full, is currently in submission for publication of the material. *tRNA cleavage via SLFN11 inhibits ATR translation to promote DNA damage-induced apoptosis*. Li, Manqing*; Kao, Elaine*; Malone, Dane*; Gao, Xia; Wang, Jean Y.J; David, Michael. ***These authors contributed equally to this work.** Dane Malone is a co-first author on this material.

Chapter 2, in full, is currently being prepared for submission for publication of the material. *De-phosphorylation of SLFN11 induces down-regulation of type II tRNA*. Malone, Dane; Li, Manqing; Lardelli, Rea; David, Michael. Dane Malone is the primary investigator and author of this material.

VITA

2010 – 2011 Undergraduate Researcher, UC San Diego, La Jolla, CA
2010 – 2012 Bachelor of Science, UC San Diego, La Jolla, CA
2010 – 2012 Bachelor of Arts, UC San Diego, La Jolla, CA
2012 – 2015 BSL2+ Laboratory Manager, UC San Diego, La Jolla, CA
2015 – 2017 Doctor of Philosophy, University of California, San Diego

Malone D, Li M, Lardelli R, David M. De-phosphorylation of SLFN11 induces down-regulation of type II tRNA. In preparation.

Li M*, Kao E*, **Malone D***, Gao X, Wang J Y.G., David M. tRNA cleavage via SLFN11 inhibits ATR translation to promote DNA damage-induced apoptosis. ***These authors contributed equally to this work.** In submission.

ABSTRACT OF THE DISSERTATION

The Regulation and Activity of Schlafen11

by

Dane Michael Malone

Doctor of Philosophy in Biology

University of California, San Diego, 2017

Professor Michael David, Chair

Schlafen11 (SLFN11) is a relatively unknown human interferon stimulated gene (ISG) whose activity and regulation are poorly understood. SLFN11 is part of the SLFN family of genes, which are primarily found in mammals [1]. The first

biological function of SLFN11 was discovered by our lab in 2012, which found that SLFN11 down-regulates the speed at which human immunodeficiency virus (HIV) synthesizes its protein by exploiting HIV's codon usage bias [2].

In the same year, two separate reports found that SLFN11 sensitizes cancer cells upon exposure to DNA damage agents (DDAs) [3][4], however the mechanism for this sensitization had not been uncovered. In chapter 1 of this dissertation, we show that SLFN11 down-regulates translation of an important DNA damage signaling protein, ataxia telangiectasia and Rad3 related (ATR), as well as prove that the down-regulation of ATR is responsible for the sensitization. We then found that upon treatment of camptothecin (CPT), a topoisomerase 1 (Top1) inhibitor, SLFN11 down-regulates type II transfer RNA (tRNA) (Leucines and Serines) via cleavage. Lastly, SLFN11 appears to inhibit translation of proteins whose codon profile prefer Leu(TTA) and Leu(CTT) codons due to the low abundance of their corresponding tRNA. Our discovery of this novel mechanism is important not only for advancing knowledge of SLFN11's role in cancer, but also in helping to pave a path in the development of novel chemotherapeutics.

One aspect of SLFN11 that has not been explored and is poorly understood is its regulation. My work seeks to uncover this uncertainty, specifically furthering our understanding of how phosphorylation regulates SLFN11. In chapter 2 of this dissertation, we show three putative phosphorylation sites of SLFN11 via mass spectrometry and mutagenic analysis. At these three sites, SLFN11 loses the ability to down-regulate type II tRNA and subsequently

protein translation inhibition when the site is mutated to Aspartic acid (phosphor-mimic), but retains its activity when mutated to Alanine (un-phosphorylatable residue). We further found that the SLFN11 Alanine mutants exploit the codon profile of a protein with complete bias towards Leu(TTA) and Leu(CTT) codons, but not Leu(CTG). This work furthers our knowledge of SLFN11 regulation, and aids in the discovery of the phosphatase(s) and kinase(s) activating or inhibiting SLFN11 respectively.

BACKGROUND

The innate immune system and interferons. Animals have evolved extremely efficient mechanisms to defend against pathogens such as viruses or bacteria. Antigen presenting-cells (APCs) such as macrophages and dendritic cells constantly engulf particles to determine if they are self or non-self [5]. Epitopes of pathogens are presented to lymphocytes by dendritic cells, which then in turn triggers an immune response that up-regulates small protein messengers known as cytokines [6][7]. There are different kinds of cytokines which trigger certain events; for example, upon Human Immunodeficiency Virus (HIV) infection, dendritic cells will release type 1 interferon (IFN-1) [8]. Interferons (IFN) are a special type of cytokine that increase immune defenses; IFN can up-regulate specific genes that interfere with viral replication [9][10]. In the case of HIV infection, the interferon regulatory 3 (IRF3) pathway is activated by sensing HIV's ssRNA genome, which in turn up-regulates expression of interferon stimulated genes (ISGs) such as apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) and SAM domain and HD domain-containing protein 1 (SAMHD1) (HIV restriction factors) [11].

The characterization of schlafen (SLFN) genes. My work pertains to another HIV restriction factor and ISG known as schlafen11 (SLFN11), which belongs to the *SLFN* family of genes that are found primarily in mammals, with the exception of poxvirus [1]. Currently there are six human and nine murine isoforms of SLFN that differ in length, ranging from 337 to 910 amino acids, and

share no significant sequence similarity with other proteins. All SLFNs share a conserved NH₂-terminus putative ATPase associated with diverse cellular activities (AAA) domain. AAA proteins typically form hexamers, utilize ATP hydrolysis for enzymatic function, and are reported to be involved in many different cellular processes such as DNA replication and repair, protein degradation and unfolding, and cell motility [12]. On the other hand, only the long SLFNs, such as SLFN11, contain motifs of a DNA/RNA helicase domain in their C-terminal domain. Notable RNA helicases such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) have been reported to recognize viral RNA, subsequently triggering an immune response by up-regulating IFN-1 [13].

The history of SLFN. *SLFN* genes were discovered at the University of California, San Diego in Dr. Stephen Hedrick's laboratory in 1998. Their lab first found that in mice, *SLFN* genes are expressed mostly in primary lymphoid tissues such as the spleen, thymus, and lymph nodes [14]. Different thymic subsets were examined and it was determined that *SLFN* genes are not only differentially regulated during thymocyte development, but their expression disrupts cell growth in fibroblasts and thymoma cells. Furthermore, early ectopic expression of SLFN1 in thymocytes greatly reduced cell growth and development (the number of thymocytes in mice reduced to 1-30% compared to normal mice), however, SLNF1 knockout mice had no significant phenotype.

In the next twelve years, various findings about SLFNs were discovered, however, a biological function/mechanism would not be elucidated for years to

come. In 2004, Geserick et al. were the first to discover that SLFNs were IFN stimulated genes and are IRF-1 dependent [15]. They also found that in mice, transgenic T-cell specific murine SLFN8 expression greatly impaired T-cell development and appeared to be cell type specific. Furthermore, they found that ectopically expressing many of the murine SLFN family members in fibroblasts did not disrupt cell growth. The following year, it was shown that overexpression of murine SLFN1 inhibits Cyclin D1 by interfering with the Cyclin D1 promoter, thus causing G1 cell cycle arrest, again implicating SLFN's involvement with cell growth [16]. In 2007, Gubser et al. reported that Camelpox virus contains remnants of a *SLFN* gene which alters its virulence [17]. Essentially, they found that in lung tissue there was an increase in lymphocyte recruitment mediated by the viral SLFN; however, these cells appeared to be in a less activated state. In 2009, the induction of murine SLFNs via IFN-1 was studied, finding that the fold increase varies greatly among different murine SLFNs [18]. In the same publication, it was also found that knockdown of SLFN2 in mouse cells caused an increase in cell proliferation, suggesting SLFN2 to be a negative regulator of malignant cell growth. In the subsequent year, it was discovered that a mutation in murine SLFN2 caused monocytes and T-cells to become activated and die, subsequently conferring immune-deficiency and high susceptibility to viral and bacterial infection [19], further corroborating other observations of SLFNs having a role in altering T-cell development. Lastly, it should be noted that it was reported in 2008 that SLFN1 and SLFN2 were not found to have growth inhibition

capabilities, and that the results of some of the previously published experiments were not reproducible [20].

SLFN11 and HIV. In 2012, our lab published findings in *Nature* suggesting a mechanism in which human SLFN11 inhibits HIV viral synthesis by exploiting the rare codon usage bias of the virus, thus slowing its translation [2]. The first interesting detail that led to this discovery was that HEK293 cells, when infected with vesicular stomatitis virus (VSV)-G pseudotyped HIV (HIV^{VSV-G}), produced significantly less HIV viral protein than HEK293T cells. Many laboratories around the world utilize HEK293 and HEK293T cells, the difference between them being that HEK293T cells are typically used as packaging cells for retroviruses as well as express large T antigen. Both cell lines were profiled for ISGs and it was determined that mRNA expression of SLFN family members varied greatly between the two cell lines, and to our surprise, HEK293T cells did not express mRNA of many SLFNs while HEK293 cells did. These un-expressed SLFNs were then transfected into HEK293T cells and infected with HIV^{VSV-G}. One SLFN, SLFN11, dramatically inhibited HIV viral protein expression. Furthermore, an shRNA SLFN11 knockdown stable cell line in HEK293 cells showed a significant boost in HIV synthesis, while reconstituting SLFN11 back in the stable cell line recovered the inhibition capability. This ultimately confirmed that SLFN11 was somehow mediating the inhibition of HIV viral protein.

Interestingly, it was observed that viral RNA levels in cells either with or without SLFN11 were not affected, suggesting that SLFN11 played some kind of translational inhibitory role. This, and the fact that there was no global shut down

of protein synthesis observed, lead to the hypothesis that SLFN11 might be targeting the codon usage bias of HIV. It is known that certain viruses such as influenza and HIV have Adenine rich genomes [21][22]. Lentiviruses exhibit codon usage bias compared to the host genome and prefer A/U in the third position for the *gag* and *pol* genes, which leads to a low GC content and inefficient translation [23][24][25]. Li et al. showed that specific viral proteins such as reverse transcriptase (RT), *vif*, *vpu*, and *vpr* (*gag* gene derived proteins) were inhibited in the presence of SLFN11, while other proteins such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enhanced green fluorescent protein (EGFP), and luciferase were not inhibited. It was further shown that wtGFP protein was inhibited in the presence of SLFN11 while EGFP was unaffected. Both GFPs code for the same amino acids but differ in the majority of their codons; wtGFP has a biased codon profile similar to HIV, while EGFP was specifically designed to have an even more optimized codon profile than highly expressed human genes. This shows that SLFN11 is specifically affecting the translation of genes which have lesser codon optimization. A similar experiment was performed where the entire *gag* gene of HIV, which is AT rich and thus exhibits codon usage bias, was synthesized to have optimized codon usage (mimicking the codon usage of EGFP for example). It was found that SLFN11 inhibited protein expression of the *gag* vector mimicking HIV, while the expression of the optimized *gag* vector was allowed greater expression of *gag* proteins, proving that the inhibition is in fact based on codon usage.

Soon after the publication of this paper, the Pillai lab at University of California, San Francisco were studying factors that may be important in HIV viral suppression among Elite controllers (persons are able to suppress HIV viremia levels for years without anti-retroviral therapy (ART)). 34 host restriction factors were tested for protein synthesis in different populations, and it was found that SLFN11 had a significantly higher up-regulation than other restriction factors in elite controllers compared to two HIV infected populations, non-controllers and ART-suppressed individuals [26]. This paper suggests that SLFN11s exploitation of HIVs codon usage could possibly play a leading role in the reason elite controllers are able to suppress the virus without ART.

SLFN11 and Cancer. The same year that our lab published on SLFN11's ability to inhibit HIV, two independent reports emerged implicating SLFN11's role in altering cancer cell sensitivity. The first report was from Barretina et al., which began as a genome wide study that compared gene copy number across hundreds of human cancer cell lines, and the efficacy of 24 chemotherapeutics across 479 cell lines [3]. A correlation of drug efficacy and gene copy number was observed, and three strong correlations were determined, one being SLFN11 and topoisomerase I (Top1) inhibitors. They found that high SLFN11 expression sensitized cancer cells to exposure of Top1 inhibitors (topotecan and irinotecan, two camptothecin (CPT) derivatives). Topoisomerases work to relieve tension in chromosomal DNA during DNA replication by cutting one strand of the DNA and then re-ligating it over the uncut strand [27]. The idea is that if topoisomerase activity is inhibited and therefore not relieving tension

during DNA replication, the DNA will incur single and double stranded breaks, as well as have stalled replication forks [28]. Barretina et al. also found that Ewing's sarcoma had the highest SLFN11 expression out of the cell lines tested, and thus the greatest sensitivity to Top1 inhibitors. The second group that reported on SLFN11 and cancer sensitivity was Zoppoli et al., whom also performed a genome-wide array and matched it with anti-cancer agent efficacy, except this time they tested expression of genes in the National Cancer Institute Antitumor Cell Line panel (NCI-60) [4]. What they found was that high SLFN11 expression was correlated with sensitization of cancer cells when treated only with DNA damaging agents (DDAs) (Top1 and Top2 inhibitors, alkylating agents, and DNA synthesis inhibitors). However, the cells expressing high levels of SLFN11 were not affected by other chemotherapeutics such as kinase inhibitors or tubulin poisons. They further observed that high SLFN11 expression could predict the survival of a group of ovarian cancer patients. These two papers suggest that SLFN11 expression in certain cancers may be a biomarker for sensitivity to DDAs.

Our work. The two independent studies of cancer cell sensitization correlated with high SLFN11 expression led us to hypothesize that SLFN11 could be inhibiting a crucial DNA damage response (DDR) protein in the same way that it exploits HIV's codon usage bias. We tested this hypothesis by measuring the protein expression of various DDR proteins when treating cells with CPT (Top1 inhibitor). Two proteins that are part of the phosphatidylinositol-3-kinase-like family (PIKK) were clearly down-regulated in SLFN11 positive cells upon

treatment of CPT; Ataxia telangiectasia and Rad3 related (ATR) and Ataxia telangiectasia mutated (ATM) proteins both showed inhibited protein expression but unaffected mRNA expression [Chapter 1]. ATM and ATR are DNA damage transducers proteins that activate a signaling cascade of phosphorylation to help repair double or single stranded DNA breaks [29].

In this dissertation, we discuss the role SLFN11 plays in sensitizing cancer cells upon DNA damage agent treatment. We show that SLFN11 down-regulates ATR protein expression, and that the down-regulation of this protein is responsible for DNA damage induced apoptosis. We also show that SLFN11 mediates the cleavage of type II tRNA, and that genes with Leu(TTA) and Leu(CTT) codon bias are most affected by SLFN11 translation inhibition because of the decrease in type II tRNA. Furthermore, we report that SLFN11 is activated via putative de-phosphorylation at three separate sites, mediating type II tRNA down-regulation which leads to translational inhibition of genes with codon profiles favoring Leu(TTA) or Leu(CTT) [Chapter 2]. These findings, of which we attributed to SLFN11, introduce novel mechanisms which may further our understanding of cancer and HIV, as well as help to uncover new therapeutics in chemotherapy and HIV infection.

CHAPTER 1: tRNA CLEAVAGE VIA SLFN11 INHIBITS ATR TRANSLATION TO PROMOTE DNA DAMAGE-INDUCED APOPTOSIS

Abstract

DNA-damaging agents (DDAs) represent the largest group of cancer drugs, but primary or secondary resistance severely impacts their effectiveness. Two recent studies identified human Schlafen11 (SLFN11) - a protein we had previously shown to inhibit HIV replication and viral protein expression based on the distinct codon-usage bias of the virus – as a key factor determining cell fate after exposure to DDAs [3][4]. However, the mechanism by which loss of SLFN11 expression causes resistance to DDAs remained elusive. Here we show that SLFN11 inhibits ATR and ATM translation in response to DDAs to enhance cell killing. This discerning inhibition of ATM and ATR translation is due to the prominent use of specific Leu codons. We demonstrate that SLFN11 inhibits protein synthesis when Leu is encoded via codon TTA or CTT, but not when synonymous Leu codons are employed. We further present SLFN11-dependent cleavage of a distinct tRNA subset including tRNA Leu-TAA and tRNA Leu-AAG in response to DDAs. Our results uncovered a novel mechanism of tRNA codon-specific regulation of translation by SLFN11 in the DNA damage response and support the notion that SLFN11-deficient cancer cells can be (re)sensitized to DDA therapy by targeting ATR or distinct Leucine tRNAs.

