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INVESTIGATING THE ESCAPE MECHANISM OF SRE BEARING MRNA TRANSCRIPTS DURING VIRAL HOST SHUTOFF

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A Dissertation Presented

By

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DEDICATION

I would like to dedicate this thesis to my wife Jewel and my son Ashton, both of whom have supported and inspired me to become a better person and scientist. To my wife, who has stood by me as I have aspired for this path in life and supports my pursuit of knowledge. Your kind heart and laughter help brighten my day, even when I am grumpy. You remind me that kindness and an open heart are a pathway to happiness. To my son, whose endless curiosity has reinvigorated my own, I hope that I can guide and inspire you to see that this world has endless wonder even if it sometimes takes a microscope to see it. To whatever your path becomes I hope that you pursue it with passion and an open mind.

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Next, I would like to thank my lab members for helping support me and the lab. As we grew together as a lab we helped one another in our pursuit of science, bouncing ideas off one another, pushing each other to try new things and making science fun even during times of failure and troubleshooting.

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ABSTRACT

INVESTIGATING THE ESCAPE MECHANISM OF SRE BEARING MRNA TRANSCRIPTS DURING VIRAL HOST SHUTOFF

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During viral infection, the virus and host clash for control over gene expression in an evolutionarily arms race that has raged for thousands of years. During lytic replication, Kaposi's sarcoma associated herpesvirus (KSHV) triggers a massive RNA decay event known as host shut off. This causes over 70% of all RNA to be degraded suppressing the host antiviral response while freeing resources for viral replication. Our lab focuses on a subset of transcripts that escape from this viral degradation event using a cis acting 3' UTR element known as a "SOX resistant element" or SRE. Although we have identified a couple of these transcripts and some of the proteins involved in their protection, the complete mechanism of protection from host shutoff has yet to be elucidated. In the first chapter, we used m^6A-eCLIP to identify m6A modifications on SRE transcripts. We also characterized that this modification is necessary for SRE escape. In the second chapter we further characterized NCL/SRE densities during host shutoff through subcellular localization and mass spec and RNA sequencing. Finally in the third chapter we explored a method of SRE

transcripts escaping from host shutoff induced nuclear retention of new transcripts through their interaction with a CRM-1 export pathway.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Kaposi's sarcoma associated Herpesvirus (KSHV)

1.1.1 KSHV Infection and Gene Expression

Kaposi's sarcoma-associated herpesvirus (KSHV) is from the γ-herpesvirus family and is the caustic agent of its namesake Kaposi's sarcoma, as well as primary effusion lymphoma (PEL) and multicentric Castleman's disease [1-3]**.** This pathogen was discovered and isolated in the sarcoma lesions of AIDS patients in 1994 by Dr. Yuan Chang and Dr. Patrick Moore [4]**.** KSHV has a similar double stranded linear DNA genome that is roughly 165 kb, with a central coding region of approximately 137 kb and flanked by highly GC-rich terminal repeat (TRs) [5-7]**.** There is a large diversity of clinical and molecular phenotypes for KSHV depending on whether it is in the B cell or endothelial lineage. KSHV's genome expressed nearly 100 open reading frames (ORFs) however KSHV maintains a biphasic lifecycle and expresses its genes based in a temporal pattern [3, 5, 8, 9]. KSHV mainly persists in its latent period with bursts of lytic viral replication (**Figure 1**) [5, 6, 8, 10]. KSHV begins its primary infection with an enveloped virion that enters the cell by interacting with cell receptors followed by clathrin and/or actin mediated endocytosis [5, 9, 11]**.** As the nucleocapsid enters the cytoplasm it releases the viral genome, which travels to the nucleus entering through the nuclear pore complex (NPC)**.** From there the linear viral genome will circularize and becomes a viral episome, which is

maintained throughout latency. During latency only a subset of viral genes is expressed in order to promote viral genome replication along with host division, genome fidelity and finally host immune evasion [5, 8, 9, 11, 12]. Some of these latently expressed genes help promote cell survival and proliferation to ensure their spread. The critical gene of latency is a master regulator protein named Latency-associated Nuclear Antigen or LANA [8, 13-16]**.** LANA functions as a genome wide suppressor of lytic gene expression, as KSHV has the capability to enter the lytic phase after primary infection or at anytime once latency has been established. LANA is also responsible for the episomal tethering and segregation of the viral genome. During mitosis KHSV episome must replicate and efficiently segregate into the daughter cells. LANA will simultaneously bind the viral episome at the terminal repeat (TR) and the host chromatin by interacting with several proteins. Some of these include H2A/B, MeCp2, Brd4 and CENP-F to name a few. LANA indirectly and directly binds to other transcription activators and repressors to regulate the master lytic phase regulator ORF50 which encodes the protein, Replication and Transcription activator (RTA) [8, 16, 17]**.**

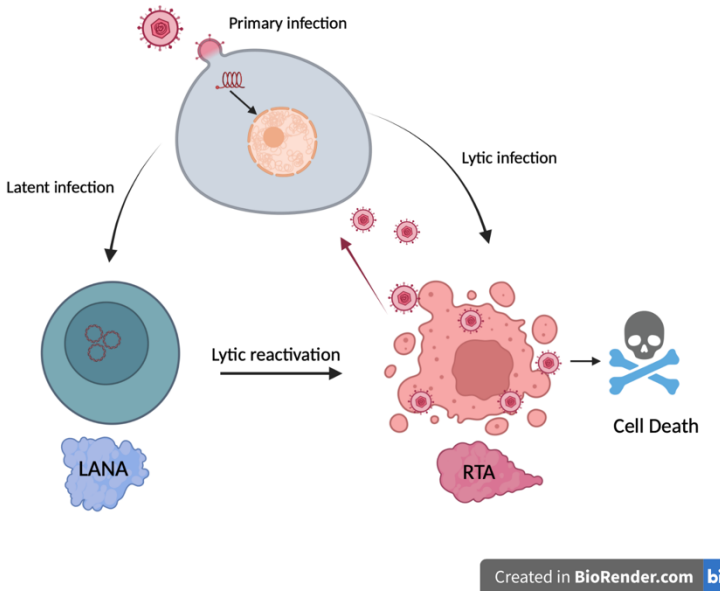

Figure 1: KSHV life cycle. Primary infection begins with virions entering a cell before going into lytic or latent infection. Latent infection can last years until reactivated. Lytic infection induces activation of many genes that will cause cell death and the new virions. Adapted from Broussard et al. 2020 [8]

If LANA is considered the master regulator for latent KSHV, then KSHV's protein RTA would the master regulator of the lytic phase. RTA has been shown to be a viral transcription factor that binds to RTA response elements (RTE) to usher and control the latent to lytic phase switch by binding to both viral and host gene promoters. For herpesviruses, depending on the cell type can either enter the lytic cycle right after primary infection or once latency has been established. However, in the instance of KSHV it's been confirmed that the virus proceeds to latency after primary infection. Usually this occurs when the host encounters some stressor and is more common in immunocompromised individuals [5, 9, 17]. There RTA helps trigger and guide the temporal cascade that is lytic gene expression. RTA expression is both sufficient and necessary for lytic reactivation and RTA binds to an RRE in its own promoter to cause a positive feedback loop for its expression [8, 9, 17]. KSHV's lytic gene expression can be broken down into early (0-24 hours post reactivation (HPR)), delayed early (24-48 HPR), and late (48-96 HPR) [8, 9, 13]. The early and delayed early set of genes are responsible for establishing proper control over host resources and expressing and coopting proteins for viral DNA replication. The late KSHV genes that are expressed consist mostly of capsid and tegument proteins. At the end of this time frame there should be many instances of viral DNA encapsidation in the nucleus followed by virion maturation and finally virion egress leading to cell death. In summary KSHV utilizes a variety of methods to gain and retain control over the host gene expression environment for its own propagation. Careful manipulation of its viral gene expression with LANA and RTA as well as host immune systems allows KSHV to propagate inside us for years.

1.1.2 RNA Decay and Viral Host Shutoff

From the synthesis of a messenger RNA (mRNA) all the way to its inevitable decay it can be said that the rate of that turnover, or steady state is a crucial regulator in gene expression. In a normal cell the series of complex pathways of transcription, modification, localization, translation, and decay can be summarized in the term "RNA fate". RNA decay is a crucial step in RNA fate that helps regulate how long a gene's message will be translated. Once it has fulfilled its purpose, degradation machinery will help recycle that material into other nucleic acid products. The half-life of mRNA varies from transcript as well as the organism, though generally housekeeping genes tend to have longer half-lives than most [18]**.** As a testament to its importance in eukaryotic systems there are multiple parallel and redundant mRNA decay pathways that operate in the cell. There are multiple types of proteins that have the capability to degrade proteins, however the two main ones are endoribonucleases, which will attach and cleave to a transcript internally and exoribonucleases, which will bind and begin degrading the transcript from one end or the other [19]. Intracellular RNA degradation machinery is primarily exonucleolytic, which allows RNAs to escape decay by protecting their 5' and 3' ends. The 5' ends of RNA polymerase II (RNA Pol II) transcripts are protected from degradation by a 7mGpppG cap modification that marks the RNA as "self" and recruits RNA and protein (RNP) complexes and is added immediately after transcription [20, 21]. These complexes also are involved in RNA processing, export, and translation initiation [20-22]. At the 3' end of an RNA a polyA tail is added which also acts as a platform for both nuclear and cytoplasmic proteins**.** Typically, when an mRNA has outlived its usefulness, and it needs to be targeted by decay, the cell will begin by deadenylating the polyA tail, decapping, displacing the RNP, or

endonucleolytic cleavage. This will expose an end of the mRNA that will allow the exonucleases to finalize the degradation.

Substantial research has been done mapping out the surveillance pathways and proteins that breakdown these flagged mRNAs in both the nucleus and the cytoplasm. The three main pathways are the nonsense mediated mRNA decay pathway (NMD), the nonstop mediated mRNA decay pathway (NSD) and the no-go mediated mRNA decay pathway (NGD). NMD detects and flags mRNAs with improper stop sites that would create truncated proteins that could be harmful to the organism [19-25]**.** NSD is the pathway that detects and degrades mRNA transcripts that lack a stop codon. Translation of these aberrant transcripts would have the ribosomes attempt to translate the polyA tail and not be able to release from the transcript causing a stalling and accumulation of ribosomes along the transcript which is deleterious to the cell [19]**.** Finally, NGD is a more recent discovery that is still being researched, currently the idea is that ribosomes who have stalled in the coding region of mRNA due to factors like rigid secondary structures. NGD will detect those transcripts, cleave them internally and allow for degradation machinery to degrade the transcript further [26]. All these surveillance pathways have series of proteins that constantly monitor mRNAs throughout their life cycle in both the cytoplasm and nucleus. However, in the end they commonly rely on the same exonucleases to finish the degradation. mRNAs that end up getting decapped or having a 5'-3' end exposed results in targeting by exonuclease from the XRN family. XRN1 localized in the cytoplasm and XRN2 which resides in the nucleus [27-29]**.** The XRN family contain two conserved regions CR1 and CR2 in the N terminus regions, with CR1 being very important for coordinating the positioning of Mg2+ cations for catalysis [27-29]**.** XRN enzymes prefer

single stranded RNA substrates with a monophosphate end, which is why the 5' cap structure along with or other sophisticated and stable secondary structures can inhibit the exoribonucleolytic activity of XRN1/2 [19, 27-29]**.** RNAs that have been deadenylated or other aberrant 3' ends are typically degraded by exosomes, a complex of ten to eleven proteins that have exonucleolytic activity as well accessory proteins like RNA helicases, GTPases and targeting proteins [19, 30, 31]**.** Exosomes exist both in the nucleus and cytoplasm with the major difference between the two being that cytoplasmic exosomes lack one subunit named Rrp6p, an exonuclease [20, 24, 30, 31]**.** Cells that lack this subunit accumulate nuclear RNAs with improperly processed 3' ends and it has been implicated in its importance for retention of mRNAs containing aberrant 3' ends [20, 24, 30, 31]. There are some cases of 3' uridylation that trigger mRNA decay by a paralogue of the exosome complex Dis3/Dis3l catalytic subunits, the Dis3L2 nuclease. Dis3l2 has been proposed to be a central player of an exosome independent mRNA decay pathway [19, 32, 33]**.** Dis3l2 has been shown to cooperate on the same pool of transcripts as XRN1 and interact with one another [32, 33]. Dis3l2 silencing resulted in 3' terminal uridine extensions, inhibition of mRNA degradation and suppression of cell death [32, 33]**.** These degradation machineries are some but not all of the ways cells degrade RNAs, and their effects are felt throughout the cell allowing for direct and speedy control over cellular gene expression.

Given the effectiveness of the host's RNA surveillance and degradation pathways, it is not surprising that viruses like KSHV have evolved ways to control these pathways for their own benefit. As stated previously, during the early and delayed early time of KSHV's lytic reactivation the virus begins to express proteins that attempt to wrest control over gene expression. Research has shown that widespread RNA decay can trigger massive

down regulation of not only translation, but it also has the capability to affect newly created transcripts in the nucleus [34-38]. The decimation of the cellular transcripts by viruses allows the RBPs and translational machinery that have been freed up to be allocated to newly made viral transcripts [38-40]. This act of "host shutoff" occurs in multiple viruses including alpha and gamma herpesviruses, influenza virus, and coronaviruses to name a few. They express an mRNA specific endoribonuclease or recruit cellular endoribonucleases to induce widespread degradation of upwards of two-thirds of the host transcriptome [36, 41-48]. In the case of KSHV, the virus orchestrates its own host shutoff event by expressing an endoribonuclease named Shutoff Alkaline Exonuclease (SOX). In 2004 Dr. Britt Glausinger and Dr. Don Ganem confirmed that upwards of 70% of cellular gene expression was reduced due to SOX specifically [49]. SOX is a member of the PD(D/E)XK superfamily which is defined by the nucleolytic domain motif along with EBV's BGLF5, Influenza A's PA-X [37, 43-45, 50-52]. SOX targets mRNAs containing a degenerate RNA motif, UGAAG [46, 53]. In 2015, Gaglia et al. was able to further define the SOX targeting motif and found that the targeting mechanism is both sequence specific and promiscuous [53]. The SOX cut sites are on mature mRNAs but are not restricted to particular regions, this information led to the discovery that most viral and human transcripts had at least one sequence that fit the SOX targeting motif. The refining of SOX targeting mechanism created the foundation for exploring the contribution of secondary structures of RNA to SOX targeting. Mendez et al. found that given the discovery of SOX cut sites on target transcripts beyond the consensus sequence stated earlier, there must be an additional mechanism to SOX targeting. Through structural exploration of the known cleavage targets they found several general stem loops and bulges flanking predicted sites

in silico. SOX substrate processing was dependent on the recognition of the loop and bulge within the RNA duplex. Crystal structure of SOX bound with RNA revealed that SOX cleavage is restricted to regions near unpaired nucleotide tracts within a loop fold [54]. Thus, illustrating the critical involvement of RNA secondary structure in SOX targeting. Once cytoplasmic, SOX targets its target transcript creating an endonucleolytic nick on mature mRNAs. This endonucleolytic cleavage is the hallmark for the activation of the host RNA decay machinery, in particular the exonucleases Xrn1 and Dis3L2 that rapidly degrade the newly exposed 5' and 3' fragments created by SOX-mediated cut [36]. This rapid and widespread process quickly reduces the amount of cytoplasmic mRNA while at the same time releasing many RNA-binding factors from these now degraded mRNA. These factors suddenly without a target mRNA to bind to are now free to shuttle in the cell and this was shown to trigger a feedback mechanism that, in response to this massive RNA decay event, halts transcription in the nucleus [34, 40]. This feedback loop was revealed to have an important pro-viral function as only host genes are affected that favor recruitment and elongation of RNAPII on viral promoters.

SOX also has another aspect of host shutoff that occurs in the nucleus that works in concert with the relocalization of RBPs due to the mRNA degradation caused by SOX induced degradation. In Lee et al. it was found that cytoplasmic poly(A) binding protein (PABPC) relocates to the cytoplasm during host shutoff where it interacts with Poly nuclear poly(A) binding protein (PABPN), and poly(A) polymerase II (PAP II) in coordination with nuclear SOX to promote hyper polyadenylation [54]. This aberrant hyper polyadenylation was linked with increased mRNA turn over, aiding in perpetuating the host shut off phenotype by preventing new transcripts from reaching the cytoplasm.

Although it is not known which ribonuclease is responsible for this particular turnover, it is known that proper 3' end formation and polyadenylation is required for mRNA export. Any defects in this process are known to trigger nuclear retention and RNA destruction by quality control pathways [20, 24]. Much research has been done in understanding SOX's role in KSHV infection, with studies expanding on the prevalence and requirement s for SOX cleavage. There is still further research that is needed to be done on how SOX and the related endonucleases from other viruses preferentially target RNA polymerase II transcripts, a feature that is not conserved in in vitro experiments with purified SOX [54, 55]**.** It is possible SOX uses some other cellular co factors for specificity. For example, Influenzas A's PA-X endonuclease interacts with cofactors that are known to be involved in mRNA maturation and processing, allowing for increased PA-X cleavage [56-58]. Exploring these possible cofactors will help expand the knowledge of how SOX chooses its targets and with that knowledge help us understand more about the populations of mRNA that are degraded.

1.2 KSHV Host Shutoff Evasion

As mentioned earlier in **Chapter 1** DNA and RNA viruses regulate the transcriptional and post- transcriptional fate of host messenger RNA (mRNA) to gain access to key resources during infection. In particular, my work focuses on KSHV which triggers widespread mRNA decay that decimates greater than 70% of the cellular transcriptome [41, 43, 55, 59]. Intriguingly however, over the past decade, our lab and others have found select mRNA transcripts that robustly escape SOX-induced decay [60-

62]. Multiple mechanisms have been hypothesized to explain what contributes to promoting escape from SOX, ranging from a lack of a SOX-targeting motif to indirect transcriptional effects [63, 64]. More recently, we have demonstrated that there is a smaller subset of cellular transcripts that actively evade SOX. These "dominant" escapees each carry a specific RNA element found within their 3' untranslated regions (UTRs) termed the SOX resistance element (SRE). The SRE confers protection to the target transcript from SOX even if the transcript contains an SOX- targeting motif [60-62]. Interestingly, the SRE can resist multiple viral endonucleases but not cellular endonucleases, making it a virus-specific RNase escape element [60-62]. To date, it is still unknown how many of these SREs are present in the genome, their mechanism of action against viral endonucleases, or what becomes of the SRE-containing transcripts once they are spared from degradation. So far, three SRE- containing "escapees" have been identified: interleukin-6 (IL-6), growth arrest DNA damage-inducible 45 beta (GADD45B), and Shiftless (SHFL) [60-62]. Although there is little sequence homology among known SREs, they share similarities in their secondary structures, bolstering the idea that the SRE may act as a platform for the recruitment of a protective protein complex [60, 61]. Furthermore, it was observed that the SRE is only active when located in the 3' UTR region of a transcript, suggesting that this RNA element likely functions in conjunction with proteins to modulate RNA stability [60, 61]. Previous mass spectrometry screens have identified several host proteins that can bind to the SRE, and intriguingly, a few of these proteins are N^6-methyladenosine (m^6A) readers [60].

1.3 N ^{6}-methyladenosine (m^6A) modification

1.3.1 m^{6}A Roles in the Cell

m^{6}A is the most prevalent mRNA modification out of the over hundred different known RNA modifications [65-68]. m^{6}A impacts virtually every stage of posttranscriptional mRNA fate from splicing, localization, translation, and decay [65, 66, 68-72]. Deposition of m^{6}A occurs cotranscriptionally via an m^{6}A writer complex that consists of a catalytic methyltransferase subunit, such as METTL3, and other cofactors, such as METTL14/16 and WTAP [65, 73-78]. The writer complex recognizes a DRACH (D = G/A/U, R = G/A, H = A/U/C) motif for methylation. Although a transcript can have several DRACH motifs, not all will be methylated [73, 74, 79]. What determines which motif will be chosen is still unknown but has implicated to be tied to splicing sites [73, 80, 81]. Some transcripts can have one m^{6}A site whereas some mRNAs can contain 20 or more m^{6}A sites [66, 68]. Demethylases or erasers like FTO and ALKBH5 add a layer of reversibility to the m^{6}A epitranscriptome [66, 68, 82, 83]. The presence or absence of these modifications can

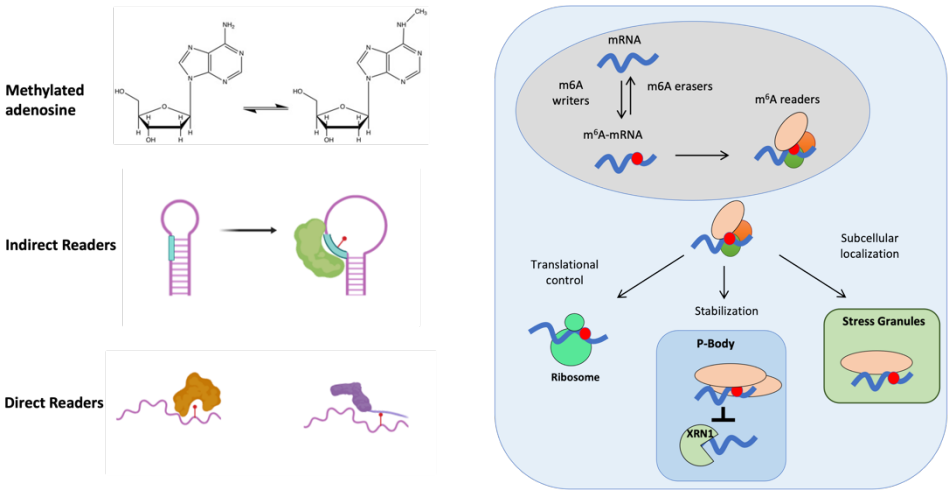

Figure 2: m^{6}A regulates RNA fate. m6A is a modification that can recruit reader proteins either directly or indirectly to mRNA. Depending on the readers and their binding locations various effects on RNA fate are produced as seen on the diagram to the right.

change the secondary structure of mRNA and create platforms for m^{6}A reader proteins. Reader proteins then recognize the modification and promote specific RNA fates in turn (**Figure 2**) [67-72].

