Regulation of Human ERV in Lymphoma by Epigenetic Modifying Agents

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Abstract of Thesis

Regulation of Human ERV in Lymphoma by Epigenetic Modifying Agents

The lymphatic system is a subset of the immune system, a multifaceted organization that has responsibilities ranging from protecting the body from invaders and removing cellular waste to maintaining body fluid levels and absorbing dietary fat from our intestines. More notably, it's responsible for the white blood cells our body uses to fight infection and keep ourselves healthy. There are several types of cancers that can impact the functioning of this system, divided into Hodgkin's and non-Hodgkin's lymphomas. Mantle cell lymphoma, a rare aggressive subtype of NHL, occurs in the mantle zone of lymphatic nodules. By the time the presence of NHL is detected, it often has already metastasized, complicating treatment, and negatively affecting the patient's prognosis. Current treatment options consist of both traditional chemotherapies and non-chemotherapeutic options but still fall short of desired patient outcomes. As novel therapies are required, it is time to look elsewhere.

An emerging field of study, epigenomics, may hold the key. Epigenomics is the study of genetic regulation and the changes in gene expression that can have profound influences on an organism's phenotype. Without changing the actual genetic code, the expression of certain genes can be tuned up and down either by external or internal factors. Often, though, these influences come from within. Most of the human genome does not code directly for amino acids, rather, much of it subtly influences the expression of sections that do. Some of these non-coding regions are sections of DNA that we've accumulated

through evolution over millions of years or are otherwise left over from our ancestors. Other sections include the induction of DNA and RNA from ancient retroviruses that have slowly integrated into our own genome. These sections of our genome, those that are the result of ancient exogenous retroviruses, are colloquially known as endogenous retroviruses, or ERVs.

Human endogenous retroviruses, HERVs, are thought to play a significant role in genetic regulation. In recent years, scientists have been able to link the abnormal regulation of some of these HERVs to several types of cancer ranging from colorectal to pancreatic. Given these findings, it is pertinent to investigate whether these genetic sections may also be linked to mantle cell lymphoma, as the potential correlation is not yet well known. Epigenetic medications, those that aid in the external up or down-regulation of genetic transcription, may serve to be a point of leverage in the fight against adverse patient outcomes. Should a concerning link be found between a HERV and a type of malignancy, these epigenetic drugs may serve to address these concerns. As this broad class of epigenetic drugs function through different mechanisms, we opted to test a variety of different subclasses to examine their effect on our target ERVs. In this study, we hope to scout for any potential correlations between abnormal HERV expression and NHL development and examine the effects of several epigenetic drugs on our target HERVs.

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List of Symbols & Nomenclature

1 Non-Hodgkin's Lymphoma (NHL)

2 Mantle Cell Lymphoma (MCL)

3 Standard Operating Procedures (SOP)

4 Ribonucleic acid (RNA)

5 Deoxyribonucleic acid (DNA)

6 Complementary DNA (cDNA)

7 Ribonuclease (RNAse)

8 Quantitative Real-Time Polymerase Chain Reaction (q RT-PCR) (qPCR)

9 Cycle Threshold (Ct)

10 Fetal Bovine Serum (FBS)

11 Phosphate Buffered Saline (PBS)

12 Endogenous Retrovirus (ERV)

13 Human Endogenous Retrovirus (HERV)

14 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

15 DNA Methyltransferase (DNMT)

16 Bromodomain and Extra-Terminal motif (BET)

17 Histone Methyltransferase (HMT)

18 Peripheral Blood Mononuclear Cells (PBMC)

19 Cluster of Differentiation 19 (CD-19)

20 Bromodomain-Containing Protein 1-3 (BRD1-3)

21 Bromodomain Testis-Specific Protein (BRDT)

Chapter 1: Introduction and Background

1.1 Lymphoma

Lymphoma is a cancer of the lymphatic system, a complicated and multifaceted organization within the body responsible for immunity, waste removal, and fluid level maintenance. Cancers of this system are divided into Hodgkin's and Non-Hodgkin's lymphoma, differentiated based on the presence of Reed-Sternberg lymphocytes; white blood cells that are deformed in appearance and may contain more than one nucleus **(Figure 1)**.[1] These abnormal lymphocytes are present only in Hodgkin's lymphoma (HL). Non-Hodgkin's lymphoma (NHL) can occur in B or T cells, the two types of lymphocytes. B-cell lymphomas include Diffuse Large B-cell Lymphoma, Follicular Lymphoma, Chronic Lymphocytic Leukemia, Mantle Cell Lymphoma, Marginal Zone Lymphoma, Burkitt Lymphoma, and more rarely, Waldenstrom-Macroglobulinemia.[2] MCL is a rare aggressive subtype of non-Hodgkin's lymphoma with relatively limited treatment options and a poor prognosis. This type of malignancy is found in the mantle zone of the lymph nodes, hence its name. Morphologically, it appears like other low-grade lymphomas and so it was initially thought

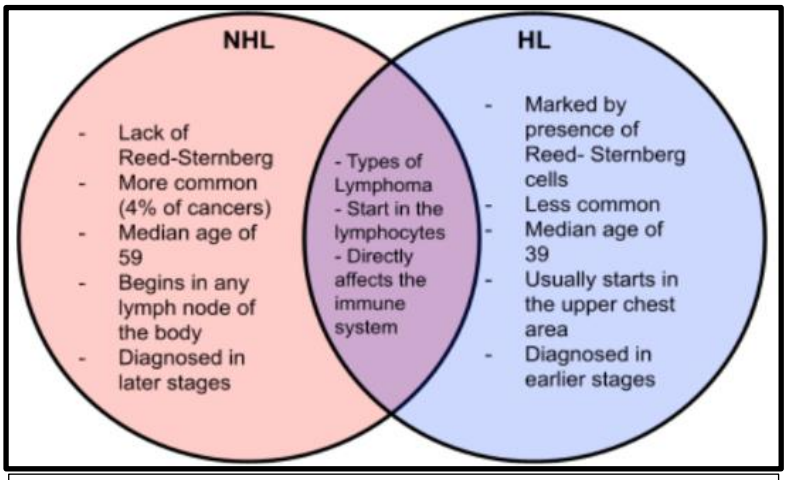

Figure 1. A Venn-diagram comparing the different types of lymphoma.
Both types impact the immune system but differ in their appearance and locations. Mantle cell lymphoma is a subtype of non-Hodgkin's lymphoma.

to be similarly lax and slow growing.[3] Unfortunately, this is not the case, as patients often experience a poor prognosis following detection and diagnosis.

Current therapies include traditional chemotherapeutic drugs alongside non-chemotherapeutic options like ibrutinib, acalabrutinib, and venetoclax, which instead act on the mechanisms of replication. Nevertheless, patient prognosis is often poor, with many experiencing recurrence and further complications.[4] There is a need for novel therapies to help curb the impact of this aggressive subtype of non-Hodgkin's lymphoma and it is becoming increasingly likely that one's genetics and epigenetics may hold the key.

1.2 Genetics and Cancer

It has been well established that genetics play an important role in determining the likelihood one has of developing cancer. Mistakes in our genetic code, inherited or otherwise, can accumulate over time and turn healthy cells cancerous as they replicate out of control. These unfortunate genetic mutations can be further spurred on by exposure to carcinogenic agents or may even happen randomly during transcription. Once enough errors have collected in a cell's genome, particularly in sections that control cell growth and development, the gene may begin to promote rapid, uncontrolled cell division. This defective, cancer-causing gene is referred to as an oncogene. Conversely, genes that can suppress tumor development and growth are tumor suppressors. Genetic regulation is a process in which the cell can increase or decrease the expression of its genes based on either external or internal pressures, and thus, affect one's phenotype. Decreasing the expression of an oncogene or increasing the expression of a tumor suppressor may be

beneficial to those wanting to discourage cancer development or even fight against a developing metastasis, and thus deserves more study.

