UNIVERSITY OF CALIFORNIA SAN DIEGO

HIV-1 Vpr Affects Alternative Splicing by Modulating SRPK Activity

A Thesis submitted in partial satisfaction of the requirements
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in

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by

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The Thesis of Karen A. William is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

To Mama and Soso, the strongest women I know, for believing in me when I didn't, for supporting and pushing me to be and do better, and for always inspiring me.

EPIGRAPH

I believe in the power of the written word and the transforming impact it can have on people's lives.
I dream - I find that reality springs from dreaming - to bring such transformation through my own written words.

Sandra William

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* Indicates that these Figures are taken from previous work and are used to provide visual presentation across several topics needed to understand this thesis.

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
E1A	Adenovirus early region 1A
FACS	Fluorescence-Activated Single Cell Sorting
Gp120	Glycoprotein 120
HEK-293	Human embryonic kidney 293 cells
HIV	human immunodeficiency virus
SR	Serine and Arginine
SRPKs	serine/arginine-rich specific kinases
Vpr	Viral protein R

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ABSTRACT OF THE THESIS

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by

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Professor Mary K. Lewinski, Chair
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Alternative splicing, an essential post-transcriptional mechanism, is a target for HIV. HIV employs this process to produce multiple viral proteins from a single mRNA. Vpr, an accessory viral protein, supports HIV infection by manipulating cellular activities - inducing G2/M arrest, promoting viral protein expression, regulating apoptosis and cytotoxicity. Previous proteomic studies demonstrated that Vpr modulates the activity of serine/arginine-rich specific kinases (SRPKs). SRPKs regulate splicing by phosphorylating serine and arginine-rich (SR) proteins and regulating their intracellular location. Thus, we hypothesized that Vpr

influences alternative splicing by modulating SRPK phosphorylation directly or via a secondary effect of Vpr-induced G2/M arrest.

We confirmed that Vpr enhances Env protein expression, then probed whether this could be due to Vpr-induced increases in Env mRNA. We examined the effects of Vpr on cellular splicing, first confirming that Vpr modulates SRPK1 phosphorylation, then employing the E1A splicing reporter assay to assess Vpr's effects on SRPK-dependent splicing. We also evaluated whether the Vpr-dependent effects we saw were secondary to G2/M arrest triggered by Vpr or a result of other direct effects of Vpr. These findings lay the foundation for further studies of Vpr's modulation of viral and cellular splicing.

INTRODUCTION

Background:

In 1983, scientists discovered that the acquired immunodeficiency syndrome (AIDS) is induced by a virus now known as HIV (human immunodeficiency virus) that destroys the human immune system, leaving those who have been infected defenseless. This causes them to acquire other slowly progressing, often incurable, and fatal diseases. Despite over 35 years of research answering numerous questions about HIV, a cure is still out of reach. Several treatments which demand constant monitoring and upkeep are available; however, these treatments simply delay the immune system from failing rather than get rid of HIV.

Successful infection by HIV is highly dependent on bypassing host defenses and hijacking cellular metabolism. The virus optimizes its own expression to replicate at the expense of the host's gene expression and function (Hu & Hughes, 2012). In conjunction with causing a global change to the host's gene expression, HIV also produces viral accessory proteins which enable its evasion of the immune system (Collins & Collins, 2014). Although viral accessory proteins have no enzymatic activity, they redirect and/or prohibit normal host functions to benefit the virus's survival (Strebel, 2013).

HIV has remained a major global public health issue. According to the World Health Organization, in 2020 roughly 37.7 million people are living with HIV worldwide and the virus claimed 680,000 lives. By continuing to investigate how the virus disrupts cellular processes, we are one step closer to discovering a treatment to prevent HIV from spreading and hopefully eradicating its control over so many lives.

HIV infection and Replication:

In the viral replication cycle, new copies of the viral RNA are produced, encapsulated, then bud out of the host cell and into the bloodstream to infect other host cells (Collins & Collins, 2014) - illustrated in Fig. 1. Once inside the human body, the encapsulated virus targets a subtype of white blood cells, CD4+ T helper cells, known to fight off infections.

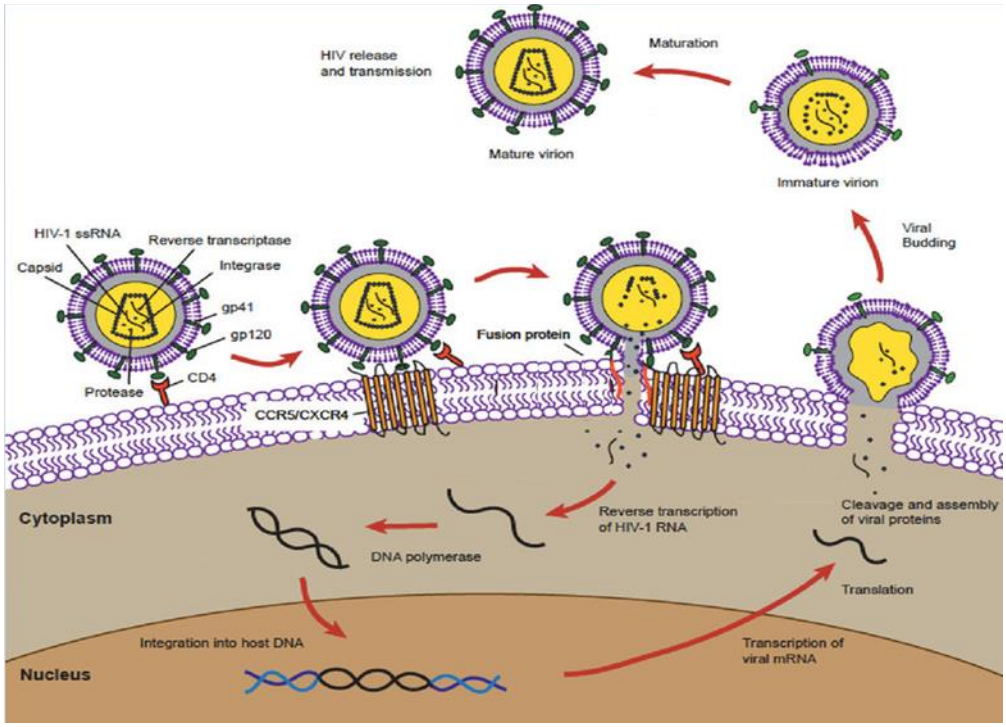

Figure 1: HIV Replication Cycle. (Yavuz et al., 2018) - with modification.

The viral lipid envelope, which surrounds the capsid (CA)-a protective coat for the viral genetic material, is studded with an envelope protein (Env) composed of two subunits: surface glycoprotein (gp120) which binds to receptor molecules, and transmembrane glycoprotein (gp41) which mediates fusion of the viral membrane with the plasma membrane - seen in Fig. 2. Fusion begins when Env attaches itself onto the CD4 receptor, permitting gp120 to attach onto one of the two chemokine receptors (CCR5 or CXCR4) present on the host cell; thus, triggering the activation

of gp41 to fuse the viral membrane with the cellular membrane (Wilen et al., 2012). Membrane fusion and viral entry, the first step of the viral replication cycle, delivers two copies of a positive single-stranded viral RNA genome (+ssRNA), multiple copies of both reverse transcriptase (RT) and integrase (IN), accompanied with other enzymes and proteins, into the host cell.

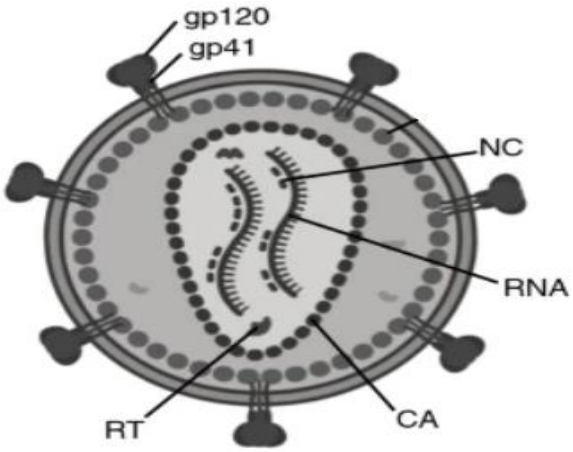

Figure 2: Virion Structure (Aiken & Rousso, 2021)

Now two copies of the single-stranded viral RNA genome (+ssRNA), reverse transcriptase (RT), and integrase (IN) are present in the host cell. Using the viral +ssRNA as a template, the viral reverse transcriptase (RT) reverse transcribes the genomic viral RNA to make double-stranded DNA (dsDNA). RT has three enzymatic activities: RNA-dependent DNA polymerase, RNase H, and DNA-dependent DNA polymerase. First, the RNA-dependent-DNA polymerase transcribes the viral RNA genome template to produce the RNA-DNA hybrid. Next, RNase H cleaves RNA out of the RNA-DNA hybrid leaving a single-stranded DNA (ssDNA). The DNA-dependent DNA polymerase replicates the ssDNA to produce the double-stranded DNA complex (dsDNA) (Hu & Hughes, 2012; Sarafianos et al., 2009). The newly produced viral dsDNA translocate into the nucleus and integrates into the host genome via the viral integrase (IN). The integrated viral dsDNA, referred to as a provirus, hijacks the host's cellular machinery to produce

viral messenger RNA (mRNA). The mRNA either becomes genomic RNA for newly produced virions or is variably spliced and translated into viral proteins, some of which are known as viral accessory proteins: Vif, Vpr, Vpu, and Nef (Sarafianos et al., 2009). For HIV to generate its RNA genome and proteins, it co-opts cellular machinery. Specifically, HIV's ability to produce various viral proteins from its small genome depends on regulating a crucial mechanism known as alternative splicing.