The best-known function for m^{6}A was to cause mRNA instability with direct m^{6}A reader protein from the YTH family, YTHDF2 [68, 84, 85]**.** Numerous studies have also linked m^{6}A to translation regulation through three mechanisms. First involves YTHDF1, which is proposed to recruit eukaryotic translation initiation factor eIF3, a multi protein complex that recruits the small ribosome subunit to mRNAs to enhance translation [68, 86]**.** Another mechanism involves the deposition of m^{6}A to the 5' UTR of mRNA which is able to recruit eIF3, bypassing the normal requirement for eIF4E, the 7-methylguanosine-containing mRNA cap binding protein that recruits eIF3 [68, 86]**.** Finally, the third mechanism involves the typically nuclear METTL3 traveling with an m^{6}A mRNA to the cytoplasm. Once in the cytoplasm METTL3 binds eIF3 creating mRNA looping and induce a platform for ribosomes to reload on the same transcript after finishing. Another role for m^{6}A is mRNA splicing where studies found a limited number of genes, however it was proposed that the nuclear YTHDC1 is involved as it interacts with splicing regulators including SAM68, SC35, SRSF1 and SRSF3, suggesting a link between m^{6}A and splicing [68, 87-89]**.** m^{6}A mRNAs are also regulated by liquid-liquid phase separation when stretches of a transcripts have m^{6}A sites that recruit either direct m^{6}A readers that bind to the m^{6}A site itself or indirect readers that bind to stretches of transcript that are revealed due to the presence of the m^{6}A site [90, 91]. In the case of YTHDF1-3, these proteins contain low complexity domains which are areas of amino acids that can interact with each other and 'phase separate' into gels, polymers, or droplets within the cytoplasm. These

m^{6}A droplets can then fuse with well-known regulatory phase separated droplets such as stress granules, P-bodies, and neuronal RNA granules [90, 92-95]**.** This ability for m^{6}A to phase separate drastically influences whether an mRNA will be stored, degraded, or transported. Finally, m^{6}A containing transcripts has been shown to enhance mRNA export from the nucleus by binding YTHDC1. YTHDC1 binds SRSF3 which is known to be a key adaptor for NXF1 dependent mRNA export. Cells that were deficient of the m^{6}A demethylase ALKBH5, showed increased mRNA export linking a role of m^{6}A to mRNA export pathways [68, 82]. There are many mRNAs that do not have m^{6}A sites are still exported, emphasizing the complexity of export but leaving the possibility that m^{6}A may act as a supporting or complementary signal for export. m^{6}A as a field is still growing and has made a resurgence through transcriptional studies like RNA sequencing to aid in m^{6}Amapping. There are still questions involving the rules of how m^{6}Ais added on and removed as well as other possible m^{6}Areaders out of the handful known currently. Most likely research into the m^{6}A writer components and their regulation will be instrumental in understanding the degree to which the epitranscriptome can be control gene expression.

1.3.1 m^{6}A and KSHV

Recent transcriptome-wide m^{6}A mapping of multiple viruses have been brought to the forefront research concerning a complex interplay between the m^{6}A pathways and viral replication success. Given the prevalence of m^{6}A it is not surprising that several viruses have been shown to contain m^{6}A in their RNA**.** There is a clear benefit as the modification diminishes immune recognition of m^{6}A modified synthesized RNAs [79, 96, 97]**.** However, m^{6}A in the viral context appears to be diverse and plays both pro- and anti-viral roles. In

human immunodeficient virus (HIV) m^{6}A along mRNA promotes its nuclear export and m^{6}A on Influenza A virus is read by the m^{6}A reader YTHDF2 are shown to promote viral replication [79, 98-100]**.** Interestingly the roles of certain m^{6}A readers remains diverse between viruses as well as the cell type the viruses is in [96, 97, 101]**.** Given the fact that most DNA viruses replicate in the nucleus and rely on cellular transcription machinery it is no surprise that KSHV and DNA virus strategies co-opt m^{6}A pathways in their struggle for control over gene expression.

In work done by Tan et al., it was revealed that KSHV possesses m^{6}A modifications both on latent and lytic viral transcripts. Intriguingly, during KSHV lytic reactivation, they also observed a general decrease of m^{6}A deposition on cellular mRNAs [96, 102]. In latently infected cells, hypomethylation at the 5' end of cellular mRNAs and hypermethylation at the 3' ends were observed compared to non-infected cells [96, 102]. The disproportionate amount of methylation that is deposited on the cellular transcripts during infection raises the question of what is the purpose of these modifications? Increased amount of 3' UTR m^{6}A possibly allows for enhanced 3' UTR regulation, which has been known to affect localization, translation initiation, and the stability/decay rate of mRNAs. In fact, it was revealed that numerous pathways implicated in cellular transformation and oncogenic signaling are upregulated during infection in correlation with an increased amount of m^{6}A methylation. One of the hypotheses raised by the authors is that the enrichment of methylation in the 3' UTR on cellular transcripts may help mediate targeting of miRNAs by KSHV own miRNAs that are known to be essential for KSHV-induced cellular transformation [96, 102, 103]. However, further examinations did not find any correlation between differential methylation and targets of KSHV miRNAs [96, 103]. All

this research has helped prompt a deeper dive into how KSHV is affected by m^{6}A modifications during infection and what occurs to the m^{6}A landscape when KSHV switches from the latent to the lytic portion of its life cycle. Recent studies from Ye et al. in vivo and Hesser et al. in vitro have also reported that the amount of m^{6}A -modified KSHV mRNA increases during lytic reactivation while decreasing on cellular mRNAs [84, 97, 104]. The decreased amount of cellular m^{6}A deposition may be due to viral-induced widespread RNA decay leading to a decrease in available transcripts for m^{6}A deposition and, therefore, an increase in viral transcript availability. Another possibility is KSHV influences m^{6}A deposition directly by increasing viral RNA likelihood of being methylated or by affecting the m^{6}A machinery itself. The counterpart of this shift in m^{6}A deposition towards viral mRNA is that the reduced m^{6}A deposition on cellular RNA could help dampen some host anti-viral processes by affecting host mRNA stability and/or translation. m^{6}A modifications are decoded by a collection of cellular factors that "read" this modification and enact the associated function. Depending on the localization of the modification on the mRNA, different reader proteins will be recruited and the resulting effect on the modified RNA will differ [68]. Ye et al. showed that the functions of YTHDC1 activity are co-opted by KSHV: it was found that chemically removing m^{6}A using 3-deazaadenosine (DAA), an inhibitor of METTL3, prevented pre-mRNA splicing of the KSHV major lytic switch protein, RTA (ORF50), which resulted in a reduction of viral lytic replication [104]. Although there were multiple m^{6}A sites found in the RTA pre-mRNA, it was revealed that a single methylation site in exon 2 was responsible for enhancing splicing by using its m^{6}A site to recruit YTHDC1 which in turn recruits SRF3 and SRS10 to aid in splicing. However, RTA pre-mRNA is known to encompass many different splice variants, so it is possible that the other

m^{6}A sites found by Ye et al. interact with other m^{6}A nuclear reader proteins such as hnRNP-C1/2 and hnRNP-A2B1 to regulate differential splicing. More recently, a new class of readers from the "Royal" family, specifically Staphylococcal nuclease domain-containing protein 1 (SND1), was shown to bind directly to m^{6}A modifications similarly to YTH domain proteins [105]. It was discovered that when SND1 was knocked down, the amount of RTA RNA was reduced by 50%, suggesting that SND1 affects the stability of unspliced RTA RNA [105]. Given the extensive role of m^{6}A in aiding the recruitment of splicing factors, uncovering more m^{6}A readers on novel m^{6}A sites will reveal a complex growing regulatory network of co-transcriptional regulators that will help us better understand cellular and KSHV RNA fate.

As stated previously, in the cytoplasm m^{6}A readers have been shown to affect the stability, localization, and translation of mRNAs. One such reader that has come up as an important regulator of the cytoplasmic viral mRNA pool is YTHDF2. This reader binds directly to m^{6}A -modified mRNA and promotes their degradation by localizing them to RNA granules known as processing bodies (P-bodies). Hesser et al. found that depleting YTHDF2 in the KSHV positive cell line ISLK.219 drastically restricts KSHV lytic cycle and subsequent virion production. Notably, it was uncovered that this mechanism is mediated by restricting the amount of RTA production, which suggests a pro-viral role of m^{6}A in promoting lytic reactivation [84]. In stark contrast, another study in the iSLK cells published by Tan et al. suggested that depletion of YTHDF2 instead results in increased KSHV replication and thus appears to posit an anti-viral role for this m^{6}A reader. On the other hand, Hesser et al. also noted that contrary to their iSLK results, in another KSHV positive cell line, TREX-BCBL1s, depletion of YTHDF2 resulted in an accumulation of

RTA, which suggests that m^{6}A in this case would have an antiviral role [84]. It is still unclear why various studies and cell lines appear to have contradictory results, but it would suggest that m^{6}A deposition and/or m^{6}A -mediated functions are highly dynamic. One possibility is that m^{6}A regulation is dependent on temporal factors and that fine-tuning of viral mRNA stability could be a requirement for proper progression of the viral life cycle. Furthermore, mapping of m^{6}A sites within viral mRNA revealed that despite some differences, many of the m^{6}A peaks are consistent across multiple cell types: the same transcripts overall are methylated but the position of this methylation along the transcript can vary. This suggests that the viral mRNAs are not necessarily interfacing with host m^{6}A methyltransferase machinery differently between cell types but instead the contradictory results further enforce the idea that m^{6}A deposition is dynamic and does not always occur on every possible m^{6}A motif. Localization of the m^{6}A mark can be tied to the rate of RNA Pol II elongation where a slow elongation leads to enhanced m^{6}A modification and eventually decrease of translation efficiency [68, 80, 81, 106]. It is thus possible that the speed of RNA pol II–dependent KSHV transcription will similarly impact m^{6}A deposition and thus be highly context-dependent, possibly explaining this variability. Understanding where and what m^{6}A depositions mean on an mRNA may help in understanding their effects on KSHV replication. The continued research into KSHV's pervasive use of cellular regulatory pathways has revealed new insights into how KSHV enhances its infection as well as new aspects of cellular RNA fate. Although this research has given more insights into KSHV regulation, more questions remain. New roles for m^{6}A in the context of KSHV have been elucidated, however, there are conflicting roles depending on cell type and the reason for this remains to be discovered.

1.4 RNA Nuclear Export

Eukaryotic cells have various organelles that aid in various cellular functions, with the largest being the nucleus which stores DNA in the form of chromatin and is surrounded by a nuclear envelope. The transcription of these genes into RNA as well as the subsequent processing occurs in the nucleus. Whereas the translation of mRNAs is done in the cytoplasm with the aid of ribosome and other cofactors. This separation aids in gene expression control by carefully regulating both the RNA and protein and their interactions with certain areas of the cell. In order to surmount this physical barrier mRNAs are typically exported through nuclear pore complexes (NPCs) which perforate the nuclear envelope (**Figure 3**). The NPC is composed of about 30 proteins that are known as nucleoporins [107-110]. The nucleoporins form an octagonal

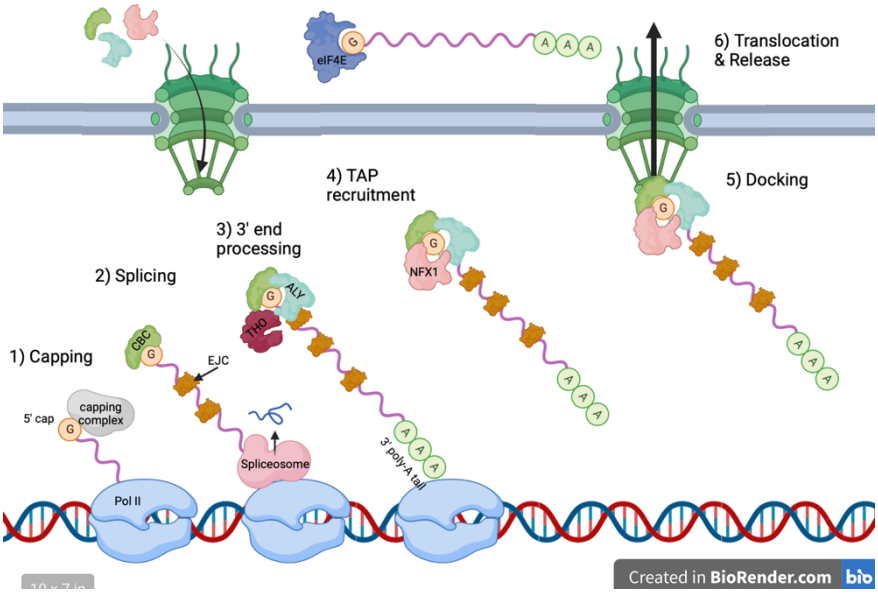

Figure 3: Nuclear bulk mRNA processing and export. new mRNAs follow processing steps that include a methyl 7-guanosine cap (1); splicing (2); and 3' end processing (3); The mRNA is considered mature. The mRNA creates an RNP with the bulk transporter NXF1 (4), where it will then dock on the nuclear basket of the NPC (5). This interaction will allow translocation through the nuclear channel before it is released along the cytoplasmic fibrils and an RNP change occurs (6). Adapted from Delaleau et al. 215(110)

structure which contains a central channel, cytoplasmic fibrils, and a nuclear basket REF. A portion of these proteins fill the central transport channel and contain phenylalanine-glycine (FG) repeat sequences, creating a dense hydrophobic network functioning as a barrier to prevent unauthorized traffic between the nucleus and the cytoplasm [107-109]. To be exported mRNA transcripts must be processed properly with steps including capping, splicing and proper 3' end formation. From there the proper RBPs allow for an RNP that is recognized by the NPC for export.

The two most common protein transporters for mRNA are NXF1 and CRM1 [21, 110]. Both proteins utilize other protein adaptors to increase affinity for RNA, as with NXF1, or associate with the target RNA, as with CRM1. NXF1 mediated the bulk of mRNA nuclear export, associated with key factors REF/ALY and proteins in the THO complex to bind to target mRNAs. In order bind to REF/ALY, NXF1 first must associate with the transcription export complex (TREX) (**Figure 3**) [21, 107, 110]**.** For these factors to come together proper mRNA processing is required as factors in the TREX complex require both splicing and capping to be done. For example, UAP56, a subunit of TREX is also a component of the exon junction complex and is deposited on the mRNA a marker of completed splicing [21, 107, 110]**.** After processing an export competent NXF1-mRNP and can associate with the TPR protein at the nuclear basket of the NPC [21, 107, 110]**.** Once docking translocation occurs via interactions with the FG nucleoporins. Nup98 and Rae1 of the central chamber are key for this process to occur [21, 107, 110]**.** The specific mechanism of mRNP translocation is still being determined but several models have been proposed [21, 110]. Once exiting the channel, the mRNP associates with the cytoplasmic fibrils of the NPC, through the actions of RANBP2, DDX19 and Gle1, where the mRNA

is released and most of the export factors are recycled back into the nucleus. In the cytoplasm the mRNP is remodeled as new RBPs come and bind the mRNA transcript to guide the transcript for the rest of its fate. As a final note for NXF1 pathway, by altering the composition of its RNP, NXF1 can enable the selection of specific mRNA cargoes [21, 110].

Not all the transcripts use the bulk pathway to associate with the NPC, some mRNAs use CRM1. Typically reserved for protein export of proteins with leucine rich nuclear export signals, CRM1 also helps export small nuclear RNAs (UsnRNAs), ribosomal RNAs (rRNAs) and a subset of mRNAs [110]. Through the association of an adaptor with leucine rich nuclear export signals CRM1 will bind its cargoes in the presence of Ran-GTP. Then in the cytoplasm the cargo release requires enzymes that enable the GTP hydrolysis of Ran-like RanGAP [110]. As mentioned, there are specific adaptors in an mRNA's RNP that permit their export with CRM1, one of those is human antigen R (HuR). HuR is a promiscuous RBP that plays a variety of roles by associating with transcripts containing AU rich elements (ARE) in the 3' UTR [111-113]. This was discovered using the inhibitor Leptomycin B, which prevents CRM1 from binding its cargo, which led to the accumulation of ARE transcripts but not bulk mRNA [111-113]. However, there are other transcripts that use CRM1 that are not HuR dependent. For instance, CRM1 aids in eIF4E-dependent mRNA export. Although eIF4E traditionally acts in the cytoplasm for cap dependent translation, it was found that a subset of mRNAs used it in lieu of the cap binding complex (CBC) and aided in export [114-116]. Target mRNAs contained a 3'UTR secondary structure of approximately 50nts called 4E-sensitivelty element (4ESE) [114-116]. This element recruits an export adaptor LRPPRC which in turn the eIF4E protein,

LRPPRC also binds to CRM1 which then allows transport through the NPC. Mysteriously endogenous 4ESE containing transcripts are targets for both bulk and eIF4E dependent export pathways [114-116]. In the end the export of mRNAs is elegantly selective with their own cis-acting elements and trans-acting factors. Through export the cell can control the availability of transcripts to the translation machinery, managing the response of the cell to various stimuli. This is a small taste of the importance of mRNA export, there is plenty more research that has been done and still to do to understand the mechanisms of how certain transcripts and RNPs are formed. It will be very interesting to decipher as more RNP complexes are understood and their effect on export and translation. Research has also just begun exploring the effects of modifications of protein and RNA along with nuclear export.

CHAPTER 2

RNA MODIFICATION M^{6}A IS ESSENTIAL FOR ESCAPE IN VIRAL INDUCED RNA DECAY

2.1. Introduction

In this chapter, I will discuss the research into the role of m^{6}A on transcripts escaping viral induced host shutoff. As previously discussed in **Chapter 1**, many viruses including alpha and gamma herpesviruses, Influenza A virus and SARS coronavirus can include widespread mRNA decay with their own endonucleases [41-45, 51, 117]**.** In KSHV's case, the viral protein SOX can use its endoribonucleolytic activity to trigger host shutoff [43, 118]**.** Although KSHV's host shutoff event degrades upwards of 70% of mRNA, there are a remaining approximately 30% of RNA that can escape SOX cleavage [43, 118]**.** Research into the method of escape has been difficult as there are multiple mechanisms that could contribute to this outcome. RNA sequencing only provides us with a snapshot into RNA steady state, so indirect transcriptional effects could compensate for SOX-mediated decay for example. Furthermore, not all transcripts bear an optimal SOX targeting motif, therefore, many mRNA remain "invisible" to SOX and simply escape passively. However, we have been focusing on a subset of transcript that should be degraded but stringently and actively evade SOX cleavage [60-62, 119]**.** This feat of active evasion has been termed "dominant escape", and research into this mechanism has revealed that these RNA contain a specific RNA element in their 3' UTRs that protects against SOX

cleavage [60-62]. This SRE (SOX Resistant Element) has been shown to be effective against a broad range of viral endonucleases from different viruses. Interestingly the SRE can be attached to mRNA that would normally be degraded to induce protection, implying its sufficiency [60, 61]. To date there have only been three mRNA transcripts found to be SRE bearing: the host interleukin-6 (IL-6), the growth arrest and DNA damage-inducible 45 beta (GADD45B) and Shiftless (SHFL) [60-62, 119]. Identifying SRE bearing transcripts has been difficult however, as there is no sequence similarity between these transcripts [62]. However, there appear to be similarity in the secondary structure of this short 200nt element, which is predicted to fold into a long hairpin with a middle bulge. Secondary structures in RNA have been associated with effective recruitment of RNA binding proteins. Therefore, it is believed that the SRE may act as a platform for the recruitment of a protective complex from either SOX recognition or cleavage [60, 61]. To address this hypothesis, Muller et al. explored the possible proteins binding the known escapees IL6 and GADD45B by a Comprehensive Identification of RNA binding Proteins by Mass Spectrometry (ChIRP-MS) and found that a partially overlapping RNP complex forms on the SRE. Several of these SRE binding proteins were found to be necessary and in both IL6 and GADD45B like nucleolin (NCL) and human antigen R (HuR) [60, 61]. Most intriguingly, a particular class of proteins appeared to preferentially bind the SRE: m^6A reader proteins. As described in **Chapter 1**, m^6A modifications are widespread and have a significant impact on RNA fate, and depending on the deposition of m^6A different m^6A reader proteins provide a variety of functions to those modifications [68-70]. In comparing the ChIRP-MS of the two SRE bearing transcripts we found a handful of m^6A

reader proteins. In an effort to elucidate the SRE's mechanism of escape we explored whether the SRE is m^6A modified, and if it was does it aid in escape from host shutoff.

In this chapter, taking advantage of the novel advances in the field of epitranscriptomics, we hoped to identify whether our m^6A modification and in particular the recruitment of specific m^6A readers could contribute to the SOX escape phenotype. Here, we show that the IL-6 mRNA is m^6A modified in its 3' UTR during KSHV lytic infection and that removal of this m^6A mark is enough to restore susceptibility to SOX-mediated degradation. We further show that the m^6A reader YTHDC2 binds to the IL-6 SRE in an m^6A -dependent manner and that down regulation of YTHDC2 is sufficient to abrogate resistance to SOX. Taken together, these results demonstrate that the m^6A pathway is pivotal in the regulation of gene expression during KSHV infection, highlighting the viral–host battle for control of RNA stability.

2.2 Results

2.2.1 KSHV Infection Reshapes the m^6A Landscape in Cells

Since KSHV reactivation broadly affects RNA fate and extensively remodels the host gene expression environment, we hypothesized that m^6A modifications may be broadly redistributed upon KSHV lytic reactivation from latency. We mapped

transcriptome-wide m^{6}A modification sites with single-nucleotide resolution using m^{6}A - eCLIP.