1.3 Epigenomics

Epigenomics is the field of study that examines how changes in genetic regulation can affect one's phenotype. Epigenetic factors, unlike one's DNA, are malleable, and respond to the environment, changes in medication, aging, infections, and notably, cancer **(Figure 2)**.[5] Interestingly, much of the human genome is made up of non-coding DNA; DNA that does not directly code for any amino acids, the building blocks of proteins. Rather, much of this remaining DNA aims to epigenetically influence the frequency at which the coding DNA is expressed. Nearly 10% of the human genome is made up of noncoding DNA remnants from ancient previously-exogenic viruses that have since integrated into our genetic code. These sections are now referred to as ERVs or HERVs, human endogenous retroviruses. While these regions are no longer capable of replicating and creating new viral particles, they may still have relevant epigenetic effects that are worth examining.[6]

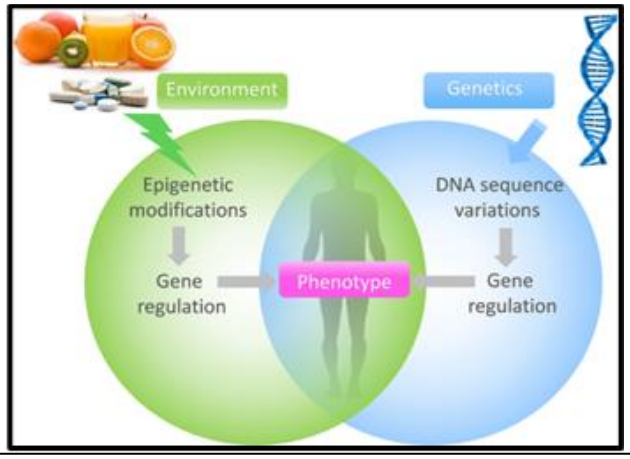

Figure 2. Genetics and genetic regulation.
The combination of genetic regulation and one's intrinsic DNA are both vital in determining phenotype.

1.4 Human Endogenous Retroviruses (ERVs)

As discussed, human endogenous retroviruses refer to a family of DNA in our genome that likely originated from exogenous retroviral RNA millions of years ago.[7] Though these portions of our genome are noncoding, they've long been thought to epigenetically influence the expression of other regions responsible for tumorigenesis or tumor suppression **(Figure 3)**.[8] Of note, HERV-3 has been found to be upregulated in colorectal, lung, liver, and prostate cancer, though these levels returned closer to baseline following treatment. Hodgkin lymphoma cells also express higher levels of HERV-3 RNA than their normal-type counterparts.[9] Additionally, HERV-K upregulation has been found to correlate with several cancers, including breast, pancreatic, melanoma, and brain cancer, and may serve to be a therapeutic target in efforts to combat these metastases.[10] Given that several HERVs have been found to be epigenetically linked to cancer development, it's worth examining how they may relate to our aggressive subtype of Non-Hodgkin's Lymphoma, Mantle Cell Lymphoma.

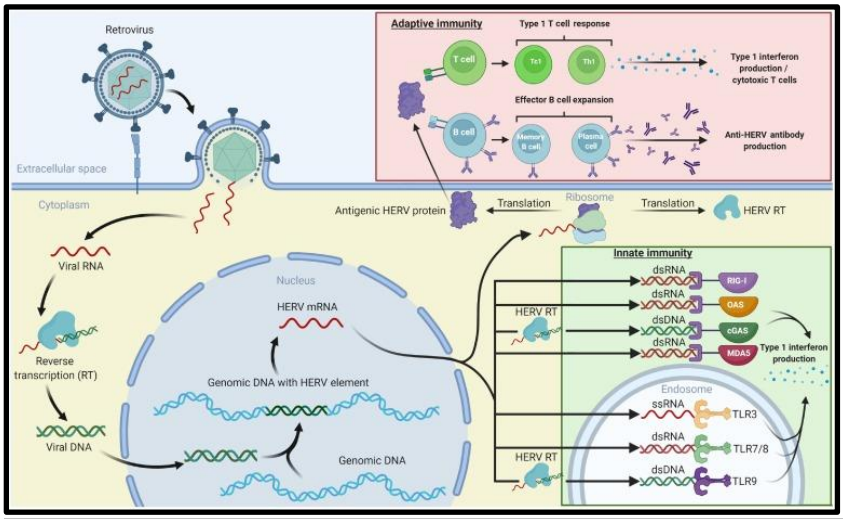

Figure 3. Integration of retroviral RNA into the human genome. *This endogenous retroviral RNA can regulate to protein translation and adaptive immunity.*

1.5 Classes of Epigenetic Drugs

Epigenetic augmentation through the administration of medication or other targeted therapies is nothing new. This class of medication either serves to promote or inhibit epigenetic changes like DNA methylation, histone methylation and acetylation, and others, to regulate genes of interest precisely up and down.[11] Many drugs have been developed specifically to epigenetically affect oncogenes and tumor suppressing genes to help combat the development and metastasis of cancer. For this endeavor, we examined drugs from three classes of epigenetic drugs. DNMT inhibitors, like 5-Azacytidine, help to reactivate the genes silenced by erratic activity of DNA methyltransferase. In this way, it may be able to un-suppress tumor-suppressing genes and serve as a bolster against tumorigenesis.[12] Another class, Bromo- and Extra-Terminal Domain Inhibitors (BETi) act by inhibiting the activity of bromodomains, which serve as promoters of a target gene. Should this target gene be oncogenic in nature, inhibition of its promoter may be beneficial for the patient.[13] **(Figure 4).**[14] Histone Methyltransferase Inhibitors (HMTi) was the next class to examine. G9a is an enzyme that acts on histone H3 Lysine 9, abbreviated as H3K9, by adding 1-2 methyl groups. Methylated H3K9 serves to both actively and passively repress transcription of a target gene, which may translate to therapeutic benefit for the patient should the gene be oncogenic or otherwise problematic.

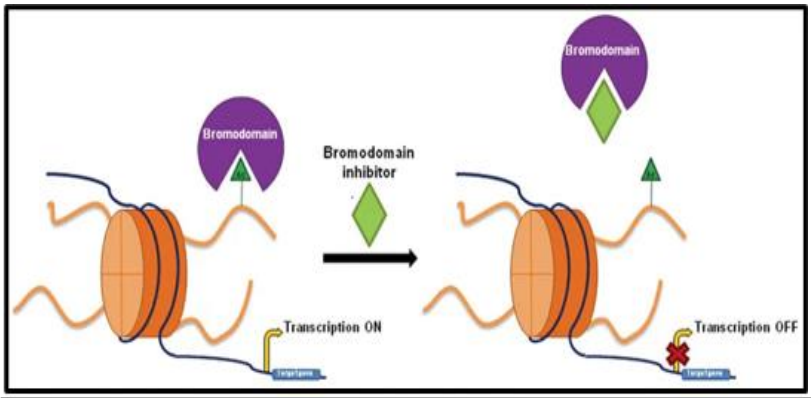

Figure 4. A bromodomain inhibitor blocking transcription.
Targeted use of bromodomain inhibitors can serve to inhibit the transcription of target genes, leading to their downregulation.

