UNIVERSITY OF CALIFORNIA

Santa Barbara

Inhibition of the eIF4E-kinase MNK as a Strategy for Controlling Kaposi's Sarcoma-associated Herpesvirus Replication

A Thesis submitted in partial satisfaction of the

requirements for the degree Master of Arts

in Molecular, Cellular and Developmental Biology

by

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December 2021

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September 2021

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Carolina Arias for taking a chance on a graduating senior and allowing me to take the time to grow and learn as a scientist. The transition from an undergraduate mentee to a graduate researcher was difficult and you taught me how to take ownership of my project and my science, which are skills I will carry on throughout my career. You opened my eyes to the world of virology and gave me a chance to contribute to it. To Dr. Diego Acosta-Alvear, thank you for fostering my love for biochemistry. Your class helped me learn how to think about science not just as facts in a book to be accepted, but something that is to be questioned and explored. To Dr. Kathy Foltz, your advice and support as graduate student advisor and as a committee member has been invaluable. Thank you so much for all you do for our MCDB program. To Dr. Fritzi-Braig Karzig, you have been instrumental in making sure our lab runs smoothly and I will always be grateful to you.

I would also like to thank Guillermo Najarro for being not only an amazing first mentor, but for being a great friend. I will always treasure not only the scientific knowledge and skills that you passed on to me, but our friendship. I am also grateful for the help given to me by Kevin Brackett when generating my double knockdown cell line. You developed the parental cell line that I used and were my go-to person for questions and help I needed regarding the establishment of the cells. Although the experiments I performed with Zika virus ultimately did not end up in my thesis, thank you to Duncan Proctor for helping me with the design of the experiments and letting me use your resources. You have been not only a great lab mate to have, but also a good friend. Also, to Zach Aralis, despite the data not being in my thesis, thank you for your help and guidance when I was working with

HSV-1. To the members of the Arias lab and the Acosta-Alvear lab past and present: Sabrina Solley, Adriana Ramirez, Dr. Jose Carlos Ponce Rojas, Dr. Francesca Zappa, Nerea Muniozguren, Soham Chowdhury, Michael Costello, Michelle Grunberg, Dylan David, and Alyse Ptacek, thank you for your contributions during lab meetings and making this lab a great place to work.

Thank you to Hannah Ghasemi and Kirstin Rollins for being an amazing support system. I'm grateful to have you as friends and I'm excited to see you become the amazing scientists I know you will be.

Finally, thank you to my family for giving me the opportunity to pursue and achieve my dreams. Thank you for being so understanding and giving me the time to figure out my path in life.

ABSTRACT

Inhibition of the eIF4E-kinase MNK as a Strategy for Controlling Kaposi's Sarcoma-associated Herpesvirus Replication

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During viral infection, viruses hijack the host protein translation machinery to efficiently produce their own proteins for subsequent infection. Cap-dependent translation relies on the formation of the heterotrimeric cap-binding complex, eIF4F. The formation and activity of this complex is regulated by different pathways that modulate the phosphorylation status of the eIF4F components. Two such pathways, p38 and ERK, converge on the phosphorylation of the MAP kinase interacting kinases 1 and 2 (Mnk1 and Mnk2), which are the sole kinases of the cap-binding protein eIF4E, an integral component of eIF4F. These kinases are required for tumor formation and in response to cellular and environmental stress, but are negligible for normal cellular functions and development. The role of these kinases during viral infection have been thoroughly investigated. In the context of infection by herpesviruses, several studies demonstrated that MNK activity is upregulated in cells infected by Herpes Simplex Virus-1 (HSV-1), Human Cytomegalovirus (HCMV) and Kaposi's Sarcoma Associated Herpesvirus (KSHV). The phosphorylation of eIF4E is critical

for herpesvirus replication as genetic and pharmacological inhibition reduces virus production. My goal is to exploit MNK1/2 and eIF4E phosphorylation as a potential therapeutic target for KSHV.

KSHV is an oncogenic virus and the etiological agent for Kaposi's Sarcoma (KS), Multicentric Castleman's Disease, and primary effusion lymphoma (PEL), which are diseases commonly diagnosed in immunocompromised patients. KSHV is prevalent in 50% of the population in sub-Saharan Africa. Unfortunately, despite its continued occurrence, there are currently no FDA-approved therapeutics against KSHV-related diseases. Because of this, there is strong interest in finding new targets for therapeutic intervention. The eIF4E kinase MNK1/2 has been considered a promising target to reduce KSHV replication. Previous studies demonstrate that inhibition of MNK1 with the small molecule inhibitor CGP57380 (CGP) reduced viral replication; however, this compound does not have an effect on the second eIF4E kinase, MNK2, and has since been reported to have numerous off-target effects.

To better evaluate the potential of MNK1/2 as therapeutic targets for KSHV infection, I evaluated pharmacological inhibition of both MNK isoforms with the compound Tomivosertib, and genetic ablation of MNK1/2 in KSHV infected cells. My studies show that treatment with Tomivosertib downregulates the levels of the KSHV structural proteins and inhibits viral replication. Using CRISPRi technology, I established a MNK1/2 double knockdown, KSHV-infected cell line to evaluate the effect of genetic ablation of MNK1/2 on KSHV infection. As seen with the Tomivosertib treated cells, viral reactivation was reduced by over 50% in these cells. These findings suggest that MNK1 and MNK2 are promising therapeutic targets for the treatment of KSHV and its associated malignancies.

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Cellular response to stimuli can be regulated at an epigenetic, transcriptional, or translational level. Understanding how a cell responds to external stimuli such as stress, viral infection, or growth factors may allow us to develop ways to counteract the negative consequences that some of these stimuli may have on the cell and ultimately the overall health of the organism. Protein synthesis is heavily regulated at the initiation step. As translation is dysregulated in cancer and during viral infection, several studies focus on understanding the mechanisms behind translation initiation regulation in health and disease. An aspect of translation initiation which has been recently explored for the development of anti-cancer drugs is the phosphorylation of the cap-binding protein eIF4E by the MAPK Interacting Kinase (MNK). eIF4E phosphorylation is upregulated in cancer cells as it is during infection by several viruses. The effect of this phosphorylation event in translation initiation remains unclear; however, in infected cells, MNK activation and eIF4E phosphorylation are associated with higher translation rates of viral proteins and efficient viral replication.

In cells infected with Kaposi's Sarcoma Herpesvirus (KSHV), an oncogenic virus that causes life threatening malignancies in immunocompromised patients, eIF4E phosphorylation is critical for proper viral protein expression and replication. Because there is currently no approved treatment for clinical management of KSHV, the identification of novel therapeutic targets for this virus is imperative. Since MNK inhibition has been shown to mitigate viral infection and production of viral proteins, it represents an attractive potential target for controlling the replication of KSHV. The following sections provide background on eukaryotic

translation, viral translation, the MNK pathway and its dysregulation during viral infection, and KSHV.

A. Eukaryotic Translation

Eukaryotic translation is comprised of four general steps (initiation, elongation, termination, and ribosome recycling) to synthesize the proteins required for normal cell function. Translation is energetically costly, and for this reason it is mainly regulated at the initiation level (Jackson et al, 2010). Translation begins with the loading of the Met-tRNA$_i$ onto the 40s subunit of the ribosome by GTP bound eukaryotic initiation factor 2 (eIF2)—an interaction aided by eIF1, eIF1a, eIF5, and eIF3—to form the 43S pre-initiation complex (PIC) that will bind to messenger RNA (mRNA). The 43S PIC will scan the mRNA for the start codon. The interaction between the 43S PIC and the mRNA is mediated by the heterotrimeric eIF4F complex composed of the cap-binding protein eIF4E, the scaffold eIF4G, and the RNA helicase eIF4A. The m^{7}G cap present at the 5' of the mRNA is recognized and bound by eIF4E. In turn, eIF4E interacts with eIF4G and eIF4A to form the eIF4F complex. The scaffold protein eIF4G interacts with eIF3 and bridges the recruitment of the mRNA to the 43S. The 80S initiation complex (IC) is formed when the 60S subunit is recruited upon the recognition of the AUG codon by the 43S (Hinnebush, 2014) **(Figure 1)**.