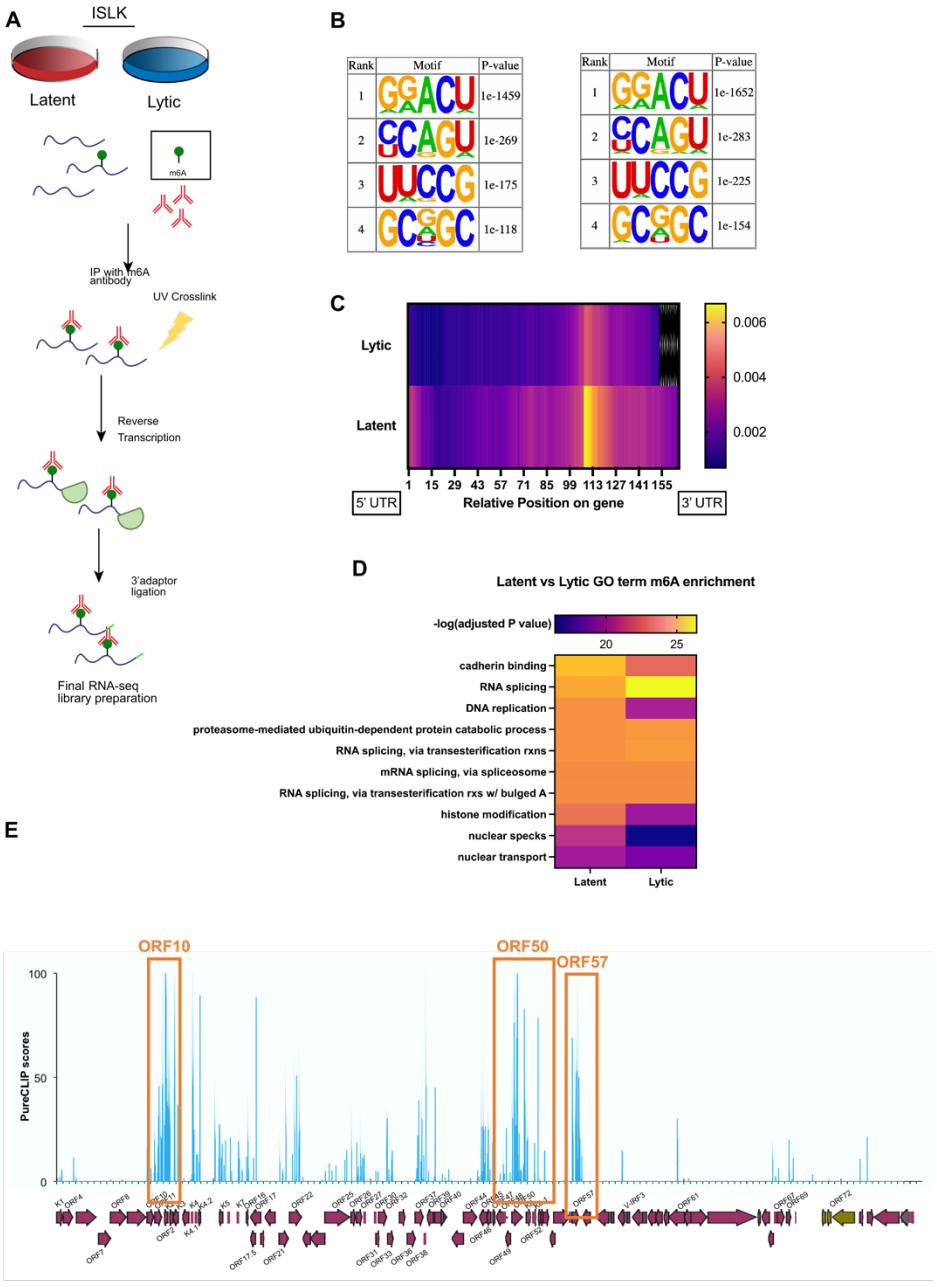

Figure 4*:* Examining iSLK m6A epitranscriptome during KSHV lytic reactivation. (A) Schematic of m6A eCLIP set up. iSLK.WT cells were either left latent or lytically reactivated with doxycycline and sodium butyrate for 48 h. Total RNA was collected then incubated with an m^6A antibody. Samples were crosslinked using ultraviolet radiation before being reverse transcribed then attached with 30 adapters in part of library preparation. Finally, m^6A-enriched samples were sequenced. (B) Most significant DRACH motifs with m^6A peaks identified by HOMER in latent and lytic cells. (C) Heat map of a metagene plot depicting the average number of sites mapped to certain genomic regions. The number of sites is calculated for each region of every gene, the lengths of the regions are then normalized, and the average number of sites for a set number of positions along the regions are calculated. (D) Heat map of the most significant m^6A-enriched functional pathways in latent and lytic cells calculated through an enrichment analysis preformed using the R package clusterProfiler. (E) m^6A PureCLIP scores of lytically reactivated KSHV genes aligned over an annotated KSHV genome. PureCLIP is the log posterior probability ratio of the m6A cross-link sites over the input samples.

RNA was isolated from KSHV-positive iSLK.219 cells either in their latent state (Lat) or lytic state (Lyt) 48-h post reactivation and an anti-m^6A antibody was used to enrich m^6A -modified RNA fragments prior to RNA sequencing of both the input and immunoprecipitated (IP) samples (**Figure 4A).** The ratio of IP and input reads were evaluated in each cluster, and clusters with IP/input enrichment greater than eightfold and associated P value < 0.001 were defined as significant "peaks." We detected a total of 2,281 peaks in the latent samples and 1,482 peaks in the reactivated samples. A tool called PureCLIP was used on these peaks to then identify over 40,000 unique, single-nucleotide–resolved sites, which 54% of sites were identical in both of our samples. As expected, the m^6A motif DRACH (in particular, [GGACU]) was enriched under the identified peaks, confirming that our m^6A deposition in these infected cells is concordant with previous observations (**Figure 4B**) (35, 36). Also, in agreement with previous data, m^6A peaks were most prevalent around the transcript STOP codon and beginning of the 3' UTR (**Figure 4C**). The overall m^6A peak deposition profiles between Lat and Lyt samples were surprisingly close; however, we observed decreased methylations in cellular mRNA 5'

UTRs upon KSHV lytic reactivation, which is in contrast with observations in other viruses such as ZIKV (44). Gene Ontology (GO) term analysis of genes with lytic-specific peaks identified an enrichment for genes with roles in RNA splicing, while genes involved in DNA replication seem to carry less m^6A modifications in KSHV lytic cells (**Figure 4D**). We also detected several m^6A peaks within viral genes, many of which have been characterized before (**Figure 4E**) (35, 36, 43). All of three of our SRE bearing transcripts were modified in both their 5' and 3' UTRs. However, all three SRE transcripts received a singular m^6A site in their SRE region when switching from latent to lytic cell samples. Together, these results reinforce past observations that the m^6A profile in lytically infected cells undergoes a massive shift compared to latent cells, redistributing m^6A modifications to different host and viral genes, which likely have far-reaching consequences on modulation of gene expression.

2.2.2 IL-6 SRE Carries a Lytic-Specific m^6A Modification

We next focused our attention on the IL-6 transcript, the best characterized SOX-resistant mRNA. In latent cells, we detected several m^6A peaks between the human genome positions 22,727,199203, which correspond to IL-6 5' UTR. However, in lytic cells, IL-6 gains an additional peak at position 22,731,646, corresponding to nucleotide 74 on IL-6 30 UTR. This m^6A modification falls on the SRE region and on a strong DRACH motif. We also found m6A sites on two of the other known SRE transcripts SHFL and Gadd45B

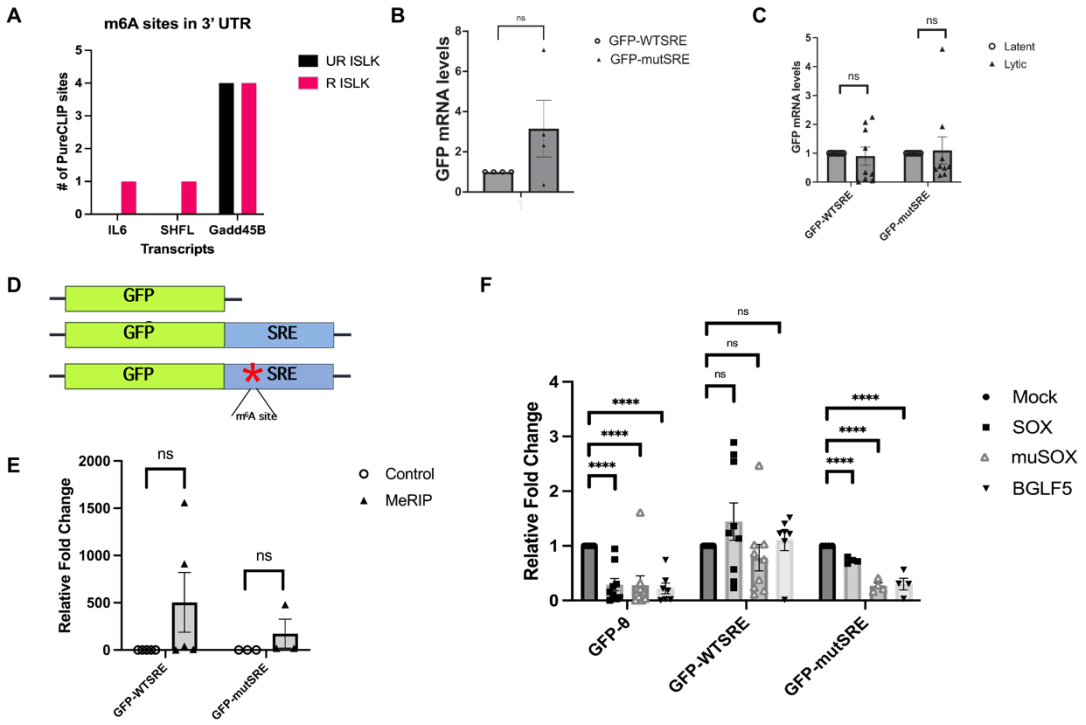

Figure 5: IL-6 SRE contains an m6A site that is necessary for viral endonuclease protection. (A) PureCLIP scores of the 3' UTR of SRE genes in latent (UR) and lytic (R/48 hpr) iSLK WT cells. (B) qPCR of GFP mRNA levels of the transfected GFP reporters in HEK293T cells showing no significant difference between the two reporters. (C) qPCR of GFP-WTSRE and GFP-mutSRE reporters transfected into Latent and 24 hour Lytically reactivated iSLK cells showing no significant difference between the reporter expression. (D) Illustrates an IL-6 SRE reporters (GFP coding region and SRE): the SRE region is in blue and the mutated methylated adenosine in red. (E) Cells were transfected with WTSRE or mutSRE GFP reporter, and total RNA was harvested 24 h later and subjected to meRIP followed by RT-qPCR using GFP primers. Fold enrichment was determined by calculating the fold change of the IP to control Ct values that were normalized through the input. (F) 293T cells transfected with one of three viral endonucleases, as indicated along with the indicated GFP reporters. RNA was collected and quantified using RT-qPCR. ****P < 0.0001; ns, not significant. phenotype

(**Figure 5A**). To confirm the presence of this m^{6}A deposition, we mutated the predicted position within the SRE (referred to as mutSRE) and performed meRIP-qPCR to assess m^{6}A deposition on the WT-SRE (wild-type SRE) compared to the mutSRE.. meRIP-qPCR confirmed the presence of the m^{6}A peak within the SRE and that mutating nucleotide 74 within the IL-6 SRE is enough to abrogate m^{6}A pull- down (**Figure 5E**). We next investigated whether this m^{6}A modification plays a role in SRE-mediated escape from

SOX-induced decay. Cells were transfected with an SOX construct (or mock) alongside a susceptible to SOX; a GFP reporter fused to a WTSRE (GFP- GFP-expressing reporter bearing no SRE (GFP-;) and thus WTSRE), expected to be protected from SOX; or fused to mutSRE (GFP-mutSRE) to test the effect of the loss of m^6A deposition on escape from SOX (**Figure 5D**). We verified that the levels of relative reporter mRNA levels among the constructs were similar (**Figure 5B/C**). As shown in **Figure 5F**, as expected, SOX efficiently degrades GFP; but GFP-WTSRE resists degradation. However, SOX-mediated decay is restored on the GFP-mutSRE reporter. Since IL-6 is known to also escape decay mediated by closely related SOX homologs, muSOX and BGLF5, we wondered whether the GFP-mutSRE would also be susceptible to these endonucleases. As shown in **Figure 5F**, a single-point mutation at position 74 in the SRE also renders transcripts susceptible to degradation from SOX homologs. Taken together, these data reveal that m^6A modification of the 3' UTR of IL-6 promotes its escape from SOX.

2.2.3 The m^6A Reader YTHDC2 Promotes the SRE Escape from SOX

A previous ChIRP-MS (comprehensive identification of RNA- binding proteins by mass spectrometry) screen had identified a number of host proteins that can bind the SRE element (15). One of these predicted interactors was the m^6A reader YTHDC2. Several reports have demonstrated that YTHDC2 directly binds to m^6A -modified mRNAs often

within 3' UTRs (45–48). YTHDC2 itself is an RNA helicase and its binding to mRNA has been associated with alteration of RNA stability (45–48).

We first confirmed the interaction between YTHDC2 and SRE-bearing mRNA by performing IPs from cells transfected with a GFP reporter fused to the WTSRE (**Figure 6A**). In agreement with our previous observations, YTHDC2 binding to the m^6A deficient mutSRE was reduced compared to the WTSRE, confirming that YTHDC2 is recruited to the SRE as an m^6A reader (**Figure 6A**). Since the m^6A modification that we identified on the SRE appears to be important to promote protection from SOX- induced degradation, we next asked whether this protective phenotype was being mediated through the recruitment of YTHDC2. We therefore used Cas9-based genome editing to generate YTHDC2 knockout clones in human embryonic kidney (HEK)293T cells (now referred to as 293TΔYTHDC2). After confirming knockout efficiency (**Figure 6B**), we used this cell line to assess how the lack of YTHDC2 expression would affect the SRE stability in the face of SOX-mediated decay. 293TΔYTHDC2 were transfected with our GFP-WTSRE

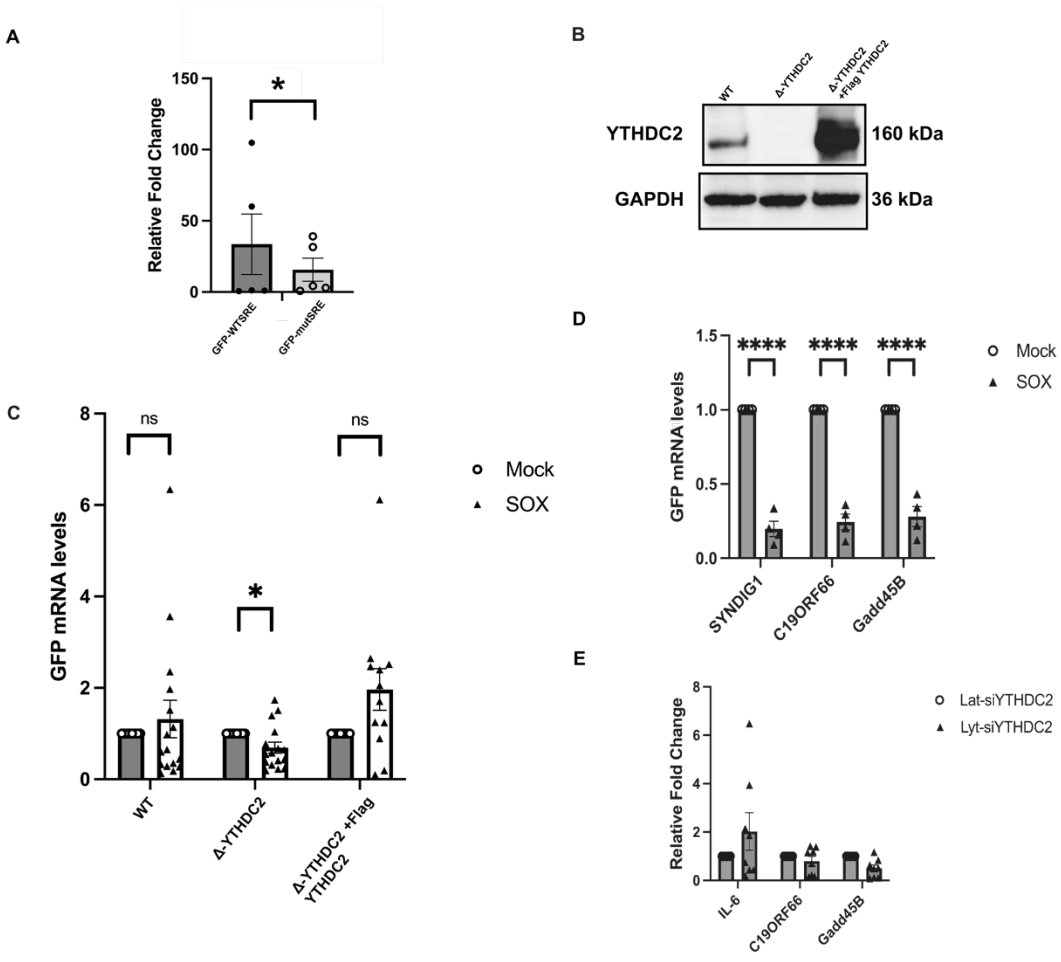

Figure 6: YTHDC2 is necessary for IL-6's evasion of SOX in vitro. (A) HEK293T cells were transfected with Flag-tagged YTHDC2 and a GFP-WTSRE reporter as indicated. Cells were cross-linked and IP using Flag-coated beads. RNA fraction was collected and used for RT-qPCR. (B) 293TΔYTHDC2 cells were obtained by stably expressing and single-cell–selecting 293TCas9 cells expressing a YTHDC2-targeting guide RNA. Cells' clones were tested for knockout efficiency by Western blot using a YTHDC2 antibody and GAPDH as a loading control. YTHDC2 expression in these cells was rescued by transfecting Flag-tagged YTHDC2 on a plasmid. (C) 293TΔYTHDC2, 293TΔYTHDC2+Flag YTHDC2 or WT cells were transfected with SOX (or mock), along with a GFP-WTSRE reporter. RNA was then collected and used for RT-qPCR. *$P < 0.05$; ns, not significant. (D) qPCR of GFP reporters using the SREs of other known escapees as indicated. These reporters were transfected into 293T-ΔYTHDC2 cells with either Mock or SOX plasmids transfected as well. (E) qPCR of endogenous transcripts in either latent or lytically reactivated iSLK.219 cells that had been treated with siYTHDC2 prior to reactivation.

reporter along with SOX (or mock), and RNA was extracted and used for RT-qPCR (**Figure 6C**). As expected, SOX does not affect the RNA levels of the GFP-WTSRE

reporter in WT 293T control cells. However, SOX-mediated decay is restored when YTHDC2 expression is knocked down. We observed the same loss of protection on other known SOX escapees like C19ORF66 and GADD45B (**Figure 6D**). To ensure that this defect in protection from SOX was not due to off- target effects of generating the 293TΔYTHDC2 cell line, we rescued YTHDC2 using ectopic expression **(Figure 6B).**

In these cells, the GFP-WTSRE stability was rescued to normal levels, even in the presence of SOX (**Figure 6C**). We also investigated the effect of YTHDC2 knockdown in the KSHV-positive iSLK cells. We checked the endogenous levels of several known SOX escapees in the YTHDC2 knockdown cells **(Figure 6E).** When YTHDC2 expression is knocked down, both C19ORF66 and GADD45b mRNA levels are reduced, indicating that YTHDC2 also modulate their stability in iSLK cells undergoing lytic reactivation and,

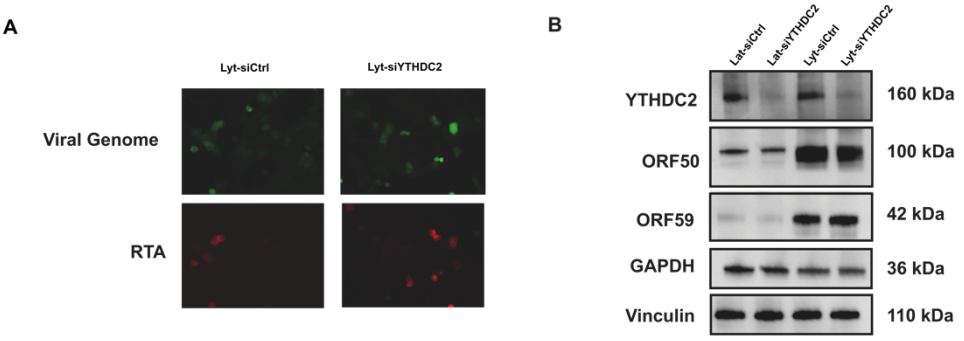

Figure 7: Loss of YTHDC2 has no effect on lytic reactivation. (A) YTHDC2 expression was knocked down in iSLK.219 cells and Western blots were performed to assess knocked down as well as its effect on ORF50 and ORF59. YTHDC2 depletion had no effect on these viral genes. (B) iSLK.219 were treated with an siRNA control or targeting YTHDC2. Latent cells fluoresce green because of a GFP marker on the viral genome and red when reactivated because of an RFP marker under the PAN promoter. Knock down of YTHDC2 does not affect viral reactivation.

thus, when SOX exerts its strongest effect on mRNA stability. We measured the green and red fluorescence of these cells as markers of KSHV latent and lytic phases, respectively (**Figure 7A**). We also investigated the effect of YTHDC2 on proper progression on the viral life cycle and measured the expression of several viral genes upon YTHDC2

knockdown (**Figure 7B**). We did not notice any significant changes in the absence of YTHDC2. Taken together, these results support a role for the m^{6}A reader YTHDC2 in protecting transcripts from SOX degradation.

2.3 Discussion

Herpesviruses extensively manipulate the fate of host transcripts during lytic reactivation using virally encoded endonucleases. In the case of KSHV, the viral endonuclease SOX targets a wide array of mRNAs via a sequence- specific degron and cleaves around 70% of mRNAs in the cell [42, 43, 120]. This allows the virus unfettered access to the host expression machinery for viral replication. Previous work has shown that among the 30% of transcripts that escape this SOX- mediated decay, there is subset of transcripts that carry an RNA stability element located in their 3' UTR that specifically enables this resistance phenotype against viral but not cellular endoribonucleases known as the SRE [60, 61, 63, 119]. While this escape mechanism remains largely uncharacterized, it is known that this RNA element is not conserved in sequence among escaping transcripts but rather adopts a common RNA structure which has been hypothesized to serve as a protein recruitment platform. Past studies have explored proteins bound to this RNA element and found several m^{6}A readers within the SRE RNA–protein complex [60]. We thus hypothesized that the RNA modification m^{6}A , which is prevalent and integral to both host and viral transcript fate, may be involved in viral endonuclease escape. This led us to preform m^{6}A eCLIP sequencing on KSHV latent and lytic cells. The m^{6}A eCLIP confirmed previous results seen in which upon reactivation there is an overall

decrease in methylation on host transcripts and a massive increase in methylation of viral transcripts [84, 103, 121].

