UNDERSTANDING THE MOLECULAR REGULATORY MECHANISMS OF KAPOSI SARCOMA ASSOCIATED HERPESVIRUS AND CHARCOT MARIE TOOTH DISEASE TYPE 1A

By

JACQUELYN CARYN SERFECZ

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2018
To my parents, Susan and Frank Serfez
ACKNOWLEDGMENTS

This dissertation would not have been possible without the contributions from many individuals. I would first like to thank my mentor, Dr. Rolf Renne, for encouraging me to achieve greatness. He has been an excellent advisor, committed to sharing his love of virology. My committee Dr. Chan, Dr. Lewin, and Dr. Bungert have all been very supportive and offered crucial scientific advice. Our collaborator, Dr. Notterpek, gave me the opportunity to research a potential therapeutic for a neurodegenerative disorder affecting over a hundred thousand individuals. Thank you to all the Renne lab members. Lauren Gay, Sunantha Sethurman, Vaibhav Jain, Pete Turner, Natalie Martinez, Yuan Hong, and Ozlem Calbay have all been good friends, offering advice in lab meetings. A special thanks to Pete Turner, who edited many presentations and documents throughout my dissertation. I would also like to thank my family and loved ones. My brother, Jason Serfecz, has been an inspiration. Clayton Santiago, my fiancé, has been my source of scientific counsel and emotional support for many years. Most importantly, my mother and father have dedicated their lives to my happiness and success.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ............................................................................................................. 4  
LIST OF TABLES .................................................................................................................. 7  
LIST OF FIGURES ................................................................................................................. 8  
LIST OF ABBREVIATIONS .................................................................................................... 10  
ABSTRACT .............................................................................................................................. 13  

## CHAPTER

### 1 INTRODUCTION .............................................................................................................. 15  
  
  Significance ......................................................................................................................... 15  
  Background .......................................................................................................................... 17  
    RNAs In Eukaryotes And Viruses ....................................................................................... 17  
    KSHV .................................................................................................................................. 20  
      Innate immune system ..................................................................................................... 22  
      KSHV tumorigenesis ....................................................................................................... 24  
      DExD/H box helicases .................................................................................................... 26  
    Charcot-Marie-Tooth Disease Type 1A ........................................................................... 27  

### 2 THE IMPACT OF EUKARYOTIC DEXD/H BOX HELICASES ON REACTIVATION OF KAPOSI’S SARCOMA ASSOCIATED HERPESVIRUS ............... 31  
  
  Results .................................................................................................................................... 31  
    Knockdown Of DExD/H Box Helicases to Screen For Effects On KSHV  
      Reactivation ..................................................................................................................... 31  
    DDX24-DDK And DDX49-DDK Stably Transfected BCBL-1 Cells .................................... 32  
    DDX24-DDK And DDX49-DDK Reactivation Assays .......................................................... 33  
    DHX29 KD Stably Transduced BCBL-1 Cells .................................................................... 34  
    DHX29 KD Reactivation Assays ........................................................................................ 34  
    Stably Transduced TREx BCBL1-Rta Cells Expressing DDX24-Avi And DDX49-Avi .......................................................... 35  
    RNA Immunoprecipitation Of TREx BCBL1-Rta Cells ................................................... 37  
  
  Discussion .............................................................................................................................. 40  
    Materials and Methods ..................................................................................................... 46  

### 3 REGULATION OF HUMAN PERIPHERAL MYELIN PROTEIN 22 BY MIR-29A IN CHARCOT-MARIE-TOOTH DISEASE TYPE 1A .............................................................. 73  
  
  MiR29a Mimic Reduced PMP22 3’UTR Reporter Activity In Transiently Transfected Cells ......................................................................................................................... 74
Inhibition Of PMP22 In Patient-Derived Fibroblasts ........................................... 74
AAV Vector Construction ....................................................................................... 76
Discussion .............................................................................................................. 77
Materials and Methods ......................................................................................... 79

4 CONCLUSIONS AND FUTURE DIRECTIONS ......................................................... 91

LIST OF REFERENCES .............................................................................................. 94

BIOGRAPHICAL SKETCH ....................................................................................... 107
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>The 22 DExD/H box helicase targets of the knockdown screen</td>
<td>52</td>
</tr>
<tr>
<td>2-2</td>
<td>Pooled siRNAs used in the Vero knockdown screen</td>
<td>53</td>
</tr>
<tr>
<td>2-3</td>
<td>Pool of RNAi (TRC) shRNAs used to generate DHX29 KD BCBL-1 stably transduced cells</td>
<td>54</td>
</tr>
<tr>
<td>2-4</td>
<td>List of samples tested for the DExD/H box RNA Immunoprecipitation analysis</td>
<td>55</td>
</tr>
<tr>
<td>2-5</td>
<td>List of genes with highly enriched regions from RNA-IP analysis</td>
<td>56</td>
</tr>
<tr>
<td>3-1</td>
<td>List of Studied Cells with Donor Information</td>
<td>83</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>2-1</td>
<td>KSHV genome showing the chronological expression of lytic genes</td>
<td>57</td>
</tr>
<tr>
<td>2-2</td>
<td>Mitochondrial antiviral signaling pathway</td>
<td>58</td>
</tr>
<tr>
<td>2-3</td>
<td>Flow chart of siRNA screen of 22 DExD/H box helicases in Vero cells infected with rKSHV.219</td>
<td>59</td>
</tr>
<tr>
<td>2-4</td>
<td>Quantification of siRNA screen of 22 DExD/H box helicases in Vero cells infected in rKSHV.219</td>
<td>60</td>
</tr>
<tr>
<td>2-5</td>
<td>KSHV genome copy number of Vero cells during DExD/H box siRNA screen at 48 hours post-induction</td>
<td>61</td>
</tr>
<tr>
<td>2-6</td>
<td>Validation of stably transfected BCBL-1 cells overexpressing DDX24-DDK and DDX49-DDK</td>
<td>62</td>
</tr>
<tr>
<td>2-7</td>
<td>Expression of latent, immediate early, and late lytic genes in BCBL-1 cells overexpressing DDX24-DDK and DDX49-DDK during a 2 mM Sodium Butyrate induction time course</td>
<td>63</td>
</tr>
<tr>
<td>2-8</td>
<td>Intracellular KSHV genome copy number in BCBL-1 DDX24-DDK and DDX49-DDK during a 2 mM NaB induction time course</td>
<td>64</td>
</tr>
<tr>
<td>2-9</td>
<td>Validation of Stably Transduced DHX29 KD BCBL-1 cells</td>
<td>65</td>
</tr>
<tr>
<td>2-10</td>
<td>Expression of KSHV latent, immediate early, and late lytic genes in DHX29 KD BCBL-1 cells during an induction time course</td>
<td>66</td>
</tr>
<tr>
<td>2-11</td>
<td>Intracellular KSHV genome copy number of DHX29 KD stably transduced BCBL-1 cells during 48 hours post induction with 2 mM NaB and 20 ng/mL TPA</td>
<td>67</td>
</tr>
<tr>
<td>2-12</td>
<td>Validation of Stably Transduced TREx BCBL1-Rta cells overexpressing DDX24-Avi, DDX49-Avi, and DDX58-Avi</td>
<td>68</td>
</tr>
<tr>
<td>2-13</td>
<td>Validation of TREx BCBL1-Rta reactivation and immunoprecipitation conditions for RNA Immunoprecipitation analysis</td>
<td>69</td>
</tr>
<tr>
<td>2-14</td>
<td>Deep Sequencing analysis of DDX24 and DDX49 associated KSHV partners and control immunoprecipitated RNA upon Rta-induced reactivation</td>
<td>71</td>
</tr>
<tr>
<td>2-15</td>
<td>KSHV microRNAs miR-K12-6 and miR-K12-12 putatively target DDX24 in Telomerase Immortalized Vein Endothelial cells</td>
<td>72</td>
</tr>
<tr>
<td>3-1</td>
<td>Alignment of the miR-29a binding site and its PMP22 target sites</td>
<td>84</td>
</tr>
</tbody>
</table>
3-2 Dual luciferase assay demonstrating that the human PMP22 3’UTR is a target of miR-29a. Dual luciferase assay performed in 293 cells validates that the human PMP22 3’UTR is a target of miR-29a...

3-3 SiGlo Red Indicator 48 hours post-transfection...

3-4 Steady state PMP22 mRNA levels of patient and age matched healthy individual fibroblasts following transfection...

3-5 Fibroblasts treated with miR-29a from patients GM05165 (P4) and GM05167 (P2) demonstrated a decrease in PMP22 protein...

3-6 The pTR2 plasmid map containing primary miRNA & siRNAs to be expressed...

3-7 Dual luciferase assay demonstrating the effect of miR-29a and siRNA on PMP22 3’UTR reporter...
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Adeno-asssociated virus</td>
</tr>
<tr>
<td>ALT</td>
<td>Antisense-to-latency transcripts</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>BCBL-1</td>
<td>Body cavity-based lymphoma cell line 1</td>
</tr>
<tr>
<td>BCL-2/vBCL2</td>
<td>B-cell lymphoma 2 gene / viral BCL-2</td>
</tr>
<tr>
<td>CCL/vCCL</td>
<td>Chemokine ligand / viral CCL</td>
</tr>
<tr>
<td>cGAS</td>
<td>Cyclic GMP-AMP synthase</td>
</tr>
<tr>
<td>circRNA</td>
<td>Circular RNA</td>
</tr>
<tr>
<td>CLASH</td>
<td>Cross-linking, ligation, and sequencing of hybrids</td>
</tr>
<tr>
<td>CMT1A</td>
<td>Charcot-Marie-Tooth Disease type 1A</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DED</td>
<td>Death-effector domain</td>
</tr>
<tr>
<td>E</td>
<td>Early lytic genes</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-barr virus</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FLIP/vFLIP</td>
<td>FLICE-inhibitory protein / viral FLIP</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HNPP</td>
<td>Hereditary Neuropathy with Liability to Pressure Palsy</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate-Early lytic genes</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-6/vIL-6</td>
<td>Interleukin 6 / viral IL-6</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosomal Entry Site</td>
</tr>
<tr>
<td>IRF/vIRF</td>
<td>Interferon Regulatory Factors / viral IRF</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator of transcription</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi’s Sarcoma</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s Sarcoma-associated Herpesvirus</td>
</tr>
<tr>
<td>L</td>
<td>Late lytic genes</td>
</tr>
<tr>
<td>LANA</td>
<td>Latency-associated nuclear antigen</td>
</tr>
<tr>
<td>IncRNA</td>
<td>Long non-coding RNA</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial Antiviral Signaling protein</td>
</tr>
<tr>
<td>MCD</td>
<td>Multicentric castleman disease</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MHV-68</td>
<td>Murine gammaherpesvirus 68</td>
</tr>
<tr>
<td>MIP/v-MIP</td>
<td>Macrophage inflammatory protein / viral MIP</td>
</tr>
<tr>
<td>MIR</td>
<td>Modulator of Immune Recognition</td>
</tr>
<tr>
<td>miR</td>
<td>microRNA</td>
</tr>
<tr>
<td>NaB</td>
<td>Sodium Butyrate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAN</td>
<td>Polyadenylated nuclear RNA</td>
</tr>
<tr>
<td>pCMV</td>
<td>Promoter of Cytomegalovirus</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEL</td>
<td>Primary effusion lymphoma</td>
</tr>
<tr>
<td>PFA</td>
<td>Phosphonoformic acid</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PMP22</td>
<td>Peripheral myelin protein 22</td>
</tr>
<tr>
<td>PNGase F</td>
<td>Peptide:N-glycosidase F</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent protein</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RIP</td>
<td>RNA Immunoprecipitation</td>
</tr>
<tr>
<td>RIP-1</td>
<td>Receptor-interacting protein 1</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RLRs</td>
<td>RIG-I like receptors</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luminometer units</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>snRNA</td>
<td>Small nuclear RNA</td>
</tr>
<tr>
<td>snoRNA</td>
<td>Small nucleolar RNA</td>
</tr>
<tr>
<td>TIVE</td>
<td>Telomerase-immortalized human umbilical vein endothelial</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TPA</td>
<td>Tetradecanoyl phorbol acetate (Phorbol Ester)</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis post-transcriptional regulatory element</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
</tr>
</tbody>
</table>
Kaposi’s Sarcoma-associated herpesvirus (KSHV) is an oncogenic gammaherpesvirus that is the causative agent of primary effusion lymphoma and Kaposi’s Sarcoma. In healthy carriers KSHV remains latent, but a compromised immune system can lead to lytic viral replication that increases the probability of tumorigenesis and transmission rates. RIG-I-like receptors (RLRs) are members of the DExD/H box helicase family of RNA binding proteins that recognize KSHV to stimulate the immune system and prevent reactivation from latency. To determine if other DExD/H box helicases can affect KSHV lytic reactivation, we performed a knock-down screen that revealed DHX29-dependent activities support viral replication but in contrast, DDX24 and DDX49 seem to have antiviral activity. When DDX24 or DDX49 are overexpressed in BCBL-1 cells, transcription of all lytic viral genes and genome replication is repressed upon induction. RNA immunoprecipitation of tagged DDX24 and DDX49 followed by next generation sequencing revealed that the helicases bind to mostly immediate-early and early KSHV mRNAs. Our findings reveal a new molecular mechanism by which host DDX24 and DDX49 can recognize gammaherpesvirus transcripts and repress lytic reactivation.
I was also involved in a microRNA therapeutics project. Charcot Marie-Tooth disease (CMT1A) is the most common inherited peripheral neuropathy. It is caused by duplication of the Peripheral Myelin Protein 22 (PMP22) gene, a dose-sensitive membrane protein primarily expressed in myelinating Schwann cells. The restricted tissue expression of the PMP22 protein in comparison to its ubiquitously detected mRNA supports a role for microRNA mediated post-transcriptional regulation. Bioinformatically, we determined that miR29a is conserved between species and targets the 3’UTR of PMP22. A luciferase reporter assay demonstrated that transient transfection with a miR29a mimic is associated with an approximately 20% reduction in PMP22 reporter activity. In dermal fibroblasts from CMT1A patients, we detected an elevated expression of PMP22 mRNA, which was effectively repressed by approximately 28-50% after transient transfection with a miR29a mimic. In congruence with the observed mRNA inhibition, immunoblots revealed a 20-24% reduction at the protein level. Together, these results support further studies with miR29a as a potential therapeutic to modulate the expression level of PMP22 in Schwann cells from CMT1A patients.
CHAPTER 1
INTRODUCTION

Significance

Kaposi’s sarcoma associated herpesvirus (KSHV) is an oncogenic gammaherpesvirus that infects endothelial cells to give rise to Kaposi’s Sarcoma (KS) and B cells to give rise to Primary Effusion Lymphoma (PEL) or Multicentric Castleman’s Disease (MCD). The seroprevalence of KSHV infection in the general population varies from <5% in the United States and Asia, to over 50% in sub-Saharan Africa, where it is estimated that 44,000 new cases of KS are discovered per year. Human gammaherpesviruses including Epstein-barr virus (EBV) and KSHV have been implicated in numerous cancers and autoimmune disorders. There is no cure or preventive vaccine for gammaherpesviruses, necessitating investigation of the molecular biology and life cycle of the virus in order to develop antiviral drugs.

When the immune system is weakened, individuals who are infected with KSHV are more likely to develop KSHV related malignancies, suggesting a critical role in the immune system for suppressing cancer development. The innate immune system regulates gammaherpesvirus-induced lytic reactivation in cell culture and in the gammaherpesvirus mouse model. RIG-I and RIG-I like receptors, members of the DExD/H box helicase family, are known to mount an innate immune response against gammaherpesviruses to inhibit reactivation. In order to identify if other DExD/H box helicases can act as Pattern Recognition Receptors (PRRs) or adaptors of the innate immune system during KSHV viral reactivation, we performed RNA immunoprecipitation followed by high-throughput sequencing analysis on two helicase targets, DDX24 and DDX49. This thesis describes how we came to discover that these helicases inhibit KSHV lytic reactivation and anticipates their function. These data support a role of DDX24 and DDX49 in nucleic acid recognition of KSHV immediate-early (IE) and early (E)
genes to prevent lytic reactivation. Understanding the molecular mechanisms that allow DDX24 to inhibit reactivation could lead to prevention of gammaherpesvirus malignancies.