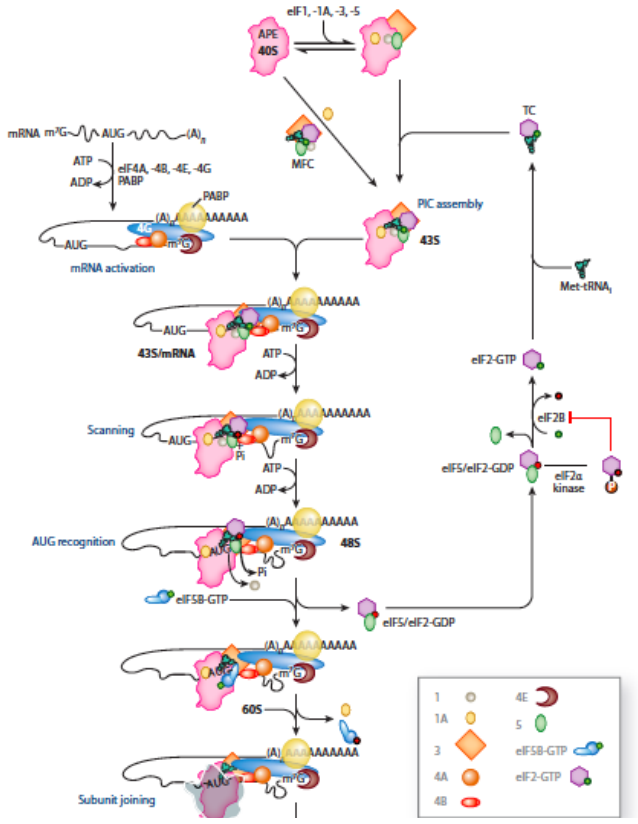

Figure 1. Cannonical eukaryotic cap-dependent translation pathway. Figure from Hinnebusch, 2014.

Once the 80S IC has been formed, translational elongation, which involves the loading of aminoacyl-transfer RNAs (aa-tRNAs) corresponding to the mRNA codon on the open reading frame (ORF) occurs. Elongation uses three main steps to load the proper amino acid to the growing peptide chain: tRNA selection, peptide bond formation, and translocation of the mRNA-tRNA complex. These three processes are aided by elongation factors (EFs) and powered by GTP hydrolysis reactions, performed by the GTPase eEF2, as the peptide chain is translocated to the respective A, P, and E sites on the ribosome (Schuller and Green, 2018) **(Figure 2).** Elongation will continue along the ORF until one of the three stop codons is recognized, and this will initiate the final step of eukaryotic protein translation: termination.

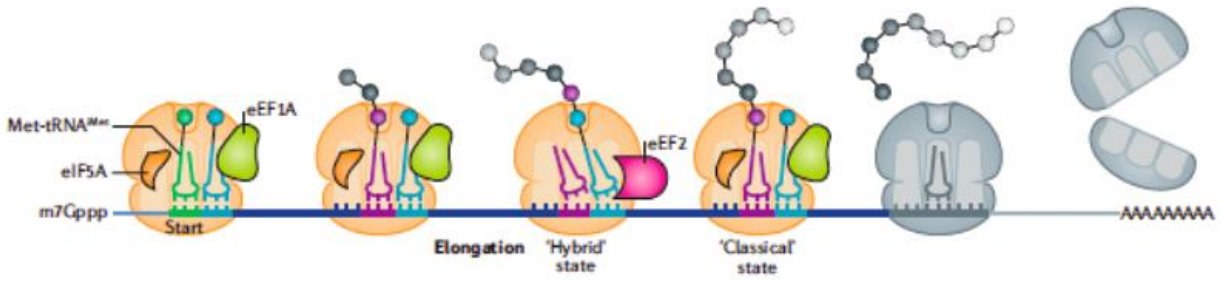

Figure 2. Overview of eukaryotic translation elongation and termination. Adapted from Schuller and Green, 2018.

During termination, the release factor eRF1 will recognize one of the three stop codons (UAG, UAA, UGA), which will cause the release of the nascent peptide. Termination is

dependent on the hydrolysis of GTP, performed by the GTPase eRF3 (Schuller and Green, 2018). Following termination, eukaryotes are capable of performing ribosome recycling, an important factor for continual protein synthesis. Eukaryotic ribosome recycling is an active area of investigation; however, it has been found that the ABC type ATPase (ABCE1) is necessary and essential for this process.

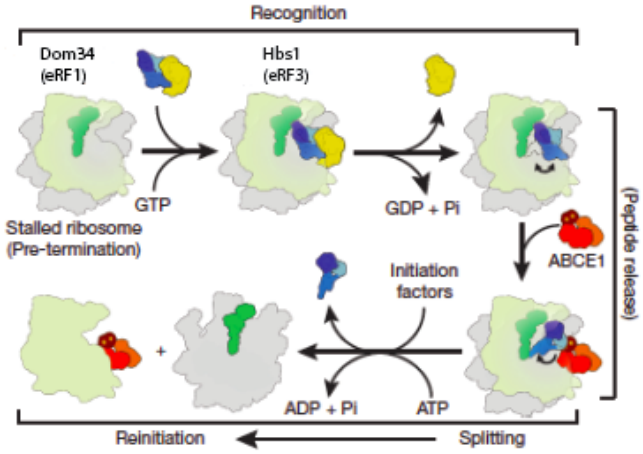

Figure 3. Eukaryotic ribosome recycling. Adapted from Becker et al, 2012.

For recycling initiated due to termination, ABCE1 in association with various eukaryotic initiation and release factors (eRF1, eIF2, eIF3, and eIF5) are utilized. Dom34 and Hbs1 are used for rescue and recycling of a stalled ribosome (Becker et al, 2014) **(Figure 3)**.

B. MAPK interacting kinases 1 and 2 (Mnk1/2) and Phosphorylation of eIF4E

The extracellular signal-regulated kinase (ERK) and p38 kinase belong to the MAPK (mitogen-activated protein kinase) family and are activated by several factors such as stress, cytokines, or growth factors. These kinases serve as critical nodes in determining cell fate by modulating proliferation, differentiation, survival, and apoptosis (Ueda et al, 2004). ERK and p38 both converge on and activate MNK1/2 (MAPK interacting kinase 1 and 2), the sole kinase responsible for phosphorylation of the cap-binding protein eIF4E on Ser-209. The phosphorylation of eIF4E alters the dynamic of translation and promotes protein synthesis in

response to stress, certain viral infections and in oncogenic processes (Hay, 2010) **(Figure 4)**. The MNK1 and MNK2 protein isoforms are generated by alternative splicing of the MNK transcript and are differentially activated. MNK1 is more responsive to growth factors, mitogens, stress, and cytokines, while MNK2 has high basal activity and is less responsive to external stimuli (Joshi and Platanias, 2014).

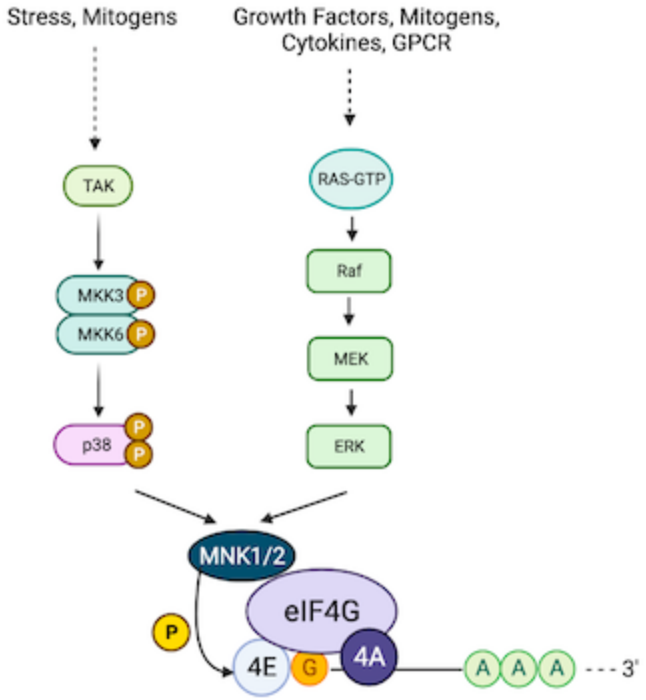

Figure 4. Pathways leading to eIF4E phosphorylation by MNK1/2.