Past epitranscriptomic studies exploring KSHV infection had also identified widespread deposition of m^6A across the KSHV transcriptome, independently of kinetic classes [84, 103]. There is large overlap between the peaks we detected here and these previous studies, suggesting that the viral m^6A profiles as well as site specificity are conserved. In lytic cells, the pool of mRNA becomes increasingly dominated by viral transcripts; therefore, it is likely that the m^6A methyltransferase machinery is more and more solicited and turned toward viral mRNA. In accordance with this possibility, we observed a 5' UTR hypomethylation and a concomitant 3' UTR hypermethylation following KSHV reactivation from latency. We know very little about the UTR of KSHV transcripts, but because of genome size constraint, they tend to be much shorter than in average human genes. Therefore, this seemingly preferential 5' UTR hypomethylation could simply reflect the changes in the pool of mRNA present in the cell at that stage of viral infection. Alternatively, m^6A modifications are known to occur mainly cotranscriptionally on the adenosines that are located within a DRACH motif by m^6A writer proteins. It is possible that this shift of m^6A deposition toward 3' UTR results from alternative splice forms being expressed during KSHV lytic infection and that, possibly, these transcripts have more favorable DRACH motifs. This can be seen in the shift of the types of transcripts being methylated in **Figure 4C**. Viruses are known to affect the global gene expression landscape, and it would, thus, not be surprising to see that those expressed during lytic infection have alternative, 3' UTR–favoring m^6A deposition. This is also in line with our observation that RNA- splicing genes are more m^6A modified during

the lytic cycle, which could suggest that they are more solicited and possibly more expressed. However, it is still unknown what dictates the m^{6}A -writing machinery to prefer one DRACH motif over another. It is possible that upon lytic reactivation a change occurs in the cell that causes m^{6}A writing to change its "priority." This is supported by a couple of genes like GADD45B and ARMC10, whose m^{6}A transcript landscape shifts during lytic reactivation as well as the DRACH motifs that are methylated (**Figure 4C**). Interestingly, we know that the m^{6}A deposition on the IL-6 SRE occurs independently of whether this SRE is in the context of the full transcript or simply fused to GFP. Indeed, our results indicated that the presence of the SRE on a GFP reporter is enough to mediate the same "SOX-blocking" effect as in the endogenous mRNA. This suggests that the m^{6}A machinery is likely more influenced by a DRACH motif in the proper context and/or presented in the proper structure than other determinants far away from the DRACH motif chosen. Since m^{6}A is mainly deposited cotranscriptionally perhaps the difference we see in DRACH motif preference is due to transcriptional rate. Another alternative is that, given the increase of RNA splicing, m^{6}A writers preferentially recognize DRACH motifs in actively spliced RNA as a result. Of note, it appears that while pulling down the mutSRE construct was virtually impossible and thus confirming that the SRE only carries one site for methylation, the pulldown for the WTSRE seemed to vary in efficiency. One possibility is that not 100% of the SRE ends up m^{6}A -modified, especially in the context of overexpression of the GFP reporter. It would be interesting to quantify which fraction of the SRE-containing mRNA pool is modified and whether a certain threshold needs to be attained in order to carry the full protection from SOX. Furthermore, we showed that this reduction in protection extends to SOX homologs muSOX and BGLF5 from MHV68 and EBV, respectively. This would

indicate that this m^{6}A "tagging" mechanism may be used widely in the context of infection with gamma herpesviruses to control certain key transcript expression. We also note that, in the context of SOX, there is still some protection of the GFP-mutSRE. SOX's effect on RNA decay is notoriously less prominent than that of its homolog in the closely related other gamma herpesviruses, so this leftover "protection" may be due to SOX's lower efficiency. However, it could also reflect that protection from SOX may rely on more than m^{6}A deposition and need other RNA- binding proteins recruited along the SRE.

We were able to show that the m^{6}A site in the IL-6 SRE recruits YTHDC2 and further demonstrate that the recruitment of this m^{6}A reader is necessary for its protection from SOX. We were also able to see the loss of the protective phenotype for other documented, dominant escapees like C19ORF66 and GADD45B when YTHDC2 was knocked down either in the ΔYTHDC2 or in the iSLK cells. This suggests that the role of YTHDC2 may be conserved among the escapees. It would be interesting to understand both its binding pattern to these mRNAs as well as its role in regulating RNA stability. Moreover, we observed that YTHDC2 depletion does not hinder proper progression of KSHV replication. Interestingly, IL-6 is known to be important for the survival of KSHV-infected cells and play an important role in the establishment of KSHV- associated carcinogenic conversion of infected cells. Therefore, one would predict that affecting IL-6 stability would not have a direct impact on KSHV replication but rather on a global scale and may have an impact on these later stages of infection. It would therefore be interesting to investigate the status of YTHDC2 expression, or lack thereof, in the long-term infection model or in patient tumor samples. YTHDC2 comes from the YTH family, which boast a YTH binding domain to interact with m^{6}A directly, albeit with low affinity. Interestingly

all the other YTH proteins are around 500 to 750 aa and composed of primarily low-complexity disordered regions, while YTHDC2 is close to 1,400 aa in length and has several other known domains besides the canonical YTH domain: an R3H, helicase, ankyrin repeats, HA2, and OB-fold domains [122, 123]. Little is known about the function of the canonically cytoplasmic YTHDC2. It has been reported that it may contribute to increased RNA decay by binding select transcripts and XRN1 [123, 124]. Other studies have shown that it enhances translation efficiency, unwinding RNA transcripts while bound to the ribosome [91, 122]. This puts it in direct contrast with YTHDC1, which is nuclear and has roles in RNA splicing and chromatin modification [70, 88, 89]. YTHDC2 functions more in line with the cytoplasmic YTHDFs 1 to 3, which have been shown to bind m^6A -containing transcripts and enhance translational activity or mRNA decay [86, 95, 125, 126]. What many of the YTH proteins have in common when binding their transcripts is that they function in complex with other proteins. This is consistent with our hypothesis that although YTHDC2 is necessary for the protection of IL-6 from SOX-mediated decay, it is most likely not sufficient. A previous study has shown that IL-6 binds with nucleolin, HuR, and AUF-1 in a protective complex [60, 61, 119]. It is likely that YTHDC2 works in concert with these proteins and possibly others to either occlude SOX targeting via their presence or by relocalizing the transcript where SOX cannot target IL-6. There is also a possibility that the YTHDC2 helicase function may be necessary for protection, and perhaps, the unwinding of the IL-6 transcript removes an internal mRNA secondary structure that is essential for SOX targeting. YTHDC2 binding to the SRE may also extend beyond the m^6A requirement. We previously investigated the secondary structure of these SRE and showed that the SRE fold is the most conserved feature of all

escaping transcripts, even beyond sequence conservation. By playing with the dynamism of m^{6}A deposition, the structure of the SRE may be modified and therefore impact recruitment of proteins more globally [60]. It would be interesting to investigate the extent of the YTHDC2-binding target to understand whether protection from SOX is more reliant on the presence of YTHDC2 or presence of an m^{6}A modification. Investigating the secondary structure of the WT-SRE versus the mutant SRE could also reveal how the presence of this modification influences hairpin formation. Recent studies have shown that m^{6}A can influence the formation of double-stranded (ds) RNA and that, during viral infection, this could help prevent detection by dsRNA sensor–like RIG-I (retinoic acid-inducible gene I) [76, 127]. Therefore, one can anticipate that m^{6}A deposition on the SRE could similarly influence RNA fate. Furthermore, while our data supports the role of m^{6}A as an important contributor to SOX resistance, it also emerges that this is not the sole answer of SRE protection. We did not find a consistent pattern in lytic-specific m^{6}A deposition in other known or predicted, SOX-resistant transcripts. These escaping mRNAs either had no change in their m^{6}A status upon lytic infection or had lytic-specific peaks outside of their 3' UTR. This indicates that it is not lytic infection per se that triggers this escape phenotype and/or directs m^{6}A deposition but rather that some m^{6}A -modified mRNAs are compatible with assembling a protective complex against SOX. Therefore, not all m^{6}A transcripts turn out to be SOX resistant, which is in line with our observations that only select transcripts among the 20% spared from SOX decay are actively escaping degradation. We thus hypothesize that these m^{6}A modifications must be in the proper context and recruit a specific set of protective proteins to be active. However, now that we have a clearer idea of what m^{6}A reader may be involved in this mechanism, it would be

interesting to reverse our question and search for new escapees using either their m^6A pattern and/or by investigating what transcripts are bound by YTHDC2 during KSHV lytic infection. Furthermore, given that the regulation of RNA fate is a crucial step in hijacking the host cell, it is perhaps unsurprising that several viruses use widespread RNA decay to take over their hosts. It would be interesting to investigate the contribution of m^6A modifications and the YTHDC2 role in these other viral families that also deploy host shutoff to overtake the host.

CHAPTER 3

SRE BEARING TRANSCRIPTS COLOCALIZE WITH NUCLEOLIN IN RNA GRANULES

3.1 Introduction

Nucleolin a multifunctional RNA binding protein

Nucleolin (NCL) is one of the most abundant proteins in the nucleolus but has been found in all areas of the cell including the cell surface allowing for a plethora of functions. NCL is a

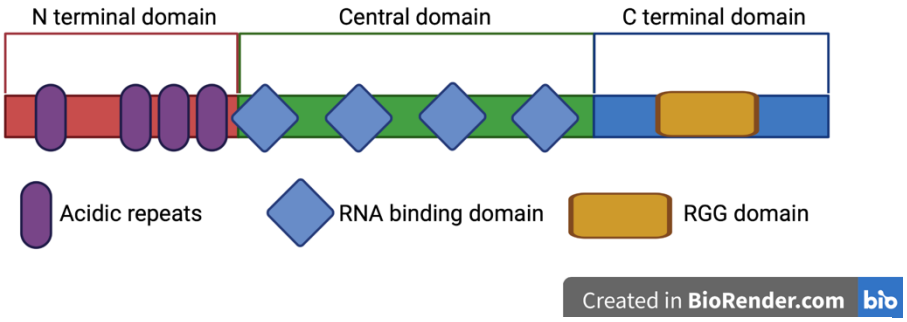

Figure 8: Nucleolin structure. Schematic representing the primary sequence of human NCL protein adapted from Jia et al. 2017 (128)

phosphoprotein whose primary function has been attributed to controlling RNA metabolism, ribosome biogenesis, as well as cellular functions such as gene silencing, cytokinesis, cell proliferation and growth [128, 129]. NCL is highly conserved, and the mammalian version consists of 707 amino acids and sequence comparison amongst other species shows a high degree of evolutionary conservation [128, 129]. Biochemical research has shown that NCL is composed of three main structural domains the N-terminal domain, the central domain, and the C-terminal domain (**Figure 8)** [128-130]. The N-terminal domain contains acidic regions that can be sites of phosphorylation and has been

found to participate in the regulation of transcription of rRNA while also regulating rRNA transcription through interactions with chromatin and UTRs [128, 130, 131]. The central domain has four RNA-recognition motifs (RRMs) which are RNA binding domains and has been the focus of many studies [132-134]. These studies have revealed that this domain is involved in processes like RNA packaging, pre-mRNA splicing and polyA tail processing [128, 135-139]. Finally, the C-terminal domain encompasses an RGG domain, rich in arginine and glycine residues which can interact with nucleic acids [128, 135, 138]. The RGG domain facilitates interaction with the RRM domains for large RNA and is also considered a protein interaction domain to help control things such as ribosome assembly or import of ribosomal proteins into the nucleus [135, 140].

Although NCL's distribution is ubiquitous including the nucleolus, nucleoplasm, cytoplasm, and the cell membrane, we will be focusing primarily on the nucleoplasmic and cytoplasmic functions. While 90% of cellular NCL exists in the nucleolus, there is a population in the nucleoplasm that has been found to associate with RNA polymerase II (RNA Pol II) and mRNAs. During instances of stress, NCL is relocalized from the nucleolus to the nucleoplasm in a p53 dependent manner, which affects DNA replication and repair temporarily [141]. NCL then undergoes a post translation modification of phosphorylation to interact with Replication Protein A (RPA) which further prevents initiation and elongation during DNA replication [141, 142]. Acetylated NCL has also been shown to colocalize to nuclear speckles with the splicing factor SC35, indicating that the NCL may also be involved in splicing [143]. Cytoplasmic NCL is mostly known for its shuttling properties for proteins and RNA between the nucleus and the cytoplasm [128, 144]. Specifically, NCL has been shown to bind and transport rRNA across the nuclear

envelope to create ribosomal subunits. NCL has also been implicated in the process of internalization, by colocalizing with cytoplasmic smooth vesicles, specifically EEA1-associated vesicle, a marker specifically associated with early endosomes [145, 146]. Due to properties of NCL associating with the actin cytoskeleton, NCL may be essential for the endocytic pathway from the membrane to the nucleus [145]. These findings were reinforced by that found the motor protein Kif5a, a motor component of the kinesin 1 complex, bound to NCL's GAR domain in neuronal cells [144]. NCL and Kif5a interaction is critical for neuronal growth by aiding in mRNA transport from the nucleus along axons.

Given its multifunctionality it is no surprise that NCL is also a prime target for viruses. In particular, DNA viruses rely on extensive reorganization of the nucleus to accommodate the formation of viral replication compartments. For example, Adenovirus protein V was shown to induce relocalization of NCL to the cytoplasm, or the human cytomegalovirus protein UL84 has a subcellular localization shift during infection which is dependent on direct and indirect interactions with NCL [147]. The Dengue capsid C protein colocalizes with NCL during its replication and that treatment with an siRNA for NCL results in a significant reduction in viral titers. This was attributed to NCL role of organizing proteins within the viral replication compartments, which is required for efficient viral DNA synthesis revealing how vital NCL is for Dengue virus replication [148]. NCL is a crucial multifunctional protein that has been shown be deeply intertwined with RNA fate. Due to its significance, viral systems have evolved methods of coopting NCL for their own benefit, reinforcing NCL's importance.

NCL aids SRE transcript escape from SOX induced degradation

Previous work showed that NCL is one of the SRE-associated RNP complex. Knocking down NCL resulted in a significant decrease in SRE mediated protection. The ability of NCL to contribute to the escape mechanism was tracked down to its RRM domains, showing that NCLs ability to bind RNA is crucial for SRE-mediated escape from SOX. Given that SOX's cleavage of mRNAs occurs in the cytoplasm, NCL mutants that either localized to the nucleus or cytoplasm revealed that cytoplasmic NCL was sufficient for protection from degradation [61]. Further study revealed that lacking NCL's RGG domain removed SRE protection, indication that NCLs ability to recruit other proteins was crucial for SRE protection [61]. Co-immunoprecipitation of proteins bound to NCL between latent and lytic KSHV infection revealed that NCL selectively binds with translation initiation factor eIF4H during KSHV lytic reactivation [60, 61]. However, there was no additional differences in the interaction profile for other mRNA cap and tail binding proteins like eIF4G, eIF4E, eiF4B or PAPBC. The use of an RNase reinforced the idea that NCL and eIF4H interaction is not stable and are brought together with an mRNA bound NCL via a long-range interaction [60, 61]**.** eIF4H also failed to bind the NCLdeltaRGG mutant in co-immunoprecipitation assays. While NCL function as an RBP with multiple functions has made it an interesting candidate to explore when trying to decipher how SRE bearing transcripts escape. In this chapter, we explored the localization of SRE bearing constructs in the presence of SOX induced host shutoff and their colocalization with NCL. Further investigation into the existence of NCL RNA granules which were revealed to be non-canonical RNA granules and prompted study into characterization RNA granules. By isolating these NCL RNA granules paired with RNA seq and MS techniques a list of RNA and proteins under these viral endonuclease conditions. Taken together we were able to

deepen the pool of knowledge of RNA and protein interactions during viral host shutoff and highlight some important players that may reveal the reason for the SRE transcript's ability to escape in the future.

3.2 Results

IL6 SRE localizes to NCL based noncanonical granules

In **Chapter 2**, it was shown that YTHDC2 does have a significant effect on protecting SRE bearing transcripts in in vitro, while in in vivo with infected cells there seemed to be a lesser effect. To explore further the ability for SRE transcripts to escape we turned to the use of immunofluorescence and confocal microscopy. We ordered fluorescent Stellaris probes that would bind to the GFP coding region of our SRE reporter mRNA. The probes' location would allow us to visualize the location of our SRE bearing transcripts without interfering with the SRE transcript's ability to escape When paired with fluorescent

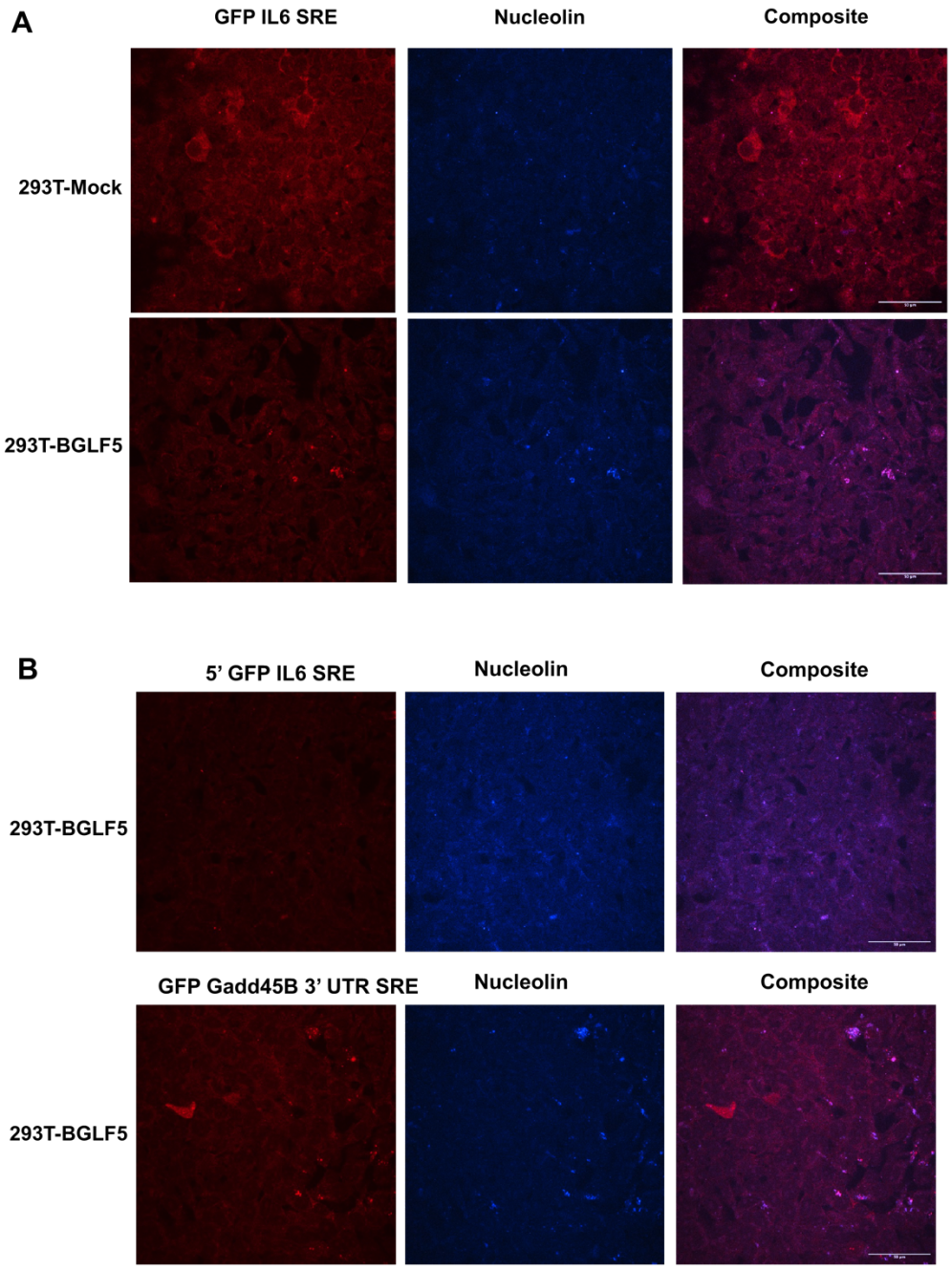

Figure 9: SRE transcripts colocalize with nucleolin in puncta. (A) HEK293T cells were transfected with BGFL5 or empty (mock) vector along with a GFP fused with IL6 SRE RNA reporter. Cells were subjected with FISH probes for GFP CDS (red) and immunoflouresence for nucleolin (blue). (B) HEK293T cells were transfected with BGFL5 with a GFP RNA reporter fused with IL6 SRE in the 5' of the CDS or GADD45B's SRE. Cells were subjected with FISH probes for GFP CDS (red) and immunoflouresence for nucleolin (blue).

labeling of other proteins, it allowed us to gain information on SRE subcellular localization

and colocalization.

Using 293T cells that were transfected with a mock vector, or a viral endo SOX or BGLF5 and an IL6 SRE containing reporter we observed interesting localizations. We decided to add in a condition using another known viral endonuclease from the gamma herpesviruses Epstein Barr virus (EBV). SOX and BGLF5 are known to have very similar methods of host shutoff, we wished to compare if there was a conserved method of escape across gamma herpesviruses. 24 hours post transfection of the Mock condition showed a large amount of diffused SRE transcript (**Figure 9A)**. In the conditions where the BGFL5 viral endonuclease is expressed, there was a severe reduction in diffuse cytoplasmic transcripts, but small puncta of transcripts were observed to form (**Figure 9A)**. We hypothesized that perhaps these puncta are packaged in a manner that prevents the targeting or cleavage by BLGF5. Interestingly, when exploring proteins that may colocalize with this phenomenon, YTHDC2 did not seem to colocalize with these puncta, suggesting the possibility that its role could be in transport to the puncta. Further exploration into other proteins known to associate with the IL6 SRE found that NCL colocalized strongly with the IL6 SRE puncta. We also found that visually we had a better puncta formation in cells expressing BGLF5 compared to SOX. This is most likely due to BGLF5 has been known to be a more efficient host shutoff protein in in vitro experiments compared to SOX, which may need additional viral processivity factor. Strengthening past results, we predictably saw a lack of puncta formation on transcripts that did not contain an SRE or if the SRE was in the 5' UTR instead of the 3' UTR (**Figure 9B**). We also saw that in those instances endogenous NCL was primarily nuclear and that the presence and most likely abundance of SRE reporter induced a cytoplasmic relocalization. Transfection with a reporter containing GADD45B

3’UTR another known SRE transcript recapitulated the puncta formation suggesting that this specific localization is conserved among SRE-bearing transcripts (**Figure 9B**).