Chapter 2: Methods

2.1 Human Lymphoma Cell Lines

Human lymphoma cell lines JVM2, Jeko-1, Mino, and Granta were obtained from the American Type Culture Collection (ATCC) in Manassas, VA, USA. Cells were cultured in RPMI-1640 with 10% Bovine Serum, 1mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37C in an Incubator containing 5% CO2. Cell lines were authenticated by STR Profiling performed at ATCC, and at Genetica in Burlington, NC, USA. Normal-type B-cells were purified from human PBMCs (from normal donors) using EasySep Human CD19 Positive Selection Kit II according to the manufacturer.

2.2 Reagents and Drugs

RNA Extraction reagents sourced from QIAGEN RNeasy Mini Kit. cDNA synthesis was performed using Thermo Fisher SuperScript III First-Strand Synthesis Supermix shortly after RNA extraction. qPCR performed using SsoAdvanced Universal SYBR Green Supermix on a BioRad CFX Connect RT-PCR machine. 5-Azacytidine and JQ1 were purchased from Sellekchem at a concentration of 10mM in DMSO. Drugs were aliquoted and stored at -20C before serially diluted to target concentration before use. G9a Inhibitor UNC0638 was similarly purchased from Sellekchem as solid 10mg powder, dissolved in DMSO to a concentration of 10mM, and stored at -20C.

2.3 Treatment and Incubation

Culture population and viability were measured using BioRad TC20 Automated Cell Counter using the Trypan Blue exclusion method. 0.4% Trypan Blue sourced from

ThermoFisher Scientific. Cells pelleted at 1000 RPM for 5 min before resuspending in cRPMI to achieve a concentration of $2x10^6$, $3x10^6$ / mL. 1mL of this mixture was pipetted into each well of a 12-well plate per treatment or condition.

Drug aliquots were removed from -20C, thawed, and serially diluted from 100mM stock solution to achieve desired concentrations (0.5μM, 1μM, 5μM, 10μM). Test plates were incubated at 37C for 72H in a humidified incubator with 5% CO_2. After 72h each solution was pipetted into 1.5mL microcentrifuge tubes and centrifuged at 10,000 RPM for 5 min to prepare for RNA extraction.

2.4 RNA Extraction and cDNA Synthesis

RNA extraction was performed using QIAGEN RNeasy Mini Kit and eluted into 15-30μL of RNAse-free H_2O proportional to the number of cells in the initial treated condition; 15μL for 2 x 10^6 cells, 20μL for 3 x 10^6 cells, and 30μL for 5 x 10^6 cells. RNA was then quantified using a spectrophotometer in triplicate. Their concentrations were averaged and compared to ensure constant RNA volume across cell lines and treatment conditions. Typically, quantified RNA concentration resulted in a mass of ~1μg of RNA per sample ahead of cDNA synthesis.

cDNA was synthesized from extracted RNA using ThermoFisher SuperScript III First-Strand Synthesis Supermix using the entirety of extracted RNA. cDNA samples were then stored overnight at -20C before proceeding with qPCR.

2.5 Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed in triplicate using SsoAdvanced Universal SYBR Green Supermix with included protocol on a BioRad CFX Connect RT-PCR machine. GAPDH was used as the reference gene, and our target genes were ERV-3, ERV-9, ERV-K, and ERV-FC2. qPCR plates were prepared on ice before being vortexed and centrifuged at 500 RPM for 5 minutes before starting qPCR. ERV primers were a kind gift from Dr. Chiappinelli's group, sourced from IDTDNA (100μM in IDTE). Each primer was diluted to a concentration of 20μM before use in qPCR, with aliquots stored at -20C. The nucleotide sequence of the primers used can be found below. qPCR trials were run for ~45 minutes before being analyzed through the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method.

2.6 Primer sequences:

ERV-3

Forward: AAC TAA TGC CCC AAG ATA ATT TCA

Reverse: TTA AGA ACC AGA TGC TCT GAC TTG

ERV-9

Forward: CAC CAA GCG GTG AGA CTA TCA

Reverse: CGG GCA ACT GCT GAC AGC ATA

ERV-K

Forward: TTT GAC TGA AGT ATT AAA AGG TGT

Reverse: GTG ACT GCA ATT AAT CCC ATA ATC

ERV-FC2

Forward: CTC CAT TAG TAG CAG TTC CTC TCC

Reverse: GAG AAT AGT GGG ACC TGT CCT TT

GAPDH

Forward: ATC ACC ATC TTC CAG GAG CG

Reverse: CAA ATG AGC CCC AGC CTT C

2.7 Relative Expression Analysis and Comparison

Relative expression of various genes is analyzed using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method, and compared across

cell types and treatment conditions. In this way, the change in relative expression can be quantified and attributed to the change in cell line or treatment condition. "Ct" refers to the cycle threshold of the given sample. The formula is as follows:

$\Delta\Delta$Ct = ΔCt (treated sample) – ΔCt (untreated sample)

Where,

ΔCt = Ct (gene of interest) – Ct (housekeeping gene).

2.8 Statistical Analysis

Data is presented as mean ± SD from three independent experiments. P value was determined using Student's t-test, and a (*) p value < 0.05, (**) p value < 0.01, and (***) p value <0.001 were considered statistically significant.

Chapter 3: Results

3.1 RNA Yield from Lymphoma Cell Lines

Rationale:

To ensure that cDNA synthesis of the RNA extracted from the chosen MCL cells was equitable across cell lines, RNA quantification was performed after extraction and before cDNA synthesis using a spectrophotometer. Per the SOP provided in the QIAGEN RNEasy Mini kit, it is recommended that an RNA extraction of 5 x 10^6 cells would elute into 30µL of RNAse-free H2O. As we were working with lower cell counts, we remedied this disparity by eluting into smaller volumes of RNAse-free H2O: 20µL for 3 x 10^6 cells. Measuring the resulting RNA concentrations before proceeding to cDNA synthesis was integral to ensuring that the RNA concentration was sufficient despite the lower initial cell count.

Results:

Two NHL cell line pellets of 3 x 10^6 cells had their RNA extracted per protocol and measured in triplicate, analyzed, and graphed **(Figure 5)**. Concentrations of RNA remained steady at 50µg/mL across all cell lines tested in this and follow-up analyses. As the RNA samples used for cDNA synthesis had a total volume of ~20µl, each sample contained roughly 0.8-1.0µg of RNA before proceeding with cDNA synthesis. This standard was held constant for subsequent runs; all cDNA used in qPCR originated from roughly 1µg of extracted RNA.

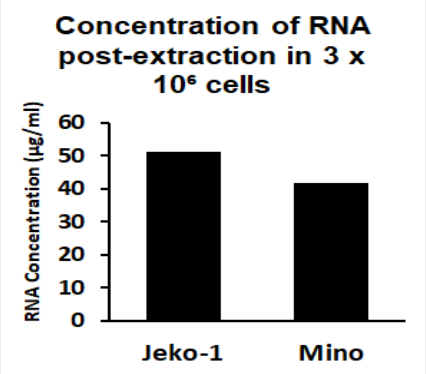

Figure 5. RNA Concentration *Measurements were conducted via spectrophotometer twice and averaged. Similar quantifications were performed on subsequent RNA extractions to ensure consistent RNA mass.*

3.2 Aim 1 - Expression of ERVs in PBMC, CD-19+ B cells, NHL cells

Rationale:

ERV deregulation has been shown to be associated with various diseases. However, ERV expression in human lymphoma is not well studied. We sought to examine the ERV expression in human NHL cell lines and normal controls such as peripheral mononuclear cells (PBMCs) and CD19+ normal B cells from normal donors.