MNK1/2 have been investigated as a potential therapeutic target for the development of anticancer drugs due to high levels of eIF4E phosphorylation seen in certain malignancies including lung, prostate, head and neck, breast and leukemias and lymphomas (Siddiqui and Sonenberg, 2015 and Carroll and Borden, 2013). The oncogenic role of MNK1/2 was demonstrated by Ueda et al, in a model of Lck-PTEN ($tPTEN^{-/-}$) mice. These mice lack the tumor suppressor PTEN (phosphatase and tensin homologue on chromosome 10) and have a higher susceptibility to tumorigenesis compared to wild-type mice. By crossing $tPTEN^{-/-}$ mice with MNK1/2 double knockdown mice ($tPTEN^{-/-}$; MNK-DKD), they observed a reduction in tumorigenesis compared to $tPTEN^{-/-}$ mice with no accompanying negative effects on the health of the host. Despite the critical role of MNK1/2 in response to stress, these kinases are not essential for normal cell function or

development, as shown by knock-down and knock-out studies in cell culture and mouse models (Ueda et al, 2004). Given these findings, MNK may be a promising therapeutic target because knockdown of MNK appears to reduce the incidence of malignancies with no deleterious effect on the host.

C. Viral Translation

Viruses lack the essential machinery for protein synthesis and must hijack the host factors for this purpose. Several mechanisms have been utilized by viruses to reduce the expression of host transcripts and shift the resources from the host to the virus, which is known as "host shut off." The two most notable methods of host shut off are cap-independent translation, first discovered in Picornaviruses, and cap-snatching as demonstrated by Orthomyxoviridae. Host shut off can also be achieved via degradation of host mRNAs by viral proteins such as those encoded by Herpes Simplex Virus-1 (HSV-1).

Viral cap-independent translation bypasses the need for recognition of the m^7G cap and precludes host translation in favor of viral translation (Stewart and Semler, 1997). Viruses in the Picornavirus family employ two different methods of cap independent translation. The first is achieved via the formation of secondary structures on the non-coding region (NCR) on viral RNAs called the internal ribosome entry site (IRES) that allows the ribosome to bind without recognition and recruitment by the eIF4F cap-binding complex. Alternatively, picornavirus infections also cause the proteolytic cleavage of eIF4G, which further disrupts host protein synthesis and favors viral protein synthesis.

Another method of host shut off occurs when viruses manipulate cap-dependent translation machinery through "cap-snatching" or through degradation of host mRNAs. Cap-

snatching is a method used by viruses in the Orthomyxoviridae family causing the depletion of the host mRNAs by cleavage of host caps and translocating them to the viral RNAs (Vlugt et al, 2018). Host shut off is also achieved when cellular mRNAs are degraded to shift the host cell's resources and energy to the translation of viral transcripts. For instance, HSV-1 encodes a viral protein VHS (Viral Host Shutoff) that is associated with a global increase in the rate of mRNA degradation, and subsequent decrease in host mRNA transcripts (He et al, 2020).

Furthermore, viruses can manipulate the formation of the cap-binding complex to inhibit host mRNA translation. Encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV) dephosphorylate 4E-BP1, the protein responsible for binding to eIF4E to inhibit its interaction with eIF4G to form the eIF4F cap-binding complex. These dephosphorylation events result in reduced translation of host cellular transcripts in favor of translation of viral transcripts. In contrast, HSV-1 utilizes the viral gene ICP0 for proteasomal degradation of 4E-BP1, and infected cells were found to have phosphorylated eIF4E (Montero, Garcia-Roman, and Mora, 2015).

D. Dysregulation of Mnk Activity During Viral Infection

Several viruses manipulate the formation, levels, and phosphorylation status of the eIF4F complex during infection. As seen during oncogenic processes, MNK1/2 activity and eIF4E phosphorylation are often dysregulated during infection. Some viruses such as ECMV and VSV dephosphorylate eIF4E, while others such as herpesviruses, poxviruses, flaviviruses and coronaviruses stimulate phosphorylation (Herdy et al, 2012, Banjeree, et al 2002, Roth et al, 2017).

Based on these observations, MNK inhibition is an attractive strategy for antiviral development. Studies by our group and others in herpesvirus infected cells (HSV-1, HCMV and KSHV) demonstrated that there is a reduction in viral protein synthesis and viral replication upon MNK inhibition. Historically, these studies were done using the most potent MNK inhibitor available at the time, CGP57380 (CGP). CGP is an effective MNK1 inhibitor, that results in robust dephosphorylation of eIF4E with no cellular toxicity or effect on p38 and ERK (Knauf, Tschopp, and Gram, 2001). CGP treatment of cells infected with HSV-1, resulted in a dramatic reduction in viral protein production and over 100-

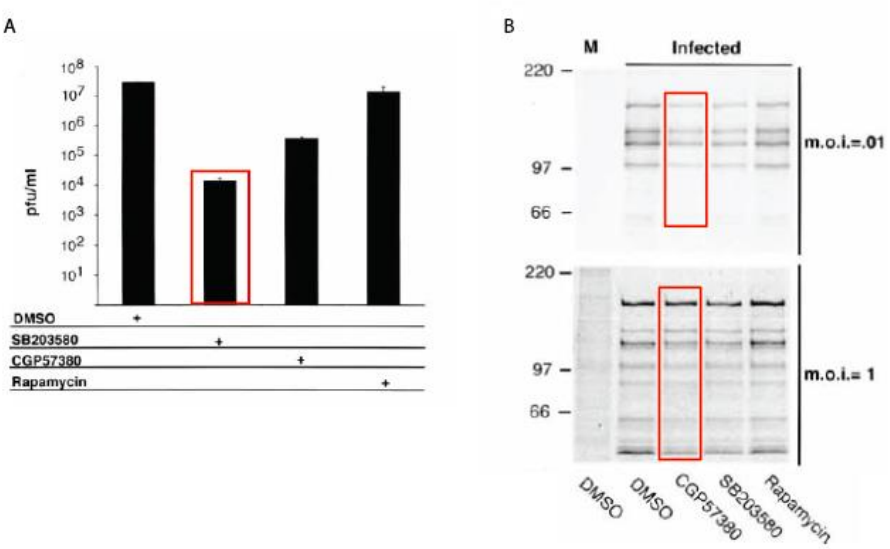

Figure 5. Inhibition of eIF4E phosphorylation by CGP results in a decrease in HSV-1 viral load and viral protein production in serum starved primary human fibroblast cells (NHDF). (A) NHDF cells infected at a low multiplicity (MOI=0.01) were treated with the p38 inhibitor SB203580, MNK1 inhibitor CGP, or MTOR inhibitor Rapamycin. 5 days post infection, viral supernatants were collected, and viral titers were done in Vero cells. (B) NHDF cells were mock (M), infected with wild-type (WT) HSV-1, or treated as in A. 14 hours post infection, cells were pulsed with ^{35}S for 1 hour. Prior to SDS-PAGE analysis, samples infected at an MOI= 0.01 were first immunoprecipitated with anti-HSV-1 antisera. Samples infected at an MOI=1 were analyzed with SDS-PAGE without immunoprecipitation with anti-HSV-1 antisera (Walsh and Mohr, 2004).

fold reduction in viral load (Walsh and Mohr, 2004) **(Figure 5 a, b)**. Similar results were obtained in a study by Arias et al in 2009 in the context of KSHV infection. Treatment with the CGP compound resulted in a robust reduction in expression of the KSHV viral proteins ORF59, the viral DNA processivity factor, and RTA (ORF50), the lytic switch protein (Arias et al, 2009) **(Figure 6 a, b)**. These results indicate that the Mnk1 protein is a potential candidate for the development of therapeutics for viruses in the Herpesviridae family.