Introduction

SLFN proteins, which share no significant homology with any other gene product, can be allocated to short (~45 kDa), intermediate (~70 kDa) or long subsets (~100 kDa) [1]. A loosely conserved N-terminal divergent AAA-domain and an equally poorly preserved C-terminal DNA/RNA helicase domain limited to the long SLFNs are the only noteworthy features in SLFN family members. The number of *SLFN* genes varies greatly among different species, e.g. there are nine *SLFN* genes in the mouse genome versus six distinct human *SLFN* genes. Despite substantial efforts, the biochemical and cellular functions of these unique proteins remained largely elusive for more than a decade.

A few years ago we reported on the ability of the human SLFN11 to inhibit HIV replication and viral protein expression based on the distinct codon-usage of the virus [2], which favors A/T in the wobble position in contrast to the C/G bias found in the mammalian genome. Meanwhile, genome-wide gene expression studies by two independent groups identified human SLFN11 as the primary factor whose expression correlated remarkably with tumor cell survival after exposure to DNA damaging agents (DDA) such as topoisomerase I and II inhibitors (Top1 and Top2), DNA synthesis inhibitors, or alkylating agents [3][4]. Our insights gained from our HIV studies led us to theorize that SLFN11 may sensitize cells to apoptosis upon DNA damage by inhibiting the synthesis of protein vital to the DNA damage response. To investigate this hypothesis, we performed basic codon usage analysis of DNA damage repair genes by calculating their Codon Adaptation Index (CAI) using 80 highly-expressed

ribosomal proteins as a reference set [30][31]. Indeed, consistent with our hypothesis, most DNA damage repair genes we examined displayed a codon usage bias similar to HIV and strikingly contrasting the codon usage inherent to the highly expressed reference genes. As such, the CAIs of the highly expressed GAPDH or b-Actin calculated as 0.802 and 0.873, respectively, highlighting a clear distinction to the CAI of 0.552 of HIV-Gag. Importantly, the two genes central to DNA repair, ATR and ATM, displayed CAIs as low as 0.603 for ATR and 0.601 for ATM. Considering the additional impact of the significantly greater length of the coding sequences for ATR (2644 a.a.) and ATM (3056 a.a.), it appeared that the translation of both ATR and ATM could indeed be a likely target for SLFN11.

Materials and Methods

Cell lines, plasmids, antibodies and chemicals. All cell lines were maintained at 37 °C, 5% CO₂ in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM L-Glutamine, 1× MEM Non-essential Amino Acid, 1 mM Sodium Pyruvate, and 50 mM 2-Mercaptoethanol. The HEK293 and HEK293T cell lines were acquired from ATCC (CRL-1573) and from Dr. Stephen M. Hedrick at Univ. of California, San Diego, respectively. Both COLO 357 FG and MIA Paca-2 cell lines were acquired from Dr. Tannishtha Reya also at Univ. of California, San Diego. HEK293 and COLO 357 FG cell lines with stable expression of shRNAs were generated using lentiviral-based vectors as previously described. To obtain HEK293 and COLO 357 FG derivative cell lines in which SLFN11 expression was obliterated using CRISPR, cells were transfected with pSpCas9 BB-2A-Puro (PX459) All-in-One CRISPR construct and selected based on puromycin resistance (for HEK293 cells: SLFN11 CRISPR guide RNA 4 - GCAGCCTGACAACCGAGAAA; for FG cells: SLFN11 CRISPR guide RNA 1 - GGCTTGACAGAGCGATCTTC; both obtained from Genscript). Surviving cells were cloned by limiting dilution and screened for SLFN11 expression by immunoblotting. The construction of pcDNA6/SLFN11/V5-His and pcDNA6.2/EGFP/Myc expression vectors were previously reported [2]. Anti-ATR antibody (N-19) (SC-1887) and anti-SLFN11 antibody (E-4) (sc-374339) were obtained from Santa Cruz Biotechnology. Antibodies against ATR (#2790S), ATM (D2E2) (#2873S), GFP (D5.1) (#2956S) and GAPDH (14C10) (2118S) were

purchased from Cell Signaling Technology. Antibody for GAPDH immunoprecipitation was acquired from Abcam (ab110305). Camptothecin was procured from EMD Millipore, and the ATR inhibitors, VE-821 and VE-822, from Selleckchem.

MTS cell viability assay. Cells were seeded at 10% confluence in 96-well tissue culture plates 24 hours prior to the indicated regimen in complete phenol red-free DMEM medium. MTS was added to cell culture at the end of the intended treatments following the manufacturer's protocol (Promega G3581). Absorbance was measured at 490 nm after incubation at 37°C for 2 hours.

Whole cell lysis and immunoblotting. Cells were lysed in 1× Cell Lysis Buffer (Cell Signaling Technology) supplemented with Phosphatase Inhibitor Cocktail Set I, Phosphatase Inhibitor Cocktail Set II, and Protease Inhibitor Cocktail Set III (all Merck Millipore). Samples were resolved by 4-12% SDS-PAGE (Invitrogen) and transferred onto PVDF membranes. After incubation with target-specific primary antibody and HRP-conjugated secondary antibody, signals were detected using Western Lightning ECL Pro (PerkinElmer) and film exposure.

Total RNA preparation and mRNAs qPCR. Total cellular RNA was isolated with TRIzol (Invitrogen) and cleaned with the TURBO DNA-free Kit (Ambion). The reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR reactions were carried out on an Applied Biosystems StepOne Plus Real-Time PCR System using iTaq Universal SYBR Green Supermix (Bio-Rad) following the

manufacturer's protocols. Relative levels of mRNAs of interest were calculated based on DCt values and subsequent normalization to GAPDH mRNA levels. The following qPCR primers were used in these assays: ATR forward 5'-cgctgaactgtacgtggaaa-3', reverse 5'-caattagtgcctgggaacatc-3'; ATM forward 5'-tttcttacagtaattggagcattttg-3', reverse 5'-ggcaattactagggccattc-3'; GAPDH forward 5'-tccactggcgtcttcacc-3', reverse 5'-ggcagagatgatgaccctttt-3'; b-Actin (ACTB) forward 5'-ccaaccgcgagaagatga-3', reverse 5'-ccagaggcgtacagggatag-3'; EGFP-tag forward 5'-CGCCGACCCAGCTTTCTTGTA-3', reverse 5'-TGATCAGCTTCTGCTCGCCG-3'.

tRNA demethylation and qPCR-based tRNA microarray assay. Total cellular RNA was prepared with TRIzol (Invitrogen), treated with TURBO DNA-free Kit (Ambion) and re-purified with TRIzol. tRNA demethylation was carried out by means of a vendor-provided protocol modified and optimized in our lab: 2.5 mg total RNA was incubated with 2.5 ml AlkB demethylase, 1 ml RNasin® Plus RNase Inhibitor (Promega) in a total of 100 µl freshly prepared tRNA demethylation buffer (50 mM MES pH 6.0, 100 mM KCl, 2 mM L-ascorbate, 1 mM α-ketoglutarate, 50 µg/ml UltraPure BSA, 300 µM Fe(NH₄)₂(SO₄)₂·6H₂O) at 37 °C for 2 hours. Reaction was terminated by sequential addition of EDTA and MgCl₂ (final concentration 1 mM each). Total RNA was further purified by phenol/chloroform extraction and isopropanol precipitation. Reverse transcription was performed using rtStar™ tRNA-optimized First-Strand cDNA Synthesis Kit (Arraystar), and qPCR for all cellular tRNAs was performed employing nrStar™ Human tRNA Repertoire PCR Array (Arraystar) and Arraystar SYBR® Green

qPCR Master Mix on a QuantStudio 7 Flex Real-Time PCR System. The data were analyzed with the Analysis tool for nrStar™ Human tRNA repertoire PCR Array - v1.01 (Arraystar) with the cut-off Ct set at 30. The p-Values were calculated by performing a two-tailed two-sample equal variance (homoscedastic) Student's t-test.

Polysome profiling by sucrose gradient. Cells were seeded 24 hours prior to a 6-hour treatment with DMSO or 40nM CPT, subsequently incubated with 100 mg/mL of cycloheximide (CHX) for 3 minutes at 37 °C and then washed with PBS containing 100 mg/mL CHX. Cells were lysed in polysome extraction buffer (0.5% Triton, 10 mM Tris pH7.4, 15 mM MgCl₂, 150 mM NaCl, 1 unit/ml RNase inhibitor, 100 mg/mL CHX), and resulting lysates were layered onto previously prepared linear sucrose density gradients (10-50%). Ultracentrifugation was performed at 35,000 rpm for 2.5 hours. Fractions were collected using ISCO Gradient Former (Model 160), total RNA from each fraction was extracted using TRIzol and reverse transcribed into cDNA. qPCR was performed using primers specific for ATR, ATM, GAPDH and β-actin.

³⁵S- protein labeling and immunoprecipitation. Cell lines were treated with DMSO or 40nM CPT as indicated, cultured in methionine and cysteine-free DMEM for 30 min at 37 °C prior to incubation with 250 mCi of ³⁵S-methionine and cysteine (PerkinElmer EasyTag EXPRESS [³⁵S]-protein labeling mix, 11 mCi/ml) for an additional 30 minutes. For pulse chase studies, labeled cells were returned into normal complete DMEM medium without ³⁵S-methionine and cysteine, and cultured for the indicated time spans. At the end of the labeling or

pulse chase experiment the cells were washed with PBS, harvested using trypsin and lysed as outlined above above. The ^{35}S -labeled cell lysates were incubated with anti-ATR (Santa Cruz Biotechnology, (N-19), SC-1887) or anti-GAPDH antibody (Abcam ab110305) for 2 hours at 4 °C. Antibody-antigen complexes were captured on Dynabeads Protein G (Life Technologies), resolved by 4-12% SDS-PAGE and transferred to PVDF membranes. The membrane was dried and analyzed using the Typhoon storage phosphorimager.

siRNA experiments. Reverse transfection of cells was performed using ON-TARGETplus SMARTpool siRNAs (GE Dharmacon) and RNAiMax reagent (Life Technologies) in 96-well plates. 72 hours post transfection cells were treated with the indicated drugs for 48 hours prior to harvesting and immunoblotting analysis. Survival rates of siRNA transfected cells were determined by means of MTS based cell viability assays.

Northern blot analysis of tRNAs. TRIzol purified total RNA was resolved on 10% TBE-Urea gels, and subsequently transferred for 14 hours onto Zeta-Probe nylon membranes (Bio-Rad) in 0.5x TBE at 25 V and 4 °C. After transfer, membranes were cross-linked in an UV Stratalinker 2400 (Stratagene), prehybridized with 10 ml ULTRAhyb-oligo hybridization buffer (Ambion) for 1 hour at 42 °C and then subject to hybridization with 10 pmol T4 PNK ^{32}P -labeled DNA oligo probes (Integrated DNA technologies). at 42 °C for at least 14 hours. Membranes were then rinsed and washed 2 times with wash-buffer (2x SSC with 0.5% SDS) at 42 °C for 1 hour, dried and analyzed using the Typhoon storage phosphorimager. The following probe sequences were chosen

based on predictions from GtRNAdb (tRNAscan-SE analysis of complete genomes, gtrnadb.ucsc.edu) [32]:

Leu-AAG: AGCCTTAATCCAGCGCCTTAGACCGCTCGGCCACGCTACC;

Leu-CAA: GGAGACCAGAACTTGAGTCTGGCGCCTTAGACCA

Leu-CAG: CACGCCTCCAGGGGAGACTGCGACCTGAACGCAGCGCCTT

Leu-TAA: CCATTGGATCTTAAGTCCAACGCCTTAACCACTC

Leu-TAG: GACTGGAGCCTAAATCCAGCGCCTTAGACC

Ser-TGA: TGGATTTCAAGTCCATCGCCTTAACCACTCGGCCACGACTAC

Ser-ACT: ATTAGTAGCAGCACGCTCTAACCAACTGAGCTAACCGGCC

Ser-AGA: GATTTCTAGTCCATCGCCTTAACCACTCGGCCACGACTAC

Ser-CGA: CCCATTGGATTTGAGTCCAACGCCTTAACCACTCGGCCA

Ser-GCT: GGATTAGCAGTCCATCGCCTTAACCACTCGGCCACCTCGTC

Thr-TGT: AGGCCCCAGCGAGGATCGAACTCGCGACCCCTGG

Val-TAC: TGGTTCCACTGGGGCTCGAACCCAGGACCTTCTGCG

Ini-CAT: CCGCTGCGCCACTCTGCT

5.8s rRNA: TCCTGCAATTCACATTAATTCTCGCAGCTAGC

EGFP Expression constructs with synonymous Leucine or Serine codons. The parental Myc-tagged EGFP pcDNA6.2/gw/d-Topo vector has been previously described [2]. EGFP coding sequences in which all Leucine or Serine residues are represented by one distinct codon were synthesized by Genscript and cloned into pcDNA6.2/gw/d-Topo using Apal and NotI. Expression of EGFP protein was visualized by anti-GFP immunoblotting, and the corresponding EGFP

mRNA levels were determined by qPCR using a primer set targeting the common C-terminal region of all EGFP constructs.

Statistical analyses. For all statistical analyses, two-sample equal variance (homoscedastic) Student's t-tests (two-tailed) were performed using Microsoft Excel. Experimental sample sizes were chosen according to commonly accepted ranges for *in vitro* studies in this field and to achieve statistical significance. For all experiments without statistical analyses, one representative result out of at least three independent experiments is shown.

Results

To investigate this potential translational control of SLFN11 on ATR and ATM, we employed two distinct cell lines with average levels of SLFN11 expression, HEK293 cells (hereafter referred to as 293 cells), and the pancreatic cancer cell line COLO 357 FG (hereafter referred to as FG cells) [33]. Using two independent lentiviral-based shRNA constructs against SLFN11 (or irrelevant control shRNAs), we generated several stable polyclonal derivatives from each parental cell line in which SLFN11 expression was permanently silenced, as we believed such a system of “matched” cell lines to be superior for our purposes compared to the use of inherently SLFN11-positive and negative cell lines of various backgrounds as utilized in previous reports. Crucially, silencing of SLFN11 expression conferred significant survival resistance upon both FG and 293 cells to the Topoisomerase I inhibitor camptothecin (CPT) (Fig. 1-1a and Extended Data Fig. 1-1a), as well as other DDAs (not shown), consistent with the behavior observed among the almost 100 distinct cancer cell lines with intrinsically varying SLFN11 levels utilized in the reported studies [3][4].

As our goal was to determine whether SLFN11 affects the protein levels of ATR or ATM in response to DNA damage, we analyzed the expression levels of both ATR and ATM after CPT treatment. Indeed, both proteins were significantly down-regulated after 24 and 48 hours of CPT exposure in control FG and 293 cells, but were barely affected in their SLFN11-lacking matched counterparts (Fig. 1-1b and Extended Data Fig. 1-1b). This down-regulation of both ATR and ATM protein expression level was not due to decreased transcription of their

genes upon CPT treatment, as both ATR and ATM mRNA levels were either unaltered in the absence of SLFN11, or actually significantly upregulated in the presence of SLFN11 (Fig. 1-1c and Extended Data Fig. 1-1c). This clear divergence between an increase in ATR and ATM mRNA levels and a contrasting concomitant decrease in their protein quantities in the presence of SLFN11 might merit a re-evaluation of conclusions drawn from studies that relied solely on the determination of ATR and ATM mRNA levels without parallel examination of their protein levels.