Vpr:

Vpr, a multifunctional accessory viral protein expressed late during the infection cycle and packaged into virions, is known to affect both viral and cellular proliferation by arresting the cell cycle at G2/M phase, activating the long-terminal repeat (LTR) viral promoter, facilitating escape from immune sensing, aiding in replication in non-dividing cells, and regulating DNA damage responses, apoptosis, and cytotoxicity (Hashizume et al., 2007; Le Rouzic & Benichou, 2005). La Rouzic provides a well-organized and thorough visualization for the role of Vpr in the HIV cell cycle seen in Fig. 3. Previous reports documented that Vpr has a global effect on both the cellular transcriptome and proteome by interacting with molecular pathways to support HIV replication in cells (Bauby et al., 2021; Greenwood et al., 2019; Lapek et al., 2017). Vpr is particularly important for infection of non-dividing cells such as macrophages, since it aids in the transport of the viral pre-integration complex into the nucleus to allow for viral integration into the host genome (Le Rouzic & Benichou, 2005). Also, in non-dividing cells, an increase in the levels of Env expression was detected in presence of Vpr indicating that Vpr may have positive effects on replication (Hashizume et al., 2007).

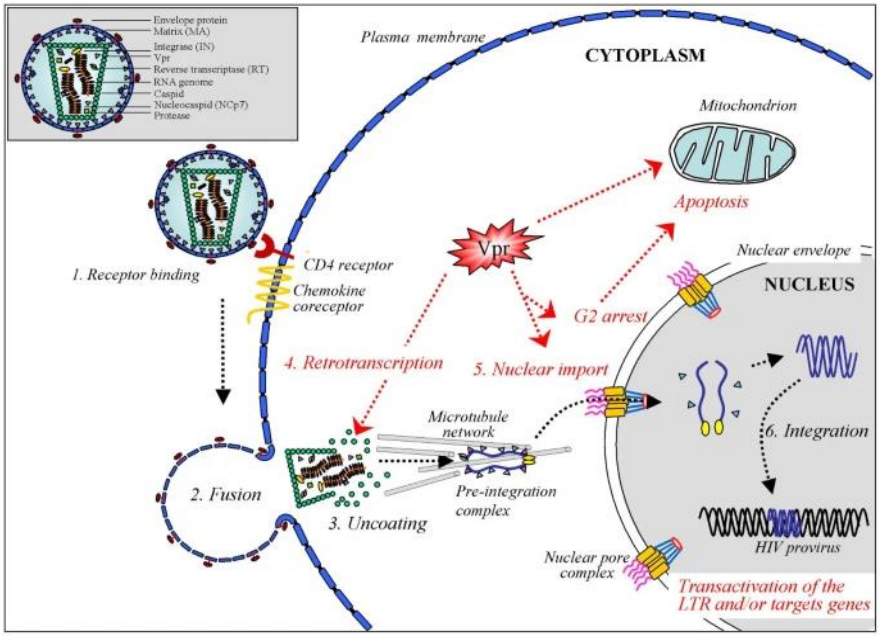

Figure 3. Role of Vpr in HIV Cycle. (Le Rouzic & Benichou, 2005)

Other studies observed that Vpr plays a role in the selective modulation of cellular splicing both *in vivo* and *in vitro* (Fabryova & Strebel, 2019). Specifically, it was reported that Vpr modulates some splicing by interacting with the spliceosomal protein SAP145 (a.k.a. Splicing factor 3B subunit 2 or SF3B2) (Hashizume et al., 2007). Multiple studies have reported that global changes in the cellular transcriptome (Bauby et al., 2021) and proteome (Greenwood et al., 2019; Lapek et al., 2017) following HIV infection are induced by Vpr. Lapek et al. conducted a study utilizing mass spectrometry and phospho-proteomics to characterize changes following HIV gene expression and identified multiple pathways that were modulated in a Vpr-dependent manner. Lapek et al. observed that the phosphorylation of the Serine-arginine protein kinase 1 was unexpectedly modulated in the presence of Vpr, increasing in the first 12 hours following HIV expression before declining (Lapek et al., 2017). SRPK1 phosphorylates multiple splicing factors leading to their activation and is itself regulated by phosphorylation. These findings suggest that Vpr by some means contributes to the regulation of RNA splicing by potentially altering the

phosphorylation and thus the activity of SRPKs (Lapek et al., 2017). However, the role of SRPK and how it is regulated in HIV infection remains unclear along with the mechanism which Vpr might mediate SRPK activity. All this research and yet we barely scratched the surface on how Vpr operates or the magnitude of its role in the HIV infection. We investigated how Vpr causes its changes and whether they result from a direct activity of Vpr on splicing machinery or are a consequence of the cell cycle block induced by Vpr.

Alternative Splicing and the Spliceosome:

Alternative splicing produces multiple proteins from the same gene. It is essential for cell cycle progression and transcriptional regulation; therefore, it is vital for the replication and proliferation of HIV (Emery et al., 2017). Splicing is the removal of all the intron regions present in pre-mRNA, a precursor of mRNA consisting of introns (non-coding) and exons (coding) regions, leaving a mature (exon only) messenger RNA (mRNA) behind (Wang & Burge, 2008). The removal of these introns is mediated by a dynamic and flexible macromolecular machine known as a spliceosome, a multi-megadalton ribonucleoprotein (RNP) complex composed of five small nuclear ribonucleoproteins (snRNPs) and numerous proteins (Will & Luhrmann, 2011).

During pre-mRNA splicing, the spliceosome removes the introns and ligates neighboring exons. Each spliceosome is composed of five small nuclear RNAs (snRNAs) – u1, u2, u4, u5 and u6, along with a range of associated protein factors, depicted in Fig. 4. Multiple steps must occur to assemble the spliceosome correctly with approximately 100 proteins participating, some of which are the serine and arginine (SR) proteins that are involved in every step of the assembly. When the snRNAs of the spliceosome are combined with various protein factors, they produce the

RNA-protein complex mentioned before - snRNP. As snRNPs combine with one another they form a larger ribonucleoprotein complex - the spliceosome.

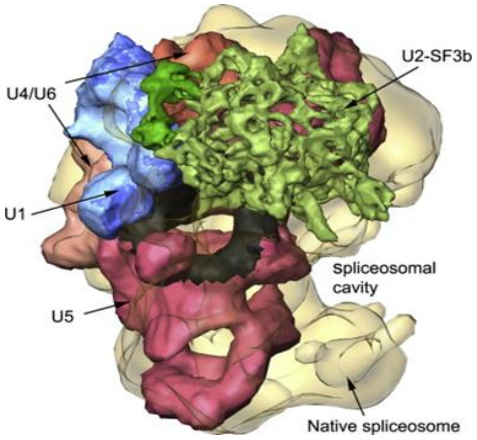

Figure 4: The Spliceosome (Frankenstein et al., 2012)

The spliceosome omits introns through the splice sites present on the pre-mRNA. These sites are specific RNA sequences that are detectable by the spliceosome with some help from the SR proteins. There are two types of splice sites acting as signal borders between the exons and introns: donor sites (D**X**) are at the start of the intron and acceptor sites (A**X**) are at the end of an intron (Wang & Burge, 2008). After successfully attaching to one of each site (D**X** and **AX**) present on the intron/s, the spliceosome wraps around the intron and merges the two neighboring exons and concurrently omits the intron/s as seen in the Figure below.

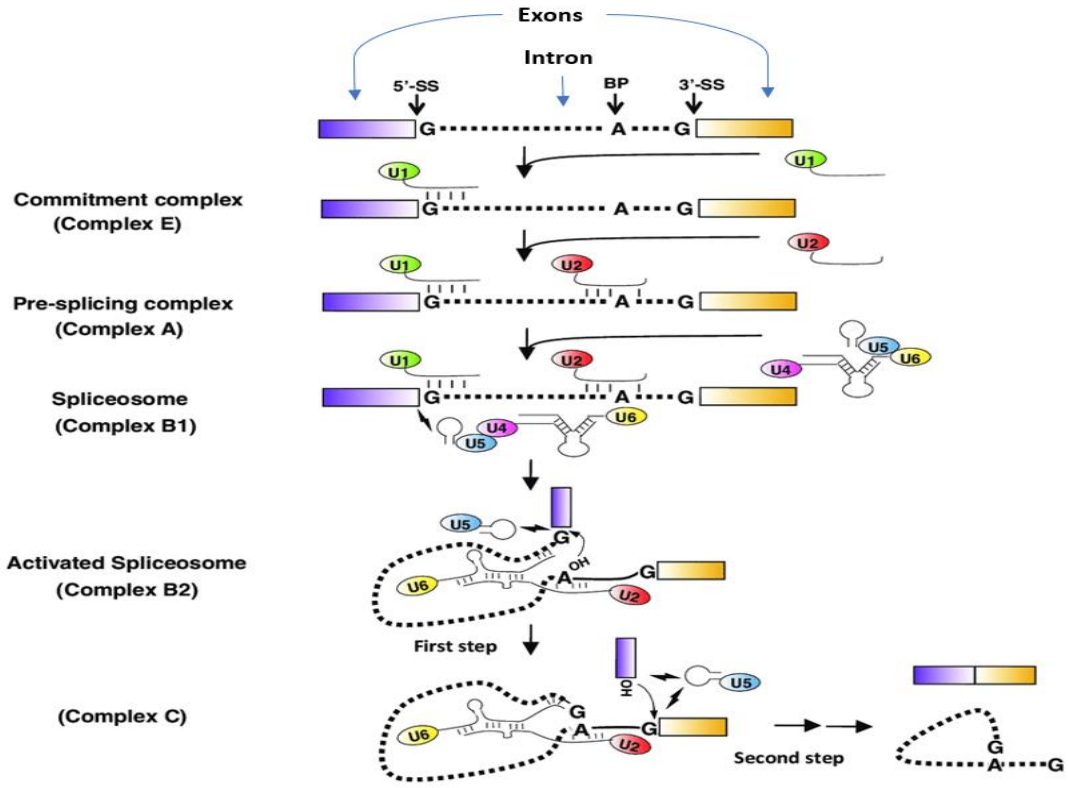

Figure 5: **Major spliceosome assembly and pre-mRNA splicing**. (Zhao et al., 2018) Dotted lines represent introns, and the boxes represent exons. The 5'splice site(5'SS)/acceptor site (A**X**), the 3' splice site(3'SS)/donor site (D**X**), and the branch point adenosine (BP) are noted in the pre-mRNA strand. Thin lines are snRNA that make up the spliceosome with their names in the ellipses.