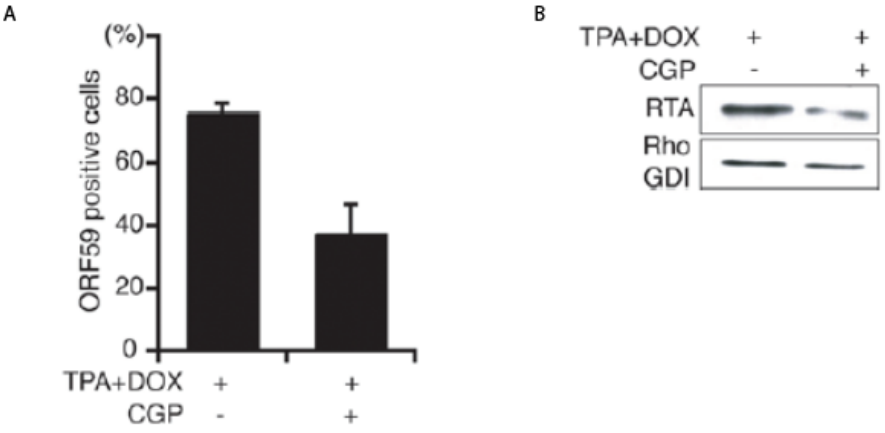

Figure 6. Inhibition of eIF4E phosphorylation in the PEL cell line TREx-BCBL1-RTA during KSHV infection results in a reduction in viral protein production. TREx-BCBL1-RTA cells were reactivated with a phorbol ester (TPA) and doxycycline (DOX) and treated with CGP for 48 hours prior analysis. (A) Quantification of an immunofluorescence assay for the KSHV ORF59 protein. Cells were fixed and permeabilized, then immunostained with antisera specific for the KSHV ORF59 protein. (B) SDS-PAGE analysis of KSHV RTA (ORF50) protein (Arias et al, 2009).

E. Kaposi's Sarcoma Herpesvirus (KSHV)

Kaposi's Sarcoma Herpesvirus (KSHV) is an oncogenic virus and the etiological agent of Kaposi's Sarcoma (KS), primary effusion lymphoma (PEL), and Multicentric Castleman's

Disease, malignancies commonly afflicting immunocompromised patients. The unusually high prevalence of KS in young homosexual men reported in 1981 was an indicator of the start of the HIV/AIDS pandemic (Beral et al, 1990). KSHV was identified as the causative agent for KS when sequences for a ɣ-herpesvirus (later named KSHV) were found in the hypervascularized tumors found in KS patients (Chang et al, 1992, Mesri et al, 2010). Since its discovery in 1994, the genome of KSHV has been sequenced and its open reading frames (ORFs) annotated. As with other herpesviruses, the life cycle of KSHV is divided in two stages: latency, a dormant viral state, and lytic reactivation, a stage characterized by a high transcriptional and translational output that leads to viral replication. Following primary infection, KSHV enters latency by the action of the Latency-Associated Nuclear Antigen (LANA), which binds the viral genome and tethers it to the host genome. The virus remains as a dormant chromatin-bound episome, with gene expression limited to a few viral products. Latency is reversed upon activation of a key viral protein; the Replication and Transcriptional Activator (RTA). This potent transcription factor elicits a temporal cascade of gene expression divided in three main stages: the immediate early, early, or late viral products (Arias et al, 2014).

Latency and lytic reactivation can be recapitulated in KSHV-infected cells in culture. In many of these models, it has been found that KSHV can undergo spontaneous lytic reactivation. To rectify this, a new cell line, iSLK.219, was developed from uninfected, epithelial SLK cells. In these cells, spontaneous lytic reactivation is minimal and entry to the lytic cycle can be controlled at will (Sturzl et al, 2012, Myoung and Ganem, 2012). iSLK.219s carry a doxycycline-inducible RTA transgene and are infected with a recombinant strain of KSHV that constitutively expresses puromycin N-acetyl transferase, a constitutive GFP reporter expressed in initial latent infection, and an RFP reporter expressed exclusively upon lytic reactivation.

iSLK.219 cells maintain a tight latent infection and can efficiently enter lytic reactivation upon induction of RTA expression via doxycycline (dox) treatment (Myoung and Ganem, 2012) **(Figure 7).**

There are currently no FDA-approved treatments in the clinic for KSHV infection; however, the critical role of MNK1/2 activity for KSHV infection and our previous observations with the MNK inhibitor CGP, prompted us to continue investigating MNK as a prospective therapeutic target. Due to the known off-target effects of MNK, and to explore the potential of MNK as an anti-KSHV target, I evaluated the effect of the dual and highly specific MNK1/2 inhibitor,

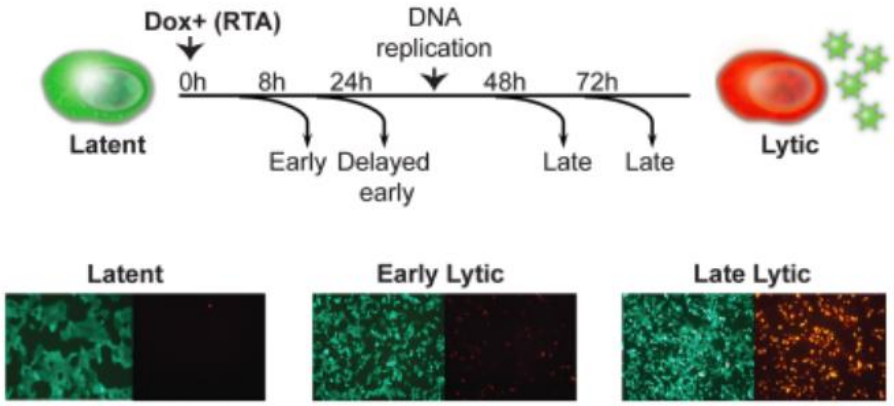

Figure 7. The iSLK.219 cell line allows for robust control of lytic reactivation with Doxycycline (Dox). Latent iSLK.219 cells will constitutively express GFP (GFP+). Treatment with doxycycline activates RTA and cells will enter the lytic cycle (RFP+) (Arias et al, 2014)

Tomivosertib (EFT508), on viral protein synthesis and replication. This small molecule inhibitor prevents the phosphorylation of eIF4E, and unlike CGP, has few off target effects at concentrations well above the IC50 (Reich et al, 2018). My results demonstrate that Tomivosertib inhibits the expression of late KSHV proteins, and the production of infectious viral particles. To confirm the importance of eIF4E phosphorylation in KSHV infection, I used CRISPRi technology to knock down MNK1 and MNK2 in iSLK.219 cells. When compared to wild-type (Wt) cells, viral reactivation in these cells is significantly reduced. My observations

confirm that MNK1/2 is an attractive therapeutic target, and opens a new avenue for the continued exploration of the potential of Tomivosertib as an antiviral drug.

II. Pharmacological Inhibition of MNK1/2 as a potential Anti-KSHV strategy

Previous studies have demonstrated that pharmacological inhibition of MNK1 using the small molecule inhibitor CGP during KSHV infection results in reduced translation of viral proteins and viral titers. While potent, CGP does not inhibit the MNK2 isoform and it shows extensive off-target effects against other kinases at low concentrations (Reich et al, 2018). In my research,

I aim to build upon previous findings and continue exploring the potential of MNK1/2 pharmacological and genetic inhibition as a mechanism to curtail KSHV infection. To investigate the effect of pharmacological inhibition of MNK, I treated cells with the dual MNK1/2 inhibitor, Tomivosertib, and evaluated its effect on viral load and viral protein production. Tomivosertib is an effective and highly specific MNK1/2 inhibitor currently in Phase 2 clinical trials as an anti-cancer agent. My results show that inhibition of MNK1/2 with this compound reduces viral late protein expression and production of infectious particles, which suggests a potential role for Tomivosertib and MNK inhibition as a strategy for KSHV treatment.

A. Treatment with Tomivosertib reduces KSHV late protein expression

1. Tomivosertib (EFT508): a promising anti-cancer compound

As the sole eIF4E-kinase, MNK has been thoroughly investigated for its role in regulating translation initiation and promoting protein synthesis in tumorigenesis and viral replication (see Chapter 1.D). While important in the context of cellular stress and pathogenesis, MNK is not essential for normal cell function and development. This is supported in MNK1/2 double knockdown studies in mice and cell culture, which demonstrate that knockdown of the kinases does not have deleterious effects. These characteristics, the role in cancer and viral pathogenesis, and the lack of requirement for normal cell function, make it a viable and attractive target for therapeutic development (see Chapter 1.B). Previous studies using the MNK1 inhibitor CGP, found increased tumor sensitivity to chemotherapy and reduction in viral load upon inhibition of MNK (Grzmil et al, 2016, Walsh and Mohr, 2004). Despite the positive and promising results of these studies, the compound was found to have off-target effects that may have contributed to the observed phenotypes. Additionally, CGP treatment does not target MNK2 activity, which may

mask the full potential of MNK1/2 inhibition. To address these caveats, novel MNK1/2 inhibitors are in development by several pharmaceutical companies.