Additionally, Charcot-Marie-Tooth disease type 1A (CMT1A) is the most common inherited peripheral neuropathy, affecting about 1:2,500 individuals in the US and Europe. It is caused by a 1.5 Mb tandem duplication of the Peripheral Myelin Protein (PMP22) gene on the short arm of chromosome 17. The PMP22 gene encodes for a dose-sensitive 22 kDa integral transmembrane glycoprotein primarily expressed in myelinating Schwann cells. Although the PMP22 mRNA is ubiquitously expressed throughout most tissue types, its protein expression is restricted to myelinating Schwann cells and glial cells, supporting a role for post-transcriptional regulation. The disease manifests itself in patients as progressive muscle atrophy over many years and numbness due to a dampened peripheral nerve response. There is currently no treatment for the disease.

Antisense oligonucleotide (ASO) therapies have been shown to be effective in rodent models of CMT1A. ASOs are typically 15-20 nucleotide long sequences that either prevent translation or lead to RNA degradation by complementary binding of deoxyribonucleotides to the sequence of a disease associated messenger RNA. The duplex RNA that is formed with the target mRNA by hybridization gets degraded by RNase H. However, ASOs interact with various cell types following systematic subcutaneous injections, are dosed at high concentrations, and are predominantly excreted by the liver. Our goal is to create a delivery system of a 20 nucleotide sequence that targets PMP22 through the RNA-induced silencing complex (RISC), is Schwann cell type specific, and functional in humans as well as mice. In collaboration, the Notterpek and Renne labs have demonstrated that PMP22 is post-transcriptionally regulated by miR-29a in rat Schwann cells. In the current study, we ask if
targeting PMP22 with miR-29a in CMT1A patient fibroblasts will lead to near-normal PMP22 levels and the repair of pathogenic phenotypes. In this work, we have been able to demonstrate that the disease associated human PMP22 transcript and protein levels are brought closer to that of normal, age-matched controls by miR-29a in CMT1A patient-derived fibroblasts.

**Background**

**RNAs In Eukaryotes And Viruses**

There are many categories of RNAs in eukaryotes and double stranded DNA viruses. Given the nature of this research, I would like to briefly compare the structure and function of the most common RNAs and highlight the contrasts of how eukaryotic RNAs differ from those seen in KSHV.

Although in eukaryotes messenger RNAs (mRNAs) comprise less than 2% of the genome, they are the focus of a lot of research because they code for proteins. Eukaryotic mRNAs require a 5’ cap and polyA tail for efficient translation. Given that KSHV relies on host Pol II for transcription throughout the entire KSHV life cycle, the viral genome also contains these conserved elements, and the vast majority of gene expression from dsDNA viruses utilize cap-dependent translation of monocistronic transcripts.

Ribosomal RNA (rRNA) is by far the most abundant RNA in eukaryotic cells. It is the RNA component of the ribosome and required for protein synthesis. Transfer RNAs (tRNAs) transport amino acids to the ribosome to lengthen the nascent polypeptide chain. KSHV utilizes cellular host ribosomes, tRNAs, and multiple host factors associated with posttranscriptional modifications and protein translation. Unique to KSHV, a single internal ribosomal entry site (IRES) is present in the coding region of the bicistronic latency mRNA, viral FLICE-inhibitory protein (vFLIP)/vCyclin, allowing for cap independent translation. Its IRES
structure contains only two stem loops and is representative of most found in the herpesviridae family 26.

Small nuclear RNAs (snRNA) are a class of non-coding RNAs located in the nucleus that mainly process primary transcripts immediately transcribed from the DNA and are required for rRNA processing23. Within the snRNA class, U-RNAs are required for precursor mRNA splicing and small nucleolar RNA (snoRNA) are able to guide chemical modifications of ribosomal subunits during assembly within the nucleolus27. Herpesviruses can hijack host machinery that is used for export and processing of snRNAs for its own viral mRNA nuclear export28-32.

MicroRNAs are a class of endogenous small non-coding RNAs about 19-25 nucleotides long that are able to inhibit gene expression, post-transcriptionally, by the RISC complex 33. The transcription of miRNAs begins in the nucleus by Pol II, generating an RNA hundreds of nucleotides long containing at least one hairpin34,35. This large primary microRNA (pri-miRNA) often contains a 5’cap for stability and a polyA tail. Drosha trims the pri-miRNA in the nucleus to form a precursor microRNA (pre-miRNA) about 70 nts long that gets exported out of the nucleus by Exportin36. While in the cytoplasm, the pre-miRNA gets cleaved by Dicer and one strand will preferentially incorporate into the RISC complex to locate its 3’UTR target and repress translation37. RISC mediates locating and binding of the miRNA to its complementary mRNA 3’UTR sequences38. The targeting depends mainly on the seed sequence of about 6 to 8 nucleotides in the complementary 3’UTR segment of an mRNA38. MicroRNA-dependent regulation of gene expression occurs in all metazoa, including mice, rats, humans, and in dsDNA viruses, highlighting their functional conservation39. In particular, KSHV encodes 12 miRNA genes encoding 25 mature miRNAs, all of which are expressed in latently infected cells and have been associated with tumorigenesis40-44.
Long non-coding RNAs (lncRNA) are categorized as any transcript longer than 200 nucleotides that does not have protein-coding capacity. LncRNAs can regulate gene expression through a diversity of mechanisms including chromatin remodeling, histone modifications, transcriptional regulation, post-transcriptional regulation, and regulating the localization of proteins. Recent evidence suggests that KSHV miRNAs may even bind to and dysregulate host, eukaryotic lncRNAs during latency. LncRNAs are expressed predominantly by the eukaryotic host. A couple of lncRNAs are known to be expressed in KSHV although there may be many more to discover transcribed antisense to known ORFs. Polyadenylated nuclear (PAN) and antisense to latency transcripts (ALT) RNAs, the characterized KSHV lncRNAs, are polyadenylated. PAN RNA is the most abundant KSHV transcript in newly lytic cells and helps to drive lytic reactivation. Even when only about 2% of the KSHV lncRNA, PAN, is associated with ribosomes, this is sufficient for lytic replication to occur. ALT is a non-capped 10 kb transcript that runs antisense to latency genes, however, its potential role in regulating viral gene expression remains unknown.

Recently, there is also considerable interest in how circular RNAs (circRNAs) function in various cellular and viral pathways. CircRNAs are generated from linear pre-mRNAs that occur through normal RNA splicing reactions. They are non-coding and are more stable than linear RNAs given their resistance to nucleases. Circular RNAs are expressed by Herpes simplex virus type 1 (HSV-1), with a potential role as miRNA sponges, capable of regulating viral and host gene expression. Other herpesviruses such as KSHV may also express circular RNA transcripts.

The formation of double stranded RNA is very common and has unique consequences depending on the length of the RNA:RNA duplex and its location in the cell. RNA helicases
bind to these segments of double stranded RNA and are required for many cellular processes including RNA transportation, localization, splicing, and translation\textsuperscript{58}. The helicases recognize their RNA targets by a combination of secondary structure and by interacting with other RNA binding protein partners\textsuperscript{59,60}. When bound, RNA helicases displace RNA:protein complexes to facilitate RNA processing and in some cases, helicases also have ability to translocate, unwinding RNA:RNA duplexes as they travel\textsuperscript{61}. During infection, certain RNA helicases also have the ability to recognize non-self dsRNAs via location, structural defects such as bulges or loops, and/or nucleotide modifications\textsuperscript{62-65}. These helicases can go on to stimulate an immune response. For example, a viral dsRNA in the cytoplasm can be recognized by RNA helicase A (RHA/DHX9) and DHX36 to activate Protein kinase R (PKR), stimulating the Nuclear Factor Kappa B (NF-\(\kappa\)B) pathway\textsuperscript{66}. A description of the innate immune response to KSHV is included below. Long (>100bp) RNA duplexes are retained in the nucleus and degraded by an unknown process\textsuperscript{56}.

**KSHV**

Kaposi’s sarcoma Associated Herpesvirus is an oncogenic gammaherpesvirus that infects endothelial cells to give rise to Kaposi’s Sarcoma (KS) and B cells to give rise to Primary Effusion Lymphoma (PEL). KS is caused by transformation of endothelial cells by the KSHV virus, which leads to the formation of new blood vessels (angiogenesis) and dark red lesions on the skin and mouth. Although KS is most common in HIV patients, other epidemiological variants occur in immune compromised individuals such as the elderly, children, or organ transplant recipients. Notably, about 1 in 200 transplant patients in the United States develop KS\textsuperscript{67}. The disease can be life threatening if KS reaches the gastrointestinal tract or lungs. Currently, the only treatment is surgical removal of the lesion or chemotherapy. In contrast, PEL is a very rare HIV-associated non-Hodgkin’s Lymphoma of B cells\textsuperscript{68}. It is a rapidly fatal body
cavity-based lymphoma (BCBL) that most likely originates from post-germinal center B-cells. KSHV DNA was originally discovered in KS lesions in 1994 and within two years, the virus was able to be isolated from a PEL biopsy, and a latently infected B cell line 1, termed BCBL-1, could be cultured.

The double stranded DNA genome of KSHV is approximately 140 kb, with an additional 20 to 30 kb of terminal repeats, which flank the ends (Figure 2-1). A common feature of the KSHV genome is that about 65% of the transcription start sites of KSHV are driven by TATA-like boxes and the approximately 90 annotated KSHV genes share polyadenylation (PolyA) sites at an average of about 2.7 genes per site. Alternative polyadenylation of KSHV genes offers another means of posttranscriptional regulation.

Like all herpesviruses, KSHV’s life cycle is biphasic, defined by latent and lytic modes of DNA replication. The establishment of latency upon de novo infection begins with KSHV entering the cell by endocytosis. The capsid is transported through the cytoplasm before the viral DNA can be delivered into the nucleus. Once inside the nucleus, the incoming linear dsDNA is immediately circularized using cellular enzymatic machinery to generate a closed-circular loop of DNA, referred to as an episome. The viral latency-associated nuclear antigen (LANA) protein is expressed very early after de novo infection and establishes latency by recruiting host Polycomb Repressive Complexes (PRCs) to the promoters of lytic genes. LANA tethers the terminal repeats of the KSHV episome to host histones. During latent infection, the KSHV episome is maintained in the nucleus with a restricted repertoire of gene expression and replicates upon cell division using host machinery without the production of progeny virions. KSHV transcribes a set of 12 precursor miRNAs, vFLIP, vCyclin, and LANA during latency.
Upon reactivation, a full repertoire of viral genes are transcribed in a temporally regulated manner to produce progeny virions\(^76\). Lytic genes are further sub-divided into three classes: immediate-early (IE), early (E), and late (L) genes (Figure 2-1). IE lytic genes are expressed immediately following reactivation and do not require de novo protein synthesis. KSHV RNAs containing self-complementary regions, including mRNAs, IncRNAs, and antisense transcripts, begin to be transcribed during this immediate early replicative cycle\(^77,78\). The first immediate-early gene, RTA, encodes for a potent master regulator that is necessary for lytic cycle activation and targets latent vFLIP for degradation. RTA transactivates the expression of the viral IncRNA, PAN (T1.1), and multiple downstream early lytic genes, which are necessary for DNA replication\(^79\). Lytic DNA replication proceeds with a KSHV DNA polymerase via a rolling circle mechanism that generates concatamers which get cleaved, leading to a 100 to 1000 fold amplification of viral DNA\(^4\). Finally, after lytic DNA synthesis, viral capsid proteins, glycoproteins, and tegument proteins are expressed during the late lytic stage. It is well established that in gammaherpesviruses, reactivation and de novo infection are regulated by innate immunity\(^80-83\). KSHV lytic reactivation is induced when the immune system is compromised and the resulting lytic gene expression contributes to lymphoproliferative disorders, an increase in the probability of tumorigenesis, and higher transmission rates\(^4,84\).

**Innate immune system**

It is becoming more evident that some of the same innate immune responses that prevent de novo KSHV infection also suppress reactivation from within the nucleus\(^83,85\). Pattern Recognition Receptors (PRRs) are host receptors that recognize molecules of a particular pathogen in the membrane or cytoplasm. In the current model of KSHV infection, PRRs including Toll Like Receptors (TLRs), RIG-I Like Receptors (RLRs), and Cyclic GMP-AMP synthase (cGAS) are all able to recognize KSHV and other herpesviruses to stimulate an innate
immune response. TLRs, such as TLR3 and TLR9, are anti-viral transmembrane proteins of the innate immune system that sense DNA and RNA complexes of invading pathogens during de novo infection at the cell surface plasma membrane and in endosomal compartments. TLRs have been shown to directly bind KSHV RNAs or viral CpG DNA motifs and are the most well characterized. RLRs, including RIG-I, MDA-5, and LGP2, are anti-viral proteins of the innate immune system able to recognize pathogen-derived RNA complexes in the nucleus and cytoplasm. During KSHV reactivation, knockdowns of RLRs lead to increased lytic reactivation in 293 cells. There are multiple RNA regions in KSHV that could act as potential virus ligands that bind to RIG-I to induce type I interferon responses. However, the mechanism by which RIG-I senses and responds to KSHV from within the nucleus during reactivation is unknown. It is uncertain whether RLRs are directly recognizing viral dsRNA since cGAS, an anti-viral DNA sensing molecule, also recognizes KSHV dsDNA and is involved in crosstalk with RIG-I to stimulate interferon (IFN) genes. Although the RIG-I recognition mechanisms of KSHV still remain uncertain, it is clear that when these PRRs are activated they stimulate the Mitochondrial Antiviral Signaling Protein (MAVS), an essential host signaling adaptor protein that participates in a broad range of innate immune pathways. MAVS activates NF-κB and Interferon Regulatory factor (IRF) signaling cascades to produce pro-inflammatory cytokines and Type I interferons, respectively (Figure 2-2). Recently, it was discovered that a few other DEAD box helicases, in addition to RIG-I regulate innate immunity by acting as Pattern Recognition Receptors or as signaling adaptors of innate immune pathways. Some examples are DDX3, DDX41, and DDX24.

NF-κB is a major regulator of KSHV reactivation and is known to be key in PEL oncogenesis (Figure 2-2). The effects of NF-κB on reactivation of KSHV are complex but
well established. When a B cell becomes latently infected, KSHV persistently activates NF-κB at higher than normal steady state levels to suppress lytic reactivation and ensure survival by inducing the expression of host Bcl-XL \textsuperscript{76,96,97}. As such, changes in NF-κB signaling is associated with increased apoptosis or lytic reactivation depending on the cell type \textsuperscript{95}. Collectively, the current data suggest that the fate of the virus is context dependent and relies on a balance of the NF-κB pathway, implying that cellular factors can tip the equilibrium between apoptosis and viral replication \textsuperscript{95}. Since NF-κB is essential for PEL cell survival, it is considered a targeting molecule for treatment.

**KSHV tumorigenesis**

Viral infections contribute to 15-20\% of all human cancers; among them is the oncogenic gammaherpesvirus, KSHV \textsuperscript{98}. In both tumor types, KS of endothelial cells and in PEL of B cells, KSHV remains predominantly latent, but at any given time 1-4\% of the tumor cell population will undergo spontaneous reactivation \textsuperscript{70,99}. Our focus is on PEL cells since current endothelial cell culture models have the ability to be infected by KSHV, but they remain latent and are not easily transfected. For this reason, we utilized the B cell culture model in our research. BCBL-1 cells are rapidly proliferating monoclonal B cells that carry markers of late stage B cell differentiation and typically contain a relatively high KSHV genome copy number (about 50-150 per cell) \textsuperscript{68}. In a healthy individual, naïve B cells develop in the bone marrow and survey the body until they recognize a pathogen peptide to become activated. Once activated, the B cells migrate to peripheral lymph nodes where they will proliferate and differentiate into memory B cells capable of surviving for many years. During infection, B cells are regularly infected with KSHV, and the virus remains latent until, by an unknown mechanism, lytic reactivation occurs.