There are two types of splicing. Constitutive splicing is the process of intron removal and exon ligation of most of the exons in the order in which they appear in a gene as seen in Fig.6(B), while alternative splicing deviates from this expected sequence by skipping certain exons. These deviations are determined by the splice sites present at the edges of each intron and the spliceosome's ability to bind. These splicing sites can be used interchangeably; by binding to a donor site at one intron with an acceptor site on another intron, the omission of several introns at once is possible along with the exons that lie between them. Through the removal of exons in between the introns, new exon sequences that were not possible otherwise develop and give rise to various forms of mature mRNA. As seen in Fig.6., three different mRNA strands (B, C and D)

were produced from the same pre-mRNA strand (A) and will then be translated into 3 different proteins (Wang et al., 2015). This illustration depicts the importance of alternative splicing, an imperative mechanism that maximizes the production of various proteins is the perfect target for HIV to commandeer.

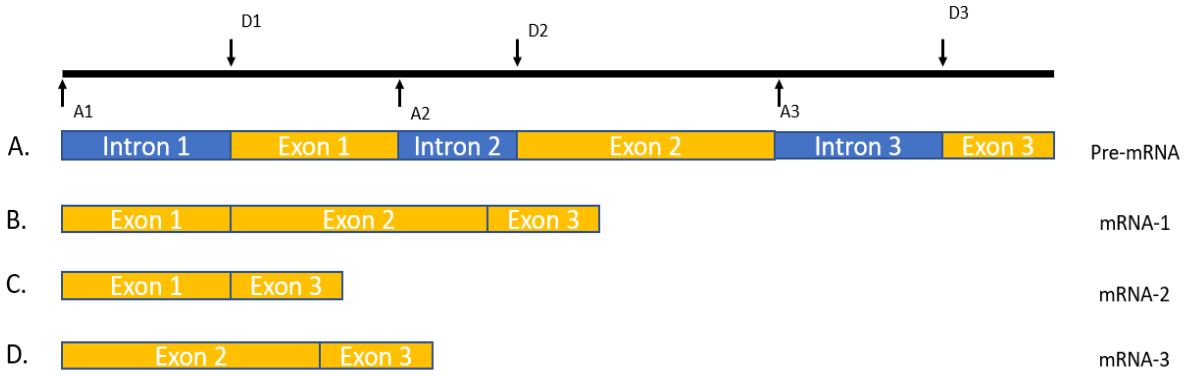

Figure 6: Demo for Alternative splicing. (A) pre-mRNA strand with three donor and three accepter sites existing on the start and end of each intron. (B) mRNA-1 strand is produced due to constitutive splicing- each intron is spliced separately: A1 to D1, A2 to D2 and A3 to D3 were removed by the spliceosome combining exon 1, 2 and 3 together. (C) mRNA-2 strand consists of only two exons since intron2 and intron3 were spliced together: removing exon 2 by binding A2 to D3 sites. (D) Exon 1 was removed amid intron 1 and 2 giving rise to mRNA-3 by utilizing A1 and D2 sites.

SR proteins and the Spliceosome:

Alternative splicing is monitored and regulated by multiple elements that are essential for the spliceosome assembly, one of which is the Serine-Arginine rich (SR) protein family. Each SR protein contains at least one RNA recognition motif (RRM) and an RS domain- long repeats of arginine and serine amino acids. These 2 components allow SR proteins to regulate both constitutive and alternative splicing by recognizing and binding to exonic splicing enhancer sequences (ESEs) in the pre-mRNA strand and consequently providing a marker for the spliceosome (Shepard & Hertel, 2009).

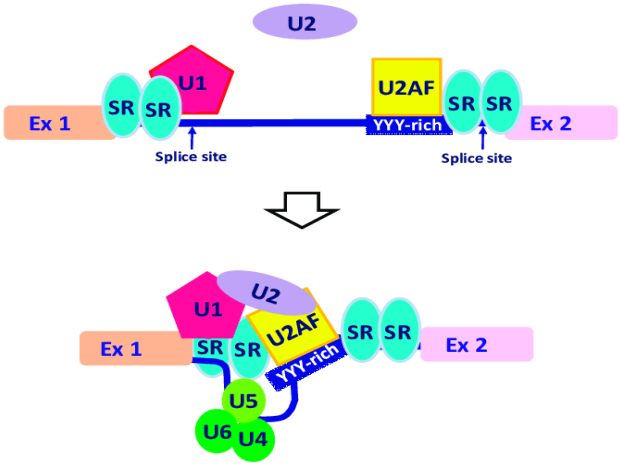

Figure 7: Assembly of spliceosome complex with the help of SR proteins. (Biamonti et al., 2019)

SR proteins have roles before, during, and after splicing; they promote intron exclusion and exon inclusion, and they contribute to post-splicing activities such as mRNA nuclear export and mRNA translation (Shepard & Hertel, 2009). SR proteins assist in the recruitment of the spliceosome to the pre-mRNA through the RRM interaction with the RNA and RS domain (Zhong et al., 2009). More specifically, they promote the binding of U1 snRNP to U2AF snRNP thereby initiating the formation of the spliceosome as seen in Figure 7. Cho's study demonstrated that the SR proteins were found to promote U1 snRNP binding to the 5' splice site and U2 snRNP binding to the 3' splice site and aid in the communication between the initial splice sites (Cho et al., 2011).

The primary kinase which phosphorylates multiple serine residues present on the RS domain of SR protein and therefore regulates the distribution of SR proteins in the cell is SR protein kinase 1 (SRPK1). The primary role of SRPK1 is activating and monitoring splicing mechanisms, however it is also extremely versatile. It has been associated in a multitude of cellular process such as cell cycle progression, mRNA maturation, innate immune response, chromosome segregation and reorganization, nuclear import and germ cell development, cell growth, cell differentiation, cell death, negative and positive regulation of viral genome replication, and

inflammation via interactions with multiple signaling pathways and transcription factors (Zhong et al., 2009).

Previous work discovered that SRPK1 is regulated based on the cellular distribution of the kinase rather than its activity since it is a constitutively active kinase partitioned between the cytoplasm and nucleus via an accessory domain- a spacer sequence that splits conserved kinase domains into two blocks (Zhong et al., 2009). The partitioning of the kinase is dependent on the ATPase activity of heat shock protein 90 (Hsp90) - a chaperone protein that assists other proteins to fold properly - since it modulates the dynamic chaperone interactions. It was also demonstrated that osmotic stress induces SRPK1 nuclear translocation by modulating the dynamic interaction of SRPK1 with the two specific HSP chaperone complexes (Hsp40 and Hsp70), thereby inducing differential SR protein phosphorylation and alternative splice site selection. These findings reveal that the regulation of SRPK is dependent upon physiological states (Zhong et al., 2009). SRPK1's involvement in numerous key pathways for cell growth and survival renders it a target for HIV. DeBoer's work revealed that the nuclear export of HIV RNA is promoted by SR proteins and SRPK1, causing an increase in HIV protein expression as well (DeBoer et al., 2018). This secondary effect suggests that the effects of the SR proteins are more widespread in HIV infection than assumed. However, there is still so much to reveal on how HIV manipulates alternative splicing, SR proteins and SRPK1 for its own benefits.

HIV splicing:

Despite its small size, the HIV unspliced genome -9.2 kilobases- can produce up to 15 proteins from 9 genes via the host's alternative splicing tools (Emery et al., 2017). Given that HIV does not have its own splicing tools, it coopts the host's cellular machinery to translate its proteins

and spread. HIV splicing utilizes 4 donor sites (D1…, D4) and 10 acceptor sites (A1…, A10) present on its RNA genome (seen in Fig.8) to construct over 50 mRNA variants that translate into different viral proteins based on the introns and exons removed (Emery et al., 2017). The genomic RNA undergoes several degrees of splicing or no splicing at all. The mRNA transcript that translates into the accessory proteins Tat and Rev requires the genomic RNA to be completely spliced. RNAs encoding Vif, Vpr and Env must undergo partial splicing while Gag, Pro, and Pol must avoid splicing. HIV must bypass several obstacles: its mRNA must not be over-spliced or full-length genomic RNA will be lost and it must overcome the cell's lack of ability to tolerate unspliced/incompletely spliced transcripts or it will not produce Vif, Vpr, Vpu and Env (Emery & Swanstrom, 2021). To replicate, HIV must monitor and control cellular mechanisms by suppressing most whilst allowing some splicing to transpire.

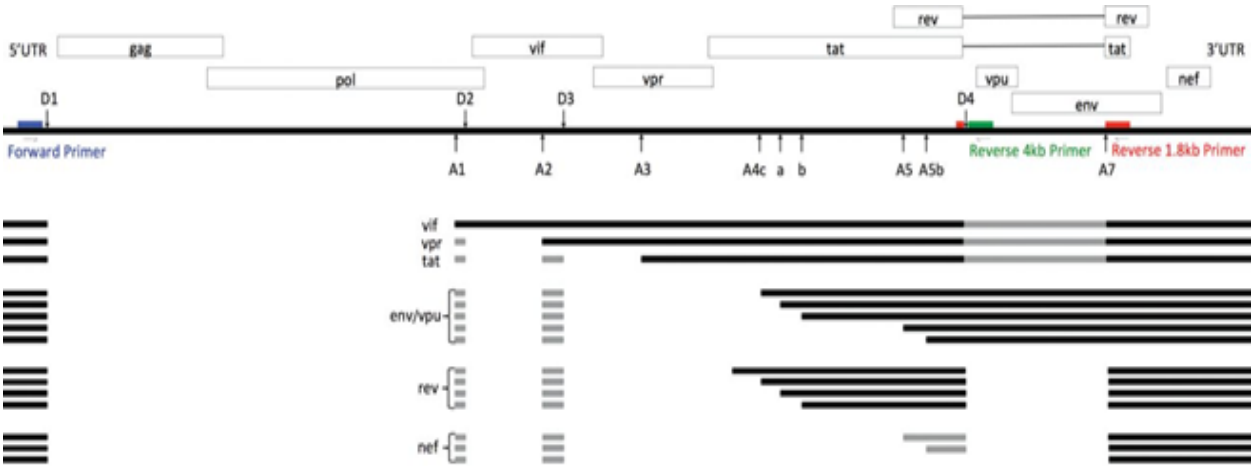

Figure 8: HIV-1 splice patterns. (Takata et al., 2018) Gray boxes are small exons or sequences present in the respective transcripts based on the different splicing sites. The white boxes correspond to the genes that will later translate into viral proteins.

Complete splicing is the default for cellular mRNAs, so initially Rev and Tat mRNA accumulate, and these are the first set of proteins to be translated. These two viral proteins contribute to viral transcription and splicing. Tat, crucial for reproducing the whole viral RNA,

counteracts the lack of HIV transcription by recruiting cellular elongation factors and permitting full-length transcription from the provirus (Dlamini & Hull, 2017). The cell's standard response is to fully splice newly formed viral RNA; however, Rev inhibits splicing by suppressing certain cellular proteins and splice sites as well as promotes the export of these unspliced or incompletely spliced viral mRNAs from the nucleus through the interaction with the cellular protein Crm1 (Dlamini & Hull, 2017). Rev simultaneously downregulates early genes and upregulates late genes present on the viral mRNA consequently downregulating its own expression. In the absence of Rev, previous studies revealed that the late viral genes (Gag, Pol, Env, Vpr, Vif and Vpu) are not translated (Dlamini & Hull, 2017). Rev is crucial for HIV's replication, yet other viral proteins have been implicated in the regulation of splicing.

Goals and Hypotheses:

Multiple regulatory elements affect HIV splicing although the regulation and recruitment of these to HIV mRNA are not fully defined. Previous work noted that a well-known kinase in cellular RNA-processing machinery, SRPK1, is phosphorylated and several splicing factors are upregulated following Vpr+ HIV expression. Vpr also was documented to affect pre-mRNA splicing of certain cellular mRNAs through its interaction with spliceosome assembly factor SF3B2. Vpr increases the accumulation of Env mRNA, possibly by interfering with pre-mRNA splicing. We aim to extend these studies and determine the effects of Vpr on viral and cellular mRNA splicing and explore whether these effects are related to Vpr-dependent modulation of SRPK activity. This study aims to shed some light on the potential connection between Vpr and HIV splicing along with the changes arising in cellular splicing due to Vpr. As Vpr is known to arrest the cell cycle in G2/M, Vpr-dependent modulation of SRPK may be a direct effect of Vpr or could be secondary to its cell cycle effects. To distinguish between these two possibilities, we

utilized and modified several assays to assess whether changes in both HIV and cellular splicing arise in the absence/presence of Vpr.

We hypothesized that Vpr enhances HIV replication by modulating alternative splicing of HIV RNA, possibly through the regulation of SRPK1 activity. We assessed the effects of Vpr on alternative splicing of HIV and non-HIV transcripts. First, we probed Env expression across different quantities of Vpr to confirm a previous report stating that Vpr increases Env expression (Zhang & Aida, 2009). Then, we tested whether the observed enhancement in Env protein expression could be due to Vpr-induced increases in Env mRNA by probing for HIV splicing transcripts including Env mRNA. We hypothesize that Vpr increases Env expression through the increase of Env mRNA.

To examine effects of Vpr on cellular splicing, we tested whether SRPK1 phosphorylation was modulated by Vpr. First, we confirmed prior results from phospho-proteomics studies which reported an increase in SRPK phosphorylation in the first 12 hours before declining (Lapek et al., 2017). To detect these changes, we analyzed shifts in SRPK phosphorylation with or without Vpr in HIV via SRPK1 and phospho-SRPK1 (Thr601) antibodies with hopes of observing an increase in Phospho-SRPK1 signal in the presence of Vpr. We then adapted an assay for cellular splicing to test Vpr effects on SRPK-dependent alterations in splicing. To assess the effects of Vpr on alternative splicing of non-HIV transcripts (cellular splicing), we first established that the Adenovirus early region 1A (E1A) splicing reporter assay (Yang et al., 1994) is a practical method to measure changes occurring to alternative splicing due to SRPK inhibition. We then utilized the E1A assay to assess whether Vpr did in fact affect alternative splicing compared to treatment of cells with an SRPK inhibitor. Lastly, to distinguish direct effects of Vpr from those secondary to its cell cycle block, we tested whether alternative splicing varied following drug-induced cell cycle

blockade with nocodazole, an anti-mitotic agent that disrupts the cell division cycle in G2/M phase. These results will lay the foundation for future studies employing Next Generation Sequencing to assess Vpr's effects on cellular splicing and HIV splicing.

CHAPTER 1

Vpr effects on HIV gene expression

HIV infection cryptically manipulates the host's cellular machinery to replicate and spread resulting in global changes in cellular gene expression. A viral accessory protein -Vpr- arrests the cell cycle at G2/M phase, although the advantages of this for viral replication remain unclear. A previous report found that Vpr increases Env expression by potentially modulating Env pre-mRNA splicing (Hashizume et al., 2007; Zhang & Aida, 2009). We aimed to confirm these findings in our system. First, we used the tet-inducible HIV system (tetHIV) (Lapek et al., 2017) employed for our proteomics studies. These cells (both HEK 293s and Jurkat T cells) harbor a tet-inducible HIV (with or without *vpr*) and express HIV RNA following treatment of cells with doxycycline. We treated these cells with doxycycline for 48 hours, then harvested and stained both cell lines to detect intracellular HIV-1 Gag p24 and envelope using fluorescence-activated single cell sorting (FACS). As seen in Figure 9, Gag/p24-positive cells from the wild-type (WT) tetHIV cell lines expressed higher levels of Env than the delta-Vpr lines. These results suggest that Vpr does indeed increase Env expression in this system, although the mechanism is unclear. We hypothesize that Vpr increases expression of Env by modulating splicing of Env mRNA (Zhang & Aida, 2009).

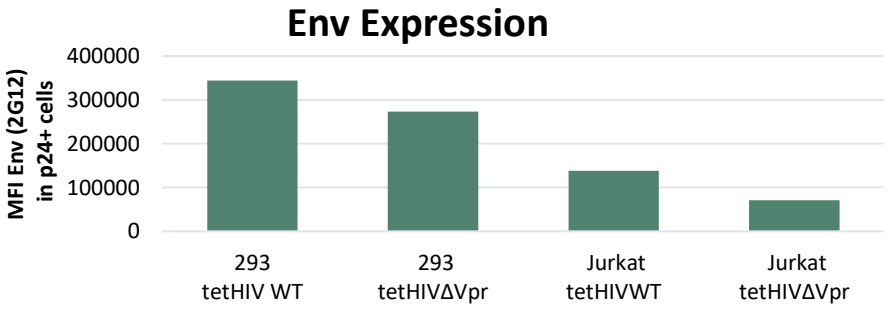

Figure 9: Vpr increases Env expression across two cell lines. HEK-293 and Jurkat tetHIV wild-type (WT) and vpr-negative (ΔVpr) cells were treated with doxycycline (Dox) to induce HIV expression**.** Utilizing FACS, Env expression in Gag/p24+ cells was quantified by mean fluorescence intensity (MFI). Error bars are standard deviations of duplicates.

We next assessed the extent to which the increase in Env expression was directly related to Vpr. We transfected HEK 293T cells with WT HIV or HIVΔVpr with varying amounts of FLAG-tagged Vpr expression plasmid and assessed Env levels in HIV Gag/p24-positive cells by FACS. Whole cell lysates were run on an SDS-PAGE gel and immunoblotted for Vpr (and GAPDH as a loading control) to confirm Vpr expression (Figure 10.A). Figure 10.B shows the Env levels in transfected cells, revealing increasing amounts of Env expression with increasing Vpr. Our results confirmed the Vpr-dependence of the increase in Env by FACS, revealing a positive correlation between Vpr quantity and Env expression.

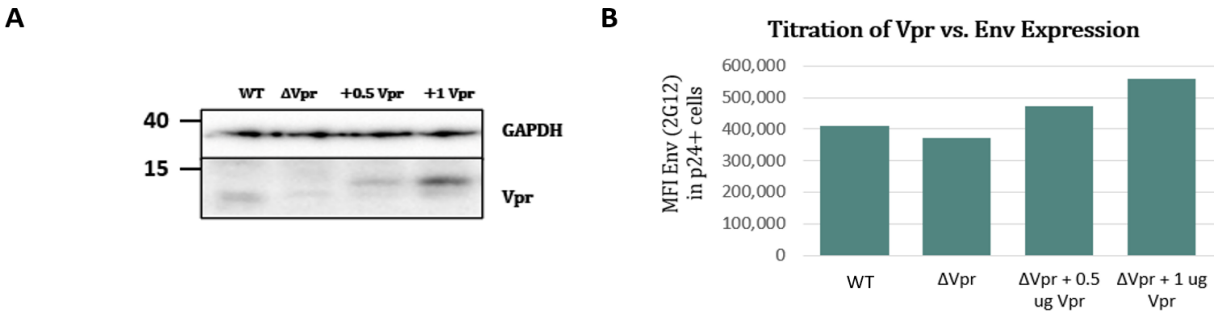

Figure 10: Titration of Vpr changes Env expression. (A) HEK-293Ts Were transfected with HIV WT or ΔVpr + varying amounts of Vpr expression plasmids (0.5 ug and 1 ug). Immunoblots for Vpr confirm expression levels compared to the loading control GAPDH. (B) Quantification of Env expression in Gag/p24-positive cells detected by FACS.

Based on these data, we hypothesized that Vpr is increasing Env expression through the increase of Env mRNA, potentially by regulating splicing of viral mRNA. To test this, we extracted RNA from the same cells evaluated above – HEK 293Ts transfected with HIV WT or ΔVpr and increasing amounts of Vpr expression plasmid. We synthesized cDNA by reverse transcription and quantitated HIV-1 splice variants by TaqMan real-time PCR using primer and probe sets specific for Gag-encoding, Env-encoding and multiply spliced transcripts. Through this we can quantify relative levels of Env mRNA. Seen in Figure 11 are the relative levels of Gag, Env and MS transcripts for HIV WT, ΔVpr + 0.5 ug Vpr plasmid and ΔVpr + 1 ug Vpr plasmid compared

to the ΔVpr alone condition. Unfortunately, this experiment did not confirm our hypothesis, as there was no apparent increase in the Env transcript with increased amounts of Vpr, although the "wild type" HIV did show an increase in Env and Gag transcripts compared to ΔVpr. Since a relative increase in Env mRNA was seen in the wild-type HIV sample (with Vpr provided in *cis*) but not in the samples where Vpr was co-transfected, we inferred that the co-transfected Vpr was not optimally expressed in the cells encoding viral transcripts. Thus, we sought to codon-optimize Vpr and repeat these experiments to determine whether Vpr expression was the culprit.

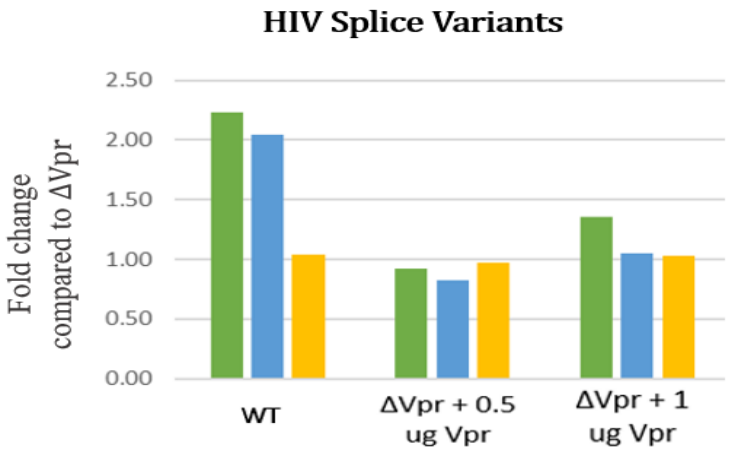

Figure 11: Changes in HIV splice variants by titrating Vpr. RNA from 293T cells transfected with HIV-1 wild-type or ΔVpr with varying amounts of +Vpr plasmids (0.5 ug and 1 ug) was quantitated by TaqMan real-time PCR. Results were normalized to ΔVpr and fold-change values were calculated using ΔΔCt analysis of wild-type or (+0.5, 1 ug) Vpr / ΔVpr.

After conducting several experiments to make human codon optimized Vpr (VPRopt), we tested if the optimized Vpr induced higher envelope protein expression detected by FACS when co-transfected with ΔVpr virus in comparison to ΔVpr alone. As seen in Figure 12.A, Env protein expression was higher with Vpr whether cis (in the virus) or trans (co-transfected as VPRopt). Figure 12.B confirms robust expression of Vpr in the VPRopt sample and shows how Env protein is increased in the Vpr-containing cell lysates (HIV WT and VPRopt) compared to ΔVpr. By

utilizing TaqMan with primer and probe sets specific for Gag-encoding, Env-encoding and multiple spliced transcripts, we examined HIV-1 splice variants to investigate whether VPRopt increased Env mRNA transcripts. However, our TaqMan results (see Fig12.C), revealed no change in Env mRNA with or without Vpr, again weighing against the hypothesis that the increase in Env protein expression detected by FACS and immunoblot (Fig 12.B) was the result of an increase in Env mRNA. The mechanism in which Vpr increases Env expression remains in question but could relate to an increase in Env protein stability induced by Vpr, or the modulation of other HIV Env splice variants that are not quantified by this TaqMan assay, which is specific for single-spliced Env mRNA, but not alternative variants that include other small exons.

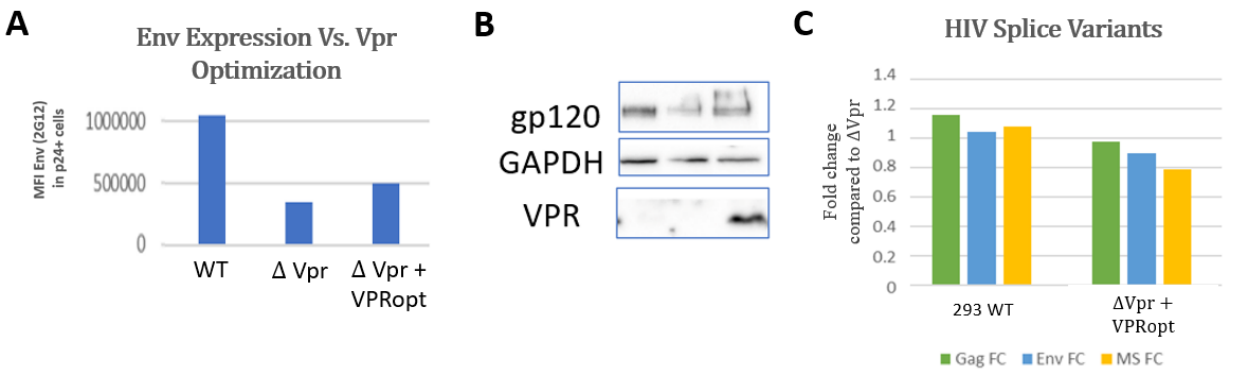

Figure 12: Test expression and function of optimized Vpr (VPRopt) (A) HEK 293T cells were transfected with HIV WT or ΔVpr +/- VPRopt expression plasmid, harvested, and stained for intracellular HIV Gag/p24 and Env. Live cells were gated for HIV Gag/p24 positivity and Env expression was quantified by FACS. (B) Immunoblots confirm Vpr expression from the VPRopt plasmid and HIV Env (gp120) expression with GAPDH as a loading control. (C) RNA extracted from samples was quantitated by TaqMan real-time PCR for Gag, Env and multiply spliced HIV transcripts normalized to GAPDH control. Results are plotted as the fold-change compared to ΔVpr values using the ΔΔCt analysis method.

Although our results confirmed the Vpr-dependence of the increase in Env by FACS in cis or trans, we fell short in defining the mechanism in which it does so. It seems Vpr is not modulating levels of the most common Env transcript, however Vpr may still manipulate Env expression in a splicing-related matter that is yet to be discovered. Future directions include assessing changes in

alternatively spliced Env transcripts, either by fluorescent primer PCR analysis which we have begun to optimize, where fluorescent-tagged primers specific to HIV splice variants are used to label PCR products followed by analysis of transcript length on a PAGE gel, or by deep sequencing of viral transcripts. Shifting gears yet centering on unraveling Vpr's contribution to alternative splicing, we dove into investigating how Vpr impacts cellular splicing.

CHAPTER 2

Vpr modulation of cellular splicing

Prior studies reported changes in the activity of SRPKs in the presence of Vpr (Iordanskiy et al., 2004; Lapek et al., 2017). SRPK1 is known to localize in both the nucleus and the cytoplasm and is thought to play a role in regulation of splicing by regulating intracellular localization of splicing factors (Fukuhara et al., 2006). To better understand the functions of Vpr, Lapek et al. evaluated how Vpr impacts the host cell proteome by quantifying over 7000 proteins and 28,000 phospho-peptides after the induction of HIV-1 gene expression with or without Vpr. Consistent with previous findings, Vpr blocked cell cycle progression by regulating spindle and centromere proteins along with modulating the Aurora kinase A. However, a new finding was also observed. They detected that the phosphorylation of serine/arginine rich protein specific kinase (SRPKs) was modulated in the presence of Vpr, with SRPK1 phospho-peptides increasing within 12 hours of HIV-1 gene expression in a *vpr*-dependent manner. Also, phosphorylation motif analysis revealed an increase in the SRPK motif RxxSP in the ΔVpr condition compared to HIV WT, suggesting Vpr-dependent inhibition of some SRPK activities (Lapek et al., 2017). This result is consistent with prior reports that HIV expression downregulates the overall activity of SRPKs (Fukuhara et al., 2006). Evidence collected by Lapek et al. suggests that Vpr mediates RNA splicing by influencing SRPKs which are known to facilitate and monitor splicing mechanisms by interacting with SR proteins (Lapek et al., 2017). We hypothesized that Vpr increases Env expression by modulating splicing via SRPK. To confirm whether Vpr increases SRPK1 phosphorylation, which could regulate its activity, we evaluated SRPK1 phosphorylation using α-phospho-SRPK1 (Thr601) and SRPK1 antibodies. For this experiment, we co-transfected HEK 293T cells with a plasmid encoding FLAG-tagged SRPK1 and either control (empty) vector, VPRopt, HIV WT or

HIVΔVpr. Transfected cells were harvested and lysed, and immunoprecipitation of FLAG-SRPK1 protein from cell lysates was performed using magnetic anti-FLAG antibody-coated beads (see Figure 13). Whole cell lysates and immunoprecipitated proteins were run on SDS-PAGE gels and assessed by immunoblot for SRPK1 and phospho-SRPK1. We hypothesized that in the presence of HIV Vpr, the phospho-SRPK1 would increase, confirming findings from our phospho-proteomics study (Lapek et al., 2017).

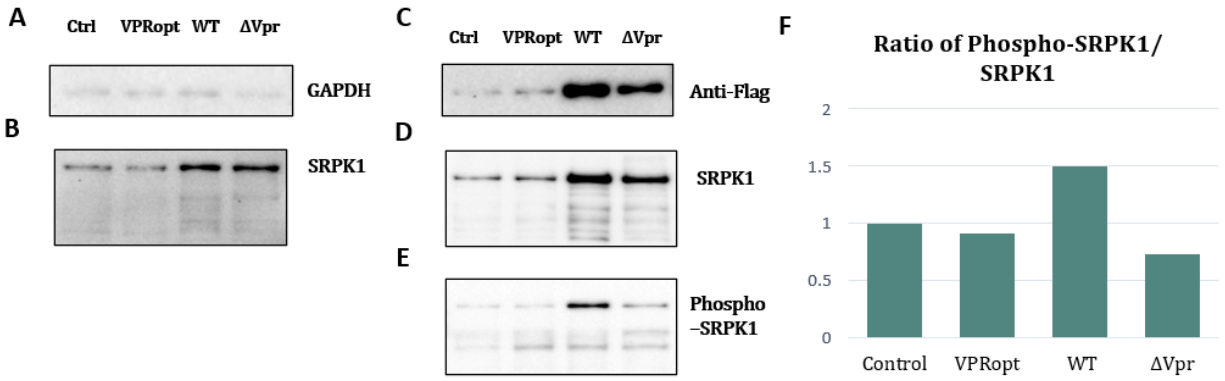

Figure 13: Observing Vpr-dependent changes in SRPK1 activity. Transfected 293T cells were harvested and lysed, and immunoprecipitation of FLAG-SRPK1 protein from cell lysates was performed using magnetic anti-FLAG antibody-coated beads (A) Whole cell lysates showing GAPDH as the loading control. (B) Immunoblot of cell lysates for SRPK1 for whole-cell SRPK levels. (C) Immunoprecipitated protein stained with anti-FLAG antibody. (D) Shows immunoprecipitated SRPK1 and (E) phospho-SRPK1 across 4 conditions (control, VPRopt, Wild-type HIV, HIV ΔVpr.). (F) Densitometry was done for blots D and E to observe changes in relative levels of phospho-SRPK1 in comparison to SRPK1.

The co-transfection of WT HIV led to increased pulldown of SRPK1 and a greater proportion of phospho-SRPK1 (compared to ΔVpr), as seen in the immunoblots of Figure 13. However, this effect was not demonstrated with VPRopt alone (compared to pcDNA). Regardless of Vpr, HIV increases the amount of SRPK1 in cells when co-transfected (Figure 13.B). We were able to deduce that there are relative HIV-induced changes to SRPK phosphorylation in the presence of Vpr leading us to question what the mechanism behind these findings is.

Determining how Vpr alters SRPK is more of a predicament than anticipated due to the varying roles of SRPKs. Previous work led us to believe that Vpr may recondition both cellular and viral pre-mRNA splicing by modulating the activity of SRPK either directly or as a secondary effect of Vpr's induction of G2/M arrest. We assessed whether Vpr regulates cellular splicing using the E1A splicing assay and in what manner it does so. The Adenovirus early region 1A (E1A) Splicing reporter assay – used by several studies (Gattoni,1991; Yang,1994) – evaluates the degree in which the adenovirus gene (E1A) undergoes alternative splicing by quantifying the splicing isoforms generated. Yang et al. employed the E1A assay as a tool to measure the impact that the A1 protein of heterogenous nuclear ribonucleoparticle (reported to modulate alternative splicing as well as favor exon skipping at the 5' splice site) has on the splicing isoforms of the E1A gene. It has been shown that an increase in the SRPK activity increases splicing of the E1A gene and can be seen via an increase in the most spliced isoform of E1A (9S) along with a decrease in the least spliced isoform (13S). To test our hypothesis, we utilized the E1A Splicing assay as a measure of SRPK-induced splicing activity to assess whether Vpr modulates the degree to which the adenovirus gene (E1A) undergoes alternative splicing.

Initially, we assessed whether the Adenovirus early region 1A (E1A) Splicing reporter assay is a feasible method to measure and evaluate changes in alternative splicing due to SRPK inhibition by utilizing an SRPK inhibitor followed by measuring changes in the extent of E1A splicing. The E1A pre-mRNA is known to have three alternative 5' splice sites that give rise to three primary mRNA species (13S, 12S, and 9S mRNAs) and has been used to assess splicing differences based on the discrepancies in quantity of mRNA species present post-splicing (Gattoni et al., 1991; Yang et al., 1994). Figure 14.A illustrates the structure of the E1A's pre-mRNA strand, the three mRNA transcripts, and their corresponding 5' splice site. The most spliced isoform is 9S

(smallest molecular weight) whilst the least spliced isoform is 13S (largest molecular weight) leaving 12S as moderately spliced. The E1A pre-mRNA can be either completely spliced or hardly spliced, with corresponding changes in levels of 9S and 13S. We extracted total RNA and subjected it to RT-PCR with E1A gene-specific primers. The PCR products were then run on an ethidium bromide/agarose gel to assess the sizes of the differentially spliced transcripts. Fig. 14.B displays a visual change between the mRNA species on the ethidium bromide gel seen as bands across two experimental conditions (DMSO vs. SRPK Inhibitor, SRPIN340). The SRPK inhibitor hinders the activity of SRPK pathway preventing the activation of SR proteins; hence, the faint, mostly absent, band for 9S and a bulkier band for 13S can be seen in Fig 14.B.

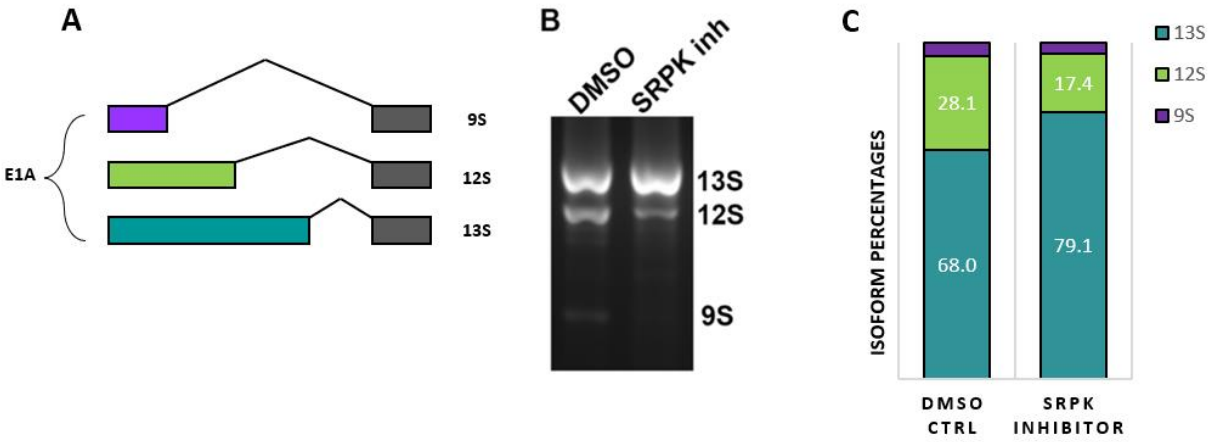

Figure 14: Alternative Splicing reduced by the presence of SRPK inhibitor observed via the E1A splicing Reporter Assay. The Adenovirus early region 1A (E1A) splicing reporter assay is used to evaluate RNA from HEK-293 (293s) cells which endogenously express the Adenovirus Early region 1A (E1A) gene. The E1A pre-mRNA of adenovirus is spliced into three mRNA species/spliced isoforms (9S, 12S, and 13S). Total RNA was extracted from 293s from each condition and subjected to RT-PCR with E1A gene-specific primers and the PCR products were run on an ethidium bromide/agarose gel to assess the sizes of the differentially spliced transcripts/ isoforms. (A) Schematic representation of Adenovirus E1A splicing producing 9S, 12S, and 13S isoforms (B) An image of E1A-specific RT-PCR bands from HEK-293 cells treated with either DMSO (as a control) or an SRPK inhibitor. The 13S, 12S, and 9S bands from (B) were quantified using Bio-Rad Image Lab 6.0 and graphed as a percentage for total intensity seen in (C).

These bands were quantified so that a more thorough comparison could be precisely drawn. The percentages of the isoforms varied between DMSO (control) and the SRPK inhibitor: compared to the control (DMSO), the SRPK inhibitor triggered a decrease in 9S and 12S, and an

increase in 13S (Fig. 14.C). In other words, inhibiting SRPK reduced the effectiveness of splicing in the E1A pre-mRNA since the most spliced isoform -9S- was reduced and the least spliced isoform of E1A -13S- correspondingly increased. The shifts seen in the E1A isoforms demonstrated that the E1A assay is receptive to SRPK inhibition thus a reasonable model to identify changes in the activity of SRPK.

Based on previous findings (Lapek et al., 2017), we hypothesized that in the presence of Vpr, SRPK activity will increase as evidenced by an increase in the isoform percentage of 9S. To isolate the effects of Vpr in comparison to other HIV accessory proteins, we utilized a controlled environment expressing only specific viral proteins (Vpu, Vif and Vpr). Cells were co-transfected with the Vpr, Vif and Vpu expression constructs. We extracted total RNA and subjected it to RT-PCR with E1A gene-specific primers, then ran the products on an ethidium bromide/agarose gel to assess the sizes of the differentially spliced transcripts. It was noted that unlike Vpu and Vif, Vpr did indeed increase 9S isoform percentages in comparison to the control (see Figure 15.A).

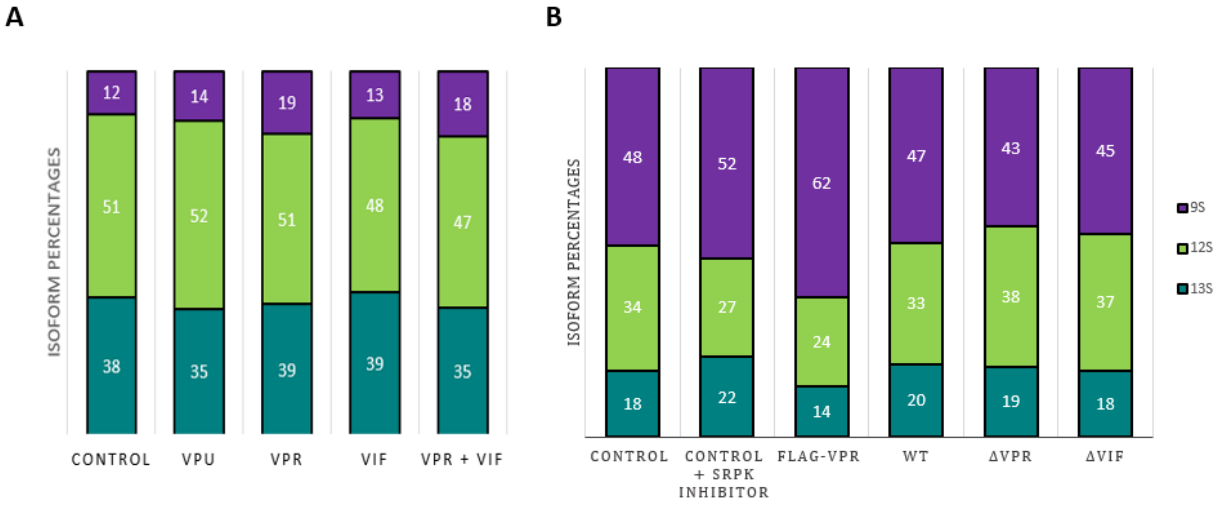

Figure 15: Changes in E1A Splicing assay due to the presence of Vpr in HEK-293s. (A) 13S, 12S, and 9S bands retrieved from the quantifications of bands seen in the ethidium bromide/agarose gel of HEK-293s that are co-transfected with the Vpr, Vif and Vpu expression constructs. (B) Presents isoform percentages for RNA extracted from cells transfected with a control plasmid, a plasmid that only expressed Flag-Vpr, wildtype HIV (WT), HIV without *vpr* (ΔVpr) or HIV without *vif*. (ΔVif).

To test the splicing effects of Vpr in the viral context, an NL4-3 (wild-type HIV-1) proviral plasmid was transfected into HEK-293 cells compared to FLAG-Vpr expression plasmid, provirus without *vpr* (ΔVpr), provirus without *vif* (ΔVif), and a control plasmid. One set of cells transfected with the control (empty) vector was also treated with an SRPK inhibitor (SRPIN340). The SRPK inhibitor unexpectedly increased the 9S isoform percentage consistent with an *increase* is splicing, suggesting that either the drug was ineffective, or the assay was not performing as expected. However, as seen in Figure 15.B, the FLAG-Vpr condition did increase the amount of 9S, suggesting a Vpr-dependent enhancement in splicing as seen in the experiment shown in Figure 15.A. Unfortunately, the Vpr-dependent change evident in the viral context was much more subtle, with a slight increase in 9S in wild-type compared to ΔVpr, but no substantial difference between ΔVif (which encodes Vpr) and ΔVpr, leaving us with more inquiries than answers. One theory for our unanticipated finding: our experiment may have been skewed because of transfection inefficiency since all HEK-293 cells carried the E1A endogenously but not all were likely transfected with Vpr.

To bypass the potential skew of results from transfection inefficiency in HEK-293 cells, we cloned the E1A gene from HEK-293 genomic DNA into an expression plasmid. This E1A construct was co-transfected into HeLa P4R5 cells in conjunction with Vpr and proviral expression constructs in the hopes of removing any background noise amplified by E1A in un-transfected cells. To clone and transfect the E1A gene, we extracted DNA from HEK-293 cell pellets, PCR amplified the E1A gene from genomic DNA and ligated it into pcDNA3.1(-) backbone plasmid. We then transformed the ligations into *E. coli*, plated the cells on LB/ampicillin plates, cultured clones, extracted pcDNA-E1A-Puro plasmid DNA, and verified the clones with sequencing. In this experiment, HeLa cells were co-transfected with the E1A expression construct and control

(empty) plasmid, Vpr expression construct, HIV(WT), or HIV without *vpr* (HIVΔVpr), and then either treated or not treated with the SRPK inhibitor.

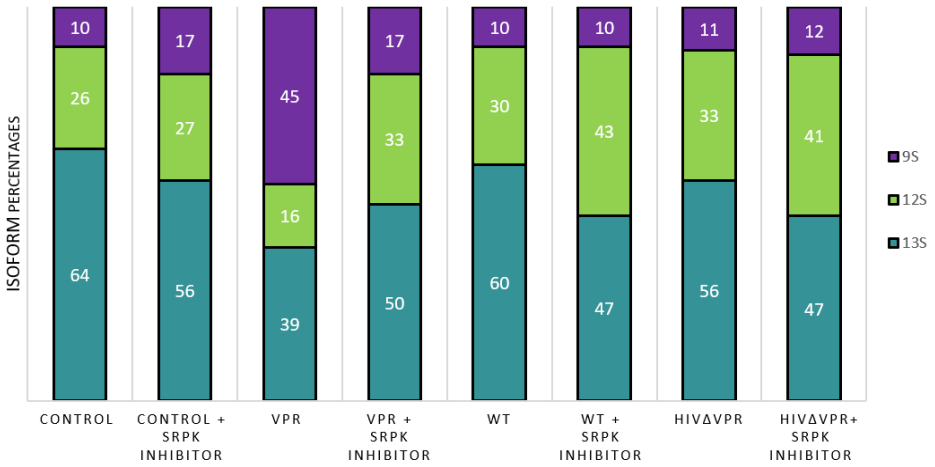

Figure 16: E1A Isoform percentages of HeLa-P4R5 cells. Percentage of 13S, 12S, and 9S isoforms calculated from the quantifications of bands seen in the ethidium bromide/agarose gel of E1A PCR products from transfected cells. For this experiment, HeLa-P4R5 cells were transfected with the cloned E1A plasmid and either control, Vpr-expression, or HIV-expressing plasmids (WT HIV and HIV without *vpr* followed by treatment (or not) with SRPK inhibitor.

Our results were again confounded by the SRPK inhibitor behaving as an inducer of splicing activity, causing an increase in 9S in all but the Vpr condition. A significant decrease in 9S was observed when cells transfected with Vpr were subjected to SRPK inhibitor in comparison to Vpr alone. In addition, Vpr generated a high amount of 9S transcripts suggesting that an overexpression of Vpr appears to favor splicing. As for the virus-expressing cells either with or without *vpr* and with or without the SRPK inhibitor, no substantial changes occurred across all 4 conditions. These results left us with several questions: was there an issue regarding the E1A transfection or is the inadequacy of the SRPK inhibitor an outlier? Or is the E1A assay not a feasible method to test variation in SRPK activity?

Setting aside the SRPK inhibitor, we investigated whether Vpr alters SRPK activity directly or via G2/M arrest. We utilized nocodazole, an anti-mitotic agent that mimics Vpr by arresting cells at G2/M phase, to determine if cycle arrest is the primary mechanism that altered E1A splicing. The following experiment aimed to assess variations in the E1A splicing caused by a cell cycle arrest at G2/M to tease out if these effects are due to Vpr directly or secondary to the cell cycle block. For this, we utilized an inducible cell culture system encoding wild-type HIV and HIV without *vpr* (ΔVpr): HEK-293 cells harboring a Tet-On transactivator and a tet-inducible HIV provirus (+/-*vpr*), which express HIV proteins upon treatment with doxycycline (Dox) (Lapek et al., 2017). First, HEK-293 tetHIV WT and ΔVpr cells were treated (or not) with nocodazole (Noc), an anti-mitotic agent, for 16 hours to induce a Vpr-like cell cycle arrest. The benefit of using an inducible system is that low transfection efficiency plays no role in the upcoming interpretation. After nocodazole was washed out, the cells were treated (or not) with doxycycline and then harvested at 0-, 12-, and 24-hours post-doxycycline treatment. This yielded eight conditions across 3 time points: tet HIV WT or ΔVpr cells (-Dox/-Noc(control), +Dox/-Noc, -Dox/+Noc, +Dox/ +Noc). We harvested cells, taking an aliquot for FACS analysis and another for RNA extraction. Cells for FACS were ethanol fixed, treated with RNase, and stained with propidium iodide (PI), which binds DNA, and analyzed for cell cycle state in each condition. From the second aliquot, we extracted Total RNA, subjected them to E1A RT-PCR, ran an ethidium bromide/agarose gel and quantified bands to assess for splicing as explained above. FACS presented below in Figure 17 confirms the expected changes in cell cycle state of the cells.

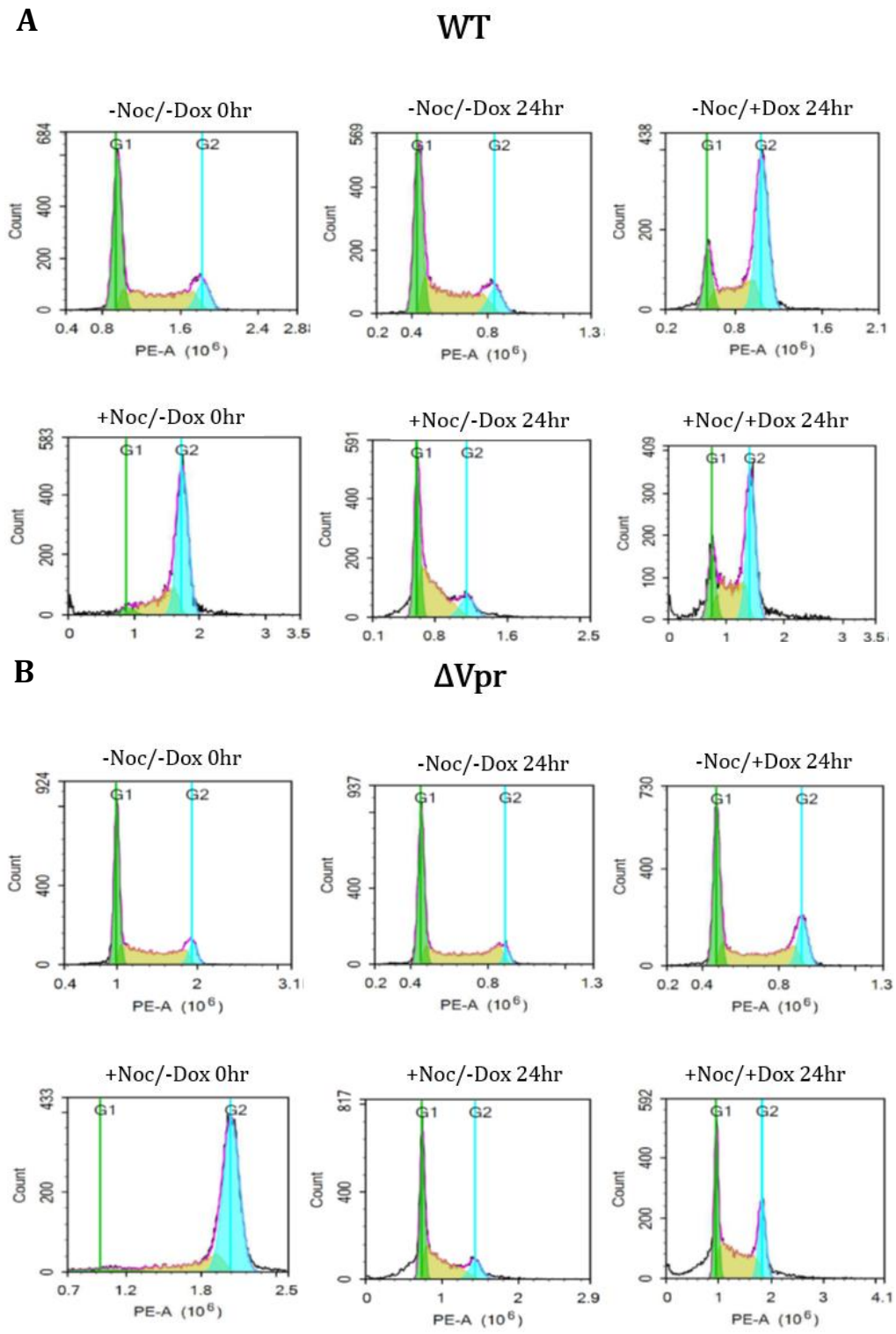

Figure 17: Cell Cycle phases across 8 experimental conditions presented via FACS.(A) Panel for WT cells.(B) Panel for ΔVpr both treated with nocodazole (Noc) and/or doxycycline (Dox).

As expected, cells harvested just after treatment with nocodazole at time 0 were arrested at G2, and by time 24 hrs. following nocodazole washout, cells had resumed normal cell cycle progression. On the other hand, after releasing cells from Noc and treating with Dox for 24 hrs., induction of wild-type HIV expression exhibited G2 arrest while its counterpart-HIV without Vpr (ΔVpr)- did not, thus confirming that Vpr plays a key role in cell cycle arrest. At time 12, all experimental conditions had similar quantities of cells in G2 phase in both HIV±*vpr* (see Fig. 18).

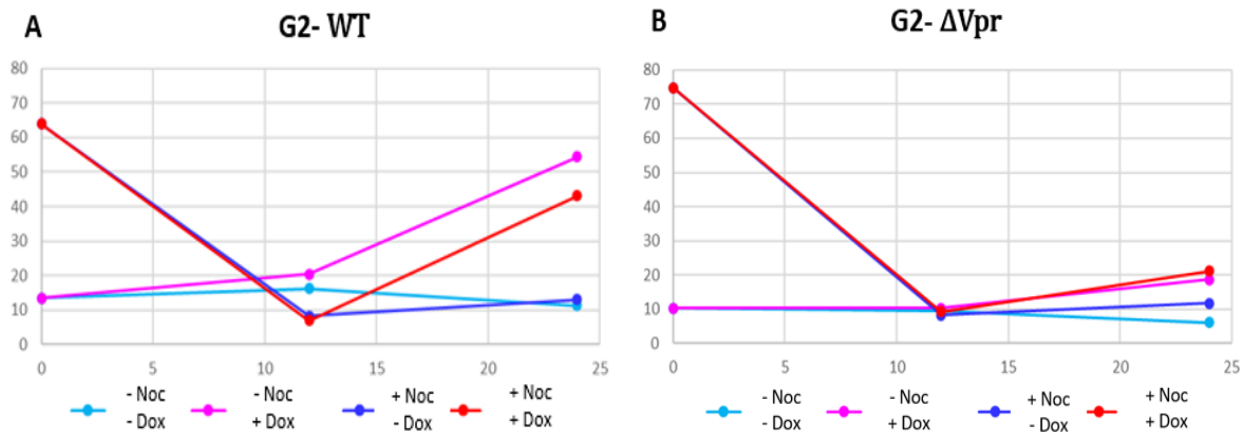

Figure 18: Trendlines across 3 different time points in G2 phase. Percentage of cells in G2 as calculated from the cell cycle analysis in PI-stained cells shown in Figure 17.

With these data on the changes in cell cycle phases, we investigated changes in 9S and 13S isoform percentages. Our findings revealed wild-type HIV cells treated with nocodazole and doxycycline (Fig. 19.A and 19.B) had the highest percentage of both 13S and 9S transcripts at hour 24. Conversely, there were no significant findings for HIVΔVpr since both isoform percentages presented mild changes; the amount of 13S (19.C) did not vary across all four samples at any time point and 9S (19.D) had a small increase when treated with nocodazole. We assessed isoform percentages in terms of cell cycle distribution to determine the extent to which cell cycle influences the E1A assay results (irrespective of *vpr* expression). In other words, if cell cycle arrest

is the exclusive cause for 13S changes then both WT +Noc 0 hr. and +Dox 24 hr. should present similar 13S levels since the percentage of G2 for +Noc conditions at 0 hr. is similar to that of WT +Dox at 24 hrs. However, our results did not support such a theory since in Fig. 19A, WT +Dox at 24 hr. had a higher percentage of 13S than WT +Noc 0 hr., suggesting that there is more at play than changes in cell cycle changes – likely Dox-induced HIV gene expression (including *vpr*). There was a divergence in 13S and 9S percentages for the 24 hr. WT conditions with or without Noc, however, despite similar cell cycle distribution profiles at that time point, the cause of which requires further study. Notably, the ΔVpr cell line showed no substantial Dox-induced changes in E1A isoform distribution, suggesting that the changes seen for WT were Vpr-dependent. Similarly, although +Noc and -Noc conditions had divergent cell cycle distributions at 0 hr., their E1A isoform profiles were similar (Figure 19). From these experiments, we concluded that cell cycle changes alone did not induce differential E1A splicing, although Vpr expression seems to.

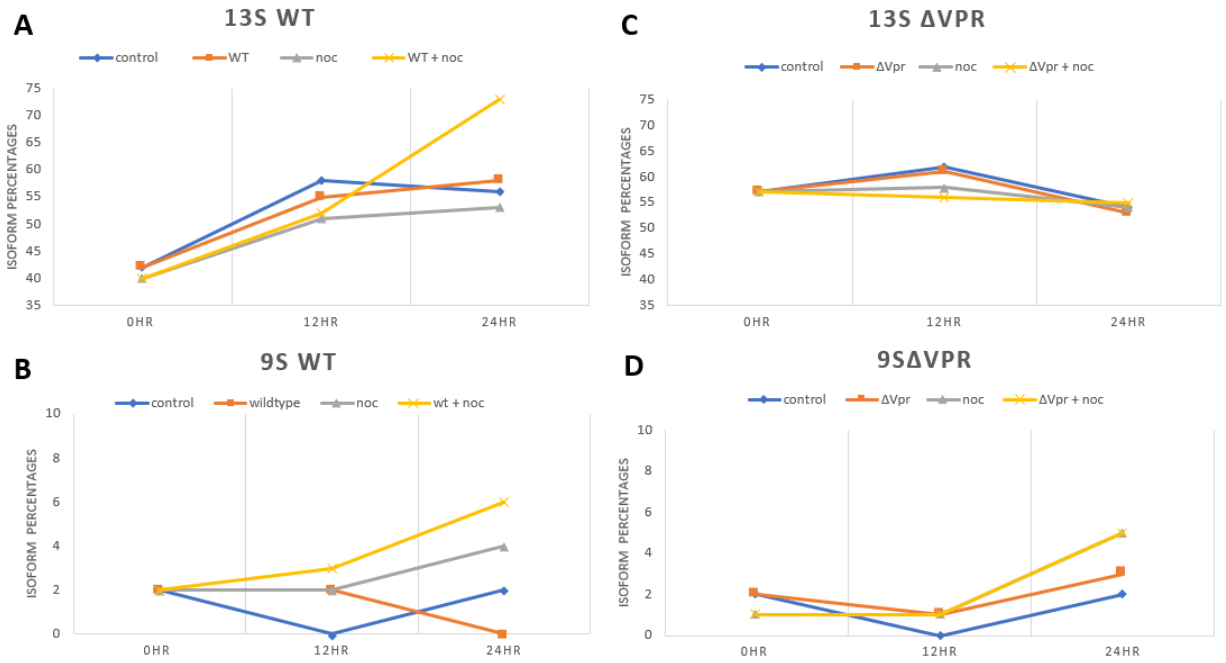

Figure 19: E1A splicing reporter assay results for cells treated (or not) with nocodazole and doxycycline. Total RNA was extracted from 293 tetHIV cells (WT and ΔVpr) following 16 hours of nocodazole treatment (or control) followed by doxycycline treatment to induce HIV gene expression for 3 different time points (0, 12 and 24 hrs.). We assessed the differentially spliced E1A transcripts/ isoforms by PCR amplification, running samples on an ethidium bromide/agarose gel and quantifying the PCR products. (A) and (B) Graphical representations of 13S and 9S isoform percentages of quantified using Bio-Rad Image Lab 6.0 across 3 different time points for 4 different conditions with wildtype tetHIV cells while (C) and (D) represented equivalent circumstances for HIV without *vpr*.

At the same time, we also assessed HIV splicing to determine if cell cycle arrest may increase Env mRNA by regulating splicing of viral mRNA and potentially be the source of increased Env expression seen with Vpr. For this analysis, we synthesized cDNA by reverse transcription from RNA extracted from tetHIV WT and ΔVpr cell lines treated +/-Noc and with doxycycline for 12 or 24 hrs. HIV-1 splice variants were quantitated by TaqMan real-time PCR using primer and probe sets specific for Gag-encoding, Env-encoding and multiply spliced transcripts. Relative changes in the HIV splice variants between the 12- and 24-hour time points for each condition were calculated using the ΔΔCT algorithm (with GAPDH as the housekeeping control) and plotted as in Figure 20. Across all four cases a parallel trend exists; there were no

significant changes in Env mRNA with or without *vpr*. Interestingly, the nocodazole treatment appears to increase relative amounts of especially the Gag (unspliced) mRNA at 24 hr. compared to 12 hr. regardless of the presence of *vpr*.