One of the newest and most promising MNK1/2 inhibitors is Tomivosertib (Compound 23, EFT508), developed by Reich et al at Effector. Their initial goal was to investigate the role of eIF4E phosphorylation on tumorigenesis using a small molecule inhibitor. By targeting the unique residues Phe159 and Cys225 in the ATP binding site of MNK1/2 **(Figure 8a)**, favorable stereochemical interactions could be made that would improve both the selectivity and the potency of the inhibitor **(Figure 8b)**. The resulting optimized compound, Tomivosertib, is a potent and specific MNK1/2 inhibitor, currently in Phase 2 clinical trials for treatment of lymphomas and solid tumors (Reich et al, 2018). However, the antiviral potential of Tomivosertib has yet to be determined.

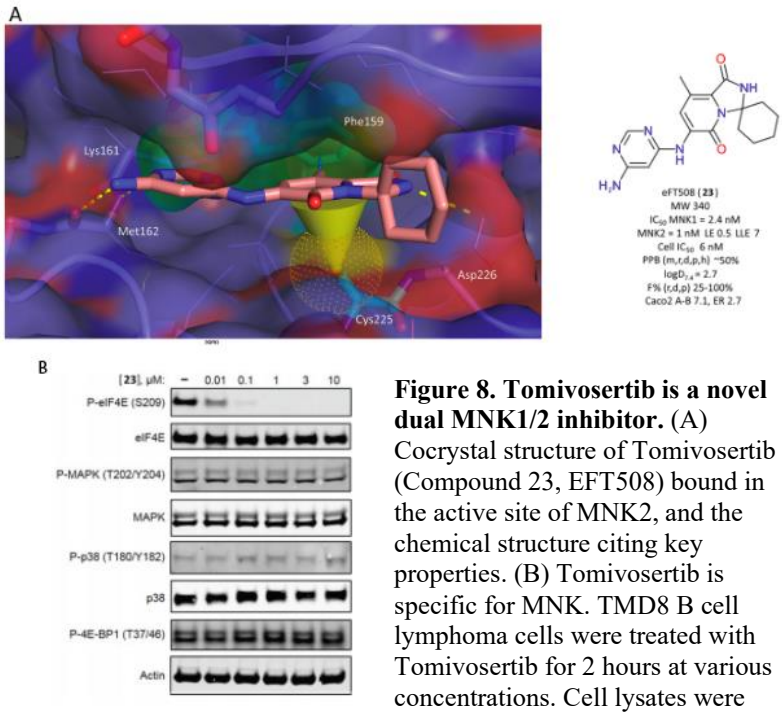

Figure 8. Tomivosertib is a novel dual MNK1/2 inhibitor. (A) Cocrystal structure of Tomivosertib (Compound 23, EFT508) bound in the active site of MNK2, and the chemical structure citing key properties. (B) Tomivosertib is specific for MNK. TMD8 B cell lymphoma cells were treated with Tomivosertib for 2 hours at various concentrations. Cell lysates were collected and immunoblotted with respective antibodies (Reich et al, 2018).

2. Treatment with Tomivosertib reduces KSHV late, but not early viral protein expression.

To evaluate the effect that Tomivosertib has on viral protein production and viral load during KSHV infection, we use a model system of KSHV infection known as iSLK.219 cells **(Figure 7)**. In these latently infected cells, KSHV can be induced to efficiently enter the lytic and

productive cycle of infection by exogenous expression of the transcription factor RTA following doxycycline (dox) treatment (see Chapter 1.E). iSLK.219 cells were pre-treated with 500nM of Tomivosertib, a concentration previously shown to down regulate MNK1/2 activity in cell culture, 6 hours prior to dox treatment. Tomivosertib was present in the media throughout the time course of viral reactivation. To evaluate the progress of viral reactivation in treated and untreated cells, I collected cell lysates to evaluate viral protein expression and viral supernatants to evaluate infectious particle production at 24, 48, and 72 hours post reactivation (see Methods).

Immunoblot analyses of Tomivosertib treated cell lysates displays a normal pattern of protein expression for two early viral proteins ORF 45, an immune modulator, and ORF 57, an RNA binding protein (Zhu and Yuan, 2020; Yuan et al, 2018). As expected for early KSHV proteins, ORF 45 and ORF57 were detected as early as 24 hours post reactivation and the levels increased by 48 and 72 hours **(Figure 9).** In contrast, immunoblot analyses of two the late, structural glycoproteins ORF62 and K8.1, exhibited a dramatic

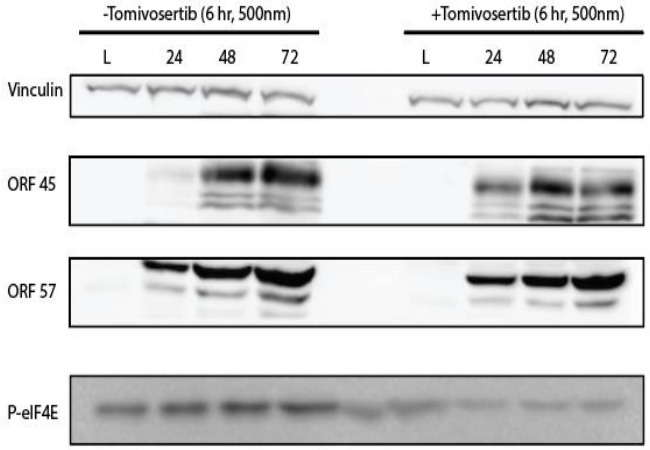

Figure 9. Treatment with Tomivosertib does not have a large effect on early protein production. iSLK.219 cells were pretreated with 500nM Tomivosertib (MNK1/2 inhibitor) for 6h, and then induced to enter the lytic cycle by exogenous expression of RTA (Dox treatment). At 24 hours post ORF 45 had an increase in expression with treatment; however, protein production recovered

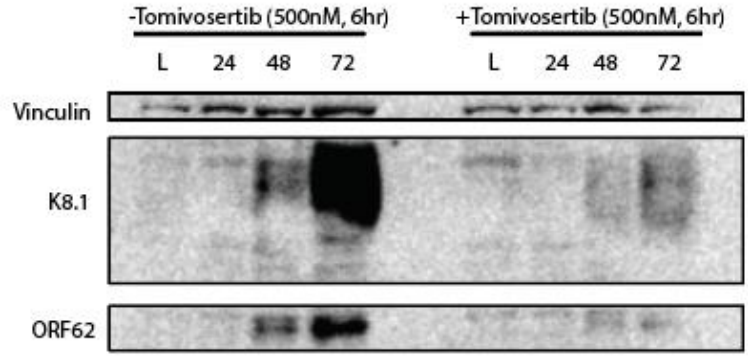

Figure 10. Pharmacologic inhibition of the eIF4E kinases MNK1/2 leads to a reduction in late protein production. Cells were treated as in Figure 9, then assayed for early protein production with immunoblot. Lysates were collected at the indicated time point post reactivation. The levels of the lytic late structural proteins K8.1 and ORF62 were reduced in the presence of the compound.

reduction in expression upon treatment with Tomivosertib **(Figure 10).** KSHV late protein synthesis requires the replication of the viral genome, as the newly replicated DNA acts as the template for transcription. My results suggest that treatment with Tomivosertib disrupts the progress of the lytic cycle. Given that proper structural protein expression is important for virion formation and subsequent KSHV infection, I determined the effect of Tomivosertib treatment on viral load.