Results:

$3x10^6$ cells from our NHL Cell lines, JVM2, Jeko-1, Mino, and Granta, were collected by centrifuge as described in Chapter 2: Methods. Additionally, stored PBMCs and CD19+ B cells were collected via pellet. Their RNA was extracted, processed into cDNA, then used in qPCR to assess the change in genetic expression of our four target ERVs: ERV-3, ERV-9, ERV-K, and ERV-FC2, in relation to our housekeeping gene GAPDH. These treatments, tests, and comparisons were carried out in five replications and collected below for cumulative analysis. When we first examine the relative expression of ERVs in various cell lines as indicated in the figure. In the case of JVM2 we observe no significant changes in ERV-3 mRNA expression, but we do see upregulation of ERV-9 compared to PBMCs **(Figure 6)**. Interestingly, both ERV-K and ERV-FC2 are downregulated. In Jeko-1, this trend continues, but the relative expression of ERV-9 is even more pronounced in that cell line. Like JVM2, both ERV-K and ERV-FC2 are less downregulated. Of note, the data variance on Jeko-1 is less than that of JVM2, suggesting that the up- and down-regulations noted may be more concretely related to the morphological differences in these cell types.

In Mino we see a stark departure from the previously observed trends. In comparison to the PBMCs tested, the ERVs tested in the Mino cell line show some downregulation with very little data variance. Though the downregulation of ERV-3 is relatively minor, the trend is noted moreso in the other ERVs tested. Specifically, ERV-FC2 shows the most downregulation in comparison to the peripheral blood mononuclear cells tested. Granta seems to have attributes of both previous trends noted. While ERV-3 is still somewhat downregulated, ERV-9 has no significant change. ERV-K and ERV-FC2 follow those of the previous cell lines in that they're both significantly downregulated in comparison to the PBMCs tested. To gain a better understanding of how these cell lines compare against one another we can observe an overview of the four NHL cell lines in comparison to the PBMCs tested. Here, apart from Mino, we observe that ERV-9 is considerably

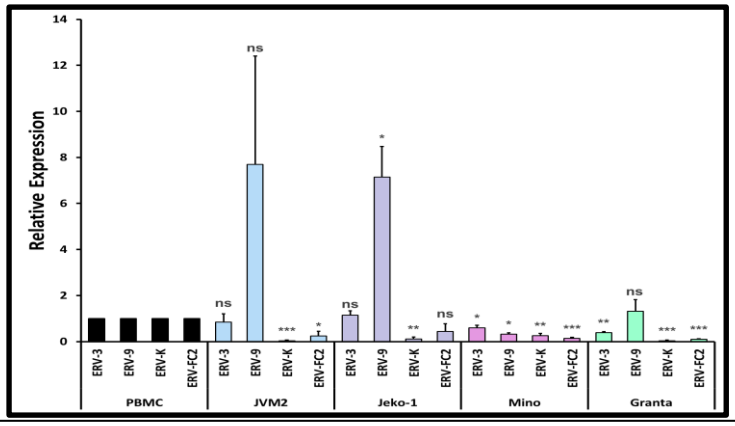

Figure 6. Relative fold-change in expression of ERVs as seen in PBMCs vs all NHL cell lines.
The relative expression of the target ERVs in the MCL cells can be seen in comparison to the peripheral blood mononuclear cells tested. Here, all PBMC ERVs have been normalized to 1 to gain an understanding of how the ERVs in the MCL lines compare.

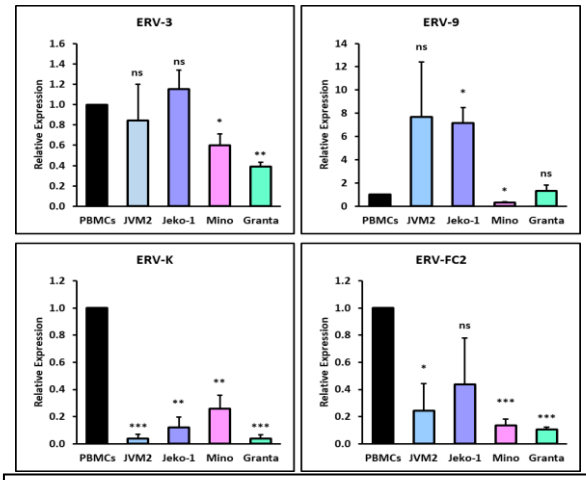

Figure 7. Relative fold-change in expression of target ERVs across cell types.
The trends in relative expression of the target ERVs can be easily visualized when broken up by ERV. Here, we can see some clear trends materialize when compared against the standardized expression in peripheral blood mononuclear cells.

upregulated in the remaining cell lines, JVM2, Jeko-1, and Granta. Viewing the data by ERV allows us to visualize the trends more easily **(Figure 7)**.

3.3 Aim 2a - Effect of DNA Methyltransferase Inhibitors (DNMTi) on NHL Cells; 5-Azacytidine

Rationale:

DNMT inhibitors act by reactivating the genes incorrectly silenced by erratic behavior of DNA Methyltransferase. These drugs can be classified into nucleoside inhibitors or non-nucleoside inhibitors, each with different mechanisms. Nucleoside inhibitors, which include 5-Azacytidine and Decitabine, act by covalently binding to DNMTs through integration into replicating DNA. Non-nucleoside inhibitors, like Curcumin and Procaine, instead bind directly to DNMTs ahead of replication. Although not clearly understood, DNA methylation can influence chemoresistance, and through utilization of DNMT inhibitors this may be brought back into alignment.

Results:

JVM2, Jeko-1, Mino, and Granta, were treated with 1μM of 5-Azacytidine, incubated for 72 hours, then collected by centrifuge as described in Chapter 2: Methods. Their RNA was extracted, processed into cDNA, then used in qPCR to assess the change in genetic expression of our four target ERVs: ERV-3, ERV-9, ERV-K, and ERV-FC2, in relation to our housekeeping gene GAPDH. These treatments, tests, and comparisons were carried out in six replications and collected below. Aggregation of the repeated tests per cell line reveal some interesting changes in genetic expression that deviate slightly between NHL cell lines. When comparing JVM2 to Jeko-1, we see a similarly slight downregulation of ERV-

3, but also a slight downregulation of ERV-9, in comparison to JVM2's slight upregulation (**Figure 8**.).This downregulation of ERV-9 is more significant given the low level of deviation in our data, suggesting that 5-Azacytidine results in a ~0.4 fold change in ERV-9 regulation in Jeko-1 when compared to the untreated sample. Interestingly, we note a larger upregulation of ERV-K and downregulation of ERV-FC2 in JVM2. When looking at Jeko-1 (**Figure 9**), we note similar slight downregulation of ERV-3 and ERV-9 in Mino (**Figure 10**), but this time an upregulation of both ERV-K and ERV-FC2 when treated with 5-Azacytidine. The low deviation in our data suggests that these regulatory changes are significant and a direct result of our 5-Azacytidine treatment. Granta shows a significant contrast in that there is significant upregulation of both ERV-3 and ERV-9 when compared to the untreated samples (**Figure 11**). Additionally, ERV-K shows a significant downregulation, when it was upregulated in the prior samples when treated with 5-

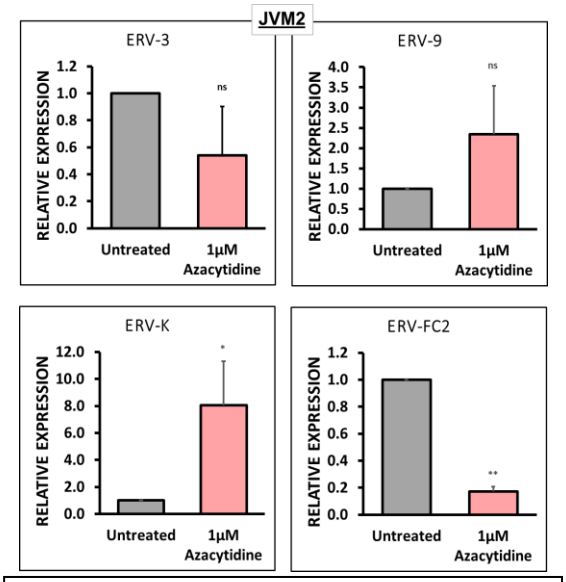

Figure 8. Relative fold-change in expression of ERVs as seen in JVM2.
The relative expression of the target ERVs in the treated samples can be seen in comparison to their untreated counterparts.