There is supporting evidence that both latent and lytic KSHV gene expression contribute to oncogenesis by perpetually manipulating immune responses and promoting angiogenesis. In
either case, potent viral proliferation plays a necessary role. During latency, cellular Pol II transcribes LANA, vFLIP, vCyclin, and 12 miRNA genes from the KSHV episome. LANA interacts with several tumor suppressor genes like p53 and pRb and is thought to play a role in oncogenesis. Cellular FLIP is a protein that binds the adapter protein Fas-associated protein with death domain (FADD) of the Fas signaling pathway via its Death Effector Domains (DEDs). FADD and RIP1 initiate apoptosis, necrosis or NF-κB signaling depending on the amount and form of FLIP present. KSHV encodes a viral counterpart to FLIP, vFLIP, during latency that contains two homologous copies of the DED domain. Similar to its counterpart, vFLIP binds to cellular FADD to prevent caspase-induced apoptosis and blocks cell death by induction of the NF-κB pathway. vCyclin shares about 50% homology with cellular cyclin D, and accordingly stimulates the cell into S phase. A viral ortholog of cellular oncogene miR-155, KSHV miR-K12-11, and a number of other KSHV miRNAs are involved in processes directly related to oncogenesis. In addition, several lytic KSHV genes are also considered to be tumorigenic. During the lytic cycle, KSHV is able to express oncolytic genes that are cellular orthologs of endogenously expressed anti-apoptotic genes, such as vIL-6 (ORF K2), viral B cell lymphoma 2 gene (vBCL-2/ORF 16), vIRFs (ORF K9, ORF K11, ORF K10), and viral chemokine ligands (vCCLs: ORF K6, ORF K4, ORF K4.1) causing the B cells to proliferate uncontrollably in several ways. vIL-6 shares 25% identical amino acid sequence with cellular IL-6 and stimulates the maturation of B cells by interacting with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, similar to cellular IL-6. Cellular BCL-2 is also a very important anti-apoptotic gene that is expressed by memory B cells in a healthy individual. This allows B cells to survive for a very long time. The KSHV viral homolog of BCL-2 (vBCL-2) shares 30-60% homology with its cellular counterpart,
predominantly in functional domains 104,108. Additionally, KSHV encodes four homologs of IRFs during the lytic cycle termed vIRFs 1-4. They share only 13% homology with the human IRFs yet are able to interact with cellular factors and modulate NF-κB and Myc signaling similar to the host 103. Finally, CCLs direct migration of B cells, T cells, and Natural Killer cells. KSHV encodes viral homologs during the lytic cycle (vCCLs 1-3) that share 26-50% homology with their cellular counterparts 106. vCCLs have been implicated in anti-apoptosis, angiogenesis, and manipulating cellular migration 106.

**DExD/H box helicases**

As mentioned earlier, RLRs are RNA helicases that act as PRRs of KSHV during reactivation to trigger MAVS and suppress the lytic cycle. MAVS goes on to initiate NF-κB signaling and Type I IRFs of the innate immune system. All three RLRs are members of the largest superfamily of RNA helicases, termed DExD/H box helicases. This family of helicases are RNA-binding proteins that utilize ATP to unwind short duplex regions of RNA and remodel RNA-protein complexes, thereby altering RNA secondary structure. They are characterized by the DExD/H box helicase domain, a conserved ATP binding motif that contains the amino acid sequence Asp-Glu-x-Asp/His 109,110. The core homologous DExD/H box domains are centered in the protein and a helicase domain is often at the C-terminal end 110. Disordered flanking segments at the C-terminal and N-terminal ends are up to hundreds of amino acids in length and are thought to contain linker regions involved in interactions with other proteins or RNAs 111.

The processivity of DExD/H box helicases is relatively poor. They become ineffective when the helix length rises above 10-15 base pairs, suggesting that their main endogenous role is conformational rearrangement, to assist folding of RNA, and to act as ribonucleoprotein scaffolds 110. The DEAD box family members often require energy from ATP hydrolysis to unwind only short RNA duplexes one at a time and are often non-processive, but members of the
DEAH box family have the ability use other NTPs and can translocate in a 3’->5’ direction\textsuperscript{112}. There are about 60 DExD/H box helicase proteins expressed in the human body\textsuperscript{113}. Based on crystal structures of DExD/H box proteins complexed with RNA, the binding sites in the helicase core contact the RNA backbone exclusively, allowing association with duplex RNA but excluding preferential binding of particular RNA sequence motifs\textsuperscript{114}. Although the core domain is highly conserved, these helicase enzymes catalyze a wide range of reactions by interacting with a variety of proteins.

In a healthy uninfected cell, DExD/H box helicases function in multiple ways throughout RNA metabolism, including translation initiation, microRNA processing, nuclear transport, alternative splicing, and long non-coding RNA structure homeostasis\textsuperscript{111,115,116}. When a cell is infected with a virus, several DExD/H box helicases that are not part of the RIG-I family have also been recently shown to act as viral PRRs or adaptors of innate immune pathways. Some examples are DDX3, DDX41, and DDX24\textsuperscript{93,111,115,117-119}.

**Charcot-Marie-Tooth Disease Type 1A**

CMT1A belongs to a group of inherited neurological disorders, termed Charcot-Marie-Tooth (CMT), named after their founders. Each form of CMT is caused by an abnormality in a different gene. The neuropathy type 1A, CMT1A, is caused by a duplication of the Peripheral Mylin Protein or PMP22 gene on the short arm of chromosome 17\textsuperscript{11}. The duplication was discovered by Dr. Lupski in 1991 using two color fluorescence in situ hybridization to directly visualize the gene duplication in CMT1A patient derived cells\textsuperscript{12}. At the polymorphic locus, sequence identity of homologous repetitive regions that flank the PMP22 gene elements result in a cross-over event of chromosomes, inserting the additional gene copy, referred to as a \textit{de novo} mutation\textsuperscript{120}. Charcot-Marie Tooth disease type 1A is an autosomal dominant genetic disorder with a prevalence of about 10 cases per 100,000 individuals\textsuperscript{121-123}. The children of a patient with
CMT1A have a 50% chance of inheriting the disorder. Since PMP22 encodes for a dose sensitive protein component of myelin, its gene duplication causes abnormal structure and function of the myelin sheath in the peripheral nervous system, leading to progressive sensory loss and muscle atrophy\textsuperscript{121,124,125}. People with the disease are typically slow-runners as children due to weakness in the lower extremities\textsuperscript{121}. As the disease progresses, foot deformities, muscle atrophy, and in rare cases respiratory muscle weakness occurs\textsuperscript{121}.

PMP22 is a dose-sensitive, disease-associated protein primarily expressed in myelinating Schwann cells\textsuperscript{124}. Schwann cells rest on top of peripheral nerves and generate myelin in their membrane, a fatty white substance that forms an electrically insulating layer that increases the nerve conduction velocity. During development and differentiation, an immature Schwann cell will begin to make myelin when it settles on an axon\textsuperscript{126}. Then, it wraps its plasma membrane in successive layers, referred to as spiral wrapping\textsuperscript{126}. When sufficient wrapping has occurred, a sheath is formed. As time progresses, the wrapping continues and the outermost layer, the neurilemma, is able to be regenerated throughout a lifetime\textsuperscript{126}.

PMP22 is a helical membrane protein component of myelin that comprises 2-5% by weight and organizes membrane ultrastructure\textsuperscript{127}. Duplication of the PMP22 gene in CMT1A leads to demyelination of the peripheral nerves. In a CMT1A affected individual, over-accumulated PMP22 protein gets marked for degradation by ubiquitin, directing the unneeded protein product to the proteasome\textsuperscript{128}. Over time, the undegraded protein cargo forms perinuclear aggregations of PMP22 in the Schwann cell\textsuperscript{128}. The over-abundant PMP22 protein is defective in reaching the cell membrane, presumably leading to the myelin sheath defects.

Severity of CMT1A is dependent on PMP22 copy number. For instance, patients with 4 copies rather than 3 have a much more severe neuropathy, whereas those with a single copy form
a different neuropathy, called hereditary neuropathy with liability to pressure palsies (HNPP). Individuals with CMT1A experience a wide range of deterioration, severity of symptoms, and age of onset, most likely due to other factors that interact with PMP22. This dose sensitivity suggests that reducing PMP22 dosage, only partially, in patient myelinating Schwann cells via small non-coding RNAs may be ideal to treat CMT1A.

The restricted tissue expression of the PMP22 protein in comparison to its ubiquitously detected mRNA supports a role for microRNA (miRNA) mediated post-transcriptional regulation. MicroRNAs (miRNA) are small regulatory molecules that can repress translation by targeting the 3’UTR of mRNAs in a reverse complementary manner. Recently, antisense oligos ASOs have been shown to be effective against neurodegenerative diseases such as CMT1A in mouse models. ASOs are similar to microRNAs with several key differences. ASOs are synthetic, 20 nucleotide sequences that are completely complementary to the sequence of a disease associated messenger RNA or microRNA. The duplex RNA that is formed by hybridization of the ASO is then degraded by RNase H, and translation or function is prevented. Sugar modifications allow ASOs to be water soluble and resistant to exonucleases. A key component is the fact that ASOs interact with various cell types following systematic subcutaneous injections, require a high concentration, and are predominantly excreted by the liver. In contrast, microRNAs are endogenously expressed, often partially mismatched, and incorporate in the AGO RNA-induced silencing complex to locate their target.

Our goal is to create a delivery system of an endogenously expressed microRNA that targets PMP22, is Schwann cell type specific, and functional in humans as well as mice. In our research we have identified miR-29a as a microRNA which targets PMP22 and is conserved among mammals. In order to achieve Schwann cell specific delivery, a construct with a Schwann
cell specific promoter will be packaged into an Adeno-associated virus (AAV) serotype that efficiently infects glia of the peripheral nervous system.
Results

Knockdown Of DExD/H Box Helicases to Screen For Effects On KSHV Reactivation

To determine if DExD/H box helicases in addition to RLRs affect KSHV lytic reactivation, we performed a transient siRNA knock-down (KD) screen of 22 individual DExD/H box helicases that were previously found to have an effect on Myxoma virus infection efficiency\(^\text{134}\) (Table 2-1). The Vero cell line latently infected with a recombinant form of KSHV (rKSHV.219) was chosen as our model because it allows us to easily detect latent and immediate-early lytic stages of infection via fluorescent markers\(^\text{135}\). The recombinant virus was constructed with an EF-1\(\alpha\) constitutive promoter that drives green fluorescent protein (GFP) expression and a KSHV lytic PAN promoter that drives RFP expression, indicating immediate-early KSHV reactivation\(^\text{135}\). Although Vero rKSHV.219 offered a simple tool for a reactivation screen, Vero cells do not produce type I interferons, one of the two downstream pathways of MAVS signaling (Figure 2-2). IFN competent cells such as BCBL-1 cells were used for further phenotypic studies in our research.

Vero rKSHV.219 cells were seeded in individual wells and a Lipofectamine transfection was performed with a pool of 5 siRNAs that target a particular DExD/H box helicase or scrambled control (Table 2-2). After the knock-down, duplex-containing media was replaced with media containing tetradecanoyl phorbol acetate (TPA) and sodium butyrate (NaB) to chemically induce lytic reactivation, and microscopy images and counts were recorded (Figure 2-3). Three random fields of vision were selected to quantify the adherent Vero cells prior to the 29 hour post-induction time point. After the 36-hour time point, lytic Vero cells were no longer adherent and could not be properly quantified.
Within 24 hours, the DHX29 knockdown demonstrated a 90% reduction in reactivation and two of the hits, DDX49 and DDX24, showed a 2-fold increase in reactivation (Figure 2-4). It should be noted that all knock-downs without induction showed little to no background reactivation. Additionally, all cells appeared viable until the chemical inducers were added to the media to activate the KSHV lytic cycle. When RIG-I alone was knocked down in Vero rKSHV.219 cells during the screen, there was no observable change in reactivation when compared to the non-specific control (data not shown).

To confirm that RFP expression truly represented lytic DNA replication, qPCR was run on intracellular DNA with primers that amplify a region encoding for viral LANA. The viral copy number at 48 hours post-induction demonstrated active KSHV replication and was in congruence with the fluorescence microscopy results. DHX29 knockdown had about 2/5 the number of genome copies, DDX49 knockdown demonstrates a 1.5 fold increase, and DDX24 knockdown demonstrated a 3-fold increase in the number of viral genome copies (Figure 2-5).

**DDX24-DDK And DDX49-DDK Stably Transfected BCBL-1 Cells**

In order to monitor KSHV reactivation in a stable transformants, we transfected human BCBL-1 cells via electroporation to overexpress either DDX24 or DDX49. BCBL-1 cells are interferon-competent, KSHV latently infected B cells that were isolated from a PEL biopsy. Overexpression was achieved by electroporating BCBL-1 cells with constructs containing DDK tagged versions of complete DEAD box open reading frames (Figure 2-6). Primers targeting the gene body demonstrated an increase of the stably transfected DDX24-DDK and DDX49-DDK cells compared to their scrambled counterparts which contain only endogenous DDX24 and DDX49 (Figure 2-6A). Although endogenous DDX24 was expressed at a relatively high abundance in the cell (based on normalization to GAPDH mRNA), significant overexpression was still observed. Additionally, primers exclusively targeting the DDK tagged 3’ end of the
cDNA demonstrated expression of the DDX24-DDK and DDX49-DDK relative transcript abundance (Figure 2-6B). An immunoblot utilizing an anti-DDK antibody confirmed protein expression of our transgenic helicases, DDX24-DDK and DDX49-DDK (Figure 2-6C). The survival of DDX24-DDK and DDX49-DDK stably transfected BCBL-1 cells during the induction time course, demonstrated that population cell death was unaffected in the stably transduced BCBL-1 cells and did not determine KSHV viral production in this experiment.

**DDX24-DDK And DDX49-DDK Reactivation Assays**

Viral latent, immediate-early, and late lytic gene expression was monitored in a time course experiment for up to 72 hours post-induction by RT-qPCR (Figure 2-7). When DDX24-DDK or DDX49-DDK were constitutively overexpressed in BCBL-1 cells, transcription of lytic viral genes was inhibited upon induction. Latent LANA transcription is responsive to the induction of Rta, and as such the fold change had also significantly changed slightly for DDX24-DDK BCBL-1 cells. The expression of immediate-early Rta, the master regulator of lytic KSHV gene expression, was inhibited the most throughout the 72 hour time course. When DDX24 or DDX49 is overexpressed, Rta gene expression is nearly non-detectable. The fact that Rta is inhibited suggests that the effects of these host helicase act very early in reactivation. Late lytic, K8.1, gene expression remained inhibited throughout the entire induction time course in the DDX49 overexpressing cells.