B. Tomivosertib reduced KSHV viral load

To monitor virus replication, we collect the infectious viral particles released in the supernatant of iSLK.219 cells following reactivation with doxycycline (dox). We then titer the virus in uninfected cells and measure the number of infected cells (see Methods). Since the KSHV strain infecting iSLK.219 contains a constitutive GFP-reporter, the infected cells will be GFP positive. When we evaluate the concentration of virus in the supernatant collected from iSLK.219 cells, we can detect viral production starting at 72h post reactivation **(**appendix **Figure S1)**. iSLK.219 cells reactivated in the presence of Tomivosertib reveal a dose dependent reduction in the viral titer at 72 hours post reactivation with doxycycline. **(Figure 11)**. This

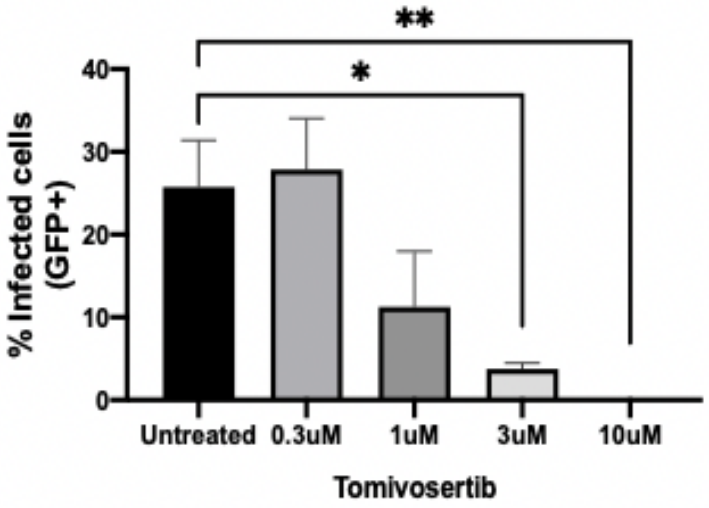

Figure 11. Dose dependent reduction in viral load. Cells were pretreated with the indicated dosages of Tomivosertib prior to reactivation. Supernatants were collected 72 hours post reactivation, then uninfected SLK cells were infected. Percent infected cells (GFP+) were determined 48 hours post infection. A robust reduction in KSHV viral replication occurred at 1, 3, and 10μM, respectively. (* P=0.01, **P=0.001)

observation supports the hypothesis that inhibition of the activity of the MNK kinases with Tomivosertib will reduce KSHV viral load.

C. Methods

1. Cell culture

iSLK.219 and SLK cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum, 1% streptomycin (1U/mL), 1% penicillin (1U/mL), and 1% glutamine. They were maintained at 37 °C with 5% CO_2. A pure iSLK.219 population was maintained using puromycin (10 mg/ml) selection.

For western blot sample collection and viral supernatant collection, iSLK.219 cells were seeded in a 6 well plate at a density of $2.5x10^5$ cells/well, then allowed to adhere overnight. They were pretreated with Tomivosertib at the indicated concentrations (0.5 μM, of 0.3μM, 1μM, 3μM, and 10μM; diluted with DMSO) 6 hours prior to reactivation with doxycycline (10 mg/mL). For viral infections, SLK cells were seeded in a 48 well plate at a density of $2.5x10^4$ cells/well.

2. Western Blot analyses

Samples were collected from a 6 well plate in 1X Laemmli Buffer (10% SDS, glycerol, 1M Tris-Cl at pH 6.8, H_2O, bromophenol blue, diluted to 1X with PBS), then boiled at ~95 °C before storage at -20°C. Prior to use, samples were thawed at room temperature, then sonicated to shear genomic DNA. 5% BME (v/v) was added to the samples, and samples were briefly centrifuged before loading onto a 12% SDS-PAGE gel. Samples were electrophoresed at 120V for ~90 minutes and wet-transferred onto a nitrocellulose membrane at 90V for 90 minutes at 4 °C. Efficacy of transfer was assessed using Ponceau staining. The membrane was blocked at room temperature for 1 hour in 5% dry milk in 1X TBST followed by three 5 minute washes with 1X TBST, and incubation in primary antibody (appendix **Table S1)** diluted to the indicated concentration in 3% BSA/1X TBST overnight at 4 °C on the shaker. After primary antibody exposure, the membrane was again washed three times for 5 minutes with 1X TBST before secondary antibody exposure. Secondary antibodies (appendix **Table S2)** were diluted with 5% dry milk in 1X TBST, then incubated on the membrane for 1 hour at room temperature. Following secondary antibody exposure, the membrane was again washed three times at 5 minute intervals before incubation with the Thermofisher Pierce ECL HRP and Peroxide Western Blotting Substrate. Imaging was done with the chemiluminescence function on the Azure Biosystems c300 imager.

3. Viral supernatant collection, viral infection, and viral titers

Viral supernatants were collected from a 6 well plate of iSLK.219 cells at their indicated time points. Supernatants were filtered (0.45 μm) to get rid of debris and ensure only viral particles were collected. SLK cells were seeded in a 48 well plate, and infected with 10-fold dilutions of viral supernatant to calculate an accurate viral titer. Dilutions were done in DMEM to achieve a

final infection volume of 250 µL. To infect, cells were spinoculated for 2 hours at 2000 RPM at 37°C. After spinoculation, the plate was placed in the incubator for 1 hour. After one hour, viral infection media was aspirated and replaced with normal DMEM.

After 48 hours, cells were detached from the plate using trypsin and resuspended in DMEM. A 10µL aliquot of the resuspended population was loaded onto each side of a countess slide, then viral titers were determined using the GFP light cube on the Life Technologies Countess II FL to identify the concentration and percent of infected (GFP+) cells. From the values obtained, fluorescent forming units per milliliter (FFU/mL) were calculated and plotted in Prism.

III. Genetic ablation of MNK1/2 in iSLK.219 cells resulted in a reduction in viral reactivation

The promising results obtained with the pharmacological inhibition of MNK1/2, encouraged me to confirm that our observations are due to abolishment of MNK activity and as opposed to off-target effects of the compound. For this purpose, I generated MNK1/2 double knockdown iSLK.219 (iSLK.219dC9 MNK DKD) cells using CRISPR interference (CRISPRi). Our results show a decrease in viral load in the MNK1/2 double knockdown cells compared to wild type iSLK.219 cells (Wt). The results of these experiments confirm that MNK activity plays a significant role in KSHV viral replication.

A. iSLK.219 dC9 MNK1/2 DKD cells inhibit phosphorylation of eIF4E

1. Generation of iSLK.219 dC9 MNK1/2 DKD cells

iSLK.219 dC9 MNK1/2 double knockdown (DKD) cells were generated using standard lentiviral transduction and CRISPRi technology (see Methods). The parental iSLK.219 dC9K cell line was developed using the techniques outlined in Brackett et al. These cells express a catalytically dead Cas9 protein fused to a KRAB repressor and BFP, and a blasticidin antibiotic selection marker. Knockdown of MNK1/2 was accomplished by transducing iSLK.219 dC9K cells with single-guide RNAs (sgRNAs) targeting the promoters of MNK1 and MNK2. The sgRNAs used in our experiments encode for a puromycin selection marker, and a BFP reporter that can be differentiated from the one in the dC9K construct. Following sgRNA transduction, the cells were selected using puromycin. Because puromycin is also necessary for maintenance of the iSLK.219 parental

cell line, fluorescent activated cell sorting (FACs) was performed twice to select for the iSLK.219 dC9 MNK DKD cells (see Methods) **(Figure 12)**.

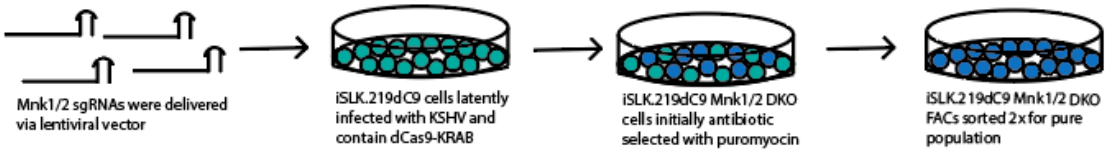

Figure 12. Generation of iSLK.219dC9 MNK DKD cell lines. iSLK.219dC9 cells were transduced with a lentiviral vector for MNK1 and MNK2 sgRNAs to generate iSLK.219dC9 Mnk1/2 DKD cells. Positive transducans were selected by FACs for high sgRNA/BFP+ expression. Cells were maintained in Puromycin for 7 days before experiments were done.

2. eIF4E phosphorylation is ablated in iSLK.219 dC9 MNK1/2 DKD

Once stable cell lines containing sgRNAs for MNK1/2 were generated, the efficacy of silencing was evaluated by immunoblotting for eIF4E phosphorylation and MNK1 expression (see Methods). Unfortunately, to date there is no commercially available antibody for MNK2; therefore, MNK1 protein levels were used to evaluate the efficiency of silencing. Thus, the levels of this protein cannot be evaluated. Compared to the iSLK.219 dC9 transduced with a non-targeting control (Gal4), in the MNK DKD cells there is a reduction in MNK1 protein expression, and eIF4E phosphorylation is below detection levels **(Figure 13)**. After confirming the knockdown of MNK1/2, I evaluated the effect of MNK1/2 ablation on viral reactivation.