Characterization of residents of NCL based RNA granules during host shutoff

Given the nature of these puncta, we sought to confirm the interaction biochemically. Rather than doing a simple pulldown and fact that the size of these RNA puncta was similar to stress granule and P-bodies, we adapted a stress granule isolation protocol to try and precipitate out our NCL granule (**Figure 10A**).

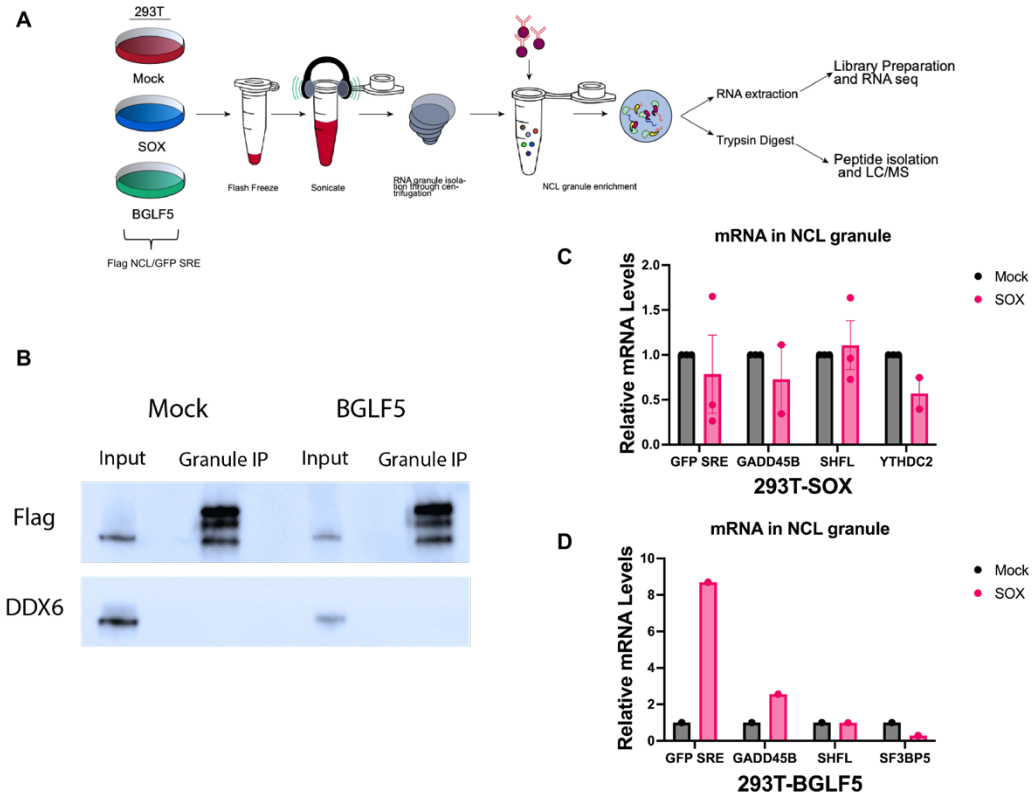

Figure 10: NCL/SRE granule isolation. (A) Diagram illustrating the set up for RNA granule isolation and subsequent characterization using RNA sequencing and Mass Spec. HEK 293T cells were transfected with either a mock, SOX or BGLF5 vector, Flag tagged NCLdeltaNLS, and GFP SRE. 24 hours after transfection cells pellets are harvested, RNA granule isolated. (B) Proteins of the granule were resolved on SDS-PAGE and immunoblotted with the indicated antibodies to confirm granule isolation. (C/D) RNA of the RNA granule were extracted and subjected to an RT-qPCR using primers targeting the indicated transcripts

Exploration into the components of these granules later revealed that they are most likely not stress granules or P-bodies as they lack some key components of those phase separations. We used the same conditional experimental set up as before, 293T cells that were transfected with a mock vector, or a viral endo SOX or BGLF5, and an IL6 SRE containing reporter. After rounds of centrifugation to isolate the specific cytoplasmic density we used antibodies for NCL to isolate any RNA granules that associated with NCL. From there we were able to isolate the proteins or RNA through Western blotting or RNA extraction to qPCR respectively. We found that in the cells transfected with SOX or BLGF5

we were no longer able to pulldown on the granule properly. We were able to restore our interaction by expressing a Flag tagged NCL deltaNLS (**Figure 10B**). We hypothesized that in this granule the composition changed and made it harder for the antibody to pulldown efficiently. The use of a flag tagged version of the NCL was able to now be pulled down and tested. From there we were able to confirm not only the presence of the NCL in our RNA granule isolation but also our SRE reporter meaning we were able to pulldown on the puncta that we had seen in some capacity. Moreover, we confirmed that endogenous SRE transcripts were also enriched in the NCL granule in the presence of SOX and BGLF5 (**Figure 10 C/D**).

Subsequently, we wanted to characterize the content of these NCL granule, both their protein and nucleic acid content by RNA sequencing and Mass Spectrometry (MS) analysis. We were able to confirm the presence of our SRE-bearing transcripts in the granule by RNA (**Figure 11C**). Interestingly, there was little to no difference in the populations of RNAs found in the granule in the presence of the viral endonucleases (**Figure 11A**). However, some specific transcripts were significantly enriched like Nesprin-2, a structural protein that tethers the nucleus to the cytoskeleton.

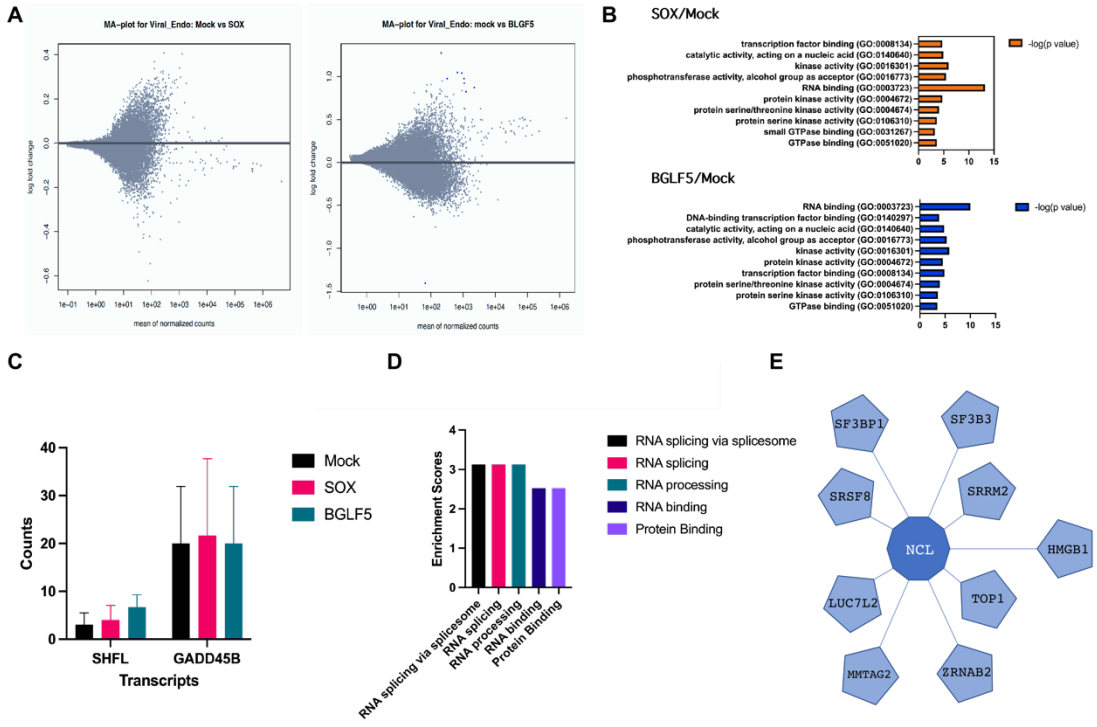

Figure 11: NCL/SRE granule RNA sequencing and Mass Spec. (A) Volcano plots comparing the log fold change of p value of NCL/RNA granule transcripts under the SOX condition over the mock condition and similarly for BGLF5 over mock. This is compared along the mean of normalized counts of transcripts. (B) Gene Ontology (GO) analyses performed using Gene Ontology Vonsortium algorithim recapitulating the enriched functions of the RNA granule isolated transcripts. (C) Total number of counts of known SRE transcrpts SHFL and GADD45B found in the isolated NCL/RNA granules. (D) Gene Ontology (GO) enrichment analysis was performed on the interacting proteins of NCL using DAVID bioinformatic database. Top enriched clusters are identified on the network. Bar graph represent the enrichment score for the most enriched GO-terms by molecular function. (E) Network generated that represents the unique interactome of NCL in the RNA granule during both SOX expression over mock.

Another was Proliferation marker protein Ki-67, an organizational protein that aids in dispersing individual chromosomes during mitosis. Analysis of the Gene Ontology terms of SOX and BGLF5 transcripts over mock condition transcripts revealed very similar functions, mostly involving RNA binding, kinase activity and transcription factor binding (**Figure 11B**). Interestingly, when comparing this RNA seq to the m^{6}A -eCLIP seq data revealed that around 90% of the m^{6}A transcripts found in a the m^{6}A eCLIP were represented

in the SOX and BLGF5 NCL granule. Therefore, it is possible that the NCL RNA granules may be a good resource to identify more SRE transcripts. Characterizing the protein content of these granules revealed 44 hits for Mock samples and 25 hits for the SOX samples. After filtering, 9 high confidence protein were shown to be significantly enriched in the granules: HMGB1, LUC7L2, SF3BP1, SF3BP3, SRRM2, SRSF8, TOP1, ZRANB2 and MMTAG2 (**Figure 11E**). The GO terminology revealed these proteins to be involved with RNA fate including RNA splicing, RNA processing, RNA, and protein binding (**Figure 11D**). Two of the most interesting proteins that were revealed were LUC7L2 and HMGB1. LUC7L2 is somewhat of a mysterious protein mostly known for biding to RNA via its Arg/Ser rich domain while HGMB1 is interesting as well as its similar to NCL as a multifunctional protein acting in multiple cellular compartments and in some cases even be secreted.

3.3 Discussion

The complex interplay between RNAs and the proteins that bind them to regulate RNA fate has long been a focus for research. By using cis acting elements along RNA, the cell can change how mRNAs are exported, translated, sequestered, and ultimately decayed. Some of these mRNAs have evolved the capacity of using these cis acting elements to recruit an RNP complex that allows them to circumvent viral induced host shutoff [60, 61]**.** An event so catastrophic that decimates over 70% of all mRNA at the expression of these viral endonucleases [41, 42, 118, 120]**.** Research in recent times has uncovered that a cis acting element, named the SRE, in the 3'UTR of some of these escaping transcripts recruits RBPs that are necessary for its protection as well as being chemically modified to aid in its

recruitment of those RBPs [60, 61, 119, 149]. Although some of these proteins have been identified, such as NCL, HuR and YTHDC2, there were still questions as to the mechanism of action of how these proteins allowed for SRE bearing transcripts to avoid viral endonuclease cleavage. We initially hypothesized that the RNP that was constructed around an SRE transcript prevented SOX from either targeting or cleaving its targets. Research into the localization of SRE bearing transcripts and members of its RNP complex showed us that during host shutoff SRE transcripts localize to RNA puncta or granules. Interestingly these granules did not colocalize with YTHDC2 but did with NCL. This is supported by previous research given NCLs role as a shuttling protein for RNP complex as well as endosomal vesicles [128, 140, 144, 146]. Further characterization did not see interactions with typical stress granule or P body components leading up to believe that the NCL RNA granule seen could be another type of phase separated entity. There is the possibility that the granule seen is some kind of transport granule for RNA like seen Xenopus oocytes, these transport granules have been known to be packaged tightly and can avoid decay until the time is right for their expression [150]**.** Similarly in that vein, NCL has been known to transport mRNA RNP complexes hitchhiking with endosomes in neuronal cells along axons [144].

Further characterization into these the RNA and protein components of these NCL granules with and without viral endonucleases being expressed. Through centrifugation and immunogenic isolation, we were able to examine the pool of RNAs that were in these NCL based granules. Our data indicated that there was no active selection on the transcripts associating with the NCL based granules in conditions with and without viral induced host shutoff. In comparing this RNA seq data with that of the m^6A eCLIP seq data revealed a

significant overlap in the amount of m^6A transcripts that ended up in the NCL based granules [151]. Perhaps NCL recruits an m^6A reader protein to enrich for these transcripts, there is also the possibly that NCL itself is an indirect m^6A reader. Further research would need to be done to verify these claims though. Given the fact that all our known SREs are m^6A modified means that at the very least that it is a direction to study. Although the m^6A pool with the NCL based granule overlapped greatly perhaps it narrows down the search for new SRE transcripts slightly for future work. There are still further questions that need to be answered given the NCL granules, are there multiple NCL/SRE granules? Are the SRE granules homogeneous or heterogenous populations of RNA? Hopefully future work can answer some of these questions.

Exploring the proteins that were exclusive to the host shutoff NCL granules yielded a small list of splicing proteins and some interesting multifunctional nucleic acid sensors. Two other specific proteins of interest are LUC7L2 and HMGB1 as stated earlier. HMGB1 has been implicated as a universal biosensor for nucleic acids [152]. In the cytoplasm functions as sensor and/or chaperone for immunogenic nucleic acids implicating the activation of TLR9-mediated immune responses, and mediates autophagy [153]**.** LUC7L2 has also been implicated in regulating HSV-1 by binding to introns and preventing splicing of genes leading to the downregulation of those RNAs [154]. The evidence of cytoplasmic based splicing factors as well as a LUC7L2, known for inducing intron retention in mRNAs is quite interesting, and we believe warrants further exploration. Normally the retention of introns leads to nuclear sequestration and decay, there have been recorded instants of intron retaining mRNAs that escape degradation for additional functions in the cytoplasm [155-161]**.** Examples of these retained intron like KCNMA1, LBR and CAMK2B, have been

shown that retained introns have conferred novel cellular function [157]**.** A more generalized function was reported in Buckley et al. where certain mRNAs contain introns called short, interspersed elements (SINE) sequences in neuronal cells that allowed the mRNAs to interact with endogenous transport mechanism [157]. Once arriving at their location, the introns are removed prior to translation [157, 158]**.** This research sounds very familiar and perhaps the SRE bearing transcripts are intron retaining transcripts and it is their ability to be localized to transport endosomes that conveys their protection from viral endonucleases. Either way more work exploring the relevance or nature of these splicing proteins to SRE based escape needs to be done.

CHAPTER 4

SRE BEARING TRANSCRIPTS ESCAPE NUCLEAR RETENTION THROUGH CRM1 MEDIATED EXPORT

4.1 Introduction

Although much of the focus for host shutoff in KSHV lytic reactivation revolves around SOX's ability to recognize and cleave upwards of 70% of RNA in the cell, SOX has other functions as well. As previously described in **Chapter 1**, nuclear SOX, utilizes newly freed RBPs from virally induced mRNA degradation to induce aberrant hyper polyadenylation [38]**.** As SOX aids in the hyper polyadenylation of newly transcribed mRNA, nuclear RNA surveillance machinery restricts its export, and they are subsequently degraded by the nuclear exonuclease [30, 31, 38]**.** Restricting newly transcribed mRNAs from reaching the cytoplasm and impeding their translation further contribute to viral-induced host shutoff and feeds into the viral control over gene expression. Due to the revisitation of SOX's nuclear function during host shut off along with the hypothesis of NCL shuttling SRE based RNPs, we explored the literature on aspects of mRNA nuclear export for any pertinent information. Interestingly in one review of mRNA export written by Delaleau and Borden highlighted a non-bulk mRNA export pathway using CRM1 [110]**.** Within that section they also wrote a specific section of a subset of mRNA that use translation initiation factor eIF4E to achieve this interaction. Furthermore, the target mRNAs for eIF4E had a 3'UTR secondary structure element of approximately 50 nucleotides that they deemed the 4E-sensitivity element (4ESE) [110, 115, 116, 162]**.** This sparked our interest as it reflected aspects of what we know to be true for our SOX resistance element.

The idea for the 4ESE was first published in 2005 by Culjkovic et al., it was found that cyclin D1 mRNA contained this 4ESE. It was not until 2017 when the same lab filled out the framework of 4ESE's role in nuclear export with CRM-1 in conjunction with a leucine rich protein adaptor named LRPPRC [116]**.** To briefly recap what was written in **Chapter 1**, 4ESE sites recruit LRPPRC, this protein recruits both eIF4E and allows it to bind to the 3' UTR as well as the 5' cap in place of the normal Cap binding complex (CBC)(**Figure 12**). This interaction of LRPPRC-eIF4E-4ESE RNA complex directly recruits CRM1 which then works to export the transcript from the nucleus [162]. Another study further piqued our interest, describing the RNA targets of this pathway in cancer cells, which revealed that known SOX escapee can use this particular export pathway possibly suggesting that they all have a 4ESE or 4ESE-like structure. we thus hypothesized that perhaps our SRE escape from host shutoff could be a two-fold escape both cytoplasmic and nuclear. While the SRE transcripts escaped detection in transport granules in the cytoplasm, they could also use CRM-1 mediated export to replenish cytoplasmic transcripts and evade the SOX induced nuclear

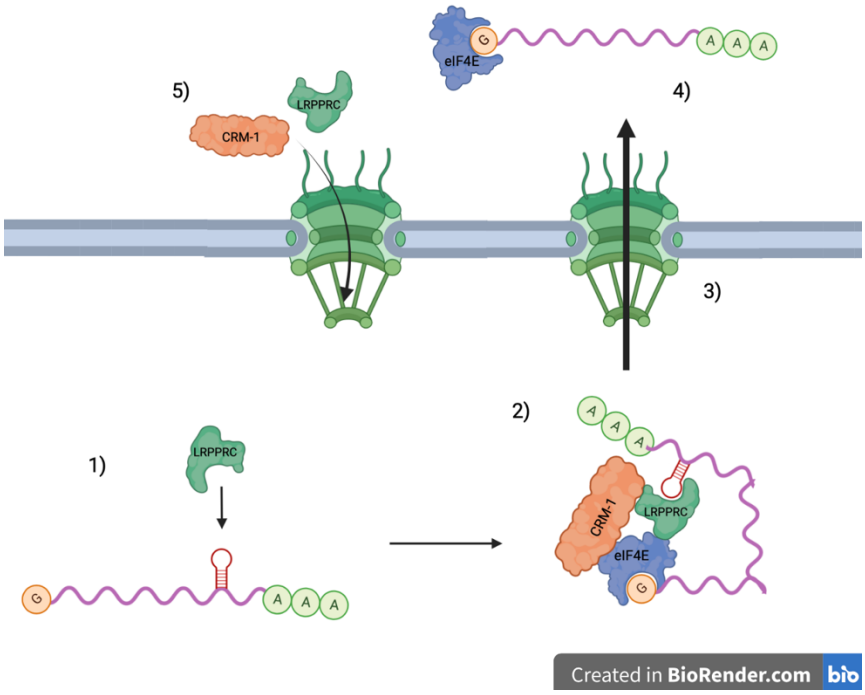

Figure 12: 4ESE/CRM1 mechanism of export.

blockade. Normally hyper polyadenylation transcripts are recognized by a subunit of the exosome, Rrp6, which occurs by binding the CBC. However, as we now know 4ESE transcripts use eIF4E in place of a CBC. Therefore, in the context of host shutoff the SRE/4ESE transcripts could use CRM-1 mediated export to supersede this processing requirement and leave the nucleus via an "emergency valve". There is some precedent for the use of CRM-1 in a viral context, even KSHV. Research has shown that CRM-1 is used as a shuttle for critical proteins LANA and ORF45 through their leucine rich domains [163]**.** It was also found that using an inhibitor of CRM1 binding that a buildup of cellular p62 occurred during infection, this cascaded into an upregulation of innate immune system and caused a severe reduction in KSHV viral titers [163]**.**

In this chapter we began to explore whether SRE bearing transcripts could mimic 4ESE bearing transcripts by assessing their interaction with CRM-1 Through the use of a CRM1 inhibitor during host shutoff we were able to confirm that the escaping transcripts accumulate in the nucleus. We were also able to confirm the interaction of CRM1 and the IL6 SRE reporter. Perturbing the known conserved stem loop in the SRE drastically reduced the affinity CRM1 has for the IL6 SRE reporter. Altogether, the data highlighted a promising start to the possibility that the SRE is also the 4ESE, and that nuclear export may play a crucial role in averting host shutoff.