Additionally, to monitor active replication, qPCR was performed on intracellular viral DNA over a time course of 72 hours. By 48 hours, both DDX24-DDK and DDX49-DDK BCBL-1 cells had about 2/3 the viral genome copy number of the empty vector control. In addition to lytic gene expression, KSHV genome replication was also reduced in these stably transfected cells, further demonstrating that reactivation was suppressed when DDX24 or DDX49 was overexpressed (Figure 2-8).
**DHX29 KD Stably Transduced BCBL-1 Cells**

In the initial screen we saw that when DHX29 was knocked down there was a decrease in the amount of KSHV reactivation. We decided to stably knock-down DHX29 in BCBL-1 cells (Figure 2-9A-C). In order to achieve a stable knockdown, we prepared lentivirus via second generation packaging of the pLKO.1 plasmid. The RNAi Consortium (TRC) at the Broad Institute designed simple hairpin shRNAs to be expressed by the pLKO.1 lentiviral vector and the University of Florida purchased these from Dharmacon. For viral production, three plasmid constructs were co-transfected into HEK 293FT cells: pLKO.1, pMD2.G, and psPAX2. BCBL-1 cells were stably transduced with either empty pLKO.1 or a construct expressing shRNAs targeting DHX29. A pool of 5 different shRNA expressing lentiviruses were used to knock down DHX29 (Table 2-3). qPCR showed about a 50% decrease in steady state transcript levels (Figure 2-9A). Additionally, immunoblots of endogenous DHX29 showed about a 40% knockdown at the protein level (Figure 2-9B and 2-9C). There was also no significant change in stably transduced DHX29 KD BCBL-1 cell survival post-induction when compared to the empty vector (Figure 2-9D).

**DHX29 KD Reactivation Assays**

In stable DHX29 Knockdown BCBL1 cells, the effect on viral gene expression was not statistically significant with an approximately 50% decrease in Rta expression at 24 hours. DHX29 KD BCBL-1 cells demonstrated a 45-55% decrease in immediate-early and early lytic gene expression at 48 hours post-induction (Figure 2-10). The phenotypic effect was not as dramatic as that seen in the DDX24-DDK and DDX49-DDK over-expressing cells but was still in congruence with that seen in the initial screen. Immediate early and early lytic KSHV gene expression was inhibited throughout the 72 hour induction time course. Unfortunately, the values between the three biological replicates varied greatly, causing the observed reduction to
be statistically insignificant (p = 0.3-0.4) in the DHX29 KD BCBL-1 cells. Measurement of intracellular genome copy number from a single pair of samples demonstrated a slight decrease in KSHV genome replication over the 72 hour time course (Figure 2-11). Since we could not reiterate the pro-viral effect in the DHX29 Knockdown cells that was seen in the initial screen, we did not move forward with DHX29 studies.

**Stably Transduced TREx BCBL1-Rta Cells Expressing DDX24-Avi And DDX49-Avi**

Based on the knockdown and overexpression data there appears to be a functional role of DDX24 and DDX49 in KSHV reactivation. We demonstrated a phenotype and wanted to characterize the biological role of these helicases during latent and lytic KSHV infection. It is well established that RIG-I interacts with RNAs during viral infection, and so we sought to discover if DDX24 and DDX49 also have the ability to interact with viral RNAs. RNA immunoprecipitation was a means for us to identify which RNAs these helicase proteins are binding. RNA immunoprecipitation was performed on doxycycline inducible B cells (TREx BCBL1-Rta cells) overexpressing our helicases of interest. There are several benefits of this cell type compared to BCBL-1 cells in high-throughput studies. TREx-BCBL1-Rta cells are BCBL-1 cells that contain the lytic Rta transcriptional regulator of KSHV downstream of a tetracycline inducible promoter. They were engineered by the Jae Jung lab using Flp-mediated site-specific recombination. The benefits of this system are that we can achieve a more robust induction. Between 60-80% of the total cell population will undergo reactivation, compared to only about 10% in induced BCBL-1 cells. In addition, Rta-specific induction avoids an increase in global cellular transcription, as is seen in TPA and NaB induction. When Pol II begins the process of transcription, it must displace the nucleosome in front of it. Histone Acetyltransferases (HATs) facilitate this process by acetylating the histone tails of the nucleosome. After Pol II has read through, histone deacetyltransferases remove the acetylation
mark on the nucleosome behind it, preventing further, unwanted transcription of the gene. Both inducers lead to a global increase in transcription, which would be detrimental to a high throughput study because it would increase non-specific RNA-helicase interactions. Finally, lytic gene expression is induced in a more temporally appropriate manner when Rta expression alone is induced.

For ribonomics experiments, much more robust expression must be achieved than that yielded by transfection by electroporation. To ensure the most efficient integration and subsequent overexpression of the construct expressing Avi/FLAG-tagged DExD/H box helicases, lentiviruses were used to transduce TREx-BCBL1-Rta cells with a construct expressing DDX24 or DDX49 driven by pCMV, a strong constitutive promoter. The Avi fusion protein contains a 15 amino acid Avi tag (GLNDIFEAQKIEWHE) at the C-terminal end for purification. The Avi tag has the advantage that it is the strongest biological interaction. A high affinity antibody is critical for pulling down these helicases which do not traditionally have selective antibodies because they share a lot of sequence homology among the endogenous proteins. The anti-Avi antibody is designed to target the 15-amino acid tag of Streptavidin and offers extremely specific and high affinity binding. Five biological groups were transduced, the four helicases plus GFP which was used as the control to monitor successful expression (Figure 2-12). Lentiviruses containing pLV constructs able to express Avi/FLAG-tagged versions of maxGFP, DDX24, DDX49, and DDX58 (RIG-I) were used to infect TREx-BCBL1-Rta cells. The day after infection, the virus containing media was replaced with media containing low concentrations of puromycin and grown under selection for 7 days. Then, the puromycin concentration was gradually increased to 1 μg/mL for another 5 days. GFP expressing cells were monitored periodically for successful expression of the transgene. The infection was performed
twice. Western blot and qPCR demonstrated the expression for the Avi-tagged helicases in TREx-BCBL1-Rta cells (Figure 2-12).

The DDX24-Avi, DDX49-Avi, and RIG-I-Avi recombinant cells were able to express their helicase of interest after both infections. When generating overexpressing cells for RNA IP analysis, DHX29-Avi cells overexpressed the least amount of tagged DHX29. The band was undetectable on western blot (Figure 2-12). For this and other reasons mentioned in the discussion, we decided not to proceed with DHX29-Avi/FLAG in the RNA immunoprecipitation (RIP) analysis.

**RNA Immunoprecipitation Of TREx BCBL1-Rta Cells**

RIP is an antibody based analytical technique that involves identifying RNA targets of a particular RNA binding protein of interest through next generation sequencing. For the RIP analysis, four biological groups were harvested in biological duplicate for the analysis. 100 million TREx BCBL1-Rta cells, either non-transduced or overexpressing DDX24, DDX49, or DDX58 were grown per sample. The non-transduced TREx-BCBL-1 cells were used as a control. One set of flasks was harvested during latency, the other was reactivated in RPMI media containing doxycycline and phosphonoformic acid (PFA) for 24 hours. The lysate was immunoprecipitated overnight with α-Avi antibody and the antibody-bound lysate was then incubated with beads binding the heavy chain. The RNA was then extracted by TRIzol (Sigma-Aldrich), DNase treated (Invitrogen), libraries were prepared, and submitted for sequencing.

RIP analysis is unique from other popular RNA sequencing techniques in that there is no crosslinking performed. This serves the purpose of isolating higher energy binding sites between the helicase and RNA target. Any given DExD/H box helicase will bind transiently to or translocate along multiple eukaryotic RNA targets within the cell. We would only like to focus on those interactions, viral or human that are legitimate. The major argument against this
technique is that nuclear:cytoplasmic or subcellular compartment interactions that otherwise would not occur endogenously, now may occur during cell lysis. However, in the case of such ubiquitous binding as that seen in RNA helicases, removing the crosslinking step ensures that when the washes are performed, we are removing as many non-specific interactions as possible.

In order to verify that the TREx-BCBL1-Rta cells were properly induced under these IP conditions, a qPCR on lytic viral RNA was performed (Figure 2-13). As evidenced by the fold change of RTA and ORF59 before and after induction, TREx-BCBL1-Rta cells were robustly induced in the presence of doxycycline and PFA after 24 hours. PFA is a compound that blocks KSHV genome replication by inhibiting the viral DNA polymerase. This allows for lytic gene expression while preventing lysis of the host cell. Additionally, in order to ensure that our immunoprecipitation is enriching for our Avi-tagged helicases, we ran an immunoblot of lysates before and after streptavidin bead selection (Figure 2-13). Avi-tagged DDX49 is barely visible in the input, which contains a multitude of proteins. After immunoprecipitation, a single, distinct band could be seen for DDX49-Avi at the correct size, 54 kDa, indicating that Avi-tagged proteins are selectively immunoprecipitated with this antibody.

Libraries were prepared using the TruSeq Stranded Total RNA kit with Ribo-Zero Gold (Illumina). The stranded library differentiates between sense and antisense RNA transcripts which is crucial for this analysis because extensive antisense transcription occurs in KSHV. The Ribo-Zero gold method of library preparation ensures that viral and cellular noncoding RNAs remain included in the analysis while selectively removing ribosomal RNAs. TruSeq Stranded Total RNA kit using Ribo-Zero Gold selectively removed cytoplasmic and mitochondrial ribosomal RNAs via a hybridization/bead capture procedure that binds rRNA species using biotinylated capture probes. The probe:rRNA hybrid was then captured by magnetic beads and
removed using a magnet, leaving the desired rRNA-depleted RNA in solution. Removing the bulk of ribosomal RNAs, followed by size selection, allowed us to capture viral and cellular IncRNAs, snRNAs, bidirectional transcription, and circular RNAs greater than 200 nucleotides long, in addition to mRNAs.

After the libraries were generated, quality control was performed with TapeStation (Agilent) to verify that enough enriched RNA was converted to cDNA for sequencing and within the appropriate size limits. Input and IP selected samples were within the sequencing range for non-transduced, DDX24-Avi, and DDX49-Avi TREx-BCBL1-Rta cells (Table 2-4). The cDNA library was below the detectable limit in the RIG-I overexpressing TREx BCBL1-Rta cells after bead selection. Sequencing was performed by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida on the Illumina HiSeq3000 platform to generate 50 million, 100 base paired-end reads.

The sequencing data were processed and analyzed as follows. First, Illumina adapter sequences were removed from the reads using Trimmomatic\textsuperscript{141}. The quality of the reads was then verified with FastQC\textsuperscript{142}. After these pre-processing steps, a paired-end alignment was performed against the KSHV BAC16 genome (GenBank accession number GQ994935.1)\textsuperscript{143} using Bowtie2\textsuperscript{144}. Once WIG files were generated from the alignment data, the peaks from the control samples, which represented non-specific background, were subtracted from those of the experimental DDX24 and DDX49 samples. The peaks were visualized using the open-source Integrative Genomics Viewer tool\textsuperscript{145}.

We observed specific enrichment of immediate early and early lytic KSHV transcripts, suggesting that DDX24 and DDX49 have a means to inhibit KSHV reactivation from within the nucleus (Figure 2-14). There is very little being pulled down on the negative strand and the
helicase binding regions are found in a mutually exclusive manner, suggesting that the helicases are probably recognizing duplex RNA regions contained in the secondary structure of the transcript and not bidirectional transcription of the KSHV genome. Additionally, the DDX24 and DDX49 helicases are recognizing identical regions of duplex KSHV RNAs during latent and lytic infection to varying degrees (Figure 2-14). Some RNA binding sites between DDX24 and DDX49 were the same and others were different, indicating some degree of specificity. As mentioned previously, DExD/H box proteins interact with RNA backbone in a sequence non-specific manner 59. The unique recognition and downstream enzymatic functions of each of the helicases in vivo were most likely determined by their protein binding cofactors. Three additional regions, shown in orange boxes, are enriched in the DDX49 pulldown, K8.1, K9, and ORF 65/66/67 (Figure 2-14).

There were 4 regions that had the greatest enrichment in both helicases; K2, ORF70/K4/K4.1/K4.2, K5, and the ORF58/59 region (Table 2-5). All of these regions include genes that encode for immediate-early or early KSHV lytic proteins involved in immune modulation, pathogenesis, or reactivation. K2 and ORF58/59 are of particular interest. K2 is an immediate-early/early gene that encodes for vIL-6. K2 activates JAK/STAT, Mitogen Activated Protein Kinase (MAPK), and Akt signaling pathways to regulate B-cell proliferation and KSHV reactivation 76. This region was most highly enriched in the latent samples. Also, ORF58 and ORF59 are unique in that they both have homologs in other herpesviruses. ORF59 is a viral DNA polymerase processivity factor that is required for the early events of KSHV infection, genome amplification, and latency establishment 146.

**Discussion**

The screen revealed that when DDX24 and DDX49 were knocked down, KSHV reactivation was increased. In contrast, the DHX29 knockdown inhibited KSHV reactivation and
replication. These data suggest that DHX29-dependent activities support viral replication. In contrast, DDX24 and DDX49 have anti-viral activity. Since these helicases serve multiple functions in a cell, we reviewed the current literature to see if any of these helicases have known functions that coincide with the mechanisms necessary for maintaining the KSHV life cycle.

DDX24 is expressed in the nucleus and the cytoplasm and has known interactions with both the innate immune system and KSHV. The DDX24 promoter region has several interferon-regulated transcription factor binding sites such as STAT1 and IRF7 and DDX24 preferentially impedes recruitment of IRF7 to the signaling complex through interaction with Receptor-interacting protein 1 (RIP-1)\textsuperscript{119}. DDX24 associates with the adaptor proteins FADD and RIP-1 of a caspase pathway known to be actively repressed by KSHV vFLIP, to attenuate NF-κB and IRF7 during infection\textsuperscript{119,147}. In a latently infected cell, vFLIP promotes cell survival and inhibits premature death-receptor mediated apoptosis which would otherwise prevent KSHV replication\textsuperscript{148}. There is already a known mechanism for how KSHV could target DDX24 in order to regulate reactivation. MicroRNAs from other herpesviruses have been recently identified to target DDX24. Ribonomics data sets collected from Renne and Tibbets labs show KSHV, Murine gammaherpesvirus 68 (MHV68), and EBV microRNAs all form hybrids with DDX24 and are a conserved means to regulate DDX24’s expression. Specifically, DDX24 is a putative target of KSHV miR-K12-6-3p and miR-K12-12-5p in Telomerase-immortalized human umbilical vein endothelial (TIVE) EX-LTC cells based on miRNA interactome Cross-linking, ligation, and sequencing of hybrids (CLASH) analysis and qPCR\textsuperscript{41} (Figure 2-15). Based on a BLAST search, the hybrids are located in the highly conserved DDX24 coding region of exon 3 and in the 3’UTR of exon 9 which prompted our interaction with the Tibbetts lab. Based on Dr.
Mehmet Kara’s CLASH data, microRNAs of other gammaherpesviruses, MHV68 and EBV, also putatively target DDX24 (Kara, Mehmet and Bullard, Whitney, unpublished results).

Steady state levels of DDX24 from TIVE cells infected with either wild type KSHV, \( \Delta \text{miR}-\text{K12}-6 \), or \( \Delta \text{miR}-\text{K12}-12 \) were analyzed by qPCR (Figure 2-15). One biological preparation remained from Lauren Gay’s studies. Interestingly, when either of these KSHV microRNAs are absent during latent infection, there was a decrease in steady state DDX24. This suggests that TIVE cells have a totally different transcriptome profile than TIVE-EX-LTC cells, the cell type in which the hybrids were discovered. Because one of the microRNA directed DDX24 target sites is in an open reading frame, it is difficult to predict how it functions. Dr. Vasudevan found that, contrary to their traditional role, microRNAs can contribute to translational activation during cell quiescence when the targeted sequences are in the 5’UTR or exonic regions\(^{149}\). Western blots in TIVE-EX-LTC and further small RNA studies will need to be performed to confirm that DDX24 is a target of gammaherpesviral microRNAs.

Until 2018, there was little experimental evidence for DDX49’s function in a cell. In March 2018, the Dhayalan group characterized DDX49 and found that it is required for the efficient export of non-spliced RNAs from the nucleus in a splicing-independent manner\(^{150}\). Interestingly, about 75% of KSHV genes are not spliced and require nucleolar localization for KSHV mRNA export\(^{151}\). DDX49 expression is elevated in a variety of cancer types, suggesting a role in oncogenic transformation of cells\(^{150}\). In particular, DDX49 expression is highly upregulated upon KSHV co-infection in huNSG Mouse-Derived EBV positive Cell Lines by about 1.7 fold\(^{152}\).