The reduced ATR and ATM protein amounts in CPT-treated, SLFN11-expressing cells suggested that SLFN11 either negatively altered the stability of these proteins, or subdued the translation efficiency of ATR and ATM, possibly due to their low CAI. To answer this question, we performed polysome profiling using sucrose density gradient centrifugation of whole cell lysates derived from our cell lines after either DMSO or CPT exposure. As early as 6 hours after CPT, but not DMSO, treatment, the majority of ATR and ATM mRNA in the FG parental cells had shifted to the lighter fractions, indicating fewer ribosomes translating the ATR and ATM mRNAs (Fig. 1-1d, top 2 left panels). This decrease of ribosome binding to the ATR and ATM mRNAs upon CPT treatment was not detectable in the SLFN11-deficient FG cells, where the response to CPT was indistinguishable from DMSO treatment (Fig. 1-1d, top 2 right panels). Importantly, no significant changes in the polysome profile upon CPT were noticeable when GAPDH or b-actin mRNA levels were analyzed, regardless of the presence of SLFN11 (Fig. 1-1d, bottom panels, and Extended Data Fig. 1-

1d).

To further corroborate our observation, we also performed ³⁵S-methionine/cysteine labeling and pulse-chase experiments to determine the synthesis rate and stability of ATR protein upon CPT treatment. As anticipated, in the presence of SLFN11, ATR protein synthesis was nearly abolished after CPT treatment in both 293 and FG cells (Fig. 1-1e, lane 4) as compared to their SLFN-deficient counterparts (Fig. 1-1e, lane 10). However, the stability of ATR protein did not seem to be affected by CPT or SLFN11 as evidenced by the unchanged levels of newly synthesized ³⁵S-labeled ATR protein in the chase-part of the experiment (Fig. 1-1e, lanes 2,3,5,6,8,9,11,12).

Finally, when comparing the effect of SLFN11 on the synthesis of ATR versus GAPDH protein as a function of time, we observed that as early as 3 hours after CPT administration the translation of ATR, but not of GAPDH, was significantly inhibited solely in SLFN11-expressing cells (Fig. 1-1f). After 6 hours of CPT treatment, slight inhibition of GAPDH translation became sometime notable, while the inhibition of ATR protein synthesis consistently escalated further (Figure 1-1f). Altogether, these results demonstrate that upon CPT treatment, a clear and prominent inhibition of ATR and ATM protein synthesis occurs. Considering our previous findings [2], the fact that translation of GAPDH, b-actin and numerous other proteins is not affected by CPT strongly suggests that their distinctive codon-usage may indeed account for the discrete susceptibility of ATR and ATM to translational suppression by SLFN11.

FIGURE 1-1

***All work in Fig. 1-1 performed by Dr. Elaine Kao**

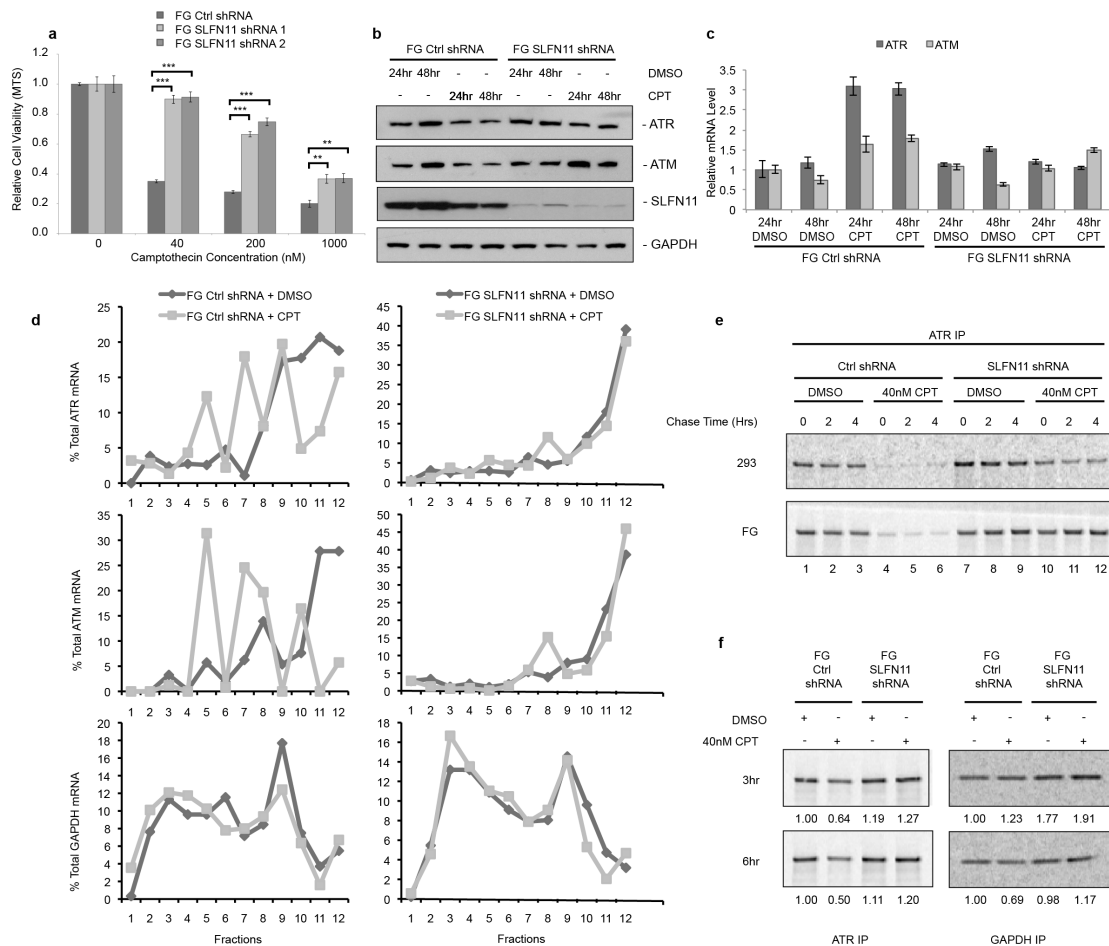


Figure 1-1: SLFN11 selectively inhibits ATR and ATM translation and promotes cell death upon CPT treatment. **a.** Relative viability of FG cells with control or SLFN11 shRNA was measured by MTS assay after 48 hours of CPT or DMSO treatment (mean \pm s.d., $n = 3$, $**P < 0.01$, $***P < 0.001$). **b.** Immunoblotting analysis of ATR and ATM protein levels after 40 nM CPT or DMSO treatment of FG cells harboring control or SLFN11 shRNAs. **c.** Relative ATR and ATM mRNA levels as determined by qPCR in FG cells with control or SLFN11 shRNA after 40 nM CPT or DMSO treatment (mean \pm s.d., $n = 3$). **d.** Polysome profiles of ATR, ATM and GAPDH after 6 hours of DMSO or CPT exposure of FG cells harboring control or SLFN11 shRNA. **e.** Pulse-chase analysis of ATR translation and stability via ^{35}S -methionine/cysteine labeling of 293 and FG cells with or without SLFN11. ATR protein synthesis is significantly impaired after 24 hours exposure to CPT in the presence of SLFN11 (lanes 1 vs 4), but not when SLFN11 is lacking (lanes 7 vs 10). Subsequent further incubation without ^{35}S -methionine/cysteine revealed similar levels of ATR protein stability independent of SLFN11. **f.** ^{35}S -methionine/cysteine protein labeling of FG cells with control or SLFN11 shRNA after 3 or 6 hours of CPT or DMSO treatments. Numbers below images represent quantified band intensity relative to DMSO treated cells containing control shRNA.

While the results thus far clearly illustrate ATR and ATM translation to be subdued by CPT treatment in a SLFN11-dependent manner, it remained unclear whether the elevated expression of ATR or ATM proteins is solely responsible for the resistance of SLFN11-deficient cells to DDAs. To address this question, we abolished the expression of ATR or ATM with corresponding siRNAs in both parental and SLFN11-deficient FG and 293 cells (Fig. 1-2b, d). As shown in Fig. 2a and c, siRNA-mediated attenuation of the increased ATR expression completely restored the sensitivity of both SLFN11-deficient cell lines to CPT-induced cell death (Fig. 1-2 a, c). In contrast, silencing of ATM expression failed to produce a similar re-sensitization effect, indicating that although ATM translation is subject to comparable restriction by SLFN11 as ATR, the biological significance of this fact seems unrelated to survival after exposure to DDAs (Fig. 1-2 a, c). For further corroboration of our results we also tested the effect of siRNA-mediated ATR suppression in the inherently SLFN11-deficient pancreatic tumor cell line, MIA Paca-2, with essentially identical results (Fig. 1-2e-g). Interestingly, when we investigated whether SLFN11-deficient cells could be re-sensitized to CPT-induced cell death by virtue of small molecule ATR inhibitors (VE-821 and VE-822), only the highest concentrations of these compounds produced a notable, but still limited effect (Extended Data Fig. 1-2a-c). This could arguably be due to a limiting potency of these inhibitors, or could be an indication that the function of ATR as a mediator of CPT-induced cell death lays beyond its kinase activity.

FIGURE 1-2

***All work in Fig. 1-2 performed by Dr. Elaine Kao**

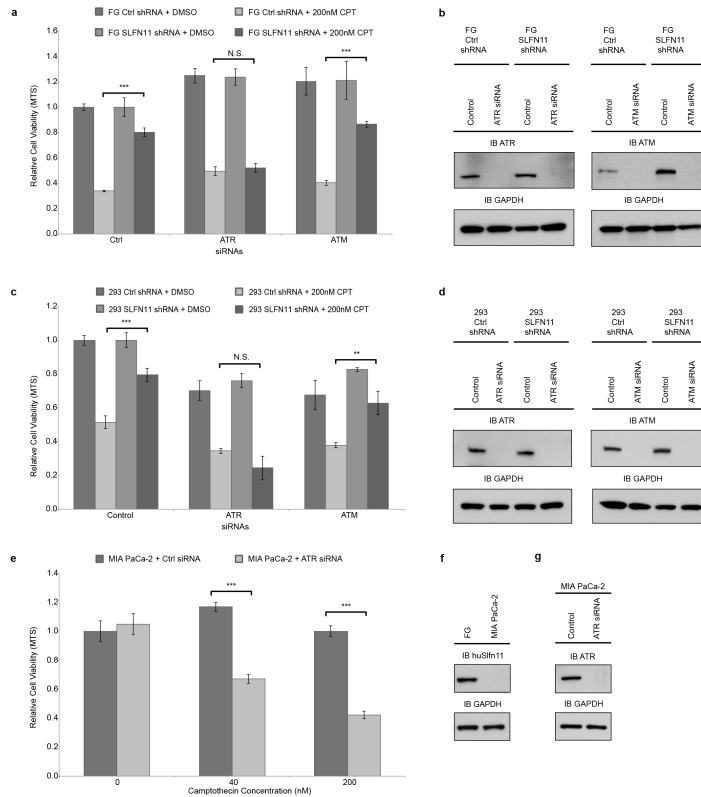


Figure 1-2: Attenuation of ATR expression re-sensitizes SLFN11 deficient cells to CPT-induced apoptosis. **a.** 72 hours after transfection of the indicated siRNAs, FG cells stably expressing control or SLFN11 shRNA were treated DMSO or CPT for an additional 48 hours. Relative cell viabilities were then measured by MTS assay. **b.** ATR and ATM expression in FG cells after transfection of respective siRNAs. **c** and **d.** as in **a** and **b**, except in 293 cells. **e** and **g.** as in **a** and **b**, except inherently SLFN11-deficient MIA PaCa-2 cells were used. **f.** lack of endogenous SLFN11 expression in MIA PaCa-2 cells (**a**, **c**, **e**, biological replicates, mean \pm SD, $n = 3$, ** $p < 0.01$, *** $p < 0.001$, N.S. = non-significant).

To facilitate our further investigations into the molecular mechanism of SLFN11 function, we established SLFN11-deficient FG and 293 cell lines using CRISPR technology to avoid any concerns relating to minute SLFN11 expression in the shRNA cell lines. Complete abrogation of SLFN11 expression via CRISPR yielded an even more profound insensitivity to DDAs in both FG and 293 cells compared to the SLFN11-directed shRNAs (Fig. 1-3a,b, Extended Data Fig. 1-3a,b).

In our previous report on the codon-usage dependent, selective translational inhibition of HIV proteins we provided the initial evidence that SLFN11 affects cellular transfer RNAs (tRNA) levels, particularly in infected cells [2]. In consideration of these discoveries we decided to investigate whether SLFN11 would potentially alter tRNA levels during a DNA damage response. The 75 to 93 nucleotides long tRNAs can be divided into two groups based on the structure and size of their variable loop: tRNAs with short variable loops of 4 or 5 nucleotides are classified as type I, whereas those harboring a variable loop at the end of a double helical stem, totaling 9-19 bases are referred to as type II tRNAs [34]. Thus, type I tRNAs display 4 stems and 3 hairpin loops, while type II tRNAs harbor an additional stem and hairpin loop.

Unpredictably, the analysis of total tRNAs abundance revealed that as early as 3 hours after the CPT addition, the levels of type II tRNAs were already beginning to be down-regulated in a SLFN11-dependent manner (Fig. 1-3c and d). After 12 hours of CPT treatment, only ~50% of type II tRNAs were still detectable in SLFN11-expressing FG and 293 cells, compared to their SLFN11-

deficient counterparts which showed no evidence of such CPT-induced changes. Strikingly, the expression levels of type I tRNAs appeared unchanged in response to the DDA regardless of SLFN11 expression (Fig. 1-3c,d, Extended Data Fig. 1-3c,d).

The type II tRNAs are comprised of all Leucine tRNAs including Leu-AAG, Leu-CAA, Leu-CAG, Leu-TAA and Leu-TAG, and most of Serine tRNAs including Ser-AGA, Ser-CGA, Ser-GCT and Ser-TGA with the exceptions of Ser-ACT, nmt-Ser-TGA and nmt-Ser-GGA [32]. To investigate whether all type II tRNAs or only specific representatives were afflicted by CPT in the presence of SLFN11, we performed RT-qPCR-based analysis on all nuclear-encoded tRNAs including type I tRNAs, as the possibility existed that a small fraction of type I tRNAs were also repressed during the DNA damage response, which may not be revealed by the experimental approach discussed above. The qPCR-based microarray analysis clearly indicated that all Leucine tRNAs as well as Ser-AGA, Ser-TGA and Ser-GGA tRNAs were significantly down-regulated after 12 hours of CPT treatment in the SLFN11-expressing FG cells (Fig. 1-3e). Intriguingly, the only type I tRNA also affected – albeit to a slightly lesser extent - was the initiator methionine tRNA Ini-CAT, whose complementarity AUG codon represents the classical translation initiation site for most mRNAs [35][36]. Most important for our investigation, however, was the finding that not a single tRNA, regardless of its type, was subdued in response to CPT in the absence of SLFN11 (Fig. 1-3f).