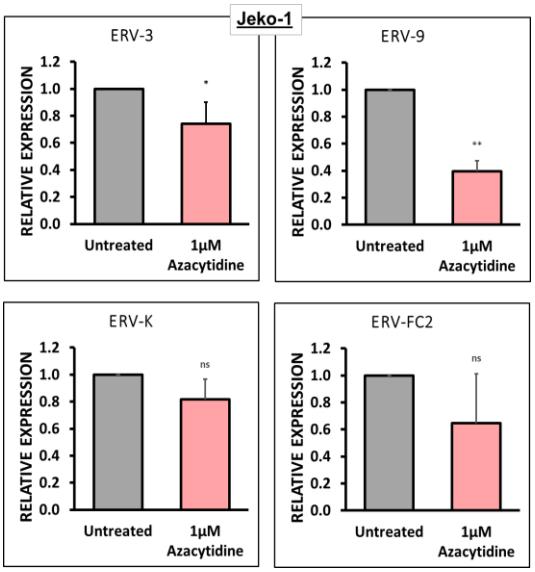

Figure 9. Relative fold-change in expression of ERVs as seen in Jeko-1.
The relative expression of the target ERVs in the treated samples can be seen in comparison to their untreated counterparts.

Azacytidine. Additionally, ERV-FC2 shows some upregulation, consistent with the other cell lines. When we view the effects of 5-Azacytidine on our various cell lines by separating them by ERVs, several trends become apparent. Granta seemed to be the only cell line with significantly elevated ERV-3 expression after treatment with 5-Azacytidine. This trend continued in ERV-9, which was similarly elevated in Granta. In contrast, JVM2 showed an upregulation of ERV-K following Azacytidine treatment. Variance of ERV-FC2 expression after Azacytidine treatment remained insignificant **(Figure 12).**

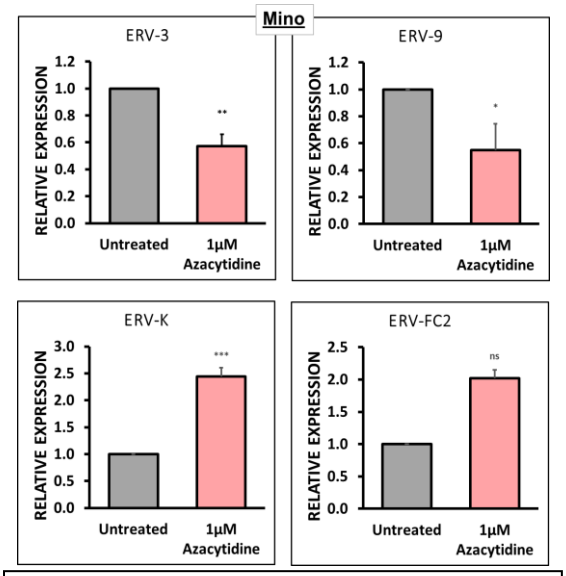

Figure 10. Relative fold-change in expression of ERVs as seen in Mino.
The relative expression of the target ERVs in the treated samples can be seen in comparison to their untreated counterparts.

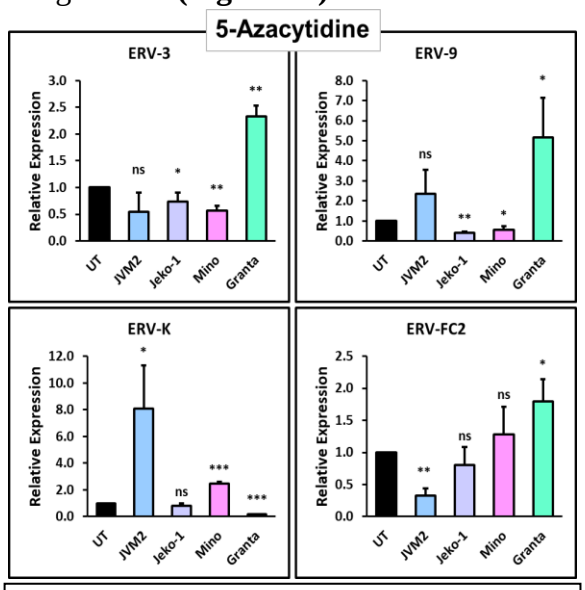

Figure 12. Effects of 5-Azacytidine on mantle cell lymphoma cell lines, by HERV.
Viewing the effects of the drug 5-Azacytidine in this manner allows easier visualization of the variation between cell lines, and highlights trends per target HERV.

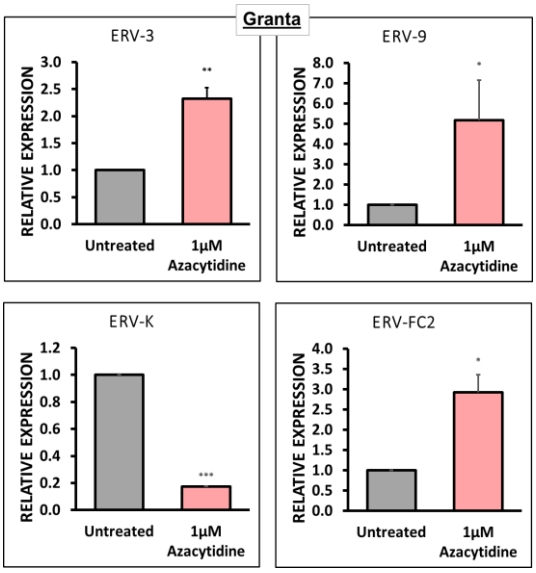

Figure 11. Relative fold-change in expression of ERVs as seen in Granta.
The relative expression of the target ERVs in the treated samples can be seen in comparison to their untreated counterparts.

3.4 Results of Aim 2b – Effect of Bromo- and Extra-Terminal Domain Inhibitors (BETi) on NHL Cells; JQ1

Rationale:

Bromodomains are protein domains composed of four α-helices connected by loop regions of varying length. These domains recognize acetylation residues, like those on the N-terminal tails of histones, and recruit transcription promoters and machinery to target and transcribe their target gene. In this way, they act as "readers" of lysine acetylation and can use this information to induce upregulation of certain genes. The bromodomain and extra-terminal domain (BET) family of proteins consists of four identified structures: BRD2, BRD3, BRD4, and BRDT. JQ1, specifically, competes for acetylation sites with BRD4, and therefore acts as a competitive inhibitor. This competition ultimately reduces activity of BRD4 and reduces the resulting transcription of its target gene, which may be oncogenic.