Finally, DHX29 is a translation initiation factor that enhances the ability of the 40S subunit to overcome 5’UTR secondary structures in a RNA target-independent manner \(^{153}\).
Homozygous knockouts of the DHX29 helicase are embryonic lethal in mice\textsuperscript{154}. Common to other translation initiation factors, DHX29 dissociates from the 40S subunit upon completion of translation initiation and monitors “entry” into the 43S complex of highly structured 5’UTRs.\textsuperscript{153} Highly structured RNAs, termed IRESs are often used by viruses to promote ribosome binding. Interestingly, vFLIP is translated from the only mRNA containing an IRES in KSHV\textsuperscript{25,155}. DHX29 senses cytosolic RNAs in human cells through a co-sensing mechanism with RIG-I and MAVS to trigger anti-viral immunity\textsuperscript{156,157}.

In our knock-down screen we observed how well KSHV is able to reactivate from latently infected Vero cells when the expression of DDX24 or DDX49 was suppressed. These data suggested that DDX24 and DDX49 may have antiviral activity in KSHV infected cells, and that DHX29-dependent activities may support viral replication. Vero cells are IFN deficient, suggesting that DDX24 and DDX49 may be acting on NF-κB to suppress reactivation. Based on current literature, the IRF7 and IRF3 pathways may also be involved and are not mutually exclusive\textsuperscript{119}. In contrast, some helicases are also required for viral life cycles, which may be the case for DHX29\textsuperscript{94}. For example, host DDX3 is required for HIV mRNA Rev-RRE export function but, by the same token, DDX3 also senses abortive HIV-1 RNAs to induce a type I interferon response through MAVS and recruit dendritic cells\textsuperscript{158,159}. Numerous inhibitors of helicases have proven to be successful against multiple viral infections and cancers, including an inhibitor of cellular DDX3 which is undergoing human phase I clinical trials. The phenotypes observed in this screen, bolstered by current literature, suggest that the innate immune system may be involved in suppressing reactivation in latently infected KSHV cells.

For further studies we generated stably transfected BCBL-1 cells. Overexpression of DDX24-DDK and DDX49 DDK in BCBL-1 cells inhibited KSHV genome replication and
KSHV lytic gene expression, suggesting that the effects of the host helicases act very early in reactivation. In theory, DDX24 and DDX49 interact with and recognize an immediate-early or early KSHV gene at the cusp of reactivation. Since both helicases are located in the nucleolus, where early lytic intronless KSHV mRNA must go in order to be exported from the nucleus, it would be interesting to see if there is a synergistic effect of the two helicases\textsuperscript{151}.

Lentiviruses expressing empty pLKO.1 or shRNAs targeting DHX29 were used to stably knockdown DHX29 in BCBL-1 cells. The results of the reactivation assays in DHX29 KD BCBL-1 cells were insignificant. It seems that in an IFN-competent cell line, such as BCBL-1 cells, the potential effects of the DHX29 KD were diminished. It stands to reason that IFN regulatory factors such as IRF3 and IRF7 may neutralize the “pro-viral” effect of DHX29 on reactivation. Additionally, both empty pLKO.1 and DHX29 KD stably transduced cells had difficulty reactivating. The stably transduced empty pLKO.1 and DHX29 KD BCBL-1 cells could not be induced into the lytic cycle as easily as the transfected DDX24-DDK or DDX49-DDK BCBL-1 cells. For this reason, we switched from using just 2 mM NaB to a combination treatment of 20 ng/mL TPA and 2 mM NaB. NaB is a chemical agent which stimulates KSHV reactivation by acting as a histone deacetylase inhibitor. TPA is a chemical agent that is the most potent inducer of lytic KSHV reactivation. In the presence of TPA, cells overexpress histone acetyltransferases and stimulate the Mitogen Activated Protein Kinase (MAPK) pathway. MAPK is a key cell signaling pathway that regulates cell proliferation, differentiation, and apoptosis. This is one of the pathways KSHV manipulates to regulate the viral life cycle. Even with this more robust chemical induction, empty pLKO.1 and DHX29 KD transduced cells showed only about 1/10 the Rta induction of electroporated BCBL-1 cells (Figure 2-7 and 2-10). Knockout of DHX29 by gene editing techniques might have demonstrated a more dramatic effect in BCBL-1
cells, but knockdown of the gene alone results in reduced protein translation and impaired proliferation of other cancer cells\textsuperscript{160}. Cellular proliferation was vastly different between the empty pLKO.1 and the DHX29 KD transduced BCBL-1 cells, making KSHV reactivation analysis difficult. The empty pLKO.1 control BCBL-1 cells grew faster than the DHX29 KD cells and the DHX29 KD cells clumped at the bottom of the flask after a few passages (data not shown). Since DHX29 is known to be involved with cellular translation of highly structured 5′UTRs, it stands to reason that knockdown would result in reduced protein translation. It is already known that knockdown of DHX29 impairs proliferation of HeLa cells\textsuperscript{160}. In short, DHX29 expression appears to be tightly controlled and toxic to the cell during knockdown or at higher expression levels.

In our research, we also attempted to isolate RIG-I knockdown BCBL-1 cell types but were unsuccessful. This is in accord with previously published data. RIG-I knockdown in epithelial-derived cells latently infected with KSHV (iSLKs) have been shown to increase reactivation, but no significant changes were seen in BCBL-1 cells when compared to the control\textsuperscript{8,161,162}. Knockdown of RIG-I may be ineffective in BCBL-1 cells because endogenous RIG-I is expressed at a low level in this cell type, and it is already established that RIG-I is targeted by KSHV\textsuperscript{9}. An α-Avi immunoprecipitation was also performed on RIG-I over-expressing cells. However, the cDNA library was below the detectable limit on TapeStation, further supporting the notion that RIG-I recognizes KSHV through cross-talk with other sensors.

Based on the RIP analysis performed in Rta-inducible TREx-BCBL1 cells, these helicases are mostly binding immediate-early and early lytic KSHV RNAs (Table 2-5), suggesting that DDX24 and DDX49 can bind KSHV transcripts from within the nucleus to prevent reactivation. Four of the five highest enrichment regions K2, ORF70/K4/K4.1/K4.2, K5,
and K9 are all involved in reactivation via immune modulation (Table 2-5). Of particular importance, K2 encodes for vIL-6, which has a short but structured 3’-UTR containing hairpins and is required for survival of KSHV infected B-cells. In a ribonomics experiment performed by the Damania lab, RIG-I was also found to bind to K2 KSHV fragments identified by Illumina deep sequencing analysis in iSLK.219 cells. The fifth high enrichment region ORF58/59, although not directly involved in reactivation is critical for viral replication and has homologs in other herpesviruses. Putative helicase binding regions are found on a single strand, in a mutually exclusive manner, suggesting that the helicases are probably recognizing duplex RNA strands contained in the secondary structure of the transcript and not bidirectional transcription of the KSHV genome. The helicases bind in identical regions during latent and lytic infection, suggesting that DDX24 and DDX49 bind to the same immediate-early transcripts at varying degrees even at low levels of expression. Three additional regions are exclusively enriched in the DDX49 pulldown, K8.1, K9, and ORF 65/66/67. ORF65 and K8.1 are late lytic, which may explain the prolonged suppression of KSHV reactivation observed in the reactivation time course. If we can further understand the mechanism of these DExD/H box helicases, we could potentially manipulate KSHV reactivation. This could lead to therapeutics that help to prevent KSHV-associated malignancies.

**Materials and Methods**

**Cell culture.** Vero rKSHV.219 cells, a gracious gift from the Michael Lagunoff lab, were maintained in DMEM with 10% FBS and 10 μg/mL puromycin, and induced with 20 ng/mL TPA & 2 mM NaB. Knockdown BCBL-1 cells were maintained in RPMI 1640 with 10% FBS and 1 μg/mL puromycin 10%, and induced with 20 ng/mL TPA and 2 mM NaB. Electroporated DExD/H box-DDK overexpressing BCBL-1 cells were maintained in RPMI 1640/10% FBS with 500 μg/mL G418, and induced with 2 mM NaB. Lentiviral transduced DExD/H box-Avi
overexpressing TREx BCBL1-Rta cells were maintained in RPMI 1640/10% FBS with 1 μg/mL puromycin, and induced with 1 μg/mL doxycycline and 0.25 mM PFA.

**Helicase siRNA screen.** Vero rKSHV.219 cells were selected because they transfect relatively well and include lytic gene associated fluorescent markers. 200 μL of Vero rKSHV.219 cells were seeded in a 48-well plate at 9.5x10^4 cells/mL in DMEM/10% FBS without antibiotics and grown overnight. ON-TARGETplus siRNA duplex pools (Dharmacon, Horizon Discovery, Lafayette, CO) targeting a particular helicase were transfected with Lipofectamine RNAiMAX (Invitrogen, Thermo-Fisher) for a final siRNA concentration of 50 nM as per the manufacturer’s instructions. After 28 hours, the knock-down of control RNAi GFP was confirmed and all siRNA containing media was replaced with 10% FBS media containing 20 ng/mL TPA and 2 mM NaB. Fluorescence images were acquired on a Leica DMI 4000 microscope and DNA was extracted at 48 hours post-induction.

**Real-time qPCR.** RNA was extracted from KSHV infected cells throughout the induction time course using RNA Bee (Tel-Test, Friendswood, TX). Total RNA was quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and 0.9 μg Vero RNA or 1.6 μg BCBL-1 RNA was Turbo DNase treated (Invitrogen, Carlsbad, CA), then converted to cDNA with the High-Capacity RNA-to-cDNA Kit (Thermo Scientific, Waltham, MA). All cDNA analyses were quantified relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

DNA was extracted using a QIAamp DNA Mini kit (QIAGEN, Valencia, CA). KSHV genome copy numbers from 30 ng of total DNA were quantified utilizing LANA N-terminus primers based on a standard curve constructed from known amounts of pcDNA3.1 plasmid DNA. Quantitative, real-time PCR (qPCR) was performed on an ABI StepOnePlus (Applied
Biosystems, Foster City, CA) for the Vero intracellular cDNA/DNA and a Roche Light Cycler 96 (Roche, Indianapolis, IN) for the BCBL-1 intracellular cDNA/DNA (Table 2-4). DNase-treated RNA was converted to cDNA using RNA-Quant™ cDNA synthesis kit (System Biosciences, Palo Alto, CA) in order to include RNA classes with strong secondary interactions.

**Transfection by electroporation for BCBL-1 overexpression.** 1x10^7 BCBL-1 cells were grown in antibiotic-free RPMI. The next day, they were pelleted and rinsed twice in PBS. The cell suspension was transferred into a 0.4 cm electrode gap cuvette with 10 μg of TrueORF Gold pCMV6-Entry DNA vector (Origene, Rockville, MD) containing the Myc-DDK (FLAG)-tagged helicase of interest (Origene, Rockville, MD) or pmaxGFP (AddGene, Cambridge, MA). Cuvettes were treated 2 times at 250 V, 950 μF with the Gene Pulser Xcell device (Bio-Rad, Hercules, CA). Electroporated BCBL-1 cells were grown for 48 hours in non-selective RPMI medium. After the 48 hours, media was replaced with RPMI containing 400 μg/mL G418 for 4 weeks.

**Antibodies and reagents.** Monoclonal mouse antibody against DDK (TA50011) was from Origene (Rockville, MD). Monoclonal mouse antibody against Avi was from Genscript (Piscataway, NJ). Goat αDHX29 (sc-107197) and goat αGAPDH (V-18, sc-20357) were from Santa Cruz (Santa Cruz, CA). Mouse monoclonal α-Tubulin (CP06-100UG) was from Oncogene/EMD Millipore (Burlington, MA). IRDye 800-labeled IgG and IRDye 680-labeled IgG Donkey α-goat (925-68074) and α-mouse (925-32212) secondary antibodies were from Li-Cor Biosciences (Lincoln, NE).

**Western blot analysis.** 8x10^5 cells were pelleted by centrifugation, washed in cold PBS, and resuspended in 80 μL RIPA buffer containing 150 mM NaCl, 50 mM Tris (pH 8), 1% (v/v) NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, and 0.1% SDS with protease inhibitor cocktail
(Roche, Indianapolis, IN). Pierce BCA protein assays were performed (Thermo Fisher, Waltham, MA). 20-40 μg aliquots (25 μg for overexpressing BCBL-1 cells, 20 μg for DHX29 KD, and 40 μg for TREx BCBL1-Rtas) of total protein were separated by 10% SDS-PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with Odyssey blocking buffer (TBS) from Li-Cor Biosciences (Lincoln, NE) at 4°C overnight and then subsequently incubated with primary antibodies (1:2000 α-DDK, 1:200 α-GAPDH for overexpressing BCBL-1 cells; 1:200 α-DHX29, 1:1000 α-tubulin for DHX29 KD; 1:1000 α-Avi, 1:1000 α-GAPDH for Trex BCBL1-Rtas) for 1 hr and secondary antibodies (1:15,000) for 1 hr at room temperature. After washing, the membranes were scanned with an Odyssey CLx infrared imaging system (Li-Cor, Lincoln, NE) at wavelengths of 700 and 800 nm, and the molecular sizes of the developed proteins were determined by comparison with pre-stained protein markers (Bio-Rad, Hercules, CA).

**Lentiviral transduction for BCBL-1 knockdown.** Hairpin shRNAs in the pLKO.1 lentiviral vector designed by The RNAi Consortium (TRC) targeting DDX24, DDX49, DHX29, and DDX58 DExD/H box helicases were cloned (Dharmacon, Horizon Discovery, Lafayette, CO). Plasmids were isolated using the Qiagen Plasmid Midiprep kit and sent for Sanger sequencing. 5 μg of each shRNA vector, 2.5 μg psPAX2, and 2.5 μg pMD2.G were co-transfected with TransIT-293 Transfection reagent (Mirus Bio, Madison, WI) in 9 cm plates containing 5x10^6 HEK 293FT cells, as per the manufacturer’s protocol. 18 hours after transfection, plasmid-containing media was replaced with fresh DMEM. At 72 hours post-transfection the supernatants were collected. All lysates were centrifuged at 3000 rpm for 5 minutes, 0.45 μm filtered, and stored at -80 °C prior to infection. 5.0x10^6 BCBL-1 cells were resuspended in a 5 mL pool of pure viral supernatant, each containing 1 mL of lentiviral stock
targeting a particular DExD/H box helicase for 24 hours. All lentivirus infected cells were selected in RPMI/10% FBS containing 1 μg/mL puromycin for 13 days and frozen in liquid nitrogen for future analysis. shDHX29 lentiviral particles were successfully used to knock down the expression of DHX29 in BCBL-1 cells (Table 2-3).

**Lentiviral transduction for TREx BCBL1-Rta overexpression.** Lentiviral pLV constructs expressing Avi-FLAG-tagged versions of maxGFP, DHX29, DDX24, DDX49, and DDX58 (designated by NCBI accession number) from VectorBuilder (Santa Clara, CA). TREx BCBL1-Rta cells were grown under 200 μg/mL hygromycin B selection to a density of 5x10^5 cells/mL. A total of 2x10^6 TREx BCBL1-Rta cells were infected at an MOI of 10 with 8 μg/mL polybrene. The next day, the virus containing media was replaced with 0.25 μg/mL Puromycin RPMI and grown under selection for 7 days. Then, the Puromycin concentration was gradually increased to 1 μg/mL for another 5 days.