FIGURE 1-3

***All work in Fig. 1-3 performed by Dr. Manqing Li**

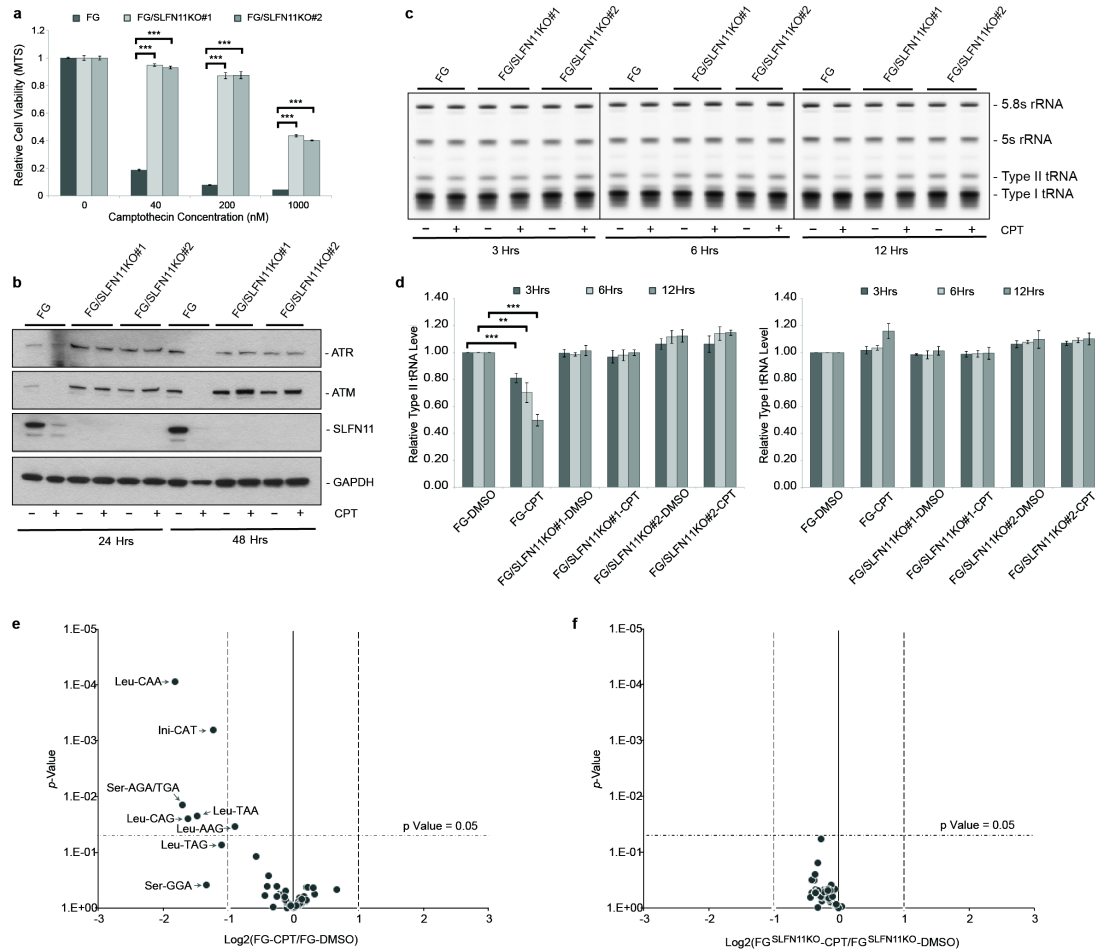


Figure 1-3: SLFN11-mediated reduction of type II tRNAs upon CPT treatment. **a.** Relative viability of parental FG cells and FG SLFN11-KO (CRISPR) cells was analyzed via MTS assay after 48 hours of DMSO or CPT exposure (biological replicates, mean \pm SD, $n = 3$, $***p < 0.001$). **b.** ATR and ATM protein expression in FG and FG SLFN11-KO cells upon DMSO or 40 nM CPT treatment. **c.** Total RNA derived from DMSO- or 200 nM CPT-treated FG cells and FG SLFN11-KO cells was resolved by 10% Urea PAGE and visualized by SYBR Gold staining. **d.** Quantified results from **c** (biological replicates, mean \pm s.d., $n = 3$, $**p < 0.01$, $***p < 0.001$; 5.8s rRNA served as the endogenous control; all data were normalized to DMSO treated FG cell samples). **e.** Volcano plot of fold change of cellular tRNA levels after 12 hours of DMSO or CPT treatment in FG cells determined by qPCR. **f.** as in **e**, except using FG SLFN11-KO cells. (log₂(mean of fold change) vs. p-Value; $n = 3$; p-Value was calculated by performing a two-tailed two-sample equal variance (homoscedastic) Student's t-test.)

As the qPCR-based microarray evaluation is a very novel, as of yet unpublished method of tRNA quantification, we decided to further confirm our findings by Northern blot analysis utilizing probes corresponding to the individual tRNAs. There, we observed again that tRNAs Leu-TAA, Leu-CAA, Leu-CAG, Ser-AGA and Ini-CAT were down-regulated as early as 3 hours after CPT administration, followed by diminution of Leu-TAG, Leu-AAG, Ser-CGA, Ser-GCT and Ser-TGA tRNAs 3 hours later (Fig. 1-4a). Once more, the attenuation of these tRNAs was only evident in cells expressing SLFN11 (Fig. 1-4a). Strikingly, the only Serine tRNA that was not affected during DNA damage in SLFN11-expressing cells was Ser-ACT, which as a type I tRNAs lacks the long stem-loop structured variable loop.

We lastly wanted to exclude the remote possibility that tRNA suppression during the DNA damage response was due to unique properties acquired during the selection of SLFN11-deficient cell lines rather than an immediate involvement of SLFN11 in the process itself (similar to the resistance of stable STAT1^{-/-} cells to TNFa-induced apoptosis, which is caused by a permanent lack of caspase expression in the absence of STAT1 rather than an involvement of STAT1 in TNFa signaling). To this end, we transiently expressed SLFN11 in HEK293T cells, which inherently lack all endogenous SLFNs [2]. As shown in Fig. 1-4b, the ectopic over-expression of SLFN11 alone was sufficient to reduce the levels of all type II Serine and Leucine tRNAs as well as tRNA Ini-CAT, whereas the type I tRNAs Thr-TGT and Val-TAC remained unchanged. These results not only corroborated our findings obtained with the selected SLFN11-deficient cell lines,

but furthermore revealed that over-expression of SLFN11 suffices for its activation, whereas tRNA suppression via endogenous SLFN11 depends on DDA exposure. A remaining question was whether SLFN11 reduced type II tRNA levels by repressing their production, or acted by virtue of degrading existing tRNA. Extended exposures of our Northern blots allowed for a smaller fragment of the type II tRNAs to become visible, whose emergence dependent on both CPT treatment and SLFN11 expression (Fig. 1-4c). This finding implicates that either SLFN11 itself, or a SLFN11-dependent factor possesses endoribonuclease activity. Considering a recent report indicating that a leporine SLFN14 N-terminal fragment in rabbit reticulocyte lysate harbors endoribonuclease activity [37], the former variant seems indeed the most probable scenario.

Our data thus far clearly illustrated that all Leucine and most Serine tRNAs were targets of SLFN11. A logical follow-up investigation encompassed therefore a likely selective impact of SLFN11 on the translation of proteins dependent on the tRNAs. To address this point, we designed a series of EGFP-encoding vectors such that in each individual construct all Leucine or Serine residues were represented by a unique codon, and the commercially available parental EGFP vector was used as a control. Each construct was then tested by transfection into the endogenous SLFN-deficient HEK293T cells, either with or without co-transfection of a SLFN11-coding vector. As we had demonstrated previously [2], expression of EGFP protein encoded by the original vector was essentially refractory to suppression by SLFN11 (Fig. 1-4d, lanes 1 and 2). Remarkably,

SLFN11 affected only the expression of EGFP_Leu(TTA), which was completely abolished, and of EGFP_Leu(CTT), which was partially subdued. EGFP protein derived from all other constructs exhibited marginal to no alteration on account of the presence of SLFN11 (Fig. 1-4d). Most importantly, any inhibition definitely occurred at the translational level, as SLFN11 did not alter the level of EGFP mRNA regardless of the deriving construct (Extended Data, Fig. 1-4).

At the beginning of this study we noted that the synthesis of proteins encoded by genes with low CAI such as ATR, ATM or HIV gag was drastically more repressed by SLFN11 than translation of gene products with optimal codon usage substantiated by their high CAI such as GAPDH or b-Actin. We therefore performed further analysis of these gene sequences which revealed that out of total 352 Leucine residues in ATR, 73 residues use the codon TTA (20.7%) and 82 residues use codon CTT (23.3%). Of the 389 Leucine residues in ATM, 91 are encoded by TTA (23.4%) and 89 residues use codon CTT (22.9%), and 14 and 5 of the 38 Leucine residues in HIV *Gag* are encoded via TTA (36.8%) or CTT (13.2%), respectively. In striking contrast, only 1 TTA and 1 CTT codon can be found among the 19 Leucine residues of GAPDH (0.05% each), whereas the b-actin gene does not exploit either codon for any of its 27 Leucine residues.

Discussion

In summary, this extension of our previously published findings indicates that not necessarily the overall codon usage bias, but more specifically, the frequency of the Leucine codon TTA, and to a lesser extent CTT, accounts for the selective translational suppression by SLFN11. An obvious challenge is the question why the SLFN11-mediated degradation of only tRNAs Leu-TAA and Leu-AAG affected the translation efficiency of mRNAs harboring the corresponding codons, while the cleavage of the other Leucine or Serine tRNAs appeared to be of little consequence. One possible explanation can be found in the apparently lower abundance of tRNA Leu-AAG (for codon CTT) and tRNA Leu-TAA (for codon TTA) we had noted in our qPCR and Northern blot analysis. Although neither technique permits a highly accurate comparative conclusion, they do support a reasonable estimation of relative tRNA abundance. For instance, as shown in the Supplementary Data 1, the relative Ct values for tRNA Leu-CAG (for codon CTG) versus tRNA Leu-TAA (for codon TTA), suggest a roughly 16-fold difference in their respective abundance levels. A similar observation could be made with regard to the relative signal intensity in our Northern Blot analysis (not shown). It would be highly coincidental for both approaches to incorrectly lead to the same conclusion. Thus, for proteins requiring tRNA Leu-TAA to support their translation, the availability of this tRNA might become the rate-limiting factor, and consequently a significant down-regulation of tRNA Leu-TAA may cause the ribosome to stall and/or detach at the corresponding TTA codon. The longer the mRNA and the higher the frequency of

TTA or CTT codons for Leucine residues, the more likely is the occurrence of a premature translation termination. In contrast, for proteins whose translation relies on more abundant tRNAs, translation initiation rates may remain the limiting factor rather than the reduction in the still sufficient supply of the required tRNAs. This notion is also supported by the fact that we did not observe an overall attenuation of protein synthesis despite the notable cleavage of the seemingly abundant initiator methionine tRNA Ini-CAT. Another, not necessarily mutually exclusive explanation for our results might be found in a translationally suppressive function of the tRNA-derived fragments (tRFs) that result from the SLFN11-mediated cleavage of the selective tRNAs. Indeed, tRFs have been implicated in cell proliferation, cancer and viral infection, and have even been suggested to act similar to microRNAs via binding to Ago, although the latter function remains uncertain. Nevertheless, the fact that SLFN11 suppressed the translation of the totally artificial EGFP_Leu(TTA) (Fig. 1-4d) renders this possibility rather unlikely.

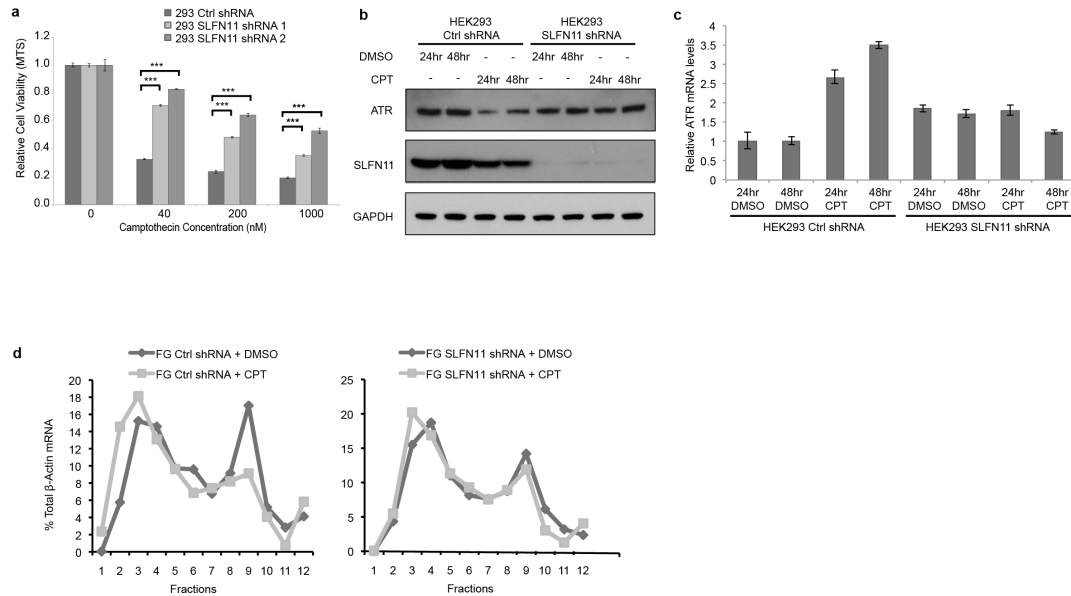
DDAs are the earliest and most widely used therapeutics for cancer treatment, accounting for almost one third of all chemotherapeutic drugs. However, many tumors are resistant to therapies based on DNA damaging approaches. Even tumors initially responsive to the regimen routinely acquire resistance over the course of the treatment (reviewed in [38]). Most recently, a study showed that in recurring small cell lung cancer, the silencing of SLFN11 expression mediated by histone modification H3K27me3 at the SLFN11 gene locus was responsible for the tumor's acquired chemo-resistance. Inhibition of

Histone-Lysine N-Methyltransferase EZH2 restored SLFN11 expression and re-sensitized the tumor cells to chemotherapy [39]. We describe here a novel molecular mechanism by which SLFN11 sensitizes cells to apoptosis upon DNA damage. The SLFN11-dependent down-regulation of type II tRNAs, importantly those contributing the amino acid Leucine via codons TTA or CTT, predisposes ATR to translational inhibition as its mRNA abundantly utilizes these specific codons. Our findings not only provide significant new insights into the molecular mechanism underlying SLFN11 function, but to our knowledge also provide the first example that modulation of a distinct tRNA subset allows for the specific targeting of proteins relying on those tRNAs for their translation. Potentially, the direct alteration of specific type II tRNAs might offer a new strategy to overcome tumor cell resistance to DDAs, or alter cellular anti-viral responses.

Extended Data

EXTENDED DATA FIGURE 1-1

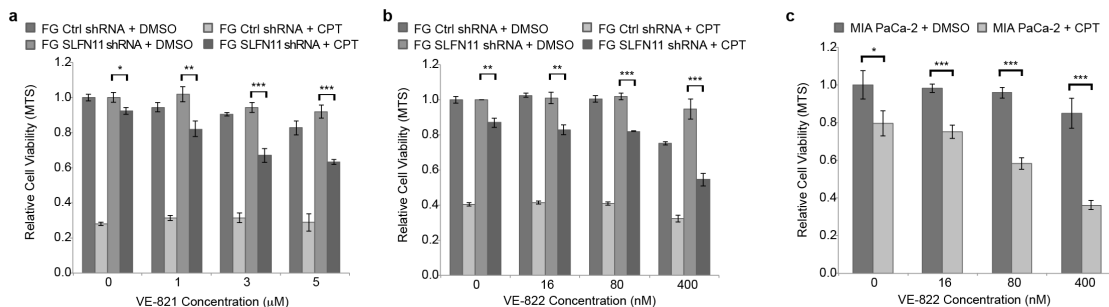
***All work in Extended Data Fig. 1-1 performed by Dr. Elaine Kao**



Extended Data Figure 1-1: SLFN11 selectively inhibits ATR translation and promotes cell death upon CPT treatment in 293 cells. **a.** Relative viabilities of 293 cells stably expressing control or SLFN11 shRNAs were determined by MTS assay after 48 hours after DMSO or 40 nM CPT treatment (biological replicates, mean \pm s.d., $n = 3$, $***P < 0.001$). **b.** ATR protein expression levels in 293 cells stably expressing control or SLFN11 shRNA after DMSO or 40 nM CPT treatment. **c.** Relative ATR mRNA levels were determined by qPCR in 293 cells with control or SLFN11 shRNA after exposure to DMSO or 40 nM CPT (technical replicates; mean \pm SD, $n = 3$). **d.** Polysome profiles of b-Actin mRNA in FG cell with control or SLFN11 shRNA exposed to DMSO or CPT for 6 hours.