Results:

NHL Cell lines, JVM2, Jeko-1, Mino, and Granta, were treated with 1μM of (+)-JQ1, incubated for 72 hours, then collected by centrifuge as described in Chapter 2: Methods. Their RNA was extracted, processed into cDNA, then used in qPCR to assess the change in genetic expression of our four target ERVs: ERV-3, ERV-9, ERV-K, and ERV-FC2, in relation to our housekeeping treatments, tests, and comparisons were conducted in six replications and collected below. JVM2 cells reacted to the treatment of JQ1 similarly to the Azacytidine treatment. ERV-3 is slightly downregulated while ERV-9 is slightly upregulated, through the change in their expression was not wholly significant (**Figure 13**). JVM2 cells reacted to the treatment of JQ1 similarly to the Azacytidine treatment. Additionally, ERV-K seems significantly upregulated when treated with JQ1, and ERV-

FC2 show significant downregulation. While the ERV-K regulation has some variation, it is interesting as it shows the same expression profile as was apparent in the Azacytidine treatment. Jeko-1 shows a bit of a disparity from its Azacytidine treatment, as ERV-3, ERV-9, and ERV-K are all upregulated **(Figure 14)**. ERV-9 shows the most significant upregulation, followed by ERV-K. ERV-FC2 has a slight downregulation, but given the high variation in the data it would be pertinent to examine this further before drawing any concrete conclusions. Mino demonstrates some downregulation of ERV-3 when treated with JQ1, more exaggerated than its similar ERV-3 downregulation when treated with Azacytidine **(Figure 15)**. We noted some inconsistent upregulation of ERV-9 as the variability in our data was significant, prompting us to take another look in the future. Granta shows somewhat significant upregulation of ERV-3, similarly to its upregulation of ERV-3 when treated with Azacitidine. We also note significant

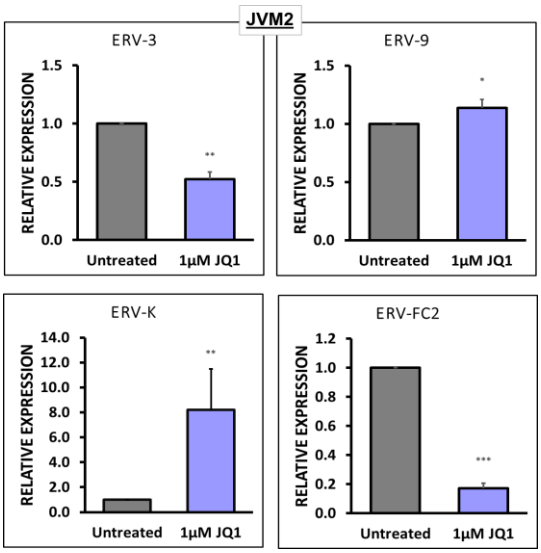

Figure 13. Relative fold-change in expression of HERVs as seen in JVM2. *The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.*

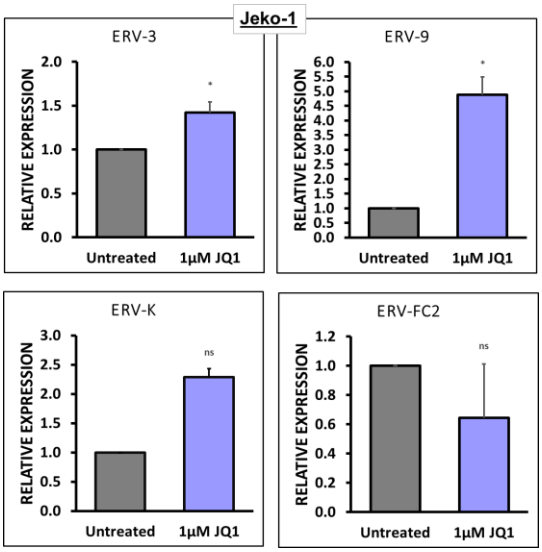

Figure 14. Relative fold-change in expression of HERVs as seen in Jeko-1. *The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.*

downregulation of ERV-K, and some upregulation of ERV-FC2 **(Figure 16)**. When we examine the effect of JQ1 on our four NHL cell lines split up by ERV, we again see some interesting trends develop. Granta again showed significant upregulation of ERV-3 upon treatment with JQ1. ERV-9 displayed some upregulation in both Jeko-1 and Mino, but with Mino's large variance in data, it would be difficult to draw any concrete conclusions. Interestingly ERV-K demonstrated some upregulation in JVM2 when treated with JQ1, though ERV-FC2 remained unremarkable in all cell lines (**Figure 17**).

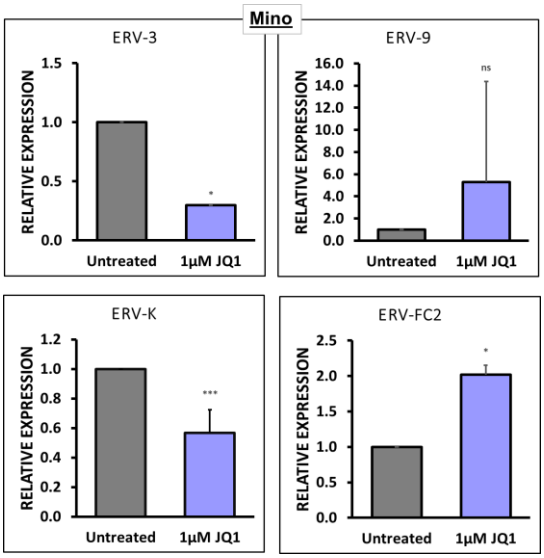

Figure 15. Relative fold-change in expression of HERVs as seen in Mino.
The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.

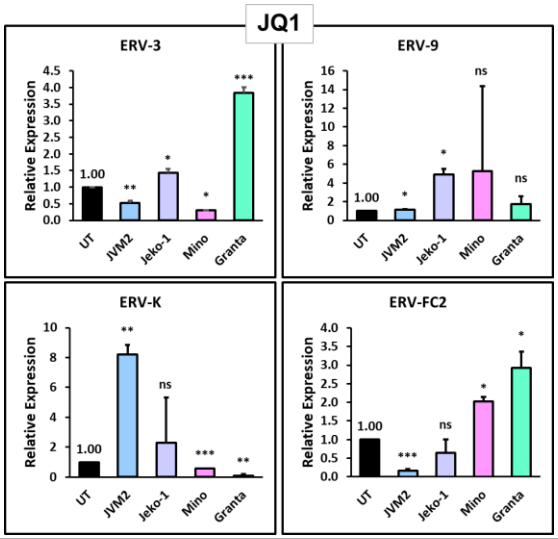

Figure 17. Effects of JQ1 on mantle cell lymphoma cell lines, by HERV.
Viewing the effects of the drug JQ1 in this manner allows easier visualization of the variation between cell lines, and highlights trends per target ERV.

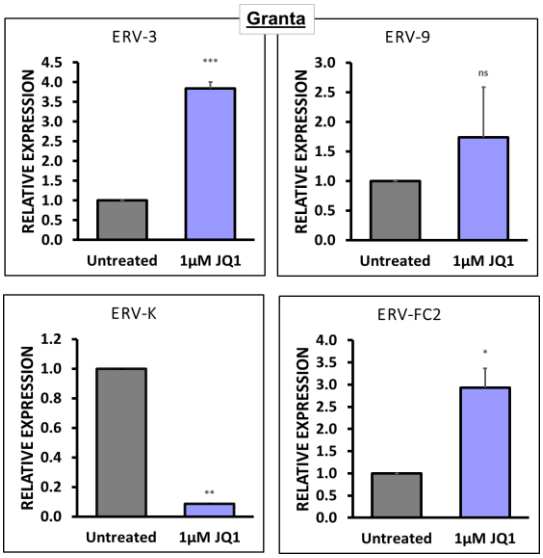

Figure 16. Relative fold-change in expression of HERVs as seen in Granta.
The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.