**RNA Immunoprecipitation.** TREx BCBL1-Rtas overexpressing maxGFP, DDX24, DDX49, or DDX58 were grown to a density of 5x10^5 cells/mL in 100 mL. One set of flasks was harvested during latency, the other was reactivated in RPMI media containing 1 μg/mL Doxycycline and 0.25 mM PFA for 24 hours. When cells were harvested, they were incubated in lysis buffer (20 mM MOPS-KOH pH7.4, 120 mM KCl, 0.5% Igepal, 2 mM β-Mercaptoethanol supplemented with 200 unit/ml RNasin (Promega) and Complete Protease Inhibitor Cocktail (Roche)) for 20 min on ice. The lysate was cleared by centrifugation and endogenous proteins were immunoprecipitated overnight at 4C with 10 μg total of α-Avi tag monoclonal mouse antibody, (2 μg/mL, Genscript, catalog# A01738) composed of a mouse IgG2a heavy chain. 5 mL of the antibody-bound RNA lysate was incubated with 200 μL of protein G MagBeads MX (Genscript, Piscataway, NJ) for 2 hours at 4 °C. The beads were washed three times with MOPS...
buffer and eluted with 0.1 M glycine, pH 2.2 as per the protocol. The RNA was isolated by TRI reagent RNA extraction (Sigma-Aldrich, St. Louis, MO), TURBO DNase treated (Invitrogen), and subjected to further analysis.

**Next generation sequencing (NGS).** RNA molecules isolated from RLR/RNA complexes were treated for library preparation using the Truseq Stranded Total RNA with Ribo-Zero Gold sample preparation kit (Illumina, San Diego, CA) according to manufacturer’s instructions. Sequencing was performed on the Illumina HiSeq3000 platform to generate 50 million paired-end 100 base reads (Table 2-4).

**Statistical analyses:** For all experiments, mean ± standard error mean (S.E.M.) were calculated and significance determined by performing unpaired two-sample Student’s t-tests, using GraphPad Prism software (GraphPad Software, La Jolla, CA). ANOVA was used for grouped analyses.
Table 2-1. The 22 DExD/H box helicase targets of the knockdown screen. siRNA pools used were a gracious gift from the McFadden lab. siRNA screen of 22 DExD/H box helicases in Vero cells infected with rKSHV.219. All transient KD Vero cells looked healthy 28 hours after siRNA transfection and prior to chemical induction, with negligible spontaneous background reactivation.

<table>
<thead>
<tr>
<th>Target DExD/H box gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIF4A1</td>
</tr>
<tr>
<td>EIF4A2</td>
</tr>
<tr>
<td>DDX3X</td>
</tr>
<tr>
<td>DDX5</td>
</tr>
<tr>
<td>DDX11</td>
</tr>
<tr>
<td>DDX18</td>
</tr>
<tr>
<td>DDX21</td>
</tr>
<tr>
<td>DDX24</td>
</tr>
<tr>
<td>DHX29</td>
</tr>
<tr>
<td>DDX31</td>
</tr>
<tr>
<td>DHX36</td>
</tr>
<tr>
<td>DHX37</td>
</tr>
<tr>
<td>DHX40</td>
</tr>
<tr>
<td>DDX41</td>
</tr>
<tr>
<td>EIF4A3</td>
</tr>
<tr>
<td>DDX49</td>
</tr>
<tr>
<td>DDX52</td>
</tr>
<tr>
<td>DDX55</td>
</tr>
<tr>
<td>DHX58</td>
</tr>
<tr>
<td>DHX9</td>
</tr>
<tr>
<td>DDX58 (RIG-I)</td>
</tr>
<tr>
<td>MDA5 (RLR)</td>
</tr>
</tbody>
</table>
Table 2-2. Pooled siRNAs used in the Vero knockdown screen (ON-TARGETplus Dharmaco)

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX24</td>
<td>5'-GCAAGAAGAACGUCGGAGA-3'</td>
</tr>
<tr>
<td>L-010397-01-0005</td>
<td>5'-AGACAAAGAAGCCGAAGGA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCGUUUAGCUCGACAGAUU-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GAACCGUCGUCCUGAGAUU-3'</td>
</tr>
<tr>
<td>DHX29</td>
<td>5'-CUCAGAUAUACGGAU-3'</td>
</tr>
<tr>
<td>L-013759-01-0005</td>
<td>5'-GAGUGAAUAUGAGCAUAA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAGAAAUUCAGCAGUUA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AAAUGUAGGGGUAGGGUAA-3'</td>
</tr>
<tr>
<td>DDX49</td>
<td>5'-CGGCCCACUUGACGAAA-3'</td>
</tr>
<tr>
<td>L-017975-01-0005</td>
<td>5'-AGGUCAACGUGGUGCGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GUAAAGAGAAAGUCCGUU-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CUGAGAAAGGUCAAGGACGC-3'</td>
</tr>
<tr>
<td>Non-targeting Pool</td>
<td>5'-UGGUUUCAUGUCGACUAA-3'</td>
</tr>
<tr>
<td>D-001810-10-05</td>
<td>5'-UGGUUUCAUGUUGUGUA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-UGGUUUCAUGUUUCUGA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-UGGUUUCAUGUUUCCUA-3'</td>
</tr>
</tbody>
</table>
Table 2-3. Pool of RNAi (TRC) shRNAs used to generate DHX29 KD BCBL-1 stably transduced cells. This pool of shRNAs was packaged individually into lentivirus and used to transduce BCBL-1 cells at a total pooled Multiplicity of Infection (MOI) of 10.

<table>
<thead>
<tr>
<th>GE Dhharmacon Clone ID</th>
<th>Mature Antisense Sequence</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRCN0000051238</td>
<td>AATAGTGGCTTGTATTTGAGG</td>
<td>all human transcript variants of DHX29 ORF</td>
</tr>
<tr>
<td>TRCN0000051239</td>
<td>ATGACAGCTATCTTTACAGGG</td>
<td>ORF of NM_001345964.1</td>
</tr>
<tr>
<td>TRCN0000051240</td>
<td>ATTGTAGGGCATACTACCAGG</td>
<td>all human transcript variants of DHX29 ORF</td>
</tr>
<tr>
<td>TRCN0000051241</td>
<td>TAATTCCTCCAAAGGTACACG</td>
<td>all human transcript variants of DHX29 ORF</td>
</tr>
<tr>
<td>TRCN0000051242</td>
<td>TAGAAGTTTCTGATACTTAGG</td>
<td>all human transcript variants of DHX29 ORF</td>
</tr>
</tbody>
</table>
Table 2-4. List of samples tested for the DExD/H box RNA Immunoprecipitation analysis

<table>
<thead>
<tr>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>lane 1</strong></td>
</tr>
<tr>
<td>DDX49-Avi latent replicate 1 input</td>
</tr>
<tr>
<td>DDX49-Avi latent replicate 2 input</td>
</tr>
<tr>
<td>DDX49-Avi lytic replicate 1 input</td>
</tr>
<tr>
<td>DDX49-Avi lytic replicate 2 input</td>
</tr>
<tr>
<td>DDX49-Avi latent replicate 1 immunoprecipitation</td>
</tr>
<tr>
<td>DDX49-Avi latent replicate 2 immunoprecipitation</td>
</tr>
<tr>
<td>DDX49-Avi lytic replicate 1 immunoprecipitation</td>
</tr>
<tr>
<td>DDX49-Avi lytic replicate 2 immunoprecipitation</td>
</tr>
<tr>
<td>TREx BCBL1-Rta latent replicate 1 input</td>
</tr>
<tr>
<td>TREx BCBL1-Rta lytic replicate 1 input</td>
</tr>
<tr>
<td>TREx BCBL1-Rta latent replicate 1 immunoprecipitation</td>
</tr>
<tr>
<td><strong>lane 2</strong></td>
</tr>
<tr>
<td>DDX24-Avi latent replicate 1 input</td>
</tr>
<tr>
<td>DDX24-Avi latent replicate 2 input</td>
</tr>
<tr>
<td>DDX24-Avi lytic replicate 1 input</td>
</tr>
<tr>
<td>DDX24-Avi lytic replicate 2 input</td>
</tr>
<tr>
<td>DDX24-Avi latent replicate 1 immunoprecipitation</td>
</tr>
<tr>
<td>DDX24-Avi latent replicate 2 immunoprecipitation</td>
</tr>
<tr>
<td>DDX24-Avi lytic replicate 1 immunoprecipitation</td>
</tr>
<tr>
<td>DDX24-Avi lytic replicate 2 immunoprecipitation</td>
</tr>
<tr>
<td>TREx BCBL1-Rta latent replicate 2 input</td>
</tr>
<tr>
<td>TREx BCBL1-Rta lytic replicate 2 input</td>
</tr>
<tr>
<td>TREx BCBL1-Rta latent replicate 2 immunoprecipitation</td>
</tr>
<tr>
<td>TREx BCBL1-Rta lytic replicate 2 immunoprecipitation</td>
</tr>
<tr>
<td>KSHV gene</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>K2</td>
</tr>
<tr>
<td>ORF70/K4/K4.1/K4.2</td>
</tr>
<tr>
<td>K5</td>
</tr>
<tr>
<td>ORF58</td>
</tr>
<tr>
<td>ORF59</td>
</tr>
<tr>
<td>K9 (only in DDX49)</td>
</tr>
<tr>
<td>ORF65/66/67 (only in DDX49)</td>
</tr>
</tbody>
</table>
Figure 2-1. KSHV genome showing the chronological expression of lytic genes (Latent=Black, Immediate early=Red, Early= green, Late=Blue). Primers used to monitor gene expression are demonstrated with arrows.
Figure 2-2. Mitochondrial antiviral signaling pathway. When RIG-I and RIG-I like receptors recognize KSHV, they activate the Mitochondrial Antiviral Signaling protein (MAVS). MAVS is an adapter molecule, located in the mitochondria, that recruits various signaling molecules to transduce downstream signaling. MAVS initiates two major downstream pathways; NF-kappa B (NF-κB) and Type I interferon regulatory factors (IRFs). In the case of canonical NF-κB signaling, MAVS triggers Fas-associated death domain (FADD) and RIP1 adaptor molecules. In an uninfected state, NF-κB is complexed with IκB. Upon infection, the I-κB inhibitor is phosphorylated and degraded, freeing NF-κB to translocate into the nucleus and initiate transcription at NF-κB response elements. In its activated form, NF-κB initiates the transcription of pro-inflammatory cytokines, such as IL-1 β, Interleukin 6 (IL-6), IL-8, and TNF-α. In the case of IRF signaling, IRF3 and IRF7 initiate Type I interferon (IFN) transcription. This image used is from Nat Rev Immunol., 2011.
1) Seed rKSHV.219 infected Vero cells

Lipofectamine transfection with a given siRNA pool that targets a particular DExD/H box helicase

2) Knock-down for 28 hours and verify transfection control

untreated | RNAi GFP

To induce, media containing 20ng/mL TPA & 2mM NaB was added

3) Take microscopy images and quantify RFP counts post-induction

4) Validate KD hits with qPCR to monitor KSHV replication

Figure 2-3. Flow chart of siRNA screen of 22 DExD/H box helicases in Vero cells infected with rKSHV.219.
Figure 2-4. Quantification of siRNA screen of 22 DExD/H box helicases in Vero cells infected in rKSHV.219. A) Fluorescence microscopy results of selected knockdowns at 24, 34, and 48 hours post-induction. All transient KD Vero cells look healthy 28 hours after siRNA transfection and prior to chemical induction, with negligible spontaneous background reactivation. B) Counts for selected knockdowns with three random fields of vision per biological replicate. Values are depicted as a ratio normalized to the scrambled control (n=4 independent trials, ***p<0.001, **p<0.01).
Figure 2-5. KSHV genome copy number of Vero cells during DExD/H box siRNA screen at 48 hours post-induction. Values based on three biological replicates (n=3), and three technical replicates of each biological replicate. **p<0.01, *p<0.1
Figure 2-6. Validation of stably transfected BCBL-1 cells overexpressing DDX24-DDK and DDX49-DDK A) Relative transcript abundance of DDX24 and DDX49. Primers target gene body of endogenous DDX24 and DDX49. Steady state transcript levels include endogenous and DDK tagged helicases. (n=1) B) Relative transcript abundance of DDX24-DDK and DDX49-DDK templates. Primers target 3’ end of the helicase Open Reading Frame (ORF) and the DDK tag (n=2). C) Immunoblots of DDX24-DDK and DDX49-DDK utilizing αDDK Ab. D) DDX24 OE and DDX49 OE stably transfected BCBL-1 cell survival during lytic reactivation of a 2mM NaB induction time course (not significant).*p<0.1, ****p<0.0001
Figure 2-7. Expression of latent, immediate early, and late lytic genes in BCBL-1 cells overexpressing DDX24-DDK and DDX49-DDK during a 2 mM Sodium Butyrate induction time course. There was a statistically significant difference in mean gene expression of LANA and Rta between the empty pCMV vector and DDX24 (n=3, LANA p=0.02, Rta p=0.0003, K8.1 not significant) There was a statistically significant difference in mean gene expression of Rta and K8.1 between the empty pCMV vector and DDX49 (n=3, LANA not significant, Rta p<0.0001, K8.1 p<0.0001)
Figure 2-8. Intracellular KSHV genome copy number in BCBL-1 DDX24-DDK and DDX49-DDK during a 2 mM NaB induction time course. There was a statistically significant difference in mean genome copy number between the empty pCMV vector and DDX24 or DDX49 throughout the induction timecourse (n=3, DDX24 p=0.03, DDX49 p=0.0002).
Figure 2-9. Validation of Stably Transduced DHX29 KD BCBL-1 cells - Lentivirus transduced BCBL-1 cells express 5 shRNAs to target DHX29. A) Relative transcript abundance of DHX29 KD to the empty vector control. n=3 B) DHX29 protein abundance in DHX29 Knock-down BCBL-1 cells set relative to α-tubulin. n=3 C) Quantification of protein band intensity. D) DHX29 KD Lentivirus transduced BCBL-1 cell survival during lytic reactivation of a 2 mM NaB & 20 ng/mL TPA induction time course (Not Significant). (**<0.01, *<0.05)
Figure 2-10. Expression of KSHV latent, immediate early, and late lytic genes in DHX29 KD BCBL-1 cells during an induction time course. KSHV gene expression of DHX29 KD stably transduced BCBL-1 cells during a 2 mM NaB and 20 ng/mL TPA induction time course. (n=3, LANA p=0.5, Rta p=0.4, ORF59 p=0.3, not significant)
Figure 2-11. Intracellular KSHV genome copy number of DHX29 KD stably transduced BCBL-1 cells during 48 hours post induction with 2 mM NaB and 20 ng/mL TPA 1 biological preparation, 3 technical replicates, p<0.0001
Figure 2-12. Validation of Stably Transduced TREx BCBL1-Rta cells overexpressing DDX24-Avi, DDX49-Avi, and DDX58-Avi for RNA IP. A) qPCR representing relative DDX24-Avi, DDX49-Avi and RIG-I-Avi from two separate lentiviral infections B) Immunoblots utilizing anti-Avi antibody representing DDX24-Avi, DDX49-Avi, and RIG-I-Avi at their appropriate relative masses.
Figure 2-13. Validation of TREx BCBL1-Rta reactivation and immunoprecipitation conditions for RNA Immunoprecipitation analysis. A) qPCR of TREx BCBL1-Rta cells during latency or induced for 24 hours with 1 ug/mL Doxycycline and 0.25mM PFA. The top figure represents the relative fold change in expression of the immediate early, Rta gene normalized with GAPDH. The below figure represents early, ORF59 expression. B) Immunoblot of lysates prior to pulldown (input) and after being pulled down with an α-Avidin antibody. 10 uL of lysate was run on an Any KD Mini-PROTEAN TGX gel, Biorad.
IE = Immediate Early
E = Early
L = Late
Figure 2-14. Deep Sequencing analysis of DDX24 and DDX49 associated KSHV partners and control immunoprecipitated RNA upon Rta-induced reactivation. RNA from A) DDX24/TREx-BCBL1-Rta and B) DDX49/TREx-BCBL1-Rta IPs on the positive and negative strand and control IP (blue, red, and green respectively) were subjected to Illumina strand-specific Next Generation Sequencing analysis. The distribution of read counts with the background TREx-BCBL1-Rta reads removed are mapped along the annotated KSHV genome on the X axis. The Y axis is showing the number of reads on the positive or negative strand of the genome. The orange boxes expended in Figure B) represent those enriched regions that were found exclusively in the DDX49 sequencing analysis. All other expanded regions are found enriched in both helicases.
Figure 2-15. KSHV microRNAs miR-K12-6 and miR-K12-12 putatively target DDX24 in Telomerase Immortalized Vein Endothelial cells. Hybrid sequences derived from Dr. Lauren Gay’s qCLASH data in TIVE-EX-LTCs are shown in the table. The mature microRNA is in bold. The underlined segments base pair with the red sequence from the DDX24 segment of the hybrid. The locations of the DDX24 hybrid binding are circled in blue. Below is a qPCR of DDX24 transcript abundance performed on lysate from TIVE cells infected with either wild type KSHV, or mutant viruses with the microRNA of interest deleted. One assay, no significance shown.
CHAPTER 3
REGULATION OF HUMAN PERIPHERAL MYELIN PROTEIN 22 BY MIR-29A IN CHARCOT-MARIE-TOOTH DISEASE TYPE 1A

In collaboration, the laboratories of Dr. Lucia Notterpek and Dr. Rolf Renne demonstrated that in rat Schwann cells, PMP22 is post-transcriptionally regulated by miR-29a\(^{14}\). In the current study, we asked if the human PMP22 transcript could be similarly inhibited by miR-29a in fibroblast cells from CMT1A patients. Recent bioinformatics predictions identified miR-29a targets within the 3’UTR of PMP22\(^{14}\). Moreover, we determined that the duplicated human PMP22 gene in CMT1A patients indeed contains the full-length 3’UTR, and the conserved seed sequence of the miR-29a binding site.