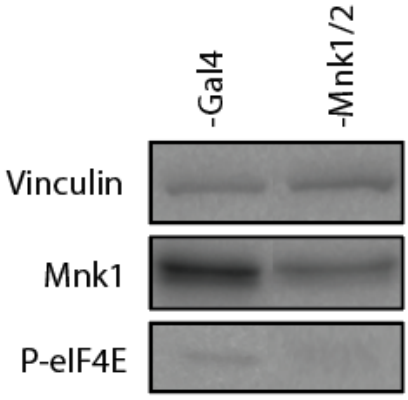

Figure 13. iSLK.219dC9 MNK DKD cell lines successfully inhibit phosphorylation of eIF4E. After selection with puromycin, Mnk 1 knockdown and inhibition of eIF4E phosphorylation was confirmed by immunoblot. Note: there is no commercially available antibody for Mnk2.

B. Genetic Ablation of MNK1 and MNK2 results in robust reduction in viral reactivation.

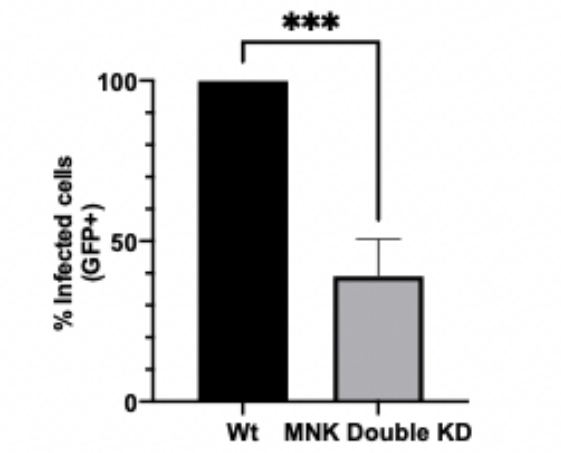

Figure 14. Genetic ablation of Mnk1 and Mnk2 reduces viral reactivation in iSLK.219 cells. iSLK.219dC9 MNK DKD cells were reactivated with doxycycline and viral supernatants were collected 72 hours post reactivation. Uninfected SLK cells were infected with supernatant, then percent infected cells (GFP+) were assayed 48 hours post infection. (*** P= 0.0008)

After confirming the knockdown of MNK1/2, I evaluated the viral titers following dox treatment of these cells. Uninfected SLK cells were infected with viral supernatant from iSLK.219 dC9K Wt or MNK DKD cells collected at 72 hours post reactivation with doxycycline (see Methods). As before, the supernatants of the cells are collected, filtered, and transferred to uninfected cells. The target cells are collected and GFP levels were evaluated as a proxy of infection. Compared to the non-transduced, wild type cells (Wt), the MNK1/2 DKD cells had about 50% less GFP+ cells, indicating a 50% reduction of the viral titers **(Figure 14)**. This reduction reflects the effects seen when wild-type iSLK.219 cells were pretreated with Tomivosertib. Thus, pharmacological inhibition or genetic ablation of MNK is a promising mechanism for controlling KSHV viral replication.

C. Methods

1. Cell culture

iSLK.219 dC9 MNK1/2 DKD cells were maintained in the same conditions as their parental iSLK.219 cell line (see Chapter 2.C.1). A pure population of iSLK.219 dC9 MNK1/2 DKD cells was selected and maintained with puromycin (10mg/mL) and blasticidin (20mg/mL) antibiotic selection.

Cell seeding concentrations for iSLK.219 dC9 cells and SLK cells for viral titers and western blot lysate collections were the same as prior experiments (see Chapter 2.C.1).

2. Cloning of Lentiviral sgRNAs

The sgRNA were designed based on the CRISPRi sgRNA library gifted from Dr. Jonathan Weissman (sequences listed in appendix **Table S3)**. The sgRNA plasmid PLG15 used for cloning was gift from Dr. Martin Kampmann. The vector was digested with two restriction enzymes BstXI and BlpI, then gel purified. The MNK1 and MNK2 oligonucleotides were annealed and ligated separately. The oligonucleotides were placed in annealing buffer (200mM potassium acetate, 60mM HEPES-KOH at pH of 7.4, and 4mM magnesium acetate), then incubated for five minutes at 95°C. Following incubation, they were allowed to gradually come to room temperature and then diluted 1:40 in water. The annealed oligonucleotides were then ligated into the backbone using New England BioLabs Quick Ligase overnight at 16°C. The plasmids were then transformed into DH5α competent bacteria. The plasmids were purified and isolated from the bacteria, then verified via sequencing.

3. Lentiviral Transduction of Mammalian Cells Using Pseudotyped Retroviruses

293METR cells were co-transfected using a packaging mixture containing Opti-MEM, Lipofectamine2000, pVSV-G and pCMVΔR8.91 helper plasmids, and the Mnk1 and Mnk2 lentiviral vectors. 6 hours after transfection, the packaging mixture was aspirated off and replaced with antibiotic free DMEM. 24 hours after transfection, the antibiotic free DMEM was replaced with viral collection media. 24 hours following media replacement (24 hours after transfection), the virus was harvested, and syringe filtered through a 0.45 μm filter to remove cell debris.

iSLK.219 dC9 target cells were seeded into a 6 well plate at the same concentrations as the iSLK.219 parental cells, then allowed to grow and adhere overnight (see Chapter 2.C.1). Target cells were infected via spinoculation (2000 RPM for 2 hours) with the harvested virus supplemented with polybrene. After spinoculation, cells were kept in the incubator to recover, then the following day were split 1:6. Antibiotic selection for MNK1/2 DKD positive transductans was performed 48 hours after splitting using puromycin and blasticidin (see Chapter 3.C.1).

4. Fluorescence Activated Cell Sorting (FACS) of iSLK.219 dC9 MNK1/2 DKD cells

iSLK.219 dC9 MNK1/2 DKD cells were FACS sorted twice to get a population of cells that contained the MNK1/2 DKD. In preparation for sorting, cells were trypsinized and resuspended in 1X PBS supplemented with 0.5% FBS. Cells were sorted based on BFP expression (data not shown). Once sorted, cells were pelleted then resuspended in DMEM growth media. They were allowed to recover then placed back under antibiotic selection at the next split.

5. Western Blot analyses

See Chapter 3.C.2

6. Viral supernatant collection, viral infection, and viral titers with iSLK.219 dC9 MNK1/2 DKD cells

See Chapter 3.C.3

IV. Discussion and Future Directions

During viral infection, viruses hijack the host protein production machinery to ensure the synthesis of functional viral proteins and successful viral infection. To ensure the correct

proteins are made at the right levels, translation is heavily regulated, particularly during the initiation stage. One such regulatory event is the phosphorylation of the cap-binding protein eIF4E by kinase MNK1/2. The phosphorylation of eIF4E is commonly dysregulated during oncogenesis and in infections by certain viruses. Because of this, MNK1/2 have been investigated as a potential therapeutic target for the development of anticancer and antiviral drugs. Previous studies in oncogenesis have demonstrated that the MAPK interacting kinases 1 and 2 (Mnk1/2) are necessary for tumor formation but are negligible for normal development and cellular functions (see Chapter 1.B). During viral infection by herpesviruses, poxviruses, flaviviruses, and coronaviruses, eIF4E is phosphorylated and in these cases this event is necessary for viral replication.

In our group, we are interested in understanding the molecular basis of virus:host interactions in cells infected with the Kaposi's Sarcoma-Associated Herpesvirus (KSHV) (see Chapter 1.D), an oncovirus that is the etiological agent of Kaposi's Sarcoma (KS), primary effusion lymphoma (PEL), and Multicentric Castleman's Disease. Unfortunately, despite its continued prevalence in sub-Saharan Africa, there is no therapeutic against it and its associated malignancies. Previous studies have shown that during KSHV infection, MNK1/2 activity and eIF4E phosphorylation are critical for protein synthesis and viral particle production. However, the mechanisms behind this requirement and the therapeutic potential of MNK1/2 modulation during infection remain underexplored. In my research, I have utilized the novel dual MNK1/2 inhibitor, Tomivosertib, and genetic ablation of MNK to demonstrate that MNK is a viable target for the development of a KSHV antiviral.