3.5 Aim 3b - Effect of Histone Methyltransferase (HMT) G9a inhibitor on NHL Cells; UNC0638

Rationale:

G9a is an epigenetic enzyme that acts on Histone H3 Lysine 9 (H3K9). As a histone methyltransferase, it is responsible for the mono- and di-methylation of H3K9, sometimes abbreviated as H3K9me and H3K9me2. H3K9me2 is strongly associated with transcriptional repression as it passively prohibits acetylation and therefore the binding of RNA polymerase, which is required for transcription. Additionally, H3K9me2 has been shown to actively repress transcription through recruitment of other transcriptional repressors. The G9a enzyme is seen to be overexpressed in many cancers and higher expression levels are correlated with poor prognosis, indicating that the target genes of this enzyme may be tumor suppressing in nature. Inhibiting G9a using a G9a inhibitor, UNC0638, may be the key to restoring proper ERV transcription.

Results:

NHL cell lines, JVM2, Jeko-1, and Granta, were treated with 1μM of our G9a inhibitor, UNC0638, incubated for 72 hours, then collected by centrifuge as described in Chapter 2: Methods. Their RNA was extracted, processed into cDNA, then used in qPCR to assess the change in genetic expression of our four target ERVs: ERV-3, ERV-9, ERV-K, and ERV-FC2, in relation to our housekeeping gene GAPDH. These treatments, tests, and comparisons were carried out in three replications and collected below. Due to time constraints and poor confluence, Mino was excluded from this trial. JVM2 shows no significant change in either ERV-3 or ERV-9 when treated with G9ai **(Figure 18.)**. In contrast, ERV-K seems significantly upregulated when treated with the inhibitor in

comparison to its untreated control. ERV-FC2 shows a slight upregulation, though with the high variance in data, it's difficult to draw any concrete conclusions. Jeko-1 displays a similar trend in ERV-3, with no significant change **(Figure 19.)**. Of note, however, it showed a significant downregulation of ERV-9. Though, there was no significant change in either ERV-K or ERV-FC2 when treated with G9ai. Granta continues the trend of having an insignificantly upregulated ERV-3 but notes a similar insignificant change in ERV-9 in contrast to Jeko-1 **(Figure 20.)**.

Interestingly, both ERV-K and ERV-FC2 show some upregulation in

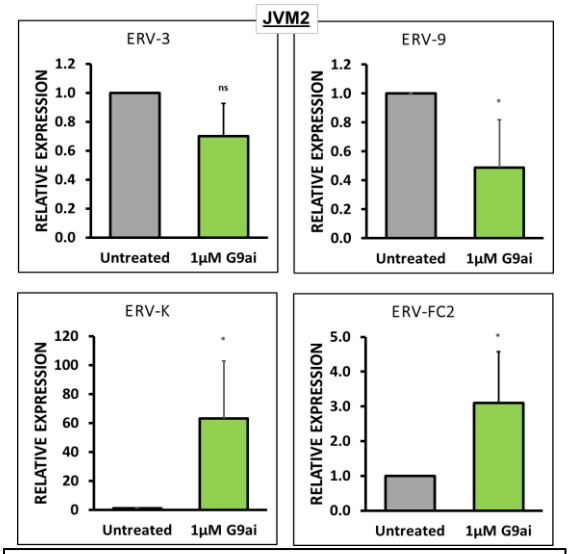

Figure 18. Relative fold-change in expression of HERVs as seen in JVM2.
The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.

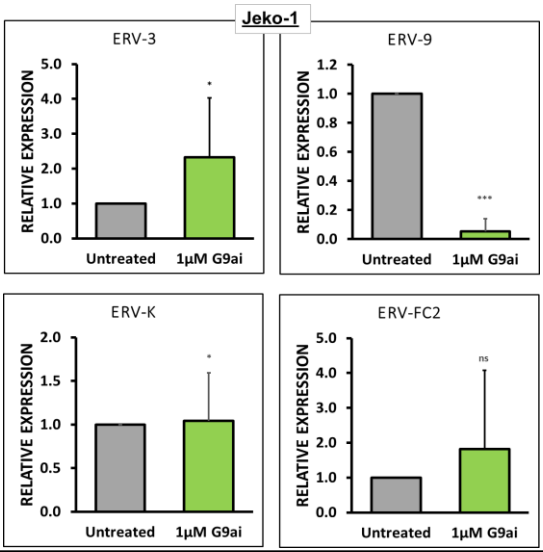

Figure 19. Relative fold-change in expression of HERVs as seen in Jeko-1.
The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.

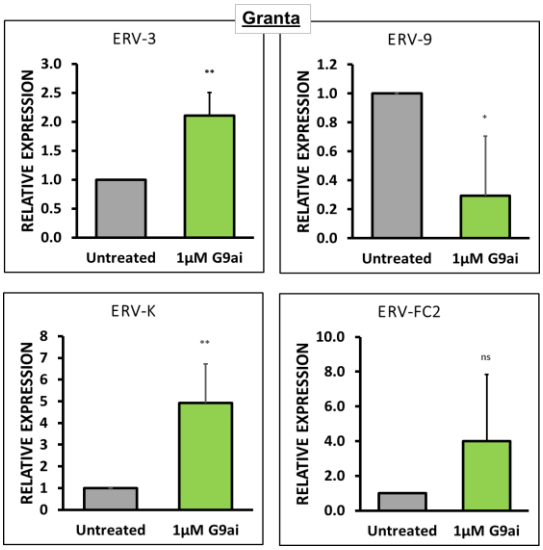

Figure 20. Relative fold-change in expression of HERVs as seen in Granta.
The relative expression of the target HERVs in the treated samples can be seen in comparison to their untreated counterparts.

Granta when treated with G9ai. When we arrange these treatments by ERV as we have with the previous drug treatments, we find it more difficult to denote trends. Due to the higher-than-normal variance in data, concrete conclusions are difficult to draw. While we do see an extreme upregulation of ERV-K in JVM2 when treated with our G9ai, we would be well to conduct more replicates to eliminate any doubt before moving forward (**Figure 21**).

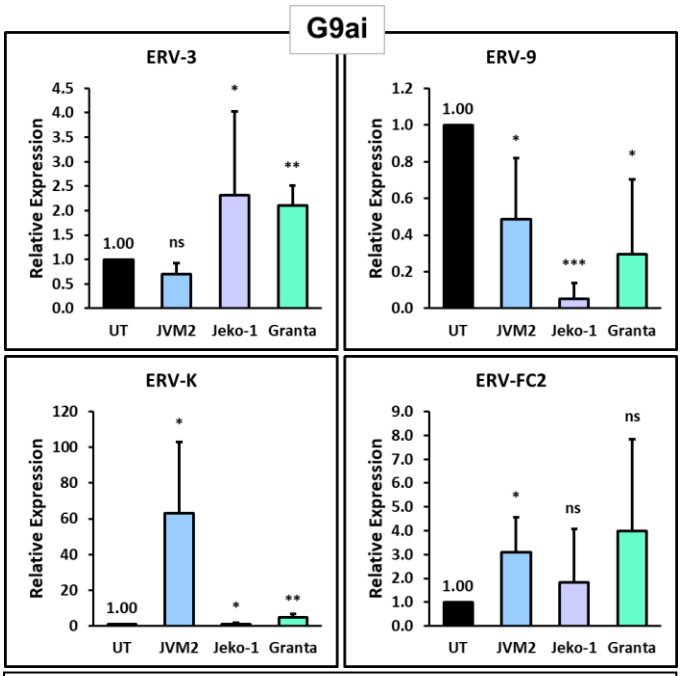

Figure 21. Effects of G9ai on mantle cell lymphoma cell lines, by ERV.
Viewing the effects of the drug G9ai in this manner allows easier visualization of the variation between cell lines, and highlights trends per target ERV.