EXTENDED DATA FIGURE 1-2

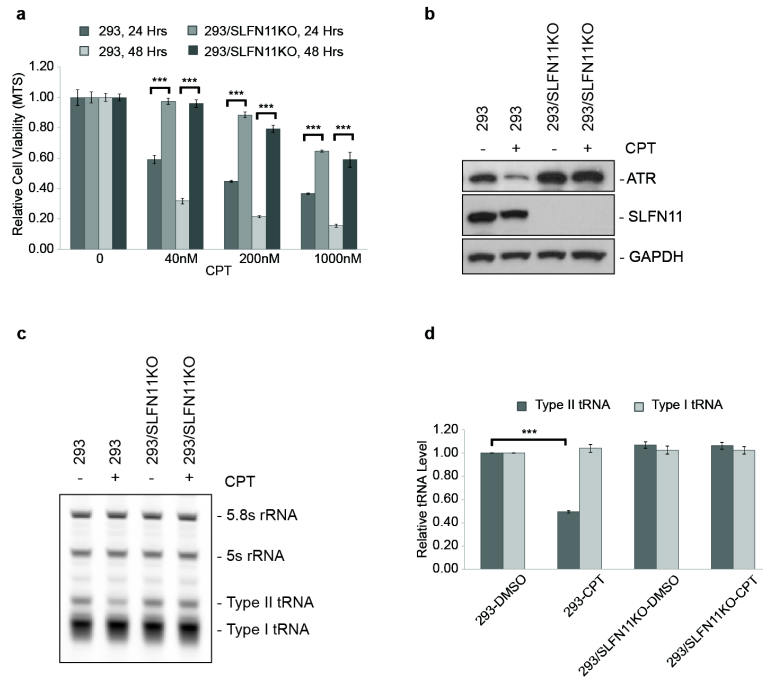
***All work in Extended Data Fig. 1-2 performed by Dr. Elaine Kao**



Extended Data Figure 1-2: Small molecule ATR inhibitors partially re-sensitize SLFN11-deficient cells to CPT-induced cell death. a. Viabilities of FG cells stably expressing control or SLFN11 shRNA were measured by MTS assay after 48 hours of DMSO or 40 nM CPT treatment in the presence or absence of the ATR inhibitor VE-821. **b.** as in a, except ATR inhibitor VE-822 was used. **c.** as in b, except MIA PaCa-2 cells were used (a, b, c, biological replicates, mean \pm SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).

EXTENDED DATA FIGURE 1-3

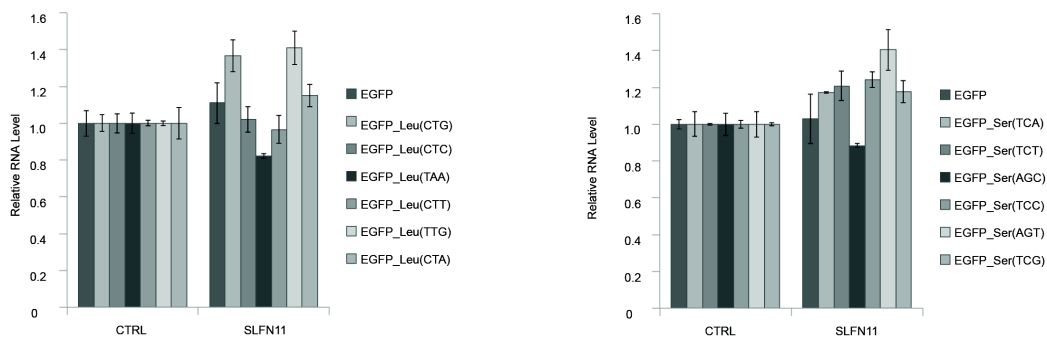
***Work in Extended Data Fig. 1-3 performed by Dr. Manqing Li and Dane Malone**



Extended Data Figure 1-3: SLFN11 mediates the down-regulation of type II tRNAs in 293 SLFN11-KO (CRISPR) cells upon CPT treatment. **a.** Relative viability of 293 cells and 293 SLFN11-KO cells were measured by MTS assay after 48 hours of DMSO or CPT treatments (biological replicates, mean \pm SD, $n = 3$, $***p < 0.001$). **b.** Ablation of SLFN11 expression in 293 cells via CRISPR abolishes CPT-induced suppression of ATR levels. **c.** Total RNA derived from 293 or 293 SLFN11-KO cells treated with DMSO or 40 nM CPT for 12 hours was resolved by 10% Urea PAGE. RNA visualization by SYBR Gold staining reveals reduction of type II tRNA by CPT only in the presence of SLFN11. **d.** Quantified results from c (biological replicates, mean \pm SD, $n = 3$, $***p < 0.001$; 5.8s rRNA served as the endogenous control; all data were normalized to DMSO treated 293 cell samples).

EXTENDED DATA FIGURE 1-4

***All work in Extended Data Fig. 1-4 performed by Dane Malone**



Extended Data Figure 1-4: Expression of SLFN11 does not significantly alter mRNA levels of EGFP regardless of codon usage. Relative mRNA levels derived from the assorted EGFP constructs used in Fig. 1-4d as determined by qPCR (technical replicates, mean \pm SD; n = 3).

Acknowledgements

Chapter 1, in full, is currently in submission for publication of the material. *tRNA cleavage via SLFN11 inhibits ATR translation to promote DNA damage-induced apoptosis*. Li, Manqing*; Kao, Elaine*; Malone, Dane*; Gao, Xia; Wang, Jean Y.J; David, Michael. ***These authors contributed equally to this work.** Dane Malone is a co-first author on this material.

CHAPTER 2: DE-PHOSPHORYLATION OF SLFN11 INDUCES DOWN-REGULATION OF TYPE II tRNA

Abstract

Human Schlafen11 (SLFN11) is an interferon stimulated early response gene (ISG) that was found in 2012 to exploit HIV's codon usage bias and thus slow its translation [2]. In that same year, SLFN11 was found in two independent studies to sensitize cancer cells upon treatment of specific DNA damage agents (DDAs) [3][4]. SLFN11 has thus been implicated to be involved with two of the most devastating diseases of our time, yet the regulation of SLFN11 is poorly understood. We have found that SLFN11 is putatively phosphorylated at three different sites, and through mutagenic studies, SLFN11 must be dephosphorylated in order to be functionally active. This activity includes the down-regulation of tRNA and subsequently the inhibition of HIV viral proteins, as well as Leu(TTA) and Leu(CTT) codon "biased" EGFP constructs. This work furthers our knowledge of SLFN11 regulation, and aids in the discovery of new chemotherapeutics as well as anti-retroviral therapy (ART).

Introduction

Schlafen (SLFN) genes are a family of ISGs found primarily in mammals [1]. These genes vary in length (around 300 to 1000 amino acids), and all SLFNs have a conserved N-terminal region that contains a AAA-binding domain, while longer SLFNs have motifs resembling an RNA helicase domain. SLFNs were first discovered in 1998, with murine isoforms characterized to be differentially regulated during T-cell development, as well as to have anti-growth properties [14]. For the next twelve years, several papers were published characterizing SLFN genes to have anti-proliferative properties. For example, it was found that transgenic T-cell expression of murine SLFN8 caused impaired T-cell development [15], and later found that murine SLFN1 caused cell cycle arrest by inhibiting induction of cyclin D1 [16]

It was not until 2012 that the first biological function of a human *SLFN* gene was discovered. Human SLFN11 was found to inhibit HIV viral synthesis by exploiting its codon usage bias during translation [2]. The same report also found that SLFN11 binds tRNA *in vitro*, however it is not known whether SLFN11 is a nuclease that degrades the tRNAs themselves. HIV (and other lentiviruses) has an A-T rich genome and thus exhibits codon usage bias [23][24][25], leading them to require rarer tRNAs; if SLFN11 is even fractionally down-regulating these rarer tRNAs, it is detrimental to any gene that utilizes this codon usage bias.

SLFN11 has since been implicated to be involved in cancer and the DNA damage response (DDR). Two papers were published by separate labs claiming that SLFN11 sensitizes cancer cells to DNA damaging agents (DDAs) such as

camptothecin (CPT) [3][4]. Our lab elucidated the mechanism of this sensitization; we found that SLFN11 down-regulates type II tRNA upon treatment of CPT, which then affects the translation of our own genes that exhibit codon usage bias, eventually leading to apoptosis [Chapter 1]. Interestingly, a DNA damage signaling protein, ataxia telangiectasia and Rad3-related protein (ATR), exhibits similar codon usage bias as HIV. In chapter 1, we showed that SLFN11 inhibits ATR protein synthesis via the down-regulation of type II tRNA cleavage, and once ATR is depleted, the cell can no longer properly repair damage and undergoes apoptosis.

It is clear that SLFN11 is important not just in HIV infection but cancer as well. To better understand SLFN11's function, as well as gain the ability to control SLFN11 through small molecule inhibitors/activators, we have been investigating the regulation of SLFN11. Our findings show, through mass spectrometry and mutagenesis, that SLFN11 must be de-phosphorylated in order to inhibit HIV viral synthesis. Furthermore, it is shown that un-phosphorylatable SLFN11 mutants down-regulate type II tRNA as well as inhibit protein synthesis of enhanced green fluorescent protein (EGFP) with complete codon bias for Leu(TTA) or Leu(CTT), but not Leu(CTG).

Materials and Methods

Cell lines, plasmids, antibodies and chemicals. All cell lines were maintained at 37 °C, 5% CO₂ in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM L-Glutamine, 1× MEM Non-essential Amino Acid, 1 mM Sodium Pyruvate, and 50 mM 2-Mercaptoethanol. HEK293T cell lines were acquired from ATCC (CRL-1573) and from Dr. Stephen M. Hedrick at Univ. of California, San Diego, respectively. To obtain HEK293 derivative cell lines in which SLFN11 expression was obliterated using CRISPR, cells were transfected with pSpCas9 BB-2A-Puro (PX459) All-in-One CRISPR construct and selected based on puromycin resistance (for HEK293 cells: SLFN11 CRISPR guide RNA 4 – GCAGCCTGACAACCGAGAAA and obtained from Genscript). Surviving cells were cloned by limiting dilution and screened for SLFN11 expression by immunoblotting. The construction of pcDNA6/CAT/V5-His, pcDNA6/SLFN11/V5-His, pcDNA6.2/wtGFP/V5-His, and pcDNA6.2/EGFP/Myc expression vectors were previously reported [2]. pNL4-3.Luc.R+E- HIV-1 vector has been described previously [40]. Murine monoclonal anti-V5 and anti-Myc tag antibodies were from Santa Cruz Biotechnology. Mouse anti-HIV-1 p24 was from Thermo Pierce. Antibodies against GFP (D5.1) (#2956S) and GAPDH (14C10) (2118S) were purchased from Cell Signaling Technology.

Creation of SLFN11 phospho-mutants. Point mutations of Alanine or Aspartic acid in pcDNA6/SLFN11/V5-His expression vector were created with GeneTailor™ Site-Directed Mutagenesis System (Invitrogen).

Mass spectrometry. pcDNA6/SLFN11/V5-His expression vector was transfected into 293T cells and immunoprecipitated with anti-V5 antibody. Sample was given to UC San Diego's proteomics facility for LC-MS-MS analysis. Mass spectrometry was performed with Lumos hybrid mass spectrometer (Thermo) interfaced with nano-scale reversed-phase UPLC (Thermo Dionex UltiMate™ 3000 RSLC nano System). Data analysis was carried out using the Byonic™ (Protein Metrics Inc.).

Whole cell lysis and immunoblotting. Cells were lysed directly in 1x NuPAGE LDS Sample Buffer (Invitrogen) containing 2.5% 2-mercaptoethanol and heated at 90 °C for 5 min. Samples were homogenized by QIAshredder (Qiagen). Samples were resolved by 4-12% SDS-PAGE (Invitrogen) and transferred onto PVDF membranes. After incubation with target-specific primary antibody and HRP-conjugated secondary antibody, signals were detected using Western Lightning ECL Pro (PerkinElmer) and film exposure.

Total RNA preparation and mRNAs qPCR. Total cellular RNA was isolated with TRIzol (Invitrogen) and cleaned with the TURBO DNA-free Kit (Ambion). The reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR reactions were carried out on an Applied Biosystems StepOne Plus Real-Time PCR System using iTaq Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's protocols. Relative levels of mRNAs of interest were calculated based on DCt values and subsequent normalization to GAPDH mRNA levels. The following qPCR primers were used in these assays:

GAPDH

Forward: 5'-TCCAAGTGGCGTCTTCACC-3'

Reverse: 5'-GGCAGAGATGATGACCCTTTT-3'

EGFP-tag

Forward: 5'-CGCCGACCCAGCTTTCTTGTA-3'

Reverse: 5'-TGATCAGCTTCTGCTCGCCG-3'

SLFN5

Forward: 59-CAAGCCTGTGTGCATTCATAA-39

Reverse: 59-TCTGGAGTATATACCA CTCTGTCTGAA-39

SLFN11

Forward: 59-AAGGCCTGGAACATAAAAAGG-39

Reverse: 59-GGAGTATATCGCAAATATCCTGGT-39

HIV p24

Forward: 59-TGCATGGGTAAAAGTAGTAGAAGAGA-39

Reverse: 59-TGATAATGCTGAAAACATGGGTA-39

wtGFP

Forward: 59-CTGGAGTTGTCCCAATTCTTG-39

Reverse: 59-TCACCCTCTCCACTGACAGA-39

Northern blot analysis of tRNAs. TRIzol purified total RNA was resolved on 10% TBE-Urea gels, and subsequently transferred for 14 hours onto Zeta-Probe nylon membranes (Bio-Rad) in 0.5x TBE at 25 V and 4 °C. After transfer, membranes were cross-linked in an UV Stratalinker 2400 (Stratagene), prehybridized with 10 ml ULTRAhyb-oligo hybridization buffer

(Ambion) for 1 hour at 42 °C and then subject to hybridization with 10 pmol T4 PNK ³²P-labeled DNA oligo probes (Integrated DNA technologies). at 42 °C for at least 14 hours. Membranes were then rinsed and washed 2 times with wash-buffer (2× SSC with 0.5% SDS) at 42 °C for 1 hour, dried and analyzed using the Typhoon storage phosphorimager. The following probe sequences were chosen based on predictions from GtRNAdb (tRNAscan-SE analysis of complete genomes, gtrnadb.ucsc.edu) [32]:

Leu-AAG: AGCCTTAATCCAGCGCCTTAGACCGCTCGGCCACGCTACC;

Leu-CAA: GGAGACCAGAACTTGAGTCTGGCGCCTTAGACCA

Leu-CAG: CACGCCTCCAGGGGAGACTGCGACCTGAACGCAGCGCCTT

Leu-TAA: CCATTGGATCTTAAGTCCAACGCCTTAACCACTC

Leu-TAG: GACTGGAGCCTAAATCCAGCGCCTTAGACC

Ser-TGA: TGGATTTCAAGTCCATCGCCTTAACCACTCGGCCACGACTAC

Ser-ACT: ATTAGTAGCAGCACGCTCTAACCAACTGAGCTAACCGGCC

Ser-AGA: GATTTCTAGTCCATCGCCTTAACCACTCGGCCACGACTAC

Ser-CGA: CCCATTGGATTTTCGAGTCCAACGCCTTAACCACTCGGCCA

Ser-GCT: GGATTAGCAGTCCATCGCCTTAACCACTCGGCCACCTCGTC

Thr-TGT: AGGCCCCAGCGAGGATCGAACTCGCGACCCCTGG

Val-TAC: TGGTTCCACTGGGGCTCGAACCCAGGACCTTCTGCG

Arg-TCT: ATTCAAAGGtTGTGGGTTTCGAGTCCCACCAGAGTCG

Ile-TAT: ATGCCGAGGtTGTGAGTTTCGAGCCTCACCTGGAGCA

iMet: CCGCTGCGCCACTCTGCT

5.8s rRNA: TCCTGCAATTCACATTAATTCTCGCAGCTAGC

EGFP Expression constructs with synonymous Leucine or Serine codons. The parental Myc-tagged EGFP pcDNA6.2/gw/d-Topo vector has been previously described [2]. EGFP coding sequences in which all Leucine or Serine residues are represented by one distinct codon were synthesized by Genscript and cloned into pcDNA6.2/gw/d-Topo using Apal and NotI. Expression of EGFP protein was visualized by anti-GFP immunoblotting, and the corresponding EGFP mRNA levels were determined by qPCR using a primer set targeting the common C-terminal region of all EGFP constructs.

Results

Mass spectrometry of immunoprecipitated SLFN11 (transiently transfected) revealed five putatively phosphorylated sites (Fig. 2-1a) that were either Serine or Threonine. To further prove phosphorylation of SLFN11, we employed mutagenic studies on three categories of sites on SLFN11. The first group of sites were those identified by mass spectrometry: S180, S219, T230, S750, and S753. The next group targeted for mutagenic studies were chosen due to their location on a phosphorylated peptide, despite not being found to be phosphorylated: S187, T220, and S770. The last group of sites chosen were based on phosphorylated motifs (S210 and S770 having an SQ motif, typical of PIKK family phosphorylation [41]), and non-conserved residues amongst primate SLFN11s (T154) [42]. Each site was mutated to either Alanine, which does not contain a hydroxyl group and is therefore not able to be phosphorylated, or Aspartic acid, which contains a negative charge that mimics phosphorylation [43]. To test these constructs, we used HEK293T cells, which do not express SLFN11 [2]. These mutant constructs were co-transfected into HEK293T cells along with an HIV pro-viral construct, pNL4-3.Luc.R+E-, and EGFP, which serves as a transfection control. HIV p24 (HIV capsid protein within the *gag* gene) expression revealed three sites, S219, T230, and S753, as complete loss of function sites when mutated to Aspartic acid, but retained complete HIV inhibition activity when mutated to Alanine (Fig. 2-1b). All three sites were found to be phosphorylation sites via mass spectrometry (Fig. 2-1a). mRNA expression profile revealed that the Aspartic acid mutant of these three sites had a boost in p24 mRNA compared

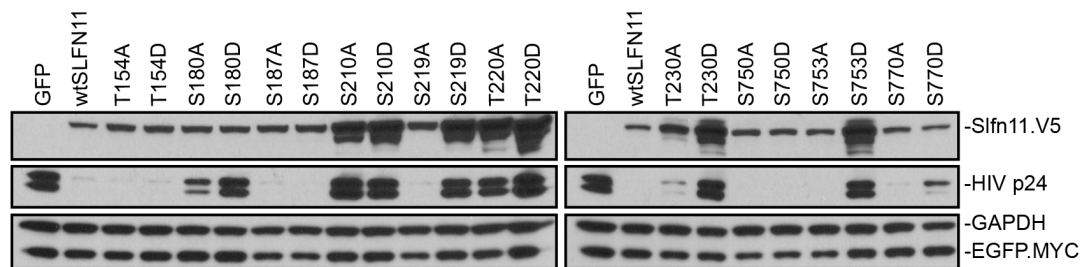
to their Alanine counterparts (Extended Data Fig. 2-1b), which would suggest that SLFN11 Alanine mutants are inhibiting HIV transcription and not translation. To prove this incorrect, the HIV p24 protein band from the western blot was quantified and normalized over mRNA to show relative amount of protein translation produced from the transcript. All three sites, S219, T230, and S753 showed significant protein expression in the Aspartic acid mutants, while the Alanine mutants levels were either low or near undetectable (Fig. 2-1c), suggesting that even though mRNA is boosted with the Aspartic acid mutants, there is still significant translational inhibition with the Alanine mutants. Furthermore, all three sites (both Alanine and Aspartic acid mutants) were co-transfected with EGFP (codon-optimized gene) and wtGFP (codon-biased gene) in order to determine if this increase in mRNA expression with the Aspartic acid mutants are isolated to only HIV. The result was that wtGFP protein expression was boosted in the presence of our Aspartic acid mutants of interest (Extended Data Fig. 2-1c), however the wtGFP mRNA was unchanged (Extended Data Fig. 2-1d). This was in contrast to HIV's protein and mRNA both being boosted.

FIGURE 2-1

a

Confidence	Peptide sequence	Modifications	Site
52.05000043	HKGVYQELPNSDPADPNSDPADLIFQK	Phospho(S)@11	S187
63.67999911	KGVYQELPNSDPADPNSDPADLIFQK	Phospho(S)@10	S187
75.66999793	GVYQELPNSDPADPNSDPADLIFQK	Phospho(S)@9	S187
99.00000095	QFSTKHFQEYVKR ^T IPEYVPAFANTGGGYLFIGVDDK	Phospho(S)@3; Phospho(T)@14	S219; T230
92.00999737	IRSNPSFNIPTGCLEVFPEAEWSQGVQGTLR	Phospho(S)@3	S750
82.81000257	IRSNPSFNIPTGCLEVFPEAEWSQGVQGTLR	Phospho(S)@3	S750
58.06000233	IRSNPSFNIPTGCLEVFPEAEWSQGVQGTLR	Phospho(S)@6	S753

b



c

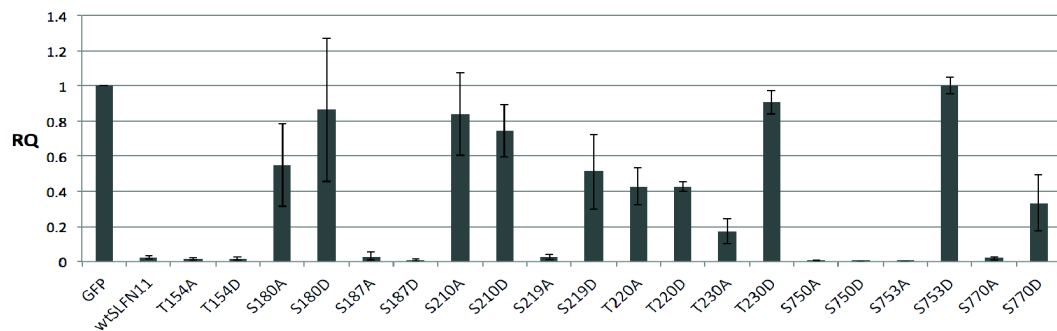


Figure 2-1: SLFN11 phospho-mimetics reveal de-phosphorylation activates SLFN11. a. Transiently transfected V5-tagged SLFN11 was expressed in HEK293T cells and immunoprecipitated. SLFN11 was then analyzed by mass spectrometry for post-translational modification. b. HEK293T cells were transiently co-transfected with SLFN11 phospho-mutants and pNL4-3.Luc.R+E-, then analyzed for subsequent protein expression via western blot. c. HIV p24 protein was quantified and divided by HIV p24 mRNA RQ.

In chapter 1 of this dissertation, we found that SLFN11 mediates the cleavage of type II tRNA (Leucines and Serines), thus down-regulating biased genes such as ATM or ATR. We also showed that the over-expression of SLFN11 in HEK293T cells was sufficient to down-regulate type II tRNA up to 50% [Chapter 1]. We believe that the SLFN11 Aspartic acid mutants are unable to inhibit codon-biased genes such as HIV or wtGFP because they lack the ability to mediate cleavage of type II tRNA. To test this hypothesis, we employed northern blot analysis of tRNAs from HEK293T cells in which our SLFN11 phospho-mutants were overexpressed. Total RNA from those cells were loaded onto a TBE-Urea gel, then stained with SYBR Gold to visually detect migrated RNA. As suspected, the “active” SLFN11 Alanine mutants down-regulated total type II tRNA more than their non-functional Aspartic acid counterparts (some differences close to 40%), while type I tRNA and 5s rRNA remained unchanged between samples (Fig. 2-2a, b). These samples were then analyzed via northern blot, probing for all type II tRNA and various type I tRNA. The result confirmed the SYBR Gold stain; when quantified, all type II tRNA were down-regulated with the Alanine mutants (up to 40% in some) while type I tRNA remained unchanged, except for iMet (Fig. 2-2a, c). It should be noted however that the transfection efficiency of SLFN11 is at most 50% (un-published data), which means that half the cells are not becoming transfected. These un-transfected cells do not have down-regulated tRNA and will dilute the overall down-regulated phenotype we observe; therefore, we are most likely seeing more than a 50% down-regulation of type II tRNA.

FIGURE 2-2

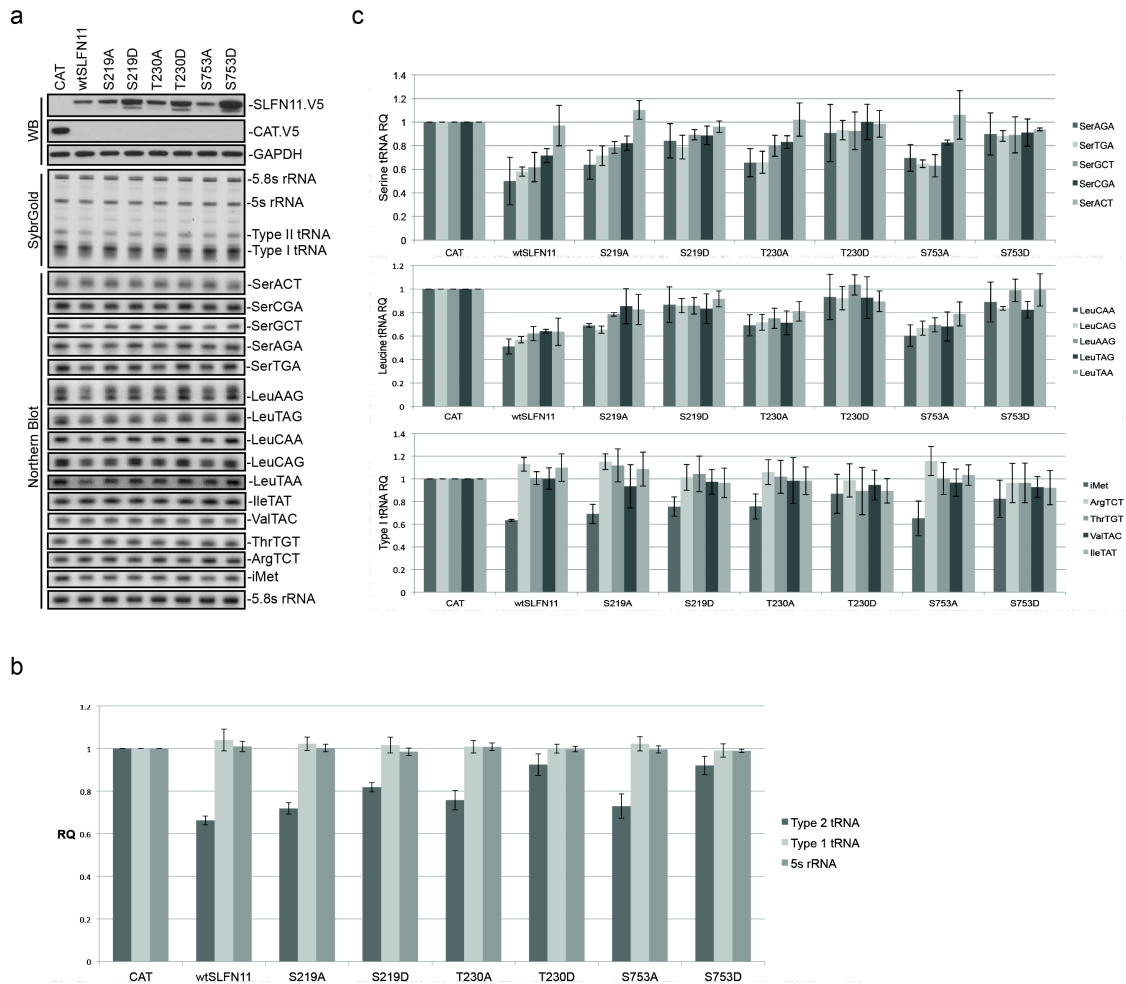


Figure 2-2: SLFN11 Alanine mutants down-regulate type II tRNA greater than SLFN11 Aspartic acid mutants. **a.** Transiently transfected wtSLFN11 and phospho-mutants were transfected and over-expressed in HEK293T cells. Protein samples were analyzed via western blot, and RNA samples were analyzed via SYBR Gold stain and northern blot. **b.** SYBR Gold stain bands were quantified and normalized to 5.8s rRNA. **c.** tRNA northern blots were quantified and normalized to 5.8s rRNA.

Thus far, the regulation of type II tRNAs via the SLFN11 phospho-mutants exactly correlates with their HIV (and wtGFP) translation inhibition capabilities, suggesting that the type II tRNA down-regulation is responsible for HIV's slowed translation. In chapter 1, we found that after SLFN11 mediated cleavage of type II tRNA, genes having certain codons are more susceptible to translational inhibition, particularly those containing Leu(TTA) and Leu(CTT). We took EGFP, a protein with extreme codon optimization, and attempted to inhibit its translation, through altering one amino acid's codons at a time. For example, we mutated all EGFP Leucine codons to Leu(CTG) or Leu(TAA). To test if our phospho-mutants affect the translation of EGFP with different codon profiles, we co-transfected our phospho-mutants with EGFP constructs designed to have all Leucines code for either Leu(CTG), Leu(TTA), or Leu(CTT). Next we determined translational repression of EGFP via western blot and qPCR. It was determined that SLFN11 Alanine mutants have no affect on EGFP Leu(CTG) protein expression, however have an enormous affect of protein inhibition on EGFP Leu(TTA) and to a smaller extent EGFP Leu(CTT), while SLFN11 Aspartic acid mutants do not have any protein inhibition activity (Fig. 2-3a). qPCR shows that mRNA was unaffected in all samples, confirming that the SLFN11 Alanine mutants are indeed acting as translational repressors of genes that prefer Leu(TAA) or Leu(CTT) codons (Fig. 2-3a).

FIGURE 2-3

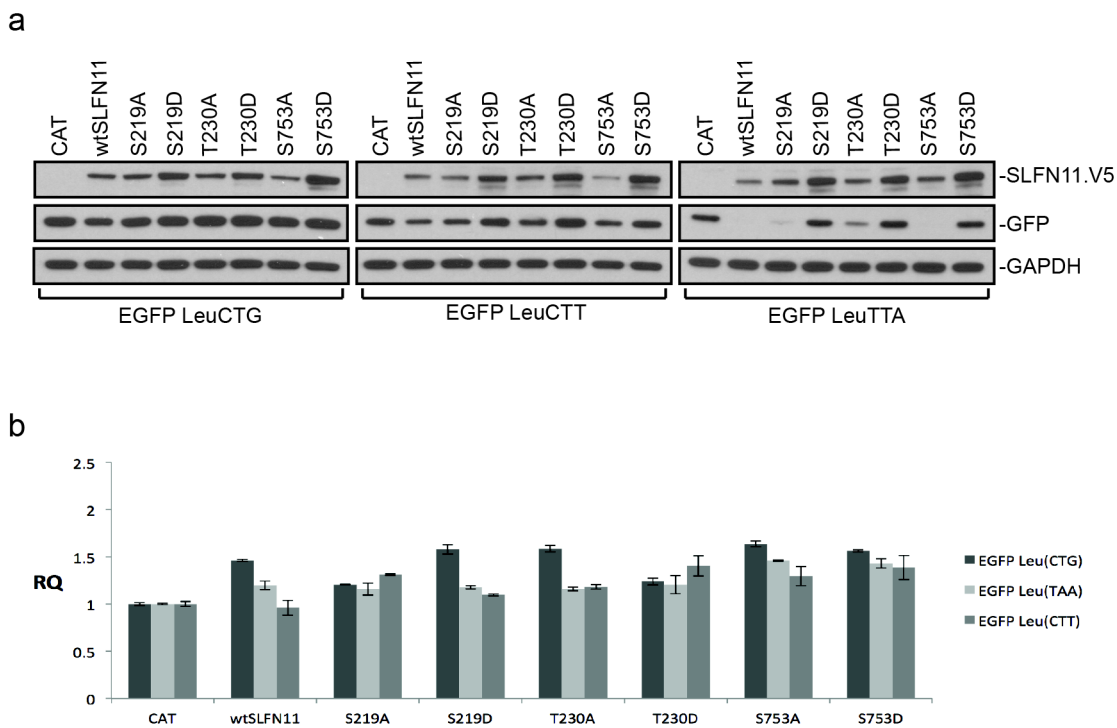


Figure 2-3: SLFN11 phospho-mutants inhibit specific EGFP Leucine codon bias constructs. **a.** HEK293T cells were transiently co-transfected with SLFN11 phospho-mutants and EGFP codon bias constructs and analyzed for subsequent protein expression via western blot. **b.** mRNA expression measured with qPCR and based on relative quantity.

Discussion

After SLFN11 was found to down-regulate HIV protein synthesis [2], we pursued the regulation of SLFN11. Our evidence suggests that SLFN11 is regulated by phosphorylation at three separate sites, S219, T230, and S753. These three sites showed significant differences in HIV protein inhibition capabilities between their Alanine and Aspartic acid mutant counterparts. Some SLFN11 sites also showed finer differences between their Alanine and Aspartic acid mutant counterparts, however, they were not sites found to be phosphorylated when analyzed by mass spectrometry. Therefore, we only further pursued S219, T230, and S753. The three most profound sites also showed to have increased HIV p24 mRNA, corresponding to an increase in HIV p24 proteins (Aspartic acid mutants); however, it was shown that when protein was quantified and normalized to mRNA expression, significantly more HIV p24 protein was produced in the presence of the SLFN11 Aspartic acid mutants compared to their Alanine mutant counterparts, suggesting that SLFN11 affects translation rather than transcription. Furthermore, wtGFP protein expression is inhibited by SLFN11; however, we do not see the boost in wtGFP mRNA. We suspect that HIV is most likely undergoing a positive feedback loop boosting its own mRNA expression. For example, HIV *tat* is known to boost HIV transcription [44]; therefore, it is possible that the more HIV proliferates and subsequently produces more *tat*, transcription of viral genes such as *gag* (including p24) will be boosted. wtGFP has no accessory proteins and has not been found to boost its own transcription. Lastly, we observed SLFN11 phospho-mutant expression

correlates with HIV p24 expression; SLFN11 itself is not a completely codon optimized gene and therefore it is safe to assume that the overexpression of SLFN11 regulates its own translation, as seen with SLFN11 mutants that were not loss of function.

Our SLFN11 Alanine mutants of sites S219, T230, and S753 all had down-regulated total type II tRNA compared to their Aspartic acid counterparts, while type I tRNA abundance and 5s rRNA remained unchanged. Type II tRNA consists of Leucines and Serines, while all type I tRNA consists of almost all other tRNA [32]. Leucines and Serines (except Ser(ACT)) contain an extra “variable” stem loop between the anticodon loop and D loop approximately 10 base pairs long [34]. As previously elucidated in chapter 1, type I tRNAs are unaffected by SLFN11 except for initiator Methionine (iMet). It is currently unknown why SLFN11 down-regulates iMet, as it does not contain a “variable” loop, however, we have observed that the down-regulation of iMet does not have an overall effect on translation. We believe this is because iMet has an initial abundance such that when SLFN11 down-regulates it, there are sufficient iMet tRNAs to maintain normal translation. Furthermore, even though each mRNA needs iMet, the overall transcriptome most likely requires more of each Leucine tRNA (each mRNA only needs one iMet, whereas usually multiple Leucine tRNAs are needed).

We previously hypothesized that the translational inhibition mediated by SLFN11 is not merely a codon-bias issue, but a bias towards specific codons paired with a strong down-regulation in cognate tRNA with low abundance. When

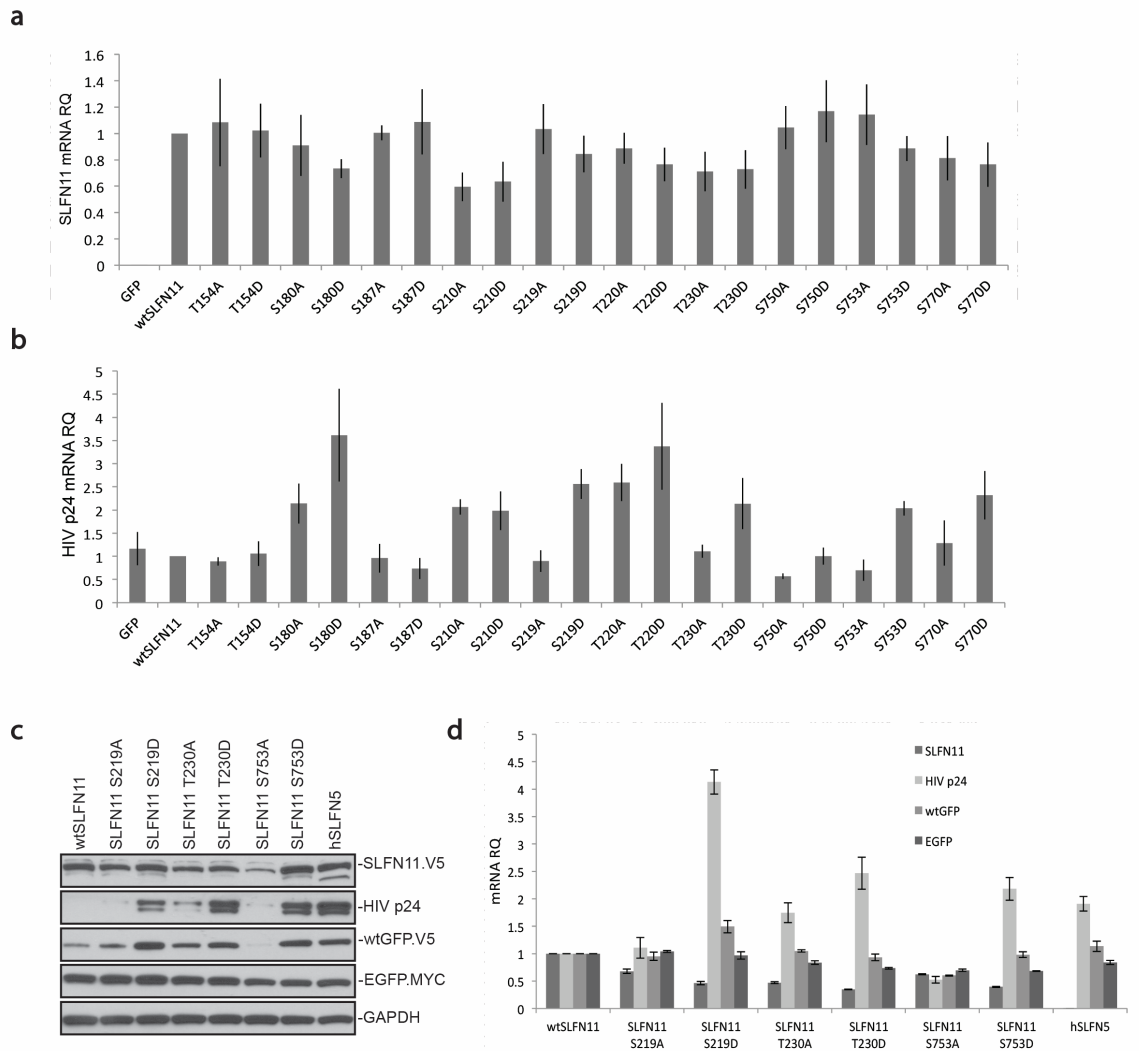
examining the frequency and raw number of Leu(TTA) and Leu(CTT) codons, HIV *gag* has 14 Leu(TTA) codons (36.8% of all Leucines), and 5 Leu(CTT) codons (13.2% of all Leucines). On the other hand, GAPDH has only 1 Leu(TTA) codon (.05% of all Leucines) and 1 Leu(CTT) codon, while b-Actin has 0 Leu(TTA) or Leu(CTT) codons [Chapter 1]. This profound result shows that the HIV *gag* gene has strong bias towards Leu(TTA) and to a lesser extent Leu(CTT) codons. When testing our SLFN11 phospho-mutants with EGFP mutant constructs, we observed no significant differences between pairs of phospho-mutants for EGFP Leu(CTG) protein expression. However, pairs had significant differences in inhibition capability when transfected with EGFP Leu(TTA) and to a lesser extent EGFP Leu(CTT). EGFP Leu(TTA) and EGFP Leu(CTT) protein inhibition via phospho-mutants predictably correlated with HIV translational inhibition. These results show that the SLFN11 Alanine phospho-mutants retain the capability to specifically down-regulate proteins that have Leu(TAA) or Leu(CTT) codon bias. Furthermore, SLFN11 phospho-mutant's ability to down-regulate type II tRNA directly predicts how well they will inhibit protein translation of biased genes.

This work has uncovered potentially three novel phosphorylation sites of SLFN11, and that SLFN11 must be de-phosphorylated in order to be active. In light of this, a phosphatase must be de-phosphorylating SLFN11, while a kinase must be phosphorylating it. Discovering the kinase that is phosphorylating SLFN11 is an important endeavor, not only because inhibiting the kinase activates SLFN11, but also because there already exist an array of small

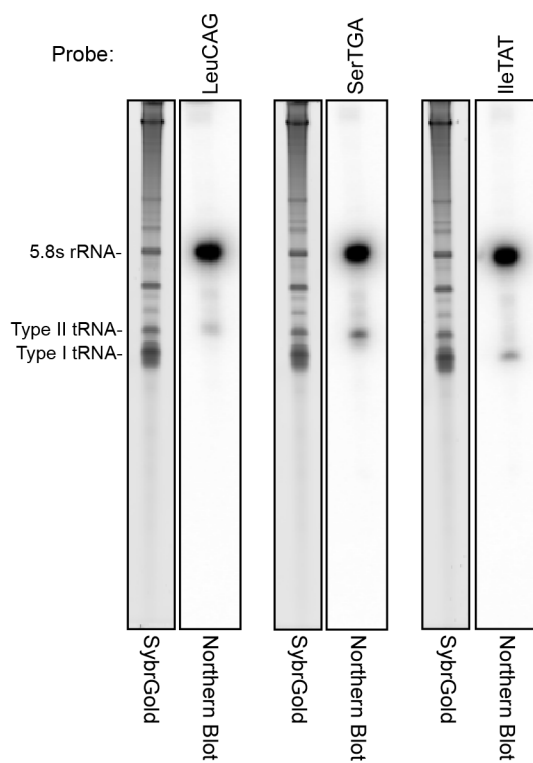
molecule kinase inhibitors, which are relatively easy to produce. Harnessing the capacity to activate SLFN11 in this manner holds great potential due to SLFN11's ability to inhibit HIV and sensitize cancer cells by exploiting ATR codon usage bias. Our work on the regulation of SLFN11 not only illuminates the inner workings of SLFN11 activity, but also paves the way in the discovery of novel chemotherapeutics and anti-retrovirals (ARTs). The potential impact of this protein in the fields of HIV and cancer are profound.

Extended Data

EXTENDED DATA FIGURE 2-1



Extended Data Figure 2-1: SLFN11 phospho-mimetics reveal de-phosphorylation activates SLFN11. a, b. mRNA expression measured with qPCR and based on relative quantity. **c.** SLFN11 phospho-mutants were co-transfected with wtGFP, EGFP, and pNL4-3.Luc.R+E-, then protein expression was determined via western blot. **d.** mRNA expression via qPCR

EXTENDED DATA FIGURE 2-2

Extended Data Figure 2-2: Verification of type I and II tRNA northern blot probes. a, b. Identical total RNA samples from HEK293T cells were migrated on TBE-Urea gel. The gels were SYBR Gold stained, then transferred as a northern blot. The membrane was then cut into strips and probed for their respective type II tRNA, then all strips were probed for 5.8s rRNA. SYBR Gold stain and northern blot scans compared side by side to show size of tRNA.

Acknowledgements

Chapter 2, in full, is currently being prepared for submission for publication of the material. *De-phosphorylation of SLFN11 induces down-regulation of type II tRNA*. Malone, Dane; Li, Manqing; Lardelli, Rea; David, Michael. Dane Malone is the primary investigator and author of this material.

CONCLUDING REMARKS AND REMAINING QUESTIONS

Our work has unraveled a biological mechanism never observed before. The cleavage of type II tRNA mediated by SLFN11 is a powerful pathway that we can possibly manipulate in order to eradicate certain cancers. However, several questions still remain. The most obvious question is if SLFN11 is itself a nuclease. In chapter 1, we found that SLFN11 down-regulates type II tRNA, however we do not have sufficient evidence to prove that SLFN11 itself has nuclease activity and thus cleaves type II tRNA itself. It was reported in 2015 that the short form of rabbit SLFN14 binds to the bottom portion of the ribosome and preferentially cleaves rRNA as an endoribonuclease [37]. The same paper also reported that the nuclease activity of rabbit SLFN14 was in the N-terminal region in the AAA domain, and since SLFN11 and rabbit SLFN14 share homology in their N-terminal domain, we believe the SLFN11 has the potential to be an endoribonuclease targeting type II tRNA. We are currently working towards proving the nuclease activity of SLFN11 by purifying recombinant SLFN11 and demonstrating tRNA cleavage *in vitro*.

The second task that follows determining SLFN11 nuclease activity is to elucidate at which sites the type II tRNA are being cleaved. Our northern blot probes target the 5' end of the tRNA, which is still able to hybridize to the smaller degradation product [Chapter 1]. We also created 3' probes of similar length, which, as we accurately predicted, did not pick up the degradation product (unpublished data). Together, this suggests that SLFN11 is mediating cleavage of

the tRNA at the 3' end, approximately 20-30 base pairs from the canonical CCA tail. If this proves correct, it is possible that this site falls within the variable arm of the tRNA, which is unique to type II tRNA [34]. To test which site SLFN11 could be cleaving, we are analyzing our cleaved tRNA with RNAseq. Furthermore, a new field of tRNA biology has come into focus recently: tRNA-derived fragments (tRFs). There are three types of tRFs, all determined by the cleavage site, which are designated as tRF-5, tRF-3, tRF-1 ranging from 18-22 base pairs [45]. We are interested in tRF-3, as this tRF is produced from the 3' end of the mature tRNA, and most closely matches our model of where SLFN11 could be mediating the cleavage. tRFs have been found to be produced by Dicer [46][47] and angiogenin [48], which are generated via stress responses [49]. tRFs have been found to act as translational repressors by displacing translational machinery from the mRNA [50][51], acting as siRNA [52], and displacing RNA binding proteins to de-stabilize mRNA [53]. To test whether or not SLFN11 is creating the previously identified tRFs, we are employing a strategy similar to the quantification of micro RNAs (miRNAs), in which adapters are utilized to make cDNA, and qPCR is used to quantify expression. There are newly developed qPCR plates specifically for tRFs, which contain primers that recognize all previously identified tRFs.

Lastly, we have determined that SLFN11 is potentially phosphorylated at three different sites; if any of the sites are mutated to Aspartic acid (phospho-mimetic), SLFN11's type II down-regulation capability is rendered ineffective, and therefore cannot affect translation. However, further evidence is needed to show

that SLFN11 is truly phosphorylated at these three sites. We have attempted to utilize phospho-specific antibodies and phospho-tag gels, but both techniques were unfruitful. We are currently testing several other methods, such as phospho-gel stains and radioactive phosphorylation labeling. An arguably more important question than the site of regulation is the identity of the kinase or phosphatase regulating SLFN11. We have evidence to suggest that either protein phosphatase catalytic subunit 1 gamma (PPP1CC) or protein phosphatase catalytic subunit 6 (PPP6C) could be de-phosphorylating SLFN11, rendering its type II tRNA down-regulation capability non-functional (un-published data). The discovery of the phosphatase or kinase is imperative because it may provide a tool for controlling SLFN11 activity through small molecule inhibitors. SLFN11 has been implicated to be involved with two of the most prominent diseases of our time, and our work has paved the way in discovering novel therapeutics in both fields of HIV and cancer.

REFERENCES

- [1] O. Bustos, S. Naik, G. Ayers, C. Casola, M. A. Perez-Lamigueiro, P. T. Chippindale, E. J. Pritham, and E. de la Casa-Esperón, *Evolution of the Schlafen genes, a gene family associated with embryonic lethality, meiotic drive, immune processes and orthopoxvirus virulence*, *Gene*, vol. 447, no. 1, pp. 1–11, 2009.
- [2] M. Li, E. Kao, X. Gao, H. Sandig, K. Limmer, M. Pavon-Eternod, T. E. Jones, S. Landry, T. Pan, M. D. Weitzman, and M. David, *Codon-usage-based inhibition of HIV protein synthesis by human schlafen 11.*, *Nature*, vol. 491, no. 7422, pp. 125–8, 2012.
- [3] J. Barretina, G. Caponigro, N. Stransky, K. Venkatesan, A. A. Margolin, S. Kim, C. J. Wilson, J. Lehár, G. V Kryukov, D. Sonkin, A. Reddy, M. Liu, L. Murray, M. F. Berger, J. E. Monahan, P. Morais, J. Meltzer, A. Korejwa, J. Jané-Valbuena, F.A. Mapa, J. Thibault, E. Bric-Furlong, P. Raman, A. Shipway, I.H. Engels, J. Cheng, G.K. Yu, J. Yu, P. Aspesi Jr, M. de Silva, K. Jagtap, M.D. Jones, L. Wang, C. Hatton, E. Palesscandolo, S. Gupta, S. Mahan, C. Sougnez, R.C. Onofrio, T. Liefeld, L. MacConaill, W. Winckler, M. Reich, N. Li, Jill P. Mesirov, S.B. Gabriel, G. Getz, K. Ardlie, V. Chan, V.E. Myer, B.L. Weber, J. Porter, M. Warmuth, P. Finan, J.L. Harris, M. Meyerson, T.R. Golub, M.P. Morrissey, W.R. Sellers, R. Schlegel & L.A. Garraway, *The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity.*, *Nature*, vol. 483, no. 7391, pp. 603–7, 2012.
- [4] G. Zoppoli, M. Regairaz, E. Leo, W. C. Reinhold, S. Varma, A. Ballestrero, J. H. Doroshov, and Y. Pommier, *Putative DNA/RNA helicase Schlafen-11 (SLFN11) sensitizes cancer cells to DNA-damaging agents.*, *PNAS*, vol. 109, no. 37, pp. 15030–5, 2012.
- [5] A. Savina and S. Amigorena, *Phagocytosis and antigen presentation in dendritic cells*, *Immunological Reviews*, vol. 219, no. 1. pp. 143–156, 2007.
- [6] P. Lacy and J. L. Stow, *Cytokine release from innate immune cells: Association with diverse membrane trafficking pathways*, *Blood*, vol. 118, no. 1. pp. 9–18, 2011.
- [7] A. Rustagi and M. Gale, *Innate antiviral immune signaling, viral evasion and modulation by HIV-1*, *Journal of Molecular Biology*, vol. 426, no. 6. pp. 1161–1177, 2014.
- [8] M. Alfano and G. Poli, *Role of cytokines and chemokines in the regulation*

- of innate immunity and HIV infection, Molecular Immunology*, vol. 42, no. 2. pp. 161–182, 2005.
- [9] S. Uematsu and S. Akira, *Pathogen recognition by innate immunity, Skin Research*, vol. 7, no. 1. pp. 1–11, 2008.
- [10] L. Fong, M. Mengozzi, N. W. Abbey, B. G. Herndier, and E. G. Engleman, *Productive infection of plasmacytoid dendritic cells with human immunodeficiency virus type 1 is triggered by CD40 ligation*, *J Virol*, vol. 76, no. 21, pp. 11033–11041, 2002.
- [11] M. Altfeld and M. Gale Jr, *Innate immunity against HIV-1 infection*, *Nat. Immunol.*, vol. 16, no. 6, pp. 554–562, 2015.
- [12] J. Snider, G. Thibault, and W. A. Houry, *The AAA+ superfamily of functionally diverse proteins.*, *Genome Biol.*, vol. 9, no. 4, p. 216, 2008.
- [13] M. Yoneyama, M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y.-M. Loo, M. J. Gale, S. Akira, S. Yonehara, A. Kato, and T. Fujita, *Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity.*, *J. Immunol.*, vol. 175, no. 5, pp. 2851–2858, 2005.
- [14] D. a Schwarz, C. D. Katayama, and S. M. Hedrick, *Schlafen, a new family of growth regulatory genes that affect thymocyte development.*, *Immunity*, vol. 9, no. 5, pp. 657–668, 1998.
- [15] P. Geserick, F. Kaiser, U. Klemm, S. H. E. Kaufmann, and J. Zerrahn, *Modulation of T cell development and activation by novel members of the Schlafen (slfn) gene family harbouring an RNA helicase-like motif*, *Int. Immunol.*, vol. 16, no. 10, pp. 1535–1548, 2004.
- [16] G. Brady, L. Boggan, A. Bowie, and L. A. J. O'Neill, *Schlafen-1 causes a cell cycle arrest by inhibiting induction of cyclin D1*, *J. Biol. Chem.*, vol. 280, no. 35, pp. 30723–30734, 2005.
- [17] C. Gubse, R. Goodbody, A. Ecker, G. Brady, L. A. J. O'Neill, N. Jacobs, and G. L. Smith, *Camelpox virus encodes a schlafen-like protein that affects orthopoxvirus virulence*, *J. Gen. Virol.*, vol. 88, no. 6, pp. 1667–1676, 2007.
- [18] E. Katsoulidis, N. Carayol, J. Woodard, I. Konieczna, B. Majchrzak-Kita, A. Jordan, A. Sassano, E. A. Eklund, E. N. Fish, and L. C. Platanias, *Role of schlafen 2 (SLFN2) in the generation of interferon α -induced growth inhibitory responses*, *J. Biol. Chem.*, vol. 284, no. 37, pp. 25051–25064,

2009.

- [19] M. Berger, P. Krebs, K. Crozat, X. Li, B. A. Croker, O. M. Siggs, D. Popkin, X. Du, B. R. Lawson, A. N. Theofilopoulos, Y. Xia, K. Khovananth, Y. M. EM, T. Satoh, O. Takeuchi, S. Akira, and B. Beutler, *An Slfn2 mutation causes lymphoid and myeloid immunodeficiency due to loss of immune cell quiescence*, *Nat Immunol*, vol. 11, no. 4, pp. 335–343, 2010.
- [20] L. Zhao, B. Neumann, K. Murphy, J. Silke, and T. J. Gonda, *Lack of reproducible growth inhibition by Schlafen1 and Schlafen2 in vitro*, *Blood Cells, Mol. Dis.*, vol. 41, no. 2, pp. 188–193, 2008.
- [21] P. L. Meintjes and A. G. Rodrigo, *Evolution of relative synonymous codon usage in Human Immunodeficiency Virus type-1.*, *J. Bioinform. Comput. Biol.*, vol. 3, no. 1, pp. 157–168, 2005.
- [22] E. H. M. Wong, D. K. Smith, R. Rabadan, M. Peiris, and L. L. M. Poon, *Codon usage bias and the evolution of influenza A viruses. Codon Usage Biases of Influenza Virus.*, *BMC Evol. Biol.*, vol. 10, p. 253, 2010.
- [23] B. Berkhout and F. J. va. Hemert, *The unusual nucleotide content of the HIV RNA genome results in a biased amino acid composition of HIV proteins*, *Nucleic Acids Res.*, vol. 22, no. 9, pp. 1705–1711, 1994.
- [24] A. C. van der Kuyl and B. Berkhout, *The biased nucleotide composition of the HIV genome: a constant factor in a highly variable virus*, *Retrovirology*, vol. 9, no. 1, p. 92, 2012.
- [25] A. Kofman, M. Graf, A. Bojak, L. Deml, K. Bieler, A. Kharazova, H. Wolf, and R. Wagner, *HIV-1 gag expression is quantitatively dependent on the ratio of native and optimized codons.*, *Tsitologija*, vol. 45, no. 1, pp. 86–93, 2003.
- [26] M. Abdel-Mohsen, R. A. S. Raposo, X. Deng, M. Li, T. Liegler, E. Sinclair, M. S. Salama, H. E.-D. a Ghanem, R. Hoh, J. K. Wong, M. David, D. F. Nixon, S. G. Deeks, and S. K. Pillai, *Expression profile of host restriction factors in HIV-1 elite controllers.*, *Retrovirology*, vol. 10, p. 106, 2013.
- [27] P. D'Arpa, P. S. Machlin, H. Ratrie, N. F. Rothfield, D. W. Cleveland, and W. C. Earnshaw, *cDNA cloning of human DNA topoisomerase I: catalytic activity of a 67.7-kDa carboxyl-terminal fragment.*, *Proc. Natl. Acad. Sci. U. S. A.*, vol. 85, no. 8, pp. 2543–2547, 1988.
- [28] N. Saleh-Gohari, H. E. Bryant, N. Schultz, K. M. Parker, T. N. Cassel, T. Helleday, S.-G. Nasrollah, H. E. Bryant, N. Schultz, K. M. Parker, T. N.

- Cassel, T. Helleday, N. Saleh-Gohari, H. E. Bryant, N. Schultz, K. M. Parker, T. N. Cassel, and T. Helleday, *Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks.*, *Mol. Cell. Biol.*, vol. 25, no. 16, pp. 7158–7169, 2005.
- [29] A. Maréchal and L. Zou, *DNA damage sensing by the ATM and ATR kinases*, *Cold Spring Harb. Perspect. Biol.*, vol. 5, no. 9, 2013.
- [30] P. M. Sharp and W. H. Li, *The codon adaptation index—a measure of directional synonymous codon usage bias, and its potential applications*, *Nucleic Acids Res.*, vol. 15, no. 3, pp. 1281–1295, 1987.
- [31] G. Wu, D. E. Culley, and W. Zhang, *Predicted highly expressed genes in the genomes of Streptomyces coelicolor and Streptomyces avermitilis and the implications for their metabolism*, *Microbiology*, vol. 151, no. 7, pp. 2175–2187, 2005.
- [32] P. P. Chan and T. M. Lowe, *GtRNADB: A database of transfer RNA genes detected in genomic sequence*, *Nucleic Acids Res.*, vol. 37, no. SUPPL. 1, 2009.
- [33] R. T. Morgan, L. K. Woods, G. E. Moore, L. A. Quinn, L. McGavran, and S. G. Gordon, *Human cell line (COLO 357) of metastatic pancreatic adenocarcinoma.*, *Int. J. Cancer*, vol. 25, no. 5, pp. 591–8, 1980.
- [34] T. Brennan and M. Sundaralingam, *Structure of transfer RNA molecules containing the long variable loop*, *Nucleic Acids Res.*, vol. 3, no. 11, pp. 3235–3251, 1976.
- [35] A. G. Hinnebusch, *Molecular mechanism of scanning and start codon selection in eukaryotes.*, *Microbiol. Mol. Biol. Rev.*, vol. 75, no. 3, p. 434–67, first page of table of contents, 2011.
- [36] S. E. Koltitz and J. R. Lorsch, *Eukaryotic initiator tRNA: Finely tuned and ready for action*, *FEBS Letters*, vol. 584, no. 2. pp. 396–404, 2010.
- [37] V. P. Pisareva, I. A. Muslimov, A. Tcherepanov, and A. V. Pisarev, *Characterization of novel ribosome-associated endoribonuclease SLFN14 from rabbit reticulocytes*, *Biochemistry*, vol. 54, no. 21, pp. 3286–3301, 2015.
- [38] K. Cheung-Ong, G. Giaever, and C. Nislow, *DNA-Damaging Agents in Cancer Chemotherapy: Serendipity and Chemical Biology*, *Chem. Biol.*, vol. 20, no. 5, pp. 648–659, 2013.

- [39] E. E. Gardner, B. H. Lok, V. E. Schneeberger, P. Desmeules, L. A. Miles, P. K. Arnold, A. Ni, I. Khodos, E. de Stanchina, T. Nguyen, J. Sage, J. E. Campbell, S. Ribich, N. Rekhtman, A. Dowlati, P. P. Massion, C. M. Rudin, and J. T. Poirier, *Chemosensitive Relapse in Small Cell Lung Cancer Proceeds through an EZH2-SLFN11 Axis*, *Cancer Cell*, vol. 31, no. 2, pp. 286–299, 2017.
- [40] R. I. Connor, B. K. Chen, S. Choe, and N. R. Landau, *Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes.*, *Virology*, vol. 206, no. 2, pp. 935–44, 1995.
- [41] A. Traven and J. Heierhorst, *SQ/TQ cluster domains: Concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins*, *BioEssays*, vol. 27, no. 4, pp. 397–407, 2005.
- [42] A. C. Stabell, J. Hawkins, M. Li, X. Gao, M. David, W. H. Press, and S. L. Sawyer, *Non-human Primate Schlafen11 Inhibits Production of Both Host and Viral Proteins*, *PLoS Pathog.*, vol. 12, no. 12, 2016.
- [43] N. Dissmeyer and A. Schnittger, *Use of phospho-site substitutions to analyze the biological relevance of phosphorylation events in regulatory networks*, *Methods Mol. Biol.*, vol. 779, pp. 93–138, 2011.
- [44] R. E. Kiernan, C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K. T. Jeang, M. Benkirane, and C. Van Lint, *HIV-1 tat transcriptional activity is regulated by acetylation.*, *EMBO J.*, vol. 18, no. 21, pp. 6106–18, 1999.
- [45] Y. S. Lee, Y. Shibata, A. Malhotra, and A. Dutta, *A novel class of small RNAs: tRNA-derived RNA fragments (tRFs)*, *Genes Dev.*, vol. 23, no. 22, pp. 2639–2649, 2009.
- [46] M. Shigematsu and Y. Kirino, *tRNA-derived short non-coding RNA as interacting partners of argonaute proteins*, *Gene Regul. Syst. Bio.*, vol. 9, pp. 27–33, 2015.
- [47] C. Cole, A. Sobala, C. Lu, S. R. Thatcher, A. Bowman, J. W. S. Brown, P. J. Green, G. J. Barton, and G. Hutvagner, *Filtering of deep sequencing data reveals the existence of abundant Dicer-dependent small RNAs derived from tRNAs.*, *Rna*, vol. 15, no. 12, pp. 2147–2160, 2009.
- [48] S. Yamasaki, P. Ivanov, G. F. Hu, and P. Anderson, *Angiogenin cleaves tRNA and promotes stress-induced translational repression*, *J. Cell Biol.*, vol. 185, no. 1, pp. 35–42, 2009.

- [49] M. M. Emara, P. Ivanov, T. Hickman, N. Dawra, S. Tisdale, N. Kedersha, G. F. Hu, and P. Anderson, *Angiogenin-induced tRNA-derived stress-induced RNAs promote stress-induced stress granule assembly*, *J. Biol. Chem.*, vol. 285, no. 14, pp. 10959–10968, 2010.
- [50] J. Gebetsberger, M. Zywicki, A. Künzi, and N. Polacek, *TRNA-derived fragments target the ribosome and function as regulatory non-coding RNA in Haloferax volcanii*, *Archaea*, vol. 2012, 2012.
- [51] A. Sobala and G. Hutvagner, *Small RNAs derived from the 5' end of tRNA can inhibit protein translation in human cells.*, *RNA Biol.*, vol. 10, no. 4, pp. 553–63, 2013.
- [52] R. L. Maute, C. Schneider, P. Sumazin, A. Holmes, A. Califano, K. Basso, and R. Dalla-Favera, *tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma*, *Proc. Natl. Acad. Sci.*, vol. 110, no. 4, pp. 1404–1409, 2013.
- [53] H. Goodarzi, X. Liu, H. C. B. Nguyen, S. Zhang, L. Fish, and S. F. Tavazoie, *Endogenous tRNA-derived fragments suppress breast cancer progression via YBX1 displacement*, *Cell*, vol. 161, no. 4, pp. 790–802, 2015.