Given this functional relationship, we wondered if there was an inverse correlation in tissue expression of PMP22 protein and miR29a. We began by looking in adult brain tissue where the PMP22 protein is not detectable, although PMP22 mRNA is still present\(^{172}\). Using the Tissue Atlas browser, we found that miR-29a tissue expression is highest in the brain, suggesting that there is a naturally occurring relationship between miR29a and PMP22 tissue expression in humans. Both miR-29a and its target sites within the 3’UTR of PMP22 are conserved between mice, rats, and humans (Figure 3-1). This suggests that human PMP22 mRNA levels could be inhibited by delivering miR-29a into CMT1A patient fibroblasts. The 3’UTR of human PMP22 mRNA is about 1.1 kb and hsa-miR-29a targets a region about 300 bp upstream from the Poly A signal. In adult tissue, the PMP22 membrane protein is predominantly expressed in myelinating Schwann cells, the principle glia of the peripheral nervous system, and the protein remains detectable only at very low levels in the central nervous system, including the cortex and brain stem\(^{172}\). MicroRNA 29a-3p is also found in a variety of tissues and is processed from one of the most highly expressed microRNA precursors in both humans and mice\(^{173}\). miR-29a-3p displays its strongest expression in the brain and relatively very low expression in skin and adipose...
derived cells, such as glia\textsuperscript{174}. This cell type specificity of PMP22 protein expression supports a role for post-transcriptional regulation of the PMP22 mRNA by hsa-miR-29a-3p. We hypothesized that the human PMP22 transcript could be inhibited by the transient transfection of miR29a mimic in primary fibroblast tissue samples from CMT1A patients, leading to near-normal PMP22 levels and the repair of pathogenic phenotypes.

**MiR29a Mimic Reduced PMP22 3’UTR Reporter Activity In Transiently Transfected Cells**

Firefly/Renilla Dual luciferase assays use a 3’UTR reporter to identify gene targets undergoing microRNA-mediated post-transcriptional regulation. The 3’UTR of PMP22 was cloned downstream of the luciferase Open Reading Frame (ORF) on a reporter plasmid. Both miR29a mimic and the recombinant plasmid were transiently co-transfected together in 293 cells. Synthetic miRNA mimics are double-stranded mature miRNAs that are widely used to study miRNA-dependent gene regulation. Upon transient transfection of HEK293 cells with a miR-29a mimic, a moderate but significant 20% decline of the human 3’ UTR PMP22 reporter-driven luciferase expression was observed (Figure 3-2). These data demonstrated that the human PMP22 3’UTR is a target of miR-29a, and that the results observed in rats may be translatable to humans. In an attempt to immortalize patient fibroblasts, lentivirus containing pLNCX2 was packaged and used for infection following the Applied Biological Materials “General Guidelines for Cell Immortalization” protocol (abm, Richmond, BC). After telomerase immortalization, the patient fibroblasts lost their PMP22 phenotype (*data not shown*).

**Inhibition Of PMP22 In Patient-Derived Fibroblasts**

While studies from a number of animal models have led to better understanding of the pathobiology of these neuropathies, there continues to be a gap in the translation of findings from rodents to humans, which is necessary for the development of therapeutic approaches. In order to demonstrate proof of concept that human PMP22 levels can be similarly inhibited in CMT1A...
patients and lead to some correction of pathogenic phenotypes, we extended our studies to human skin cell lines. Fibroblasts offer a robust primary cell line that is easy to grow in culture, relatively non-invasive to collect, and demonstrates measurable phenotypes of the CMT1A disorder.

We obtained skin fibroblasts from the Coriell Institute for Medical Research (Camden, New Jersey) for two CMT1A pedigrees with confirmed PMP22 gene duplication with a donor age range from 17-51 years old (Table 3-1). Samples from non-neuropathic individuals with ages ranging from 21-57 years were used as controls.

In order to determine if miR-29a can reduce elevated PMP22 levels in CMT1A patient fibroblasts, a transient transfection of miR-29a mimic was performed on fibroblasts from two CMT1A patients and two age-matched healthy individuals. We transfected a luminescent labeled (DY-547) smallRNA, and showed that at 48 hours post-transfection nearly 100% of cells were red (Figure 3-3). Additionally, it was confirmed that the CMT1A patient fibroblasts transfected with the non-specific mimic control still expressed 1.5- to 2.0-fold elevated levels of PMP22 mRNA as compared to the normal individual (Figure 3-4A-C). PMP22 transcript abundance in CMT1A patients was significantly reduced by 40-50% after transient transfection with the miR-29a mimic, ultimately producing the PMP22 mRNA levels observed in non-diseased fibroblasts (Figure 3-4D-G). It should be noted that the effect of miR-29a mimic repression is more robust in CMT1A patient cell lines that overexpress PMP22 than in age-matched control individuals with standard PMP22 mRNA data, suggesting that additional post-transcriptional regulatory mechanisms are potentially involved.

In congruence with the observed mRNA repression, immunoblots from Patient 2 and Patient 4 fibroblasts transfected with a miR-29a mimic revealed a 20-24% reduction in the
overall PMP22 protein level when compared to the non-specific control (Figure 3-5). Figure 3-5A is the immunoblot of PMP22 and B is the graphical representation. This experiment suggests that miR29a contributes to the decrease in steady state levels of PMP22 protein by preventing the translation in patients affected by CMT1A. Overall, these data indicate that transient overexpression of miR-29a lowered PMP22 protein steady state levels in CMT1A patient fibroblasts closer to levels seen in normal age-matched control individuals. However, in order to restore PMP22 protein levels to near normal, we would need stronger inhibition than that given by miR29a alone. So, we generated constructs either expressing miR-29a or siRNA.

**AAV Vector Construction**

We generated Adeno-associated virus serotype 2 constructs to determine if inhibiting patient PMP22 levels would lead to a correction of pathogenic phenotypes in long-term studies. Our constructs expressed either miR-29a or an siRNA-like mutant of miR29a. For the designed siRNA, 7 bases in the 3’stem were changed, making it perfectly complementary to its PMP22 3’UTR target. The endogenous form of the precursor miR-29a and the designed siRNA were cloned downstream of the splice acceptor site of the intron and upstream of the tdTomato start site into the TR2 expression vector (Figure 3-6). When miR-29a and siRNA constructs were co-transfected with the 3’UTR luciferase reporter into 293 cells, we found a significant decrease in luciferase activity (Figure 3-7). As expected, the siRNA showed the greatest inhibition at the higher concentration (80 ng siRNA). All samples were compared to the empty pEZX-MT01 plasmid, which was designated as 100 relative luciferase units (RLUs). Our luciferase data shows that using this siRNA strategy to target PMP22 brings reporter levels closer to that seen in non-neuropathic individuals making it more therapeutically desirable.
After demonstrating microRNA expression efficacy, the constructs were packaged into AAV2, a serotype which was demonstrated to efficiently infect human Schwann cells during systemic delivery and would be ideal for testing in patient fibroblast cells\textsuperscript{176}. Tomato Red fluoresced in the control vector but did not fluoresce in those cells that contained the miRNA/siRNA. The processing of the microRNA potentially cleaved the transcript prior to the marker gene. This suggests that the tomato red segment transcript may have been removed, despite expression of the microRNA and siRNA from the plasmid.

**Discussion**

In our studies we have shown some unique and promising results. In contrast to synthetic ASO therapeutics that would be delivered ubiquitously throughout the body, we are attempting to deliver an endogenously expressed microRNA directly to where it would be therapeutically beneficial. MicroRNA miR-29a is conserved among species, endogenous, and has tissue specific expression in the brain which demonstrates an inverse correlation with PMP22 protein expression. Its conservation among murine and human species allows us to perform studies in the mouse model that can be translated to humans. We have been able to show that miR-29a is able to effectively suppress steady state PMP22 mRNA levels in cell culture of CMT1A patient biopsies.

In luciferase assays, we saw a moderate decline in PMP22 3’UTR reporter expression in 293 cells transfected with the miR-29a mimic. This suggests that the results found in rat cells are translatable to human cells. To further support direct binding and translational repression of miR-29a on PMP22, mutations of miR-29a in the seed regions can be made. If the mutants are ineffective in 293 luciferase assays, it suggests that other upstream factors are not contributing to the translational repression observed with the 3’UTR reporter.
Sooyeon Lee’s work in the Notterpek lab demonstrated that human dermal fibroblasts provide an effective model to test the phenotypic effects of miR-29a on CMT1A patient cells\textsuperscript{175}. Our fibroblast transfections show that microRNA mimics work in human primary cells to suppress PMP22 at the mRNA and protein levels. We successfully showed that miR-29a downregulates PMP22 to near-normal levels, without completely silencing it, which would also be detrimental to its function if used as a therapy. This is important because we want to avoid phenotypes caused by too little PMP22 expression such as that of hereditary neuropathy with liability to pressure palsies. An ideal and reversible reduction in expression would be therapeutic as gene therapy or a long-term drug therapy\textsuperscript{124}.

Next, we wanted to demonstrate that miR-29a-dependent down-regulation of PMP22 reverts pathogenic phenotypes in patient fibroblasts. To achieve this, we need stable expression of miR29a/siRNA. To complete this task we designed an AAV vector as a rotation project with Christopher Fields. We generated an siRNA that is designed to target the 3’UTR of PMP22 in a more efficient manner. Our AAV vector expressing miR-29a and a designed siRNA proved to be effective in luciferase assays but did not fluoresce red when transfected into 293 or Schwann cells. The control pTR2 vector continued to express the full length transcript and did fluoresce tomato red. It appears that although the small RNAs of interest were expressed, the transcript was cleaved prior to the tdTomato red marker. Because the insertion site of the smallRNAs was placed directly after the promoter and prior to the tomato red, a truncated transcript was expressed from the pTR2 vector. To avoid this in the future, the insertion site will always be cloned into the intron or in the 3’UTR of the tdTomato gene, prior to the polyA signal.

Currently, the Notterpek and Renne labs are researching an AAV delivery strategy using an AAV serotype with a cell-type specific promoter that can target Schwann cells selectively,
essentially avoiding off target effects on surrounding cells. The AAV2 serotype infects multiple cell types but prefers Schwann cells. A more Schwann cell specific expression system can be achieved through a cell type-specific enhancer or promoter in the AAV construct.

**Materials and Methods**

**Dermal fibroblast cultures:** Human skin fibroblasts from four CMT1A patients were from Coriell Institute (Camden, New Jersey) and maintained in Dulbecco’s Modified Essential Medium supplemented with 10-20% fetal bovine serum (FBS). Skin fibroblasts from unaffected individuals were obtained from Coriell (GM11091), or from volunteers under an approved IRB at the University of Florida (Table 3-1). All procedures with the human cells were done in compliance with IRB-approved procedures. All fibroblast cultures used for experiments were grown at or below eight passages.

**Fibroblast transfection:** Confluent cultures were transiently transfected with human microRNA miRIDIAN hsa-miR-29a-3p mimic, Dharmacon miRIDIAN microRNA mimic Negative Control, or Dharmacon siGLO Red Transfection Indicator using Lipofectamine RNAiMAX (Invitrogen, Thermo-Fisher). Transfected cells were cultured for 48 h (RT-PCR) or 72 h (Immunoblot), then red fluorescent protein (RFP) expression was examined by fluorescent microscopy (Leica DMI 4000). Cultures were harvested in RNA-Bee (Tel Test) and total RNA was isolated according to the manufacturer’s instructions.

**Luciferase assay:** Human Embryonal Kidney 293 (HEK293) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) and supplemented with 10% Fetal Bovine Serum and 1% penicillin-streptomycin. 293 cells were seeded into 24-well plates at a concentration of 1.5 x 10^5 per mL. Lipofectamine 3000 (Invitrogen) co-transfection was performed using with 20 ng pEZX-MT01-PMP22-3’-UTR reporter plasmid (GeneCopoeia) and either the pTR2 plasmid (50 ng and 80 ng) constitutively expressing the precursor microRNA or a human microRNA
miRIDIAN hsa-miR-29a-3p mimic (2, 5, and 10 nM) (C-300504-07-0005, GE Dharmacon). pEZX-MT01 reporter vector contains the firefly luciferase gene immediately upstream of the PMP22 3’-UTR and an independent Renilla luciferase gene as an internal control for normalization. Forty-eight hours after co-transfection of HEK293 cells, a dual luciferase assay was performed using the Dual-Luciferase assay kit (Promega, USA) on a FLUOstar Optima microplate reader (BMG Labtech). The ratio of firefly to Renilla luciferase was taken and normalized relative to the cells transfected with the pEZX-MT01-PMP22-3’-UTR vector and human microRNA miRIDIAN Mimic Negative Control.

**Real time qRT-PCR:** Total RNA (900 ng) was reverse transcribed using High-capacity RNA-to-cDNA kit (Applied Biosystems). For quantitative RT-PCR analyses, undiluted cDNA and primers for PMP22 or GAPDH were analyzed with the SYBR Green FastStart PCR Master Mix (Roche). For the amplification of PMP22 the primers were designed to amplify a 237 bp region within the first coding exon of the human gene, recognizing transcripts containing both exon 1A and exon 1B \(^{177}\). PMP22 Forward Primer: 5’ GTA TCA TCG TCC TCC ACG TC 3’; PMP22 Reverse Primer: 5’ GGC AGA AGA ACA GGA ACA GA 3’. The relative expression of PMP22 to GAPDH mRNA was determined using the \(2^{-\Delta \Delta CT}\) method.

**Fluorescent immunoblot:** Primary patient CMT1A fibroblasts were transfected as above. 72 h post transfection, cultured cells were lysed in 3% SDS RIPA buffer containing complete protease inhibitors (Roche). Pierce BCA assays were performed on each lysate (Thermo Fisher). For carbohydrate modification analysis of PMP22, total cell lysates (10 µg) were treated with Peptide -N-Glycosidase F (PNGase F) (New England Biolabs) per manufacturer instructions. Proteins were separated on 12.5% polyacrylamide SDS gels and were transferred to PVDF membranes. After blocking in Odyssey blocking buffer (TBS), blots were
incubated overnight at 4 C with a 1:500 primary rabbit polyclonal anti-PMP22 antibody (Andy, Pareek, 1997). Then, the blots were incubated in a 1:15,000 secondary IRDye Donkey α-Rabbit antibody and imaged using the CLx system (LiCor).