4.2 Results

CRM1 exports SRE bearing transcripts from the nucleus

CRM1, also known an XPO1, is a fascinating nuclear export protein that binds to proteins as well as specific RNA through adaptor proteins. In order to explore whether CRM1 is important for SRE transcripts, a CRM1 inhibitor was used to block this nuclear export route [163]. KPT-8602 (Eltanexor) is a second-generation SINE compound: a type of drug that forms a reversible covalent bond with CRM1's cysteine 528, making it less cytotoxic than the previously used leptomycin B (LMB). We designed a microscopy experiment in which we plated HEK 293T cells with the intent to transfect them with SOX

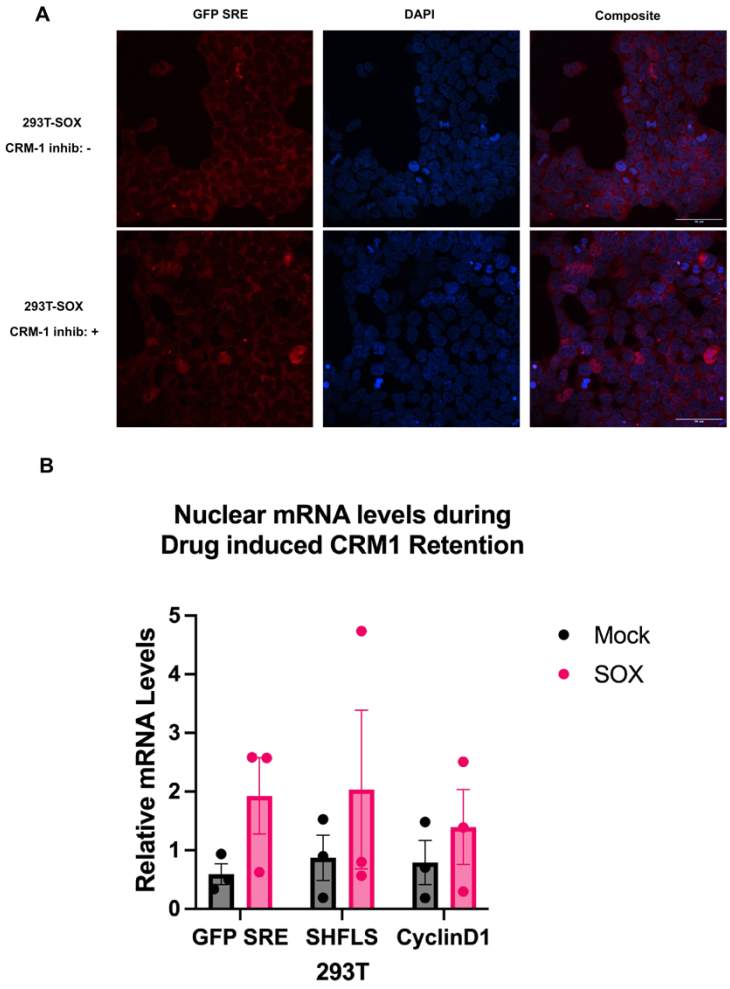

Figure 13: SRE transcripts utilize CRM1 to export from nucleus. (A) HEK293T cells were transfected with SOX and GFP SRE and subjected to either KPT-8602 or a DMSO control. Cells were subjected to FISH for GFP SRE reporter (red) and immunofluorescent stain for nuclear control (DAPI,blue). (B) HEK293T cells were transfected with mock or SOX and GFP SRE and subjected to either KPT-8602 or a DMSO control. Cells were then subjected to nuclear fractionation and RNA extraction. RNA was then subjected to RT-qPCR and primers are the ones indicated above to evaluate the nuclear retention of the RNA in question.

and a IL6 SRE reporter. One hour prior to transfection, KPT-8602 or a DMSO control was added to the cells. 24 hours post transfection, cells were fixed, permeabilized, and the SRE was detected using fluorescent probes tilling the reporter sequence. We observed that in control cells, the SRE reporter had a mix of diffuse and puncta localization in the cytoplasm (**Figure 13A**). However, in the cells treated with the inhibitor, there were clear and severe nuclear accumulation of the IL6 SRE transcript in the nucleus (**Figure 13A**). We next performed a subcellular fractionation followed by RT-qPCR to confirm this change in localization. Using the Norgen Biotek Coroporation's cytoplasmic and nuclear RNA purification kit to separate the nuclear and cytoplasmic RNA, we found that the levels of nuclear IL6 SRE reporter were significantly elevated in the presence of the CRM1 inhibitor. Furthermore, endogenous level of the known escapees had a similar defect in localization upon treatment. We found that that by adding CRM1 inhibitor to the SOX transfected cells we saw a 2-fold enrichment in the SRE bearing transcripts in nucleus (**Figure 13B**). Reinforcing the possibility that these transcripts are using both the bulk mRNA export pathway and CRM1's export pathway, and it is only under duress of a SOX induced nuclear blockade does the necessity CRM1 function.

CRM1 binds IL6 SRE in a secondary structure-based manner

Although we found CRM1 function effected IL6 SRE's localization during host shutoff, we wanted to confirm the interaction through RNA immunoprecipitation. First, we plated HEK 293T cells and transfected them with SOX or BGLF5 and IL6 SRE reporter. From there we used a CRm1 antibody to capture any RNA bound to CRM1 and looked at the levels of reporter with RT-qPCR. We had some issues with the SOX samples in the RNA quality during extraction, however the BGLF5 samples came out very clean. We saw the IL6 SRE reporter was significantly enriched indicating an interaction with CRM1 and the SRE. In order to characterize the interaction further we repeated the experiment with BGLF5 and a couple of SRE mutants. Given that CRM1 and the 4ESE interact indirectly via RNA structure we used a mutant that had been designed in a previous Muller lab paper disrupted the conserved stemloop required for protection, IL6 mutGG SRE [60, 61]. Since our previous work also found that m^{6}A was important for protection we used the IL6 mutm^{6}A SRE, that lacked the m^{6}A site [151]. Utilizing these SRE mutants we found that removing the m^{6}A site from the SRE did not reduce the affinity for CRM1 meaning that most likely the

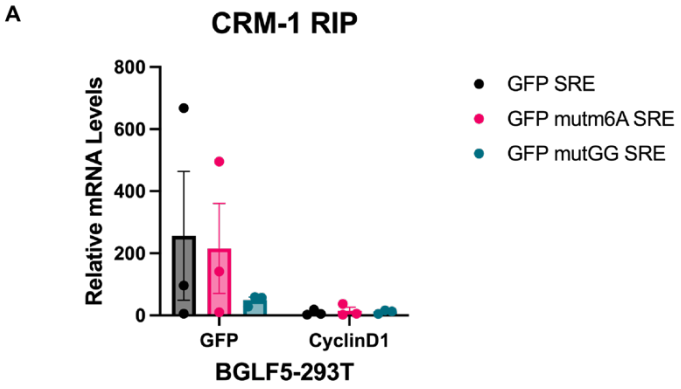

Figure 14: IL-6 SRE binds CRM1 through a conserved RNA secondary structure. (A) HEK293T cells were transfected with BGFL5 and either GFP SRE, GFP mutm6A or GFP mutGG reporter. Cells were then harvested, lysed and RAN immunoprecipitation (RIP) using CRM1 antibody conjugated beads or control beads (mock IP). Following reverse crosslinking, total RNA was harvested and subjected wot RT-qPCR using primers indicated.

modification does not aid in its indirect recruitment. On the other hand, abrogating the known conserved stem loop in the SRE drastically reduced the affinity of CRM1 (**Figure 14A**). This reinforced the idea that the adaptor that recruits CRM1 recognizes specific RNA secondary structures.

4.3 Discussion

Both SOX and BGLF5s ability to prevent the nuclear export by causing hyper polyadenylation is a critical part of the gamma herpesvirus tactic of host shutoff. Preventing new transcripts from exiting the nucleus reinforces the hijacking of the host gene expression machinery. But what about the escaping transcripts? Do they also evade this nuclear export restriction and if so, how? Excitingly, we were able to show that SRE containing transcripts indeed use the alternative, non-polyA dependent CRM-1 export pathway. We were able to confirm that under host shut off conditions, inhibiting CRM1s ability to interact with cargo resulted in a stark retention of SRE bearing transcripts in the nucleus. We also were able to confirm physical interaction of CRM1 and SRE bearing transcripts through RNA immunoprecipitation. Furthermore, through the use of key SRE mutants we were able to show that the SRE's ability to bind with CRM1 occurs through the conserved stemloop structure that is also required for cytoplasmic protection suggesting that the SRE may act as a 4ESE-like element [110, 115, 116, 162]**.** Interestingly, the lack of m^6A site did not affect CRM1 affinity highlighting the idea that the SRE's cytoplasmic function is most likely distinct from its nuclear function. Many questions remain: is the SRE acting like the 4ESE in going through the CRM-1 dependent pathway? In our case, we did not see an interaction with the common 4ESE CRM1 adapter LRPPRC, it would be

interesting to directly compare the 3D structure of the SRE to that of the 4ESE to understand what could cause this difference. Furthermore, HuR is a known SRE binding protein and has been previously shown to be another potential CRM1 adapter, so it would be interesting to see if HuR recruitment to the SRE is needed for the SRE bearing transcript to be loaded onto the CRM1 pathway.

CHAPTER 5

MATERIALS AND METHODS

5.1 Cells, Transfections and Drugs

HEK293T 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). The KSHV-infected renal carcinoma human cell line iSLK.219 (cells were supplied by Britt Glaunsinger, UC Berkeley, CA)-bearing, doxycycline-inducible RTA was grown in DMEM supplemented with 10% FBS (61). Lytic reactivation cells were induced by the addition of 0.2μg/mL doxycycline (BD Biosciences) and 110 μg/mL sodium butyrate for 72h. The 293TΔYTHDC2 knockout clone and control Cas9-expressing cells were made by transducing HEK293T cells, as previously described (62, 63). Briefly, lenti-Cas9-blast lentivirus was spinfected onto a monolayer of HEK293T cells, which were then incubated with 20 μg/mL blasticidin for a selection of transduced cells. These HEK293T-Cas9 cells were then spinfected with lentivirus made from pLKO-tet on containing the YTHDC2 sgRNA (single guide RNA) sequence, designed using the broad institute analysis tool and checked for off-target effects. After selection using and 1 μg/mL puromycin, the pool of YTHDC2 knockout cells was then single-cell cloned in 96-well plates, and individual clones were screened by Western blot to determine knockout efficiency. For DNA transfections, cells were plated and transfected after 24 h when 70% confluent using PolyJet (SignaGen). 293T cells were given a final concentration of 0.5uM of KPT-8602 (Selleck) whose stock had been dissolved in dimethyl sulfoxide (DMS).

5.2 Plasmids

The GFP-based reporters and BGLF5/SOX expression plasmids were described previously [60]. The mutGG SRE reporter was described previously in [60]. The mut m^6A SRE reporter was generated by introducing an A to T point mutation at position 74 of the WTSRE using the Quickchange site-directed mutagenesis protocol (Agilent) using the primers described in Table S1. YTHDC2 expression plasmid was supplied by Chuan He, University of Chicago, IL.

5.3 RT-qPCR

Total RNA was harvested using TRIzol according to the manufacturer's protocol. cDNAs (complementary DNA) were synthesized from 1μg total RNA using avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and used directly for qPCR analysis with the SYBR green qPCR kit (Bio- Rad). Ct values (cycle thresholds) signals obtained by qPCR were normalized to those for 18S unless otherwise noted.

5.4 Immunoblotting

Cell lysates were prepared in lysis buffer (NaCl, 150mM; Tris, 50mM; Nonidet P-40, 0.5%; dithiothreitol [DTT], 1mM; and protease inhibitor tablets) and quantified by Bradford assay. Equivalent amounts of each sample were resolved by SDS-PAGE and Western blotted with the following antibodies at 1:1,000 in Tris-buffered saline, 0.1% Tween 20, rabbit anti- YTHDC2 (Abcam), and mouse anti-GAPDH (Abcam). Primary

antibody incubations were followed by horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:5,000; Southern Biotechnology).

5.5 Immunoprecipitation

Cells were lysed in low-salt lysis buffer (150 mM NaCl, 0.5% NP-40, 50 mM Tris [pH8], 1 mM DTT, and protease inhibitor cocktail), and protein concentrations were determined by Bradford assay. At least 400 μg of total protein were incubated overnight with the designated antibody and then with protein G-coupled magnetic beads (Life Technologies) for 1 h. For FLAG NCLdeltaNLS construct pull-downs, total protein lysates were instead incubated overnight with Anti-FLAG M2 Magnetic Beads (Sigma) or G-coupled magnetic beads. Beads were then washed extensively with lysis buffer. Lastly, samples were resuspended in 4X laemmli loading dye before resolution by SDS-PAGE.

5.6 RNA Immunoprecipitation

Cells were cross-linked in 1% formaldehyde for 10 min, quenched in 125 mM glycine, and washed in PBS (phosphate buffered saline). Cells were then lysed in low-salt lysis buffer (NaCl 150 mM, Nonidet P-40 0.5%, Tris pH 8 50 mM, DTT 1 mM, MgCl2 3 mM containing protease inhibitor mixture and RNase inhibitor) and sonicated. After removal of cell debris, specific antibodies were added as indicated overnight at 4 °C. Magnetic, G-coupled beads were added for 1 h and washed three times with lysis buffer and twice with high- salt lysis buffer (low-salt lysis buffer except containing 400 mMNaCl). Samples were separated into two fractions. Beads containing the fraction used for Western blotting were resuspended in 30 μL lysis buffer. Beads containing the fraction

used for RNA extraction were resuspended in proteinase K (PK) buffer (NaCl 100 mM, Tris pH 7.4 10 mM, EDTA 1 mM, SDS 0.5%) containing 1 µL PK. Samples were incubated for 1 hour at 65°C to reverse cross-linking. Samples to be analyzed by Western blot were then supplemented with 10 µL 4×loading buffer before resolution by SDS-PAGE (sodiumdodecyl sulphate–polyacrylamide gel electrophoresis). RNA samples were resuspended in TRIzol and were processed as described in RT-qPCR.

5.7 Methylation Immunoprecipitation qPCR

HEK293T or iSLK cells were transfected as indicated and used for meRIP (methylated [m^{6}A] RNA immunoprecipitation); then, total RNA was extracted using TRIzol. Pulldowns were performed using protein G Dynabeads (Invitrogen) with 10 µg m^{6}A antibody (Sigma-Aldrich) and 100 µgRNA in meRIP buffer (50mMTrisHCl at 7.4 pH,150mMNaCl, 1mMEDTA, 0.1%Noni- det P-40, Millipore H2O) and 1 µL RNAsin (RNAse inhibitor - Promega) per sample overnight at 4 °C. After extensive washing, samples are eluted in meRIP buffer containing 6.7 mM sodium salt for 30 min at 4 °C. cDNAs were then obtained from 1µg total RNA using AMV reverse transcriptase (Promega) and used directly for qPCR analysis with the SYBR green qPCR kit (Bio-Rad). RIP.

5.8 m^{6}A eCLIP and seq

iSLK.219 cells were harvested in their latent phase or 48-h post reactivation. RNA was then extracted by TRIzol and purified as described in RT-qPCR. The samples were processed by EclipseBio as described in their user guide, performing 150 paired-end run

on NovaSeq6000 on was PolyA- selected RNA. Ratio of IP and input reads were evaluated in each cluster, and clusters with IP/input enrichment greater than eightfold and associated P value < 0.001 were defined as significant "peaks." PureCLIP was used to identify m^6A sites with a single-nucleotide resolution. This algorithm identifies cross-link sites in eCLIP experiments and assesses enrichment of DRACH motif relative to reads starts in IP and input libraries, as well as what fraction of identified cross-link sites are positioned on DRACH motifs.

5.9 RNA granule isolation

Cells were prepared in an altered stress granule isolation protocol found here [164]. After transfections cell were pelleted then flash frozen in liquid nitrogen then thawed on ice for 5 mins. Pellets were then lysed in RNA granule lysis buffer [50 mM TrisHCl pH 7.4, 100 mM KOAc, 2 mM MgOAc, 0.5 mM DTT, 50 μg/mL Heparin, 0.5% NP40, complete mini EDTA-free protease inhibitor (1 tablet/50 mL lysis buffer, 11836170001, Sigma-Aldrich), 1 U/μL RNasein Plus RNase Inhibitor (N2615, Promega)] followed by sonication. We isolated the proper a series of centrifugation as written in [164]. We preclear the RNA granule enriched fraction using Protein G magnetic Dynabeads. Following that we used either Protein G magnetic Dynabeads or Anti-FLAG M2 Magnetic Beads (Sigma) overnight to immunoprecipitated. The following day we use Wash Buffer 1 [20 mM Tris HCl pH 8.0, 200 mM NaCl, and 1 U/μL of RNasein Plus RNase Inhibitor] 3 times, wash buffer 2 [20 mM Tris HCl pH 8.0, 500 mM NaCl, and 1 U/μL of RNasein Plus RNase inhibitor] once and wash buffer 3 [RG lysis buffer with 2M Urea] once. We then elute from the beads by resuspending RNA samples in proteinase K (PK) buffer (NaCl 100 mM, Tris

pH 7.4 10 mM, EDTA 1 mM, SDS 0.5%) containing 1 µL PK. Samples were incubated for 1 hour at 65°C to reverse cross-linking. Samples to be analyzed by Western blot were then supplemented with 10 µL 4×loading buffer before resolution by SDS-PAGE (sodiumdodecyl sulphate–polyacrylamide gel electrophoresis). RNA samples were resuspended in TRIzol and were processed as described in RT-qPCR.

5.10 RNA-Seq

293T cells were transfected with herpesviral endonucleases (SOX, BLGF5) or a mock vector in conjunction with an mRNA GFP reporter attached to the IL6 SRE, and a Flag tagged NCLdeltaNLS. RNA granules were isolated according to protocol written above. Purity and integrity were assessed Agilent 2100 Bioanalyzer RNA 6000 Pico assay. Libraries were subjected to a 76-paired end sequencing using NextSeq 500 with Illumina adapter trimming. Purity analysis and Sequencing was done with the aid of the Umass Amherst Genomics Resource laboratory. Using Galaxy, reader was aligned and merged to the human genome (hg38) by STAR. Transcript assembly and quantification was done using StringTie**.** Fold change expression between mock and endonuclease condition was done by DESeq2, goseq and featureCounts. Read quality was assessed FastQC.

5.11 Mass Spectrometry

HEK 293T cells were seeded into 10-cm plates and transfected. Following 72 Hours post-reactivation, cells were harvested and followed the RNA granule isolation until the first wash step. Samples were then extensively washed with IP buffer [50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% NP40 and milliQ water], and trypsin digested

overnight. Samples were then cleaned up using a C18 column and mass spectral data obtained from the University of Massachusetts Mass Spectrometry Center using an Orbitrap Fusion mass spectrometer. Raw data was filtered based on the number of peptides for each hit and Gene Ontology (GO) enrichment analysis was performed on the human interacting proteins of SHFL using DAVID bioinformatic database. Top enriched clusters are identified on the network.

5.12 FISH and Immunofluorescence

Protocol was done in accordance with Biosearch technologies, Stellaris FISH and IF protocol. In brief, HEK293T cells were grown on coverslips and fixed in 4% formaldehyde for 20 min at room temperature. Cells were then permeabilized in 70% ethanol for 1 hour at 4^{o}C 10. Coverslips were washed in Wash buffer A for 4 mins. 100uls of Hybridization buffer containing the fluorescent probe and properly diluted protein primary antibody was added to a humidified chamber. The coverslips were then placed face down on the droplet and sealed in a dark room at 37^{o}C for 4 and a half hours. Coverslips were then transferred a plate cell side up in Wash Buffer A along with properly diluted Alexa Fluor 350 secondary antibody, before being incubated in a dark room at 37^{o}C for 30 mins once more. Coverslips were then washed in Wash Buffer B for 4 mins and then mounted in Vectashield mounting medium, that sometimes-contained DAPI (Vector Labs) to stain cell nuclei, when necessary, before visualization by confocal microscopy on a Nikon A1 resonant scanning confocal microscope (A1R-SIMe). The microscopy data were gathered in the Light Microscopy Facility and Nikon Center of Excellence at the Institute

for Applied Life Sciences, UMass Amherst, with support from the Massachusetts Life Sciences Center.

5.13 Cellular Fractionation

Nuclear RNA was obtained using Norgen Biotek Corportaion's Cytoplasmic & Nuclear RNA Purification Kit. In brief, after obtaining the cells they were lysed in ice cold Lysis Buffer J before being centrifuged for 10 mins at 12,000 x g in order to separate out the cytoplasmic and nuclear fractions. The supernatant and pellet reflecting the previously mentioned fractions are separated into their own column tubes and mixed with Buffer SK. Through rounds of washing and centrifugation the fractions are eluted and can proceed with RT-qPCR.

5.14 Statistical Analysis

All results are expressed as means ± SEMs of experiments independently repeated at least three times (individual replicate points are shown on bar graph). The unpaired Student's t test was used to evaluate the statistical difference between samples. Significance was evaluated with P values as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; and ns refers to not significant.

CHAPTER 6

CLOSING REMARKS

6.1 Use of Post Transcriptional Modifications as Viral/Host vie for Gene Expression Control

Viruses have evolved inside human hosts for thousands of years and as such have developed numerous and eclectic ways to control cell fate. DNA viruses and more specifically herpesviruses have evolved masterful ways to manipulate cellular gene expression in order to facilitate their own replication. Fascinatingly, herpesviruses have evolved a similar mechanism during lytic reactivation, where they express a viral endonuclease to induce a widespread reduction on cellular RNA that culminates in freed up gene expression resources [37, 38, 42, 44, 45, 51, 54, 120]**.** As stated in previous chapters this act of KSHV host shut off is twofold. First there is the cytoplasmic degradation of RNAs that prevents expression of proteins and frees up RBPs to enhance their own gene expression [34, 40, 43]. As for the second effect, some of these RBPs relocalized to the nucleus where they interact with SOX to induce hyper polyadenylation which causes newly made transcripts to be retained and then degraded [38]. Although viral host shutoff is a powerful tool, it does not degrade all mRNA. Some do not have viral endonuclease targeting sequences, while others utilize cis and/or trans acting factors to evade cleavage. Normally in the field of virology studies examine how the virus subverts cellular functions or evade our innate immune systems.

Our group has taken up the mantle of exploring an example of how a host mechanism is able to resist its viral invader in the evolution of RNA element, the SOX Resistance Element (SRE). Most of the characterization on the SRE was done on IL-6 and

GADD45B but in the past couple years a third was discovered in SHFL [60-62, 119]**.** The SRE has been defined as around a 100nt region in the 3' UTR of the transcript. Its position within the transcript is crucial as moving it to the 5'UTR seems to ablate its protective function. The SRE can be attached to transcripts that would normally be degraded and grant them protection. It is known that the SRE contains a conserved stemloop structure that when disrupted also ablates protective function [60, 61]. Unfortunately, there is little sequence homology between the SREs, making the discovery of more difficult. These pieces of information support the idea that the SRE serves as a binding platform for certain RBPs to build a specific RNP to avoid viral endonuclease cleavage. There has been some research done to expand the interactome of SRE transcripts, where there were partially overlapping RNP components between IL6 and GADD45B. Two of the most important proteins were HuR and NCL, lacking either one reduced the SRE protective phenotype drastically [60, 61]**.** It was also confirmed that in a SRE mutant lacking the stemloop, the SRE no longer was able to bind NCL. Reinforcing data back from 1996 where it was found that a minimal 18nt long stemloop structure that at least contained the motif UCCCGA was able to bind NCL tightly (Kd 5 to 20 nm), most often found in pre-rRNA binding sites [165]. There were a couple of other proteins that were identified as possible members of SRE protective RNP complex known as m^6Areaders.