A. Pharmacological inhibition of Mnk1/2 with Tomivosertib modulates KSHV viral reactivation

Our studies with Tomivosertib demonstrate that treatment of iSLK.219 cells with this small molecule, dual MNK1/2 inhibitor, reduces late viral protein production and viral infection and confirm previous observations in the field. To evaluate the mechanisms behind the inhibition of viral replication, I determined the impact of MNK1/2 inhibition on viral protein synthesis. Immunoblots against two early viral proteins, ORF57 an RNA binding protein that facilitates splicing and ORF45 an immune modulator, indicate that MNK1/2 inhibition has no effect on the levels of these factors and early viral protein expression is not altered. However, this is not the case for late proteins expressed at 48-72h post reactivation. Treatment with Tomivosertib had a robust effect on late protein production as demonstrated by the decrease in levels of the KSHV structural proteins K8.1 and ORF62. Proper expression and folding of structural proteins is important for the formation of infectious virions that are responsible for subsequent infection.

There are two potential mechanisms of viral protein production that could be altered in the presence of Tomivosertib that could explain the observed results. First, late protein expression is dependent on viral DNA replication because the newly replicated DNA acts as a transcriptional template for viral structural proteins. Treatment with Tomivosertib could inhibit the translation of viral and host factors required for DNA replication, thus reducing late protein synthesis. Second, there could be differential translation of host and viral transcripts in presence or absence of eIF4E phosphorylation. Selective translation of mRNAs has been previously seen when the levels, availability, or phosphorylation status of eIF4A (RNA helicase), eIF4E, eIF4B (helicase co-factor), eIF3 and other translation

initiation factors (Lin et al, 2020, Rubio et al, 2014, Sen et al, 2016) are altered. To determine which viral and host factors are selectively translated during Tomivorsertib treatment of KSHV-infected cells, I propose to use ribosome profiling (Ribo-seq), which allows for the mapping of all mRNAs that are protected by the ribosome at any given time. This experiment would reveal whether there is differential host or viral protein expression during Tomivosertib treatment. If selective translation of host or viral factors were observed, then it could lead to information about which transcripts are favored for translation and why we see a decrease in viral protein production and viral infection.

Another documented mechanism which could contribute to the reduction of viral infection is the increase in type I interferon (IFN1) following MNK1/2 inhibition. Previous studies have demonstrated that inhibition of eIF4E phosphorylation is linked to a decrease in translation of the NF-$\kappa\beta$ inhibitor I$\kappa\beta\alpha$. By reducing the production of I$\kappa\beta\alpha$, there is sustained IFN1 that results in lower viral replication in cells infected with poxviruses (Herdy et al, 2012). Our initial studies investigating if I$\kappa\beta\alpha$ is reduced in Tomivosertib treated iSLK.219 cells do not appear to show any changes in the levels of I$\kappa\beta\alpha$ in presence or absence of eIF4E phosphorylation (data not shown). An alternative pathway of interest that also activates type I interferon, is the cGAS-STING pathway, which uses phosphorylation of interferon regulatory factor 3 (IRF3) to initiate transcription of IFN stimulated genes. Depletion of the cGAS-STING negative regulator, PPP6C, has been found to inhibit viral replication of vesicular stomatitis virus (VSV), HSV-1 and KSHV (Ni et al, 2020). Similar to the mechanism by which I$\kappa\beta\alpha$ translation is decreased when eIF4E phosphorylation is inhibited, translation of PPP6C may be downregulated and result in sustained activation of IFN1 via the cGAS-STING pathway. Future experiments evaluating the levels and

activation status of PPP6C and IRF3 in Tomivosertib treated iSLK.219 cells, together with Ribo-seq and could reveal mechanistic details on the effects of MNK1/2 activation and eIF4E phosphorylation in KSHV-infected cells.

B. Genetic ablation of Mnk1/2 inhibits KSHV viral replication

Studies with pharmacological inhibitors of MNK1/2 are powerful and useful for exploring the therapeutic potential of these kinases. However, as previous studies using the MNK1 inhibitor CGP show, off-target effects could mask the true effects of eIF4E phosphorylation on viral replication. To confirm the requirement for MNK1/2 for KSHV replication, we generated an iSLK.219 MNK1/2 double knockdown (DKD) cell line. Our results confirmed that phosphorylation of eIF4E was below the limits of detection in these cells despite incomplete knockdown of the kinases as indicated by a low level of detection of MNK1 by immunoblot. Similar to the findings with Tomivosertib treatment, viral replication was reduced by about 50% when uninfected SLK cells were infected with supernatant from the iSLK.219 MNK1/2 DKD cell line. The similarities in phenotype obtained from both pharmacological and genetic studies lend validity to the potential of MNK as a therapeutic target for KSHV infection.

Given that the iSLK.219 MNK1/2 DKD cell line did not have complete knockdown of the MNK kinases, I propose to generate a new iSLK.219 dC9 MNK DKD cell line to further explore the effect of MNK1/2 genetic ablation. In our approach, we used the same antibiotic resistance marker (puromycin) in the vectors expressing the sgRNAs as in the recombinant KSHV virus present in iSLK.219 cells. To address this caveat, I propose replacing the antibiotic resistance marker in the sgRNA vector plasmid. Designing a vector

with a different antibiotic resistance marker would lead to more efficient selection of a sgRNA transduced population. In a complementary approach to enhance MNK1/2 silencing, I would use sgRNA vectors for MNK1 and MNK2 with different fluorescent markers. For example, if MNK1 had a BFP marker and MNK2 had a YFP marker, then we could select for cells that are BFP+/YFP+ by FACS. Once the MNK1/2 DKD cells are established, we would evaluate the effect the knockdown had on KSHV viral DNA replication, transcription, and translation. I would anticipate a confirmation of our observations and a more robust phenotype. Future studies to continue exploring the promising therapeutic potential of MNK1/2 inhibition of KSHV infection and related malignancies, would require testing Tomivosertib in other cell and animal models of KSHV infection, such as in cells derived from patients and in a previously published rat model for KSHV (Mutlu et al, 2007).

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Appendix

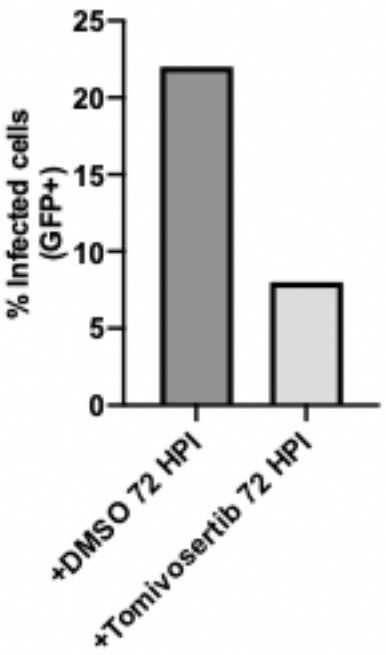

Figure S1. Viral titer data from iSLK.219 cells treated with 500nM Tomivosertib. Viral supernatants were collected 72 hours post reactivation with doxycycline and used to infect uninfected SLK cells. Percent infected cells (GFP+) was determined. Compared to untreated, there was a robust reduction in viral load; however, this data was not reproducible.

Table S1. Primary antibodies used

Antibody Name	Company and Catalog Number	Working dilution for Western Blot
K8.1	Homemade	1:500
Mnk1	Cell Signaling, 2195S	1:1000
ORF 45	Santa Cruz Biotechnology, sc-53883	1:1000
ORF 57	Santa Cruz Biotechnology, sc-135747	1:1000
ORF 62	Abgent, A01017a	1:1000
P-eIF4E	Cell Signaling, 9741P	1:1000
Vinculin	Cell Signaling, 4650S	1:1000

Table S2. Secondary antibodies used

Antibody Name	Company and Catalog Number	Working dilution for Western Blot
Mouse-HRP	BioRad, 1706516	1:2500
Rabbit-HRP	BioRad, 1706515	1:2500
Rat-HRP	Unknown	1:2500

Table S3. sgRNA sequences for CRISPRi cell lines

Target	Sequence, 5'-3'
Mnk1 T1 Top	TTGGGGAGGAGCGATCTGCAGGTGTTTAAGAGC
Mnk1 T1 Bottom	TTAGCTCTTAAACACCTGCAGATCGCTCCTCCCCAACAAG

Mnk2 T1 Top	TTGGGTCCGGGCTGCGCGACGTGGTTTAAGAGC
Mnk2 T1 Bottom	TTAGCTCTTAAACCACGTCGCGCAGCCCGGACCCAACAAG