**Plasmids:** Plasmids constructed from pTR2-CB-dTomato-3X-Myc-WPRE-MCS were generated with the GeneArt Seamless Cloning and Assembly Kit (Life Technologies). miR-29a was generated using primers that amplify miR-29a pre-miRNA from pCMV-miR-29a plasmid (Origene). An siRNA was generated using the same primers from a template which includes a hairpin which is perfectly complementary to the binding site in the 3’UTR of PMP22 (designed and ordered gBlock from IDT). Plasmids containing a luciferase reporter were constructed as follows. Linearized pTR2 was cut with XbaI and XhoI restriction enzymes for 1 h and a 6 kb fragment excised from a 1% agarose gel. These cut sites were selected to avoid interference from tdTomato fluorescence. Full miR-29a and siRNA inserts were PCR amplified with Forward 5’-CGTGTGACCGGCGGCGCATACTACACCATTTTCTATCA-3’ and Reverse 5’-ATTATCGATAAGCTGCAGGGGTTTTCTAGGTATCCG-3’ primers. The bolded sequence indicates homology with pTR2. These amplicons were generated using NEB Phusion High Fidelity DNA Polymerase with <18 amplification cycles. DNA was purified with the Monarch PCR cleanup Kit (New England Biolabs), and plasmids assembled by GeneArt Cloning. Each reaction was setup so that 20 ng of ampicon was mixed with 100 ng of pTR2 vector in 5X reaction buffer. The reaction mix was transformed into TOP10 competent cells per the manufacturer’s protocol. Clones were screened via restriction digests and analyzed. Colonies were harvested using Qiaprep Spin Mini Prep Kit (Qiagen). AAV2 plasmids were constructed as follows. Linearized pTR2 was cut with XbaI and HindIII restriction enzymes for 1 h, and a 7kb fragment was purified by agarose gel electrophoresis. Full miR-29a and siRNA inserts were PCR
amplified with Forward 5'-CGTGACCGGCATACTACACCATTTTCTATCA-3’ and Reverse 5’- AGTGATATCCAAATTGCCAGGAGTGTTCCTAGGTATCCG-3’, with the bold sequences indicating homology with pTR2. PCR reactions and GeneArt cloning were performed as above. Clones were screened by restriction digestion and sequenced. Plasmid Maxi preps (Qiagen) of those that successfully underwent recombination were submitted to the Powell gene Therapy Center Vector Core at the University of Florida. Bacteria containing the ITR plasmid were not grown longer than 14 hours and the integrity of the ITRs was checked routinely by restriction digestion with DraIII and SnaBI (ITR1) and HincII and SapI (ITR2).

**Statistical analyses:** For all experiments, mean ± standard error mean (S.E.M.) were calculated and significance determined by performing unpaired two-sample Student’s t-tests, using GraphPad Prism software.
Table 3-1. List of Studied Cells with Donor Information. Human skin fibroblasts from four CMT1A patients (GM05148, GM05167, GM05146, GM05165) were from the Coriell Institute (Camden, New Jersey). Skin fibroblasts from unaffected individuals were obtained from either the Coriell Institute (GM11091) or from volunteers under an approved IRB at the University of Florida (MD09, MD13, MD16).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age, years</th>
<th>Sample ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1 (C1)</td>
<td>17</td>
<td>GM11091</td>
</tr>
<tr>
<td>Control 2 (C2)</td>
<td>21</td>
<td>MD13</td>
</tr>
<tr>
<td>Control 3 (C3)</td>
<td>34</td>
<td>MD16</td>
</tr>
<tr>
<td>Control 4 (C4)</td>
<td>56</td>
<td>MD09</td>
</tr>
<tr>
<td>CMT1A Patient 1 (P1)</td>
<td>17</td>
<td>GM05148</td>
</tr>
<tr>
<td>CMT1A Patient 2 (P2)</td>
<td>28</td>
<td>GM05167</td>
</tr>
<tr>
<td>CMT1A Patient 3 (P3)</td>
<td>40</td>
<td>GM05146</td>
</tr>
<tr>
<td>CMT1A Patient 4 (P4)</td>
<td>51</td>
<td>GM05165</td>
</tr>
</tbody>
</table>
Figure 3-1. Alignment of the miR-29a binding site and its PMP22 target sites. The miR-29a binding site and its PMP22 target sites are conserved between the species *Homo sapiens* (hsa), *Mus musculus* (mmu), and *Rattus norvegicus* (rno). Alignments of the miR-29a binding site in the 3’-UTR of the human, mouse and rat PMP22 transcript are shown. The seed region for the miR-29a binding site is underlined. Alignment from www.microRNA.org
Figure 3-2. Dual luciferase assay demonstrating that the human PMP22 3’UTR is a target of miR-29a. Dual luciferase assay performed in 293 cells validates that the human PMP22 3’UTR is a target of miR-29a. An approximately 20% decrease in relative luciferase activity was observed when 20 ng of reporter vector pEZX-MT01 was co-transfected with the miR-29a mimic but not with the miRNA negative mimic control (NC). Two biological replicates with 10 technical replicates of each condition are shown. P-values <0.0001 (****) were considered significant.
Figure 3-3. SiGlo Red Indicator 48 hours post-transfection. Human fibroblasts from CMT1A patients P2 and P4 and age matched unaffected individuals C2 and C4 were transfected with SiGLO Red and images were taken with 5x objective, 150 msec exposure, 48 hours post-transfection.
Figure 3-4. Steady state PMP22 mRNA levels of patient and age matched healthy individual fibroblasts following transfection. Values set relative to GAPDH. A, B, C) Control (CX) cells were normalized to 1 (n=2 independent cultures per sample). A) PMP22 mRNA in control (C2) and CMT1A patient fibroblasts (P2), after transfection with scrambled control and normalization to GAPDH. B) PMP22 mRNA in control (C4) and CMT1A patient fibroblasts (P4), after transfection with scrambled control and normalization to GAPDH. C) Patient fibroblasts P2 and P4 compared to control (C4) after transfection with scrambled control demonstrate severity of PMP22 overexpression between patient fibroblasts. D, E, F, G) Control (CX) cells were normalized to 1 (n=3 independent cultures per sample, 3 technical replicates each). D) CMT1A patient 2 fibroblasts E) Control 2 age matched healthy fibroblasts F) CMT1A patient 4 fibroblasts (G) Control 4 age matched healthy fibroblasts. P-values <0.01 (**), <0.0001 (****) were considered significant.
Figure 3-5. Fibroblasts treated with miR-29a from patients GM05165 (P4) and GM05167 (P2) demonstrated a decrease in PMP22 protein. Cells were transfected with 50 nM miR-29a mimic and PNGase F treated protein was run on polyacrylamide gels. A) The respective immunoblot bands and B) quantification. Results from three biological transfection replicates are shown. P-value *<0.05.
Figure 3-6. The pTR2 plasmid map containing primary miRNA & siRNAs to be expressed. Inserts were cloned between XbaI and HindIII cut sites, downstream of the chicken β-actin intron. The predicted folding structures of the precursor miRNAs were created using Mfold software. hsa-miR-29a-3p targets the 3’UTR of PMP22. The yellow shaded boxes represent the mature 3p miRNA/siRNA that targets the PMP22 3’UTR.
Figure 3-7. Dual luciferase assay demonstrating the effect of miR-29a and siRNA on PMP22 3’UTR reporter. 293 cells were co-transfected with the PMP22 3’UTR reporter and pTR2-CB-3X-Myc-WPRE-MCS miR29a expression constructs. All miR29a pTR2 transfected 293 cell RLU values have a p value <0.0001 and are significant when compared to the reporter control. P-values <0.0001 (****) were considered significant.
Our hypothesis was that in addition to the RIG-I-like receptor family, other DExD/H box helicases prevent KSHV lytic reactivation. In Vero cells, when DDX24 and DDX49 are transiently knocked down, a 2-3-fold increase in PAN driven reactivation occurs. Conversely, when DDX24 and DDX49 are overexpressed in BCBL-1 cells, immediate early to late lytic gene expression and replication are inhibited. An RNA IP analysis identified immediate-early and early KSHV RNA targets of DDX24 and DDX49.

There are three major focuses for future studies on the DExD/H Box helicase project. The first is to identify cellular RNA binding partners of DDX24 and DDX49 from the RNA IP dataset. This will give us insight into cellular processes of the helicases. In addition to the expected snoRNAs, there may also be innate immune, tumorigenic, or IncRNA binding partners of these helicases. The second focus is to validate that microRNAs of MHV-68, EBV, and KSHV can target DDX24, potentially by binding to exon 3 in a non-canonical fashion. Quantitative PCR and western blots of DDX24 will need to be analyzed to look at translation efficiency in the presence of KSHV microRNAs. Thirdly, it would be very interesting if the cell induces apoptosis in response to DDX24 binding to KSHV RNAs. DDX24 competitively binds to FADD and RIP, preventing caspase 8 from activating NF-κB, which is necessary for KSHV reactivation\textsuperscript{119}. The same apoptotic pathway is targeted by KSHV\textsuperscript{147}. Apoptotic assays would need to be performed in these cell types to study this potential effect.

Additionally, we asked if targeting PMP22 with miR29a in CMT1A patient fibroblasts would lead to near-normal PMP22 levels and the repair of pathogenic phenotypes. Bioinformatically, we determined that the human PMP22 gene in CMT1A patients indeed contains the full-length 3’UTR, and the conserved seed sequence of the miR-29a binding site.
Using a luciferase reporter assay in HEK293 cells we demonstrated that transient transfection of a miR-29a mimic was associated with an approximately 20% reduction in PMP22-3’UTR reporter activity. Additionally, in dermal fibroblasts from CMT1A patients, we detected elevated expression of PMP22 mRNA, which was effectively repressed by approximately 40-50% after transient transfection with a miR-29a mimic. In accord with the observed mRNA repression, immunoblots from 5167 (P2) and 5165 (P4) patient fibroblasts transfected with a miR-29a mimic revealed a 20-24% reduction in the overall PMP22 protein level. Together, these results support further studies with miR-29a as a potential method to therapeutically modulate the expression level of PMP22 in Schwann cells from CMT1A patients.

Future directions intend to show miR29a-dependent down-regulation of PMP22 can revert pathogenic phenotypes such as accumulation of un-degraded protein cargo and impaired proteasome function. The Notterpek lab will continue this research by attempting to correct pathological phenotypes using constitutive expression of miR29a. After the expression of miR-29a, an antagonist should be introduced to demonstrate the ability to revert the phenotype. Reversion of the effect of miR-29a is critical in a clinical setting where reversibility is a desirable feature in clinical trials. Phenotype correction can be measured using qPCR, proliferation assays, staining for PMP22 aggregates, and immunohistochemistry using an anti-ubiquitin antibody to check for proteasome activity. An AAV construct is being considered to therapeutically deliver miR-29a in a cell type specific manner using an inducible promoter and a serotype that prefers Schwann cells.

In summation, we have begun to characterize the role of DExD/H box helicases in KSHV nucleic acid recognition to prevent lytic reactivation. Based on the RNA IP analysis performed in Rta-inducible TREx-BCBL1 cells, DDX24 and DDX49 are mostly binding immediate-early and
early lytic KSHV RNAs, suggesting that DDX24 and DDX49 can recognize KSHV nucleic acids from within the nucleus and prevent lytic reactivation. Given that DDX24 is putatively targeted by microRNAs of three gammaherpesviruses, understanding the molecular mechanisms that allow DDX24 to prevent reactivation could lead to the prevention of EBV and KSHV malignancies. Additionally, we have shown that miR-29a can suppress the disease-associated protein PMP22 closer to normal levels in CMT1A patient fibroblasts. If miR-29a can be effectively delivered specifically to Schwann cells via AAV, there is great potential for clinical application. The field of RNA biology has grown leaps and bounds in the past three decades. Understanding molecular mechanisms of disease allows us to develop novel nucleic acid therapies.
LIST OF REFERENCES


100


Awasthi, S. *et al.* DDX49 is an RNA helicase that affects translation by regulating mRNA export and the levels of pre-ribosomal RNA. *Nucleic Acids Res* (2018).


BIOGRAPHICAL SKETCH

Jacquelyn Serfecz built her foundation in science with a bachelor’s degree in biochemistry and analytical techniques at Georgetown University. Under the direction of Dr. Eagles, their group studied the Ketogenic Diet, a high fat, low carb diet that stimulates ketone body metabolism and generates energy for the brain. The diet has long been shown to reduce the severity and duration of seizures in children with epilepsy. Her assignment was to monitor glucose metabolism and the duration of chemically induced seizures in rats to determine if there was a change in the ketogenic diet’s anticonvulsant effect due to changes in protein intake.

After graduation, she worked for 5 years as an analytical chemist in pharmaceutical quality control (QC) departments. Her main responsibility at both companies was to perform High Performance Liquid Chromatography (HPLC) assays on a variety of drug products to verify that they met FDA specifications. In early 2011, she was promoted to a group leader of 4 analysts, and her assignment was to guarantee compliance of the company’s stability standard operating procedures.

While working, she went back to graduate school in the evenings to pursue a Master of Science degree. Research at Villanova University gave her hands-on experience with virulence and stress resistance of Salmonella typhimurium. She published her master’s thesis research in a peer reviewed journal and book chapter; “The Bacterial iprA Gene is Conserved across Enterobacteriaceae, Is Involved in Oxidative Stress Resistance, and Influences Gene Expression in Salmonella enterica Serovar Typhimurium” published in Journal of Bacteriology and “Recombineering and Conjugation as Tools for Targeted Genomic Cloning, Genetic Manipulation of DNA and Protein” published in Examples from Current Research. During this time, she also prepared and presented laboratory lectures for Anatomy and Physiology, led group
dissections, and graded exam material for 3 sections of 30 nursing students, for a total of 90 students per semester.

In the interim between completing her master’s thesis and applying to doctorate programs, she worked at University of Pennsylvania (UPenn) for a short time, where she generated RNA and DNA high-throughput sequencing libraries. Under the direction of Dr. Muredach Reilly, their group identified long intergenic noncoding RNAs (lincRNAs) that were differentially expressed in an inflammatory disease model of human tissue. Abnormal activation of innate immunity and subsequent chronic inflammation is a typical feature of many cardiometabolic disorders, yet little is known about the role of lincRNAs in innate immunity-related inflammation. In this study, healthy volunteers were exposed to a low dose of lipopolysaccharide (LPS), which acts as an endotoxin, promoting the secretion of cytokines in macrophages and B cells. She was responsible for isolating RNA from human blood, adipose, and monocyte tissue in order to generate over 100 RNA libraries from these individuals. Deep RNA sequencing revealed differences in the inflammatory transcriptomes (RNA profiles) of “high LPS responders” vs. “low LPS responders” of the innate immune challenge. Her research was published as “Tissue-Specific RNA-Seq in Human Evoked Inflammation Identifies Blood and Adipose LincRNA Signatures of Cardiometabolic Diseases.” in the journal of Arteriosclerosis, Thrombosis, and Vascular Biology. Concurrently, she also worked under the direction of a second PI, Dr. Brian Gregory, where she generated mRNA libraries for human cell lines infected with the *Toxoplasma gondii* parasite.

During her time as a graduate research assistant at the University of Florida in Dr. Renne’s lab she studied the impact of RNA helicases on *KSHV de novo* infection and lytic reactivation. Additionally, she collaborated with the UF Neuroscience Department on a project
that studies miRNA therapeutics in Type I Charcot-Marie-Tooth Neuropathy. She hopes to continue to expand her knowledge on viruses, bacteria, gene therapy, and the innate immune system. Jacquelyn Serfecz successfully completed her doctorate in biomedical sciences at the University of Florida through great diligence and a passion for research. Her goal is to pursue a career involving the research and design of new therapeutics for genetic disorders or microbial pathogens.