As previously explained in **Chapter 1** and **2,** m^6A is one of RNA modifications that are able to exert changes to an RNAs fate. $m^6A$is also one of the most ubiquitous and varied in its functions, acting more as a beacon for reader proteins to give function to the modification based on the protein and location its recruited to [67, 68, 70]. Given its powerful hold over RNA fate it was been discovered that m^6Ahas been shown to be coopted

by viruses in their eternal struggle for control with the host. Recruiting readers that aid cellular functions like splicing or translation to increase viral replication [84, 99, 103, 104, 166-170]. Through the use of a recent technology, we were able to screen for all of the m^{6}Atranscripts that were present during KSHV lytic reactivation as well as their sites of modification. Amazingly, all three of our known SREs were found to be m^{6}A modified. Through follow up studies on the IL6 SRE we were able to confirm its importance in protection against viral endonucleases. Further experiments revealed an importance for m^{6}A and YTHDC2, although its significance in protections was greater in an invitro setting than an in vivo one for all the SREs [151]. Further work in **Chapter 3** also highlighted the fact that YTHDC2 did not colocalize the SRE puncta. Perhaps the role of YTHDC2 is more ancillary in the SRE's ability to protect. Perhaps the m^{6}Asite recruits another still undefined m^{6}Areader. Another hypothesis is that the m^{6}Asite aids in the secondary structure formation of the conserved stem loop. Although it should also be noted that the m^{6}Asite is not located in the conserved stemloop structure. It is still a possibility, in which case NCL could be considered an indirect m^{6}Areader, which would explain the m^{6}Afunction. Through our work though we can be sure that we have expanded the knowledge of m^{6}Ause on the battlefield that is virus vs host. Additional knowledge as well that SREs seem to be m^{6}Amodified which helps a little in identifying potentially new SRE bearing transcripts.

6.2 Mechanism for SRE functions During Herpesviral Infection

In further exploration of the actual protective mechanism of the SRE on transcripts has us focus on the components of the RNP that were known to us, primarily NCL. Several studies have shown NCL to be a flexible protein capable of binding both RNAs and proteins

for a myriad of functions across the cell. NCL has also been shown to be a shuttling protein for many different types of RNAs**.** We were able to tie these functions to the SRE with the research done in **Chapter 3,** first visualizing that the IL6 and GADD45B SRE and NCL colocalize in granules during viral host shutoff. Further experiments were able to confirm the interaction of the SRE transcripts and NCL in these granules. Characterization of NCL/RNA granules of that density revealed no drastic change in RNA population between cells that were and were not experiencing host shutoff. This supports the idea that the SREs ability to protect is a passive one and not something that the cell is actively seeking. Analysis of unique proteins in the NCL/RNA granules highlighted some RNA sensing proteins and quite a few splicing proteins, including a protein known to retain introns. While splicing normally occurs in the nucleus there are instances where splicing can be delayed, and the intron allows for transportation to specific locations in the cell where it is then spliced in the cytoplasm and translated. Therefore, it could be possible that SRE bearing transcripts could be intron retaining transcripts to allow NCL to recruit the SRE transcripts for transportation. NCL has been known to bring mRNAs to locations in the cell through interactions with endosomes, could the SRE transcripts be packaged into endosomes and this method of transport prevent access of SOX to these SRE transcripts. However, there are still many questions that need to be answered by this idea, does NCL bring the transcripts into the endosomes or just on the exterior? If SRE bearing transcripts are intron retaining, is the SRE site a retained intron or is it just a marker as one? Can we examine the endogenous SRE transcripts and see if there are different populations of transcripts during host shutoff? Hopefully these are questions that can be answered going forward.

NCL's ability to shuttle across the nuclear envelope gave rise to an extensive literature dive that explored the nature of mRNA nuclear export. In that dive similarities within a class of mRNAs that contain an export element known as 4ESE that used eIF4E and a nuclear export protein known as CRM1. CRM1 control an alternative pathway to export and with eIF4E provided an explanation how some transcripts might be able to escape the nuclear blockade caused by SOX induced hyper polyadenylation. Furthermore, studies done identifying some 4ESE bearing transcripts coincided with our three known SRE bearing transcripts. Exploring this avenue revealed that in fact our SRE bearing transcripts utilized CRM1 export from the nucleus during host shutoff. Utilizing a CRM1 inhibitor resulted in nuclear retention. Furthermore, we were able to confirm not only CRM1 and IL6 SRE interaction but that this interaction is based around the conserved stemloop structure. A secondary structure not unlike the one seen by 4ESE containing transcripts. There is an issue though is that in the instance of 4ESE transcripts the eIF4E and CRM1 are recruited through a leucine rich repeat protein known as LRPPRC. Unfortunately, studies into the SRE RNP have not seen such a protein. This observation begs the questions is there another leucine rich protein that could take the place of LRPPRC? HuR has been shown to be an adaptor protein that can recruit CRM1, but not through eIF4E. HuR only has one leucine rich repeat, perhaps it could be working with another protein to recruit CRM1. NCL has also been shown to contain a leucine rich repeat in its RRM1 domain. Interestingly CRM1 has numerous adaptors for different types of RNAs, and its possible NCL could be a new one possibly working with HuR as well. Due to the varied types of RNAs CRM1 exports it could be possible that the place to look for possible SREs based on their functions but their ability to exit the nucleus with CRM1.

In conclusion, we propose a model for how mechanism of SRE escapes from viral host shutoff (**Figure 15**). During lytic reactivation, KHSV expresses its viral endonuclease

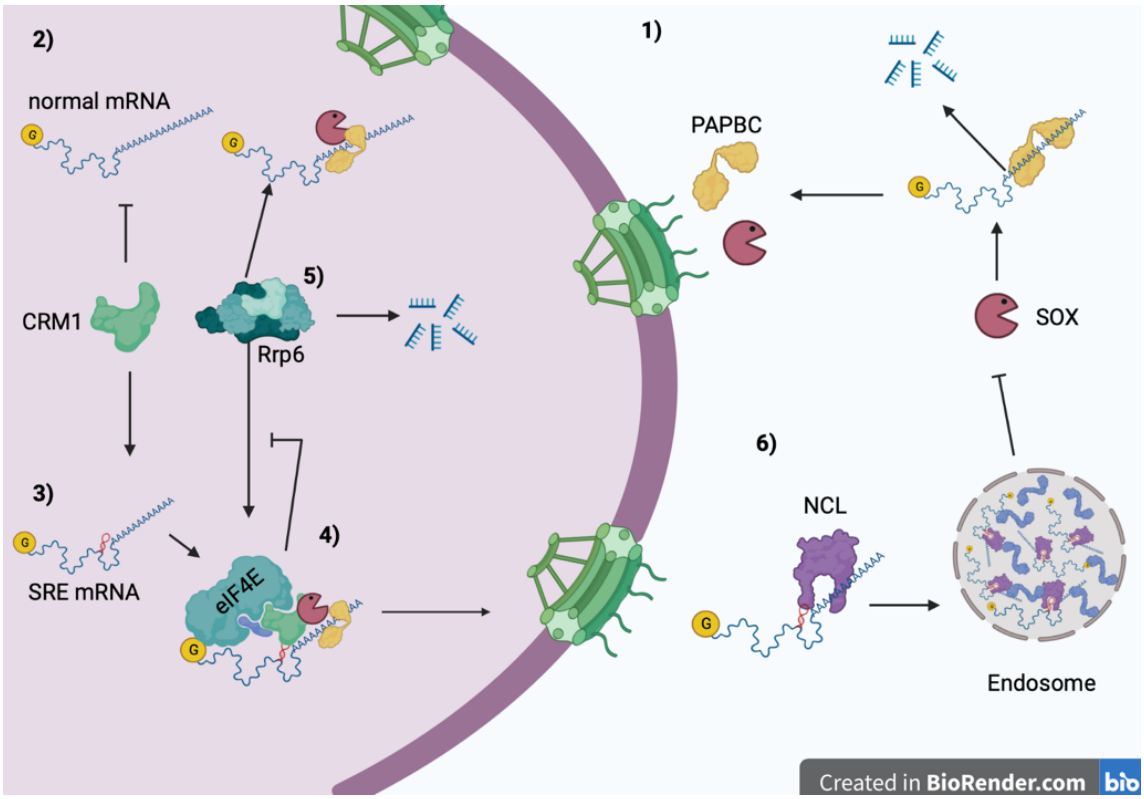

Figure 15: The SRE's mechanism of protecting mRNAs during viral host shutoff. During KSHV lytic reactivation, the virus triggers massive RNA decay event by expressing SOX. This viral protein cleaves most mRNAs causing a massive relocation of RBPs to the nucleus. There SOX, PABPC and PABPN cause hyper polyadenylation of new transcripts, which causes the transcripts to be degraded reinforcing the host shutoff event. A portion of transcripts contain an SRE, a cis-acting secondary structure in the 3'UTR. This allows recruitment of a leucine rich protein like HuR or LRPPRC which recruits eIF4E and CRM1. The combination allows exit from the nucleus where the SRE transcript's RNP changes introducing NCL. NCL then facilitates interactions with other proteins that localize the SRE/NCL RNP to endosomes and transports it to other locations in the cell where it can wait to be translated. During its transport, this packaging prevents SOX from degrading the SRE transcript.

SOX. SOX begins to cleave over 70% of the RNAs in the cytoplasm. SRE bearing transcripts are passively being transported an intron retained mRNAs in endosomes via a NCL based RNP. This transport mechanism protects them from SOX cleavage until they are released where they need to be translated. As they leave the endosome it is possible

some SRE transcripts are degraded. As other transcripts are being degraded RBPs including PABPC are relocated to the nucleus where in cooperation with nuclear SOX and PABPN cause hyper polyadenylation on new transcripts. This causes the new transcripts to be targeted for decay by the nuclear exosome, preventing any restoration of cytoplasmic mRNAs. However, SRE bearing transcripts recruit NCL and or HuR which in turn recruit CRM1 which allows them to export the nucleus where they once again enter transport endosomes. This nuclear escape supplements any loss that might occur by SOX near the end of the SRE transcripts journey resulting a relatively stable steady state compared to other transcripts. There are still gaps in this model such as how does m^6A fit in exactly does it just allow secondary structure formation or does YTHDC2 help SRE transcripts be spliced through its helicase activity? Mutually, our research does push the boundary of how the host is able to evade a catastrophic event during viral lytic reactivation. As we learn more pieces to this puzzle, we can be proud over the mastery of RNA fate as we struggle ever onwards with our unsolicited passengers.

CHAPTER 7

TABLES

Name	Sequence
qPCR	
h18s-F	GTAACCCGTTGAACCCCATT
h18s-R	CCATCCAATCGGTAGTAGCG
qPCR-GFPfwd	CAACAGCCACAACGTCTATATCATG
qPCR-GFPrv	ATGTTGTGGCGGATCTTGAAG
F-YTHDC2	CAAAACATGCTGTTAGGAGCCT
R-YTHDC2	CCACTTGTCTTGCTCATTTCCC
F-KCNK6 qPCR	GTCGTGCTTGCTAACGCTTC
R-KCNK6 qPCR	CGTTGTGTACCCATAGCCCA
IL6 qPCR Fwd	TGTTGTGCAAGGGTCTGGTT
IL6 qPCR Rev	TCTTCTCCTGGGGGTACTGG
ORF57-qPCRfwd	TTTGACGAATCGAGGGACGACG
ORF57-qPCRRv	GCAGTTGAGAACGACCTTGAGAT
ORF37-qPCRfwd	TGGGCGAGTTTATTGGTAGTGAGG
ORF37-qPCRRv	CTCCACTAGACAGCAGATGTGG
Cyclin D1- qPCR Fwd	CAATGACCCCGCACGATTTC
Cyclin D1- qPCR rev	CATGGAGGGCGGATTGGAA
SF3BP5-qPCR Fwd	ACTGACCGCTACACCATCCAT
SF3BP5-qPCR Rev	GTAGTTGAGAAGGTCGAAGTGG
sequencing	
YTHDC2 Fwd	AGACGCTGCTCGGCCTGGAC
YTHDC2 rev	GAACGTCTTCCCTTGGGAGAAA
CMV Fwd	GCAAATGGGCGGTAGGCGTG
BGH Rv	TAGAAGGCACAGTCGAGGCT
Quickchange primers	
mutSRE Fwd	CAT AGA GAA CAA CAT AAG ATC TGT GCC CAG TGG ACA
mutSRE Rev	TGT CCA CTG GGC ACA GAT CTT ATG TTG TTC TCT ATG

Table 1: The List of sequencing and qPCR primers.

REFERENCES

1. Polizzotto, M., et al., Clinical Manifestations of Kaposi Sarcoma Herpesvirus Lytic Activation: Multicentric Castleman Disease (KSHV–MCD) and the KSHV Inflammatory Cytokine Syndrome. Frontiers in Microbiology, 2012. 3.
2. Ramaswami, R., K. Lurain, and R. Yarchoan, Oncologic treatment of HIV-associated Kaposi sarcoma 40 years on. Journal of Clinical Oncology, 2022. 40(3): p. 294-306.
3. Dittmer, D.P. and B. Damania, Kaposi sarcoma associated herpesvirus pathogenesis (KSHV)—an update. Current Opinion in Virology, 2013. 3(3): p. 238-244.
4. Osborne, J., P.S. Moore, and Y. Chang, KSHV-encoded viral IL-6 activates multiple human IL-6 signaling pathways. Hum Immunol, 1999. 60(10): p. 921-7.
5. Arias, C., et al., KSHV 2.0: A Comprehensive Annotation of the Kaposi's Sarcoma-Associated Herpesvirus Genome Using Next-Generation Sequencing Reveals Novel Genomic and Functional Features, in PLoS Pathogens. 2014.
6. Sarid, R., S.J. Olsen, and P.S. Moore, Kaposi's sarcoma-associated herpesvirus: epidemiology, virology, and molecular biology. Adv Virus Res, 1999. 52: p. 139-232.
7. Cesarman, E., et al., Kaposi sarcoma, in Nature Reviews Disease Primers. 2019. p. 9.
8. Broussard, G. and B. Damania, Regulation of KSHV Latency and Lytic Reactivation. Viruses, 2020. 12(9).

9. Yan, L., et al., Towards Better Understanding of KSHV Life Cycle: from Transcription and Posttranscriptional Regulations to Pathogenesis, in Virologica Sinica. 2019, Science Press. p. 135-161.
10. Purushothaman, P., et al., KSHV Genome Replication and Maintenance. Frontiers in Microbiology, 2016. 7.
11. Veettil, M.V., et al., Interaction of KSHV with host cell surface receptors and cell entry. Viruses, 2014. 6(10): p. 4024-46.
12. Uppal, T., et al., Chromatinization of the KSHV Genome During the KSHV Life Cycle. Cancers (Basel), 2015. 7(1): p. 112-42.
13. Ruiz, J.C., et al., Kaposi's sarcoma-associated herpesvirus fine-tunes the temporal expression of late genes by manipulating a host RNA quality control pathway. 2020.
14. Juillard, F., et al., KSHV LANA acetylation-selective acidic domain reader sequence mediates virus persistence. Proc Natl Acad Sci U S A, 2020. 117(36): p. 22443-22451.
15. Uppal, T., et al., KSHV LANA--the master regulator of KSHV latency. Viruses, 2014. 6(12): p. 4961-98.
16. Di, C., et al., RTA and LANA Competitively Regulate let-7a/RBPJ Signal to Control KSHV Replication. Front Microbiol, 2021. 12: p. 804215.
17. Guito, J. and D.M. Lukac, KSHV Rta Promoter Specification and Viral Reactivation. Front Microbiol, 2012. 3: p. 30.
18. Tani, H., et al., Genome-wide determination of RNA stability reveals hundreds of short-lived noncoding transcripts in mammals. Genome Res, 2012. 22(5): p. 947-56.

19. Łabno, A., R. Tomecki, and A. Dziembowski, Cytoplasmic RNA decay pathways - Enzymes and mechanisms, in Biochimica et Biophysica Acta - Molecular Cell Research. 2016. p. 3125-3147.

20. Fasken, M.B. and A.H. Corbett, Process or perish: Quality control in mRNA biogenesis, in Nature Structural and Molecular Biology. 2005. p. 482-488.

21. Katahira, J., Nuclear export of messenger RNA, in Genes. 2015. p. 163-184.

22. Wagner, E. and J. Lykke-Andersen, mRNA surveillance: the perfect persist. J Cell Sci, 2002. 115(Pt 15): p. 3033-8.

23. Hilleren, P., et al., Quality control of mRNA 3′-end processing is linked to the nuclear exosome, in Nature. 2001. p. 538-542.

24. Moore, M.J., Nuclear RNA turnover, in Cell. 2002. p. 431-434.

25. Palacios, I.M., RNA processing: Splicing and the cytoplasmic localisation of mRNA, in Current Biology. 2002. p. 50-52.

26. Harigaya, Y. and R. Parker, No-go decay: a quality control mechanism for RNA in translation. Wiley Interdiscip Rev RNA, 2010. 1(1): p. 132-41.

27. Blasco-Moreno, B., et al., The exonuclease Xrn1 activates transcription and translation of mRNAs encoding membrane proteins. Nature Communications, 2019. 10(1): p. 1298.

28. Burgess, H.M. and I. Mohr, Cellular 5′-3′ mRNA exonuclease Xrn1 controls double-stranded RNA accumulation and anti-viral responses, in Cell Host and Microbe. 2015, Elsevier Inc. p. 332-344.

29. Nagarajan, V.K., et al., XRN 5'-->3' exoribonucleases: structure, mechanisms and functions. Biochim Biophys Acta, 2013. 1829(6-7): p. 590-603.

30. Januszyk, K., The eukaryotic RNA exosome, in Curr Opin Struct Biol. 2012. p. 997-1003.

31. Schmid, M. and T.H. Jensen, The exosome: a multipurpose RNA-decay machine. Trends Biochem Sci, 2008. 33(10): p. 501-10.

32. Luan, S., et al., Regulation of RNA decay and cellular function by 3'-5' exoribonuclease DIS3L2. RNA Biol, 2019. 16(2): p. 160-165.

33. Malecki, M., et al., The exoribonuclease Dis3L2 defines a novel eukaryotic RNA degradation pathway. EMBO J, 2013. 32(13): p. 1842-54.

34. Abernathy, E., et al., Viral nucleases induce an mRNA degradation-transcription feedback loop in mammalian cells, in Cell Host and Microbe. 2015.

35. Abernathy, E. and B. Glaunsinger, Emerging roles for RNA degradation in viral replication and antiviral defense. Virology, 2015. 479-480: p. 600-8.

36. Covarrubias, S., et al., Coordinated destruction of cellular messages in translation complexes by the gammaherpesvirus host shutoff factor and the mammalian exonuclease Xrn1. PLoS pathogens, 2011. 7(10): p. e1002339.

37. Covarrubias, S., et al., Host Shutoff Is a Conserved Phenotype of Gammaherpesvirus Infection and Is Orchestrated Exclusively from the Cytoplasm, in Journal of Virology. 2009. p. 9554-9566.

38. Lee, Y.J. and B.A. Glaunsinger, Aberrant herpesvirus-induced polyadenylation correlates with cellular messenger RNA destruction, in PLoS Biology. 2009.

39. Duncan-Lewis, C., et al., Cytoplasmic mRNA decay represses RNA polymerase II transcription during early apoptosis. Elife, 2021. 10.

40. Hartenian, E. and B.A. Glaunsinger, Feedback to the central dogma: cytoplasmic mRNA decay and transcription are interdependent processes. Crit Rev Biochem Mol Biol, 2019. 54(4): p. 385-398.

41. Covarrubias, S., et al., Host Shutoff Is a Conserved Phenotype of Gammaherpesvirus Infection and Is Orchestrated Exclusively from the Cytoplasm. Journal of Virology, 2009. 83(18): p. 9554-9566.

42. He, T., et al., Host shutoff activity of VHS and SOX-like proteins: role in viral survival and immune evasion. Virology Journal, 2020. 17(1): p. 68.

43. Hyunah Lee, A.O.M.P., Claire Bagne´ris, Hannah Ryan, Christopher M. Sanderson, Bahram Ebrahimi, Irene Nobeli and Tracey E. Barrett, KSHV SOX mediated host shutoff: the molecular mechanism underlying mRNA transcript processing

. Nucleic Acids Research, 2017.

44. Levene, R.E. and M.M. Gaglia, Host shutoff in influenza A virus: Many means to an end, in Viruses. 2018. p. 1-20.

45. Rowe, M., et al., Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proceedings of the National Academy of Sciences of the United States of America, 2007. 104(9): p. 3366-3371.

46. Rodriguez, W., et al., Fated for decay: RNA elements targeted by viral endonucleases, in Seminars in Cell and Developmental Biology. 2021, Elsevier Ltd. p. 119-125.

47. Gaglia, M.M., et al., A common strategy for host RNA degradation by divergent viruses. J Virol, 2012. 86(17): p. 9527-30.

48. Pardamean, C.I. and T.T. Wu, Inhibition of Host Gene Expression by KSHV: Sabotaging mRNA Stability and Nuclear Export. Front Cell Infect Microbiol, 2021. 11: p. 648055.

49. Glaunsinger, B. and D. Ganem, Lytic KSHV Infection Inhibits Host Gene Expression by Accelerating Global mRNA Turnover. Molecular Cell, 2004. 13(5): p. 713-723.

50. He, T., et al., Host shutoff activity of VHS and SOX-like proteins: role in viral survival and immune evasion. Virol J, 2020. 17(1): p. 68.

51. Pasieka, T.J., et al., Herpes simplex virus virion host shutoff attenuates establishment of the antiviral state. J Virol, 2008. 82(11): p. 5527-35.

52. Knizewski, L., et al., Realm of PD-(D/E)XK nuclease superfamily revisited: detection of novel families with modified transitive meta profile searches. BMC Struct Biol, 2007. 7: p. 40.

53. Gaglia, M.M., C.H. Rycroft, and B.A. Glaunsinger, Transcriptome-Wide Cleavage Site Mapping on Cellular mRNAs Reveals Features Underlying Sequence-Specific Cleavage by the Viral Ribonuclease SOX. PLoS Pathog, 2015. 11(12): p. e1005305.