Chapter 4: Discussion and Conclusion

As discussed, mantle cell lymphoma is an aggressive subtype of Non-Hodgkin's Lymphoma associated with poor prognosis and adverse patient outcomes. Current therapies and treatments, while not wholly ineffective, are not enough to address patient needs. It would be beneficial both to those suffering as well as science if novel therapies were discovered and developed. Given the growing body of knowledge on epigenetic technology and the role of gene regulation in the proliferation of cancer cells, it would serve us well to look to epigenetics for further answers, just as we have done here. As the up and downregulation of several HERVs are already linked to the development of some cancers, we would be keen to continue examining these portions of the genome for other potential correlations. As HERV-3 and HERV-K have both been found to correlate strongly with the development of several types of metastases ranging from colorectal to pancreatic, it made sense to test their correlation with the development of B-cell mantle cell lymphoma (Bustamante Rivera, Yomara Y, et al.). In Aim 1, we looked at several HERVs; ERV-3, ERV-9, ERV-K, and ERV-FC2, and noted some interesting correlations. After several repetitions of our testing, we saw insignificant upregulation of suspected oncogenes ERV-3 and ERV-K when compared to the peripheral blood mononuclear cells that serve as the precursor to B-cell development. Interestingly, though, we noted some solid trends when looking at both ERV-9 and ERV-FC2. ERV-9 seemed to be significantly upregulated in all NHL cell lines (except Mino) when compared to PBMCs. Additionally, ERV-FC2 was consistently downregulated in our NHL lines compared to PBMCs, although the data variance for this ERV was greater. Before drawing any concrete

conclusions, it would be wise to perform more testing to further enhance our body of data to draw from.

In Aim 1, we also compared the relative expression of our target HERVs in NHL cell lines to those in CD-19+ B cells. As discussed earlier, CD-19 is a known biomarker of mature B cells and serves as a way for us to denote differentiated B cells from other types of lymphocytes. In this way, we're able to closely compare the relative expression of HERVs in cancerous NHL cells to normal type B-cells. In the second half of Aim 1 we examined these differences and noted some interesting trends. While ERV-FC2 was again, unremarkable, other ERVs displayed more significant changes. ERV-3 and ERV-K, the suspected oncogenes discussed earlier, were upregulated in all our NHL cell lines when compared to their normal type counterparts. ERV-9 demonstrated even more upregulation; in some cell lines, a several-hundred-fold increase in comparison to normal type B cells. This significant upregulation of ERV-3, ERV-K, and ERV-9 in NHL cell lines compared to normal type B cells could denote a correlation between the upregulation of these HERVs and development of mantle cell lymphoma.

Of note, ERV-9 was upregulated in our NHL cell lines both in comparison to PBMCs and CD-19+ B cells, but to a higher degree in the CD-19+ B cells tested. As the cells develop into NHL B cells in the following order; PBMCs → CD-19+ B cells → NHL cells, we note an interesting trend. As NHL cell lines have significantly higher ERV-9 expression when compared to CD-19+ B cells than PBMCs, it serves to imply that somehow during the development of CD-19+ B cells from PBMCs, the ERV-9 expression decreases, only to significantly increase again when becoming NHL cells. Although it is

currently unknown what this could mean, it would be an interesting trend to look into in further studies.

In Aim 2-3 we instead look at the change in genetic expression of our target HERVs in response to various epigenetic drugs. As discussed, this class of drugs looks to tune up and down the relative expression of various coding and noncoding regions of our genome. With further research, the hope is that one day we will be more able to precisely downregulate problematic or oncogenic genes and enhance the effectiveness of those with tumor suppressing properties.[9] In the realm of epigenetic drugs, we examined the effect of a DNA Methyltransferase Inhibitor (5-Azacytidine), a Bromo- and Extra-Terminal Domain Inhibitor (JQ1), and a Histone Methyltransferase Inhibitor (G9ai) for their effects on our target HERVs. After repeated testing, we were able to note some concrete trends that may be of interest. For one, 5-Azacytidine seemed to upregulate both ERV-3 and ERV-9 in our cell line Granta, while down regulating these ERVs in our other cell lines. This may speak to the abnormal qualities of the Granta cell line in that it seemed to have a differing reaction to the drug when compared to the other cell lines. Additionally, ERV-K proved to be upregulated in JVM2 when treated with 5-Azacytidine but showed little change in the other cell lines. As ERV-3 and ERV-K have shown to be upregulated in other metastases, these bouts of upregulation need to be seriously examined in that administration of these medications, while therapeutic for some types of cancer, may have unintended effects elsewhere.

After administration of JQ1, we noted, again, a significant upregulation of ERV-3 in the cell line Granta. Continuing the prior trend, ERV-K was upregulated in our cell line JVM2 after treatment with this epigenetic drug. ERV-9, while slightly upregulated, seemed

less affected in terms of regulation than when treated with 5-Azacytidine. As these changes in regulation mirror those of the earlier drug treatment, it would be wise to revisit both JVM2 and Granta for continued examination and testing.

As neither 5-Azacytidine or JQ1 acted to counteract the ERV-9 upregulation we noted when comparing our NHL cells to our normal controls, we had to look to other options in G9ai. Interestingly, while ERV-9 displayed either no significant change or slight upregulation in our various cell lines when treated with both 5-Azacytidine and JQ1, we saw almost universal downregulation of ERV-9 when treated with G9ai. Given the noted upregulation of ERV-9 in our NHL cell lines in comparison with our normal controls, it may be wise to conduct further experimentation with G9ai as it served to address. The upregulation of our target HERVs in Granta noted in the other drug trials was much less pronounced, though the upregulation of ERV-K in JVM2 continued. As JVM2 displayed significantly upregulated ERV-K in response to treatments with all three classes of our epigenetic drugs, it encourages us to look further into either the abnormalities of that cell line specifically, or any confounding variables that may have impacted our data along the way to create this trend. The mechanisms of each of these drugs are different, so the possibility that each of them has a similar effect on JVM2 remains slim.

Given additional resources and time, it would be beneficial to conduct additional testing regarding the combinatory effects of these drugs. Looking into how the regulation of our target ERVs change when two epigenetic drugs act on them at the same time. Although we have already completed some preliminary testing on these combinatory trials, more needs to be done before any concrete conclusions can be drawn, no matter how promising the data thus far. Though the examination of our target HERVs across

developmental stages of the mantle cell lymphoma cells gave us an insight into the progression of its expression, a concrete conclusion regarding HERV response to epigenetic drugs remains to be seen. Given the wider field of science and advancing developments in epigenetics as of late, we remain optimistic for what lies ahead in science for these little retroviral pieces of our genome. Given the significant changes in ERV-9 regulation across the lifespan of our NHL cells, investigating this HERV and its connection to lymphoma may be the route forward.

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