54. Lee, H., et al., KSHV SOX mediated host shutoff: the molecular mechanism underlying mRNA transcript processing. Nucleic Acids Res, 2017. 45(8): p. 4756-4767.

55. Mendez, A.S., et al., Site specific target binding controls RNA cleavage efficiency by the Kaposi's sarcoma-associated herpesvirus endonuclease SOX. Nucleic Acids Res, 2018. 46(22): p. 11968-11979.

56. Gaucherand, L., et al., The Influenza A Virus Endoribonuclease PA-X Usurps Host mRNA Processing Machinery to Limit Host Gene Expression, in Cell Reports. 2019, ElsevierCompany. p. 776-792.e7.

57. Hayashi, T., et al., Critical Role of the PA-X C-Terminal Domain of Influenza A Virus in Its Subcellular Localization and Shutoff Activity, in Journal of Virology. 2016. p. 7131-7141.

58. Hu, J., C. Ma, and X. Liu, PA-X: a key regulator of influenza A virus pathogenicity and host immune responses, in Medical Microbiology and Immunology. 2018, Springer Berlin Heidelberg. p. 255-269.

59. Abernathy, E., et al., Gammaherpesviral gene expression and virion composition are broadly controlled by accelerated mRNA degradation. PLoS Pathog, 2014. 10(1): p. e1003882.

60. Muller, M. and B.A. Glaunsinger, Nuclease escape elements protect messenger RNA against cleavage by multiple viral endonucleases, in PLoS Pathogens. 2017, Public Library of Science.

61. Muller, M., et al., A Ribonucleoprotein Complex Protects the Interleukin-6 mRNA from Degradation by Distinct Herpesviral Endonucleases, in PLoS Pathogens. 2015. p. 1-23.

62. Rodriguez, W., K. Srivastav, and M. Muller, C19ORF66 Broadly Escapes Virus-Induced Endonuclease Cleavage and Restricts Kaposi's Sarcoma-Associated Herpesvirus. J Virol, 2019. 93(12).

63. Clyde, K. and B.A. Glaunsinger, Deep sequencing reveals direct targets of gammaherpesvirus-induced mRNA decay and suggests that multiple mechanisms govern cellular transcript escape. PLoS One, 2011. 6(5): p. e19655.

64. Glaunsinger, B. and D. Ganem, Highly selective escape from KSHV-mediated host mRNA shutoff and its implications for viral pathogenesis. J Exp Med, 2004. 200(3): p. 391-8.

65. Huang, H., H. Weng, and J. Chen, The Biogenesis and Precise Control of RNA m 6 A Methylation, in Trends in Genetics. 2019, Elsevier Inc. p. 1-9.

66. Zhu, Z.-M., F.-C. Huo, and D.-S. Pei, Function and evolution of RNA N6-methyladenosine modification, in International Journal of Biological Sciences. 2020. p. 1929-1940.

67. Fernandez Rodriguez, G., B. Cesaro, and A. Fatica, Multiple Roles of m6A RNA Modification in Translational Regulation in Cancer. Int J Mol Sci, 2022. 23(16).

68. Zaccara, S., R.J. Ries, and S.R. Jaffrey, Reading, writing and erasing mRNA methylation. Nat Rev Mol Cell Biol, 2019. 20(10): p. 608-624.

69. Lee, M., B. Kim, and V.N. Kim, Emerging Roles of RNA Modification: m6A and U-Tail. Cell, 2014. 158(5): p. 980-987.

70. Zhen, D., et al., m6A Reader: Epitranscriptome Target Prediction and Functional Characterization of N6-Methyladenosine (m6A) Readers, in Frontiers in Cell and Developmental Biology. 2020. p. 1-14.

71. Batista, Pedro J., et al., m6A RNA Modification Controls Cell Fate Transition in Mammalian Embryonic Stem Cells. Cell Stem Cell, 2014. 15(6): p. 707-719.

72. Wei, W., et al., Regulatory Role of N6-methyladenosine (m6A) Methylation in RNA Processing and Human Diseases. Journal of Cellular Biochemistry, 2017. 118(9): p. 2534-2543.

73. Garcia-Campos, M.A., et al., Deciphering the "m6A Code" via Antibody-Independent Quantitative Profiling, in Cell. 2019, Elsevier BV. p. 731-747.e16.

74. Wang, J. and L. Wang, Deep analysis of RNA N6-adenosine methylation (m6A) patterns in human cells, in NAR Genomics and Bioinformatics,. 220, Oxford University Press. p. 1-12.

75. Ping, X.-L., et al., Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. Cell Research, 2014. 24(2): p. 177-189.

76. Li, N., et al., METTL3 regulates viral m6A RNA modification and host cell innate immune responses during SARS-CoV-2 infection. Cell Rep, 2021. 35(6): p. 109091.

77. Liu, J., et al., A METTL3–METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. Nature Chemical Biology, 2014. 10(2): p. 93-95.

78. Shetty, S. and M.N. Hall, More writing: mTORC1 promotes m6A mRNA methylation. Molecular Cell, 2021. 81(10): p. 2057-2058.

79. Bayoumi, M. and M. Munir, Evolutionary conservation of the DRACH signatures of potential N6-methyladenosine (m6A) sites among influenza A viruses, in Scientific Reports. 2021, Nature Publishing Group UK. p. 1-12.

80. Uzonyi, A., et al., Exclusion of m6A from splice-site proximal regions by the exon junction complex dictates m6A topologies and mRNA stability., in Molecular cell. 2022. p. 237-251.

81. V.A.R.Barao, et al., Exon-intron architecture determines mRNA stability by dictating m6A deposition, in Braz Dent J. 2022. p. 1-12.

82. Zheng, G., et al., ALKBH5 Is a Mammalian RNA Demethylase that Impacts RNA Metabolism and Mouse Fertility. Molecular Cell, 2013. 49(1): p. 18-29.

83. Bayoumi, M. and M. Munir, Structural Insights Into m6A-Erasers: A Step Toward Understanding Molecule Specificity and Potential Antiviral Targeting, in Frontiers in Cell and Developmental Biology. 2021. p. 1-14.

84. Hesser, C.R., et al., N6-methyladenosine modification and the YTHDF2 reader protein play cell type specific roles in lytic viral gene expression during Kaposi's sarcoma-associated herpesvirus infection, in PLoS Pathogens. 2018, Public Library of Science.

85. Engel, M., et al., The Role of m6A/m-RNA Methylation in Stress Response Regulation, in Neuron. 2018. p. 389-403.e9.

86. Chen, X.-Y., et al., The m6A Reader YTHDF1 Facilitates the Tumorigenesis and Metastasis of Gastric Cancer via USP14 Translation in an m6A-Dependent Manner. Frontiers in Cell and Developmental Biology, 2021. 9(561).

87. Li, Y., et al., Flexible binding of m6A reader protein YTHDC1 to its preferred RNA motif, in bioRxiv. 2019. p. 787937.

88. Xiao, W., et al., Nuclear m(6)A Reader YTHDC1 Regulates mRNA Splicing. Mol Cell, 2016. 61(4): p. 507-519.

89. Kim, G.-W., H. Imam, and A. Siddiqui, The RNA binding proteins YTHDC1 and FMRP regulate the nuclear export of N6-methyladenosine modified Hepatitis B Virus transcripts and affect the viral life cycle, in Journal of Virology. 2021.

90. Ries, R.J., et al., m6A enhances the phase separation potential of mRNA, in Nature. 2019.

91. Mao, Y., et al., m6A in mRNA coding regions promotes translation via the RNA helicase-containing YTHDC2, in Nature Communications. 2019.

92. Asamitsu, S. and N. Shioda, Potential roles of G-quadruplex structures in RNA granules for physiological and pathological phase separation, in Journal of Biochemistry. 2021. p. 527-533.

93. Cochard, A., et al., RNA at the surface of phase-separated condensates impacts their size and number, in Biophysical Journal. 2022, Biophysical Society. p. 1675-1690.

94. Tian, S., H.A. Curnutte, and T. Trcek, RNA Granules: A View from the RNA Perspective. Molecules, 2020. 25(14).

95. Fu, Y. and X. Zhuang, m6A-binding YTHDF proteins promote stress granule formation. Nature Chemical Biology, 2020. 16(9): p. 955-963.

96. Tan, B. and S.J. Gao, RNA epitranscriptomics: Regulation of infection of RNA and DNA viruses by N6-methyladenosine (m6A), in Reviews in Medical Virology. 2018.

97. Ye, F. and C.W. Reserve, RNA N6-Adenosine Methylation (m6A) Steers Epitranscriptomic Control of Herpesvirus Replication, in Inflammation & Cell Signaling. 2018.

98. Lichinchi, G., et al., Dynamics of the human and viral m(6)A RNA methylomes during HIV-1 infection of T cells. Nat Microbiol, 2016. 1: p. 16011.

99. Kennedy, E.M., et al., Posttranscriptional m6A Editing of HIV-1 mRNAs Enhances Viral Gene Expression, in Cell Host and Microbe. 2016, Elsevier Inc. p. 675-685.

100. Courtney, D.G., et al., Epitranscriptomic Enhancement of Influenza A Virus Gene Expression and Replication, in Cell Host and Microbe. 2017, Elsevier Inc. p. 377-386.e5.

101. Kim, G.W. and A. Siddiqui, The role of N6-methyladenosine modification in the life cycle and disease pathogenesis of hepatitis B and C viruses, in Experimental and Molecular Medicine. 2021, Springer US. p. 339-345.

102. Tan, B. and S.-j. Gao, The RNA Epitranscriptome of DNA Viruses. 2018. p. 1-9.

103. Tan, B., et al., Viral and cellular N 6 -methyladenosine and N 6 ,2′-O-dimethyladenosine epitranscriptomes in the KSHV life cycle, in Nature Microbiology. 2017, Nature Publishing Group. p. 108-120.

104. Ye, F., R. Chen, and T. Nilsen, Kaposi's Sarcoma-Associated Herpesvirus Utilizes and Manipulates RNA N6- Adenosine Methylation To Promote Lytic Replication. 2017, Journal of Virology. p. 1-21.

105. Baquero-Perez, B., et al., The Tudor SND1 protein is an m 6 A RNA reader essential for replication of Kaposi's sarcoma-associated herpesvirus.

106. Zhou, K.I., et al., Regulation of Co-transcriptional Pre-mRNA Splicing by m6A through the Low-Complexity Protein hnRNPG, in Molecular Cell. 2019.

107. Tingey, M. and W. Yang, Unraveling docking and initiation of mRNA export through the nuclear pore complex. Bioessays, 2022. 44(8): p. e2200027.

108. Petrovic, S., et al., Structure and Function of the Nuclear Pore Complex. Cold Spring Harb Perspect Biol, 2022. 14(12).

109. Bensidoun, P., D. Zenklusen, and M. Oeffinger, Choosing the right exit: How functional plasticity of the nuclear pore drives selective and efficient mRNA export. Wiley Interdiscip Rev RNA, 2021. 12(6): p. e1660.

110. Delaleau, M. and K.L.B. Borden, Multiple export mechanisms for mRNAs, in Cells. 2015. p. 452-473.

111. Gallouzi, I.E., C.M. Brennan, and J.A. Steitz, Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock. RNA, 2003. 9(11): p. 1410.

112. Brennan, C.M., I.E. Gallouzi, and J.A. Steitz, Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J Cell Biol, 2000. 151(1): p. 1-14.

113. Barreau, C., L. Paillard, and H.B. Osborne, AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res, 2005. 33(22): p. 7138-50.

114. Kimura, T., et al., Novel cis-active structures in the coding region mediate CRM1-dependent nuclear export of IFN-α 1 mRNA, in Medical Molecular Morphology. 2010. p. 145-157.

115. Culjkovic-Kraljacic, B., et al., Combinatorial targeting of nuclear export and translation of RNA inhibits aggressive B-cell lymphomas, in Blood. 2016. p. 858-868.

116. Culjkovic, B., et al., eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3′UTR, in Journal of Cell Biology. 2005. p. 245-256.

117. Daly, R., D.A. Khaperskyy, and M.M. Gaglia, Fine-tuning a blunt tool: Regulation of viral host shutoff RNases. PLoS Pathog, 2020. 16(4): p. e1008385.

118. Uppal, T., et al., The DNase Activity of Kaposi's Sarcoma-Associated Herpesvirus SOX Protein Serves an Important Role in Viral Genome Processing during Lytic Replication, in Journal of Virology. 2019. p. 1-20.

119. Hutin, S., Y. Lee, and B.A. Glaunsinger, An RNA element in human interleukin 6 confers escape from degradation by the gammaherpesvirus SOX protein. J Virol, 2013. 87(8): p. 4672-82.

120. Glaunsinger, B., L. Chavez, and D. Ganem, The exonuclease and host shutoff functions of the SOX protein of Kaposi's sarcoma-associated herpesvirus are genetically separable. J Virol, 2005. 79(12): p. 7396-401.

121. Martin, S.E., et al., The m6A landscape of polyadenylated nuclear (PAN) RNA and its related methylome in the context of KSHV replication. Rna, 2021.

122. Hsu, P.J., et al., Ythdc2 is an N6 -methyladenosine binding protein that regulates mammalian spermatogenesis, in Cell Research. 2017, Nature Publishing Group. p. 1115-1127.

123. Wojtas, M.N., et al., Regulation of m6A Transcripts by the 3′→5′ RNA Helicase YTHDC2 Is Essential for a Successful Meiotic Program in the Mammalian Germline, in Molecular Cell. 2017, Elsevier Inc. p. 374-387.e12.

124. Kretschmer, J., et al., The m6A reader protein YTHDC2 interacts with the small ribosomal subunit and the 5′-3′ exoribonuclease XRN1, in Rna. 2018. p. 1339-1350.

125. Shi, H., et al., YTHDF3 facilitates translation and decay of N6-methyladenosine-modified RNA. Cell Research, 2017. 27(3): p. 315-328.

126. Zhang, Y., et al., RNA-binding protein YTHDF3 suppresses interferon-dependent antiviral responses by promoting FOXO3 translation, in Proceedings of the National Academy of Sciences of the United States of America. 2019. p. 976-981.

127. Gao, Y., et al., m(6)A Modification Prevents Formation of Endogenous Double-Stranded RNAs and Deleterious Innate Immune Responses during Hematopoietic Development. Immunity, 2020. 52(6): p. 1007-1021.e8.

128. Jia, W., et al., New perspectives of physiological and pathological functions of nucleolin (NCL), in Life Sciences. 2017, Elsevier. p. 1-10.

129. Tonello, F., M.L. Massimino, and C. Peggion, Nucleolin: a cell portal for viruses, bacteria, and toxins, in Cellular and Molecular Life Sciences. 2022, Springer International Publishing. p. 1-17.

130. Doron-Mandel, E., et al., The glycine arginine-rich domain of the RNA-binding protein nucleolin regulates its subcellular localization, in The EMBO Journal. 2021. p. 1-22.

131. Santos, T., et al., Nucleolin: a binding partner of G-quadruplex structures, in Trends in Cell Biology. 2022, Elsevier Ltd. p. 1-4.

132. Bouvet, P., et al., Recognition of pre-formed and flexible elements of an RNA stem-loop by nucleolin. J Mol Biol, 2001. 309(3): p. 763-75.

133. Bugler, B., et al., RNA binding fragments from nucleolin contain the ribonucleoprotein consensus sequence. J Biol Chem, 1987. 262(23): p. 10922-5.

134. Serin, G., et al., Two RNA-binding domains determine the RNA-binding specificity of nucleolin. J Biol Chem, 1997. 272(20): p. 13109-16.

135. Creancier, L., et al., Determination of the functional domains involved in nucleolar targeting of nucleolin. Mol Biol Cell, 1993. 4(12): p. 1239-50.

136. Ghisolfi, L., et al., The glycine-rich domain of nucleolin has an unusual supersecondary structure responsible for its RNA-helix-destabilizing properties. J Biol Chem, 1992. 267(5): p. 2955-9.

137. Lapeyre, B., et al., Protein and cDNA sequence of a glycine-rich, dimethylarginine-containing region located near the carboxyl-terminal end of nucleolin (C23 and 100 kDa). J Biol Chem, 1986. 261(20): p. 9167-73.

138. Berger, C.M., X. Gaume, and P. Bouvet, The roles of nucleolin subcellular localization in cancer. Biochimie, 2015. 113: p. 78-85.

139. Schmidt-Zachmann, M.S. and E.A. Nigg, Protein localization to the nucleolus: a search for targeting domains in nucleolin. J Cell Sci, 1993. 105 (Pt 3): p. 799-806.

140. Gaume, X., et al., Centrosomal nucleolin is required for microtubule network organization, in Cell Cycle. 2015. p. 902-919.

141. Daniely, Y. and J.A. Borowiec, Formation of a Complex between Nucleolin and Replication Protein a after Cell Stress Prevents Initiation of DNA Replication. Journal of Cell Biology, 2000. 149(4): p. 799-810.

142. Kim, K., et al., Novel checkpoint response to genotoxic stress mediated by nucleolin-replication protein a complex formation. Mol Cell Biol, 2005. 25(6): p. 2463-74.

143. Das, S., et al., Characterization of nucleolin K88 acetylation defines a new pool of nucleolin colocalizing with pre-mRNA splicing factors. FEBS Lett, 2013. 587(5): p. 417-24.

144. Perry, R.B.T., et al., Nucleolin-Mediated RNA Localization Regulates Neuron Growth and Cycling Cell Size, in Cell Reports. 2016, The Author(s). p. 1664-1676.

145. Hovanessian, A.G., et al., The Cell-Surface-Expressed Nucleolin Is Associated with the Actin Cytoskeleton. Experimental Cell Research, 2000. 261(2): p. 312-328.

146. Legrand, D., et al., Surface nucleolin participates in both the binding and endocytosis of lactoferrin in target cells. European Journal of Biochemistry, 2004. 271(2): p. 303-317.

147. Matthews, D.A., Adenovirus protein V induces redistribution of nucleolin and B23 from nucleolus to cytoplasm. J Virol, 2001. 75(2): p. 1031-8.

148. Balinsky, C.A., et al., Nucleolin interacts with the dengue virus capsid protein and plays a role in formation of infectious virus particles. Journal of Virology, 2013. 87(24): p. 13094-13106.

149. Rodriguez, W., et al., C19ORF66 Broadly Escapes Virus-Induced Endonuclease Cleavage and Restricts Kaposi's Sarcoma-Associated Herpesvirus. Journal of Virology, 2019. 93(12): p. e00373-19.

150. Neil, C.R., et al., L-bodies are RNA-protein condensates driving RNA localization in Xenopus oocytes. Mol Biol Cell, 2021. 32(22): p. ar37.

151. Macveigh-Fierro, D., et al., The m(6)A reader YTHDC2 is essential for escape from KSHV SOX-induced RNA decay. Proc Natl Acad Sci U S A, 2022. 119(8).

152. Yanai, H., et al., HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature, 2009. 462(7269): p. 99-103.

153. Ivanov, S., et al., A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood, 2007. 110(6): p. 1970-81.

154. Li, C., et al., The RNA-binding protein LUC7L2 mediates MITA/STING intron retention to negatively regulate innate antiviral response. Cell Discov, 2021. 7(1): p. 46.

155. Steiman-Shimony, A., O. Shtrikman, and H. Margalit, Assessing the functional association of intronic miRNAs with their host genes, in Rna. 2018. p. 991-1004.

156. Monteuuis, G., et al., The changing paradigm of intron retention: Regulation, ramifications and recipes, in Nucleic Acids Research. 2019, Oxford University Press. p. 1149711513.

157. Buckley, P.T., et al., Cytoplasmic intron retention, function, splicing, and the sentinel RNA hypothesis, in Wiley Interdisciplinary Reviews: RNA. 2014. p. 223-230.

158. Li, Y., et al., An intron with a constitutive transport element is retained in a Tap messenger RNA, in Nature. 2006. p. 234-237.

159. Lutter, D., et al., Intronic microRNAs support their host genes by mediating synergistic and antagonistic regulatory effects, in BMC Genomics. 2010.

160. Li, Y., et al., An NXF1 mRNA with a retained intron is expressed in hippocampal and neocortical neurons and is translated into a protein that functions as an Nxf1 cofactor, in Molecular Biology of the Cell. 2016. p. 3903-3912.

161. Rekosh, D. and M.L. Hammarskjold, Intron retention in viruses and cellular genes: Detention, border controls and passports, in Wiley Interdisciplinary Reviews: RNA. 2018.

162. Volpon, L., et al., A biochemical framework for eIF4E-dependent mRNA export and nuclear recycling of the export machinery, in Rna. 2017. p. 927-937.

163. Meng, W. and S.J. Gao, Targeting XPO1 enhances innate immune response and inhibits KSHV lytic replication during primary infection by nuclear stabilization of the p62 autophagy adaptor protein, in Cell Death and Disease. 2021, Springer US. p. 1-16.

164. Khong, A., Isolation of mammalian SG cores for RNA-Seq analysis, in Methods. 2018. p. 1-18.

165. Ghisolfi-Nieto, L., et al., Nucleolin is a sequence-specific RNA-binding protein: characterization of targets on pre-ribosomal RNA. J Mol Biol, 1996. 260(1): p. 34-53.

166. Xia, T.L., et al., N(6)-methyladenosine-binding protein YTHDF1 suppresses EBV replication and promotes EBV RNA decay, in EMBO reports. 2021. p. 1-23.

167. Lu, M., et al., N6-methyladenosine modification enables viral RNA to escape recognition by RNA sensor RIG-I, in Nature Microbiology. 2020, Springer US. p. 1-15.

168. Mcfadden, M.J. and S.M. Horner, Review N 6 -Methyladenosine Regulates Host Responses to Viral Infection, in Trends in Biochemical Sciences. 2020, Elsevier Ltd. p. 1-12.

169. Xue, M., et al., Viral N6-methyladenosine upregulates replication and pathogenesis of human respiratory syncytial virus, in Nature Communications. 2019, Springer US. p. 4595.

170. Fleming, A.M., N.L.B. Nguyen, and C.J. Burrows, Colocalization of m 6 A and G-Quadruplex-Forming Sequences in Viral RNA (HIV, Zika, Hepatitis B, and SV40)

Suggests Topological Control of Adenosine N 6 -Methylation, in ACS Central Science. 2019, American Chemical Society. p. 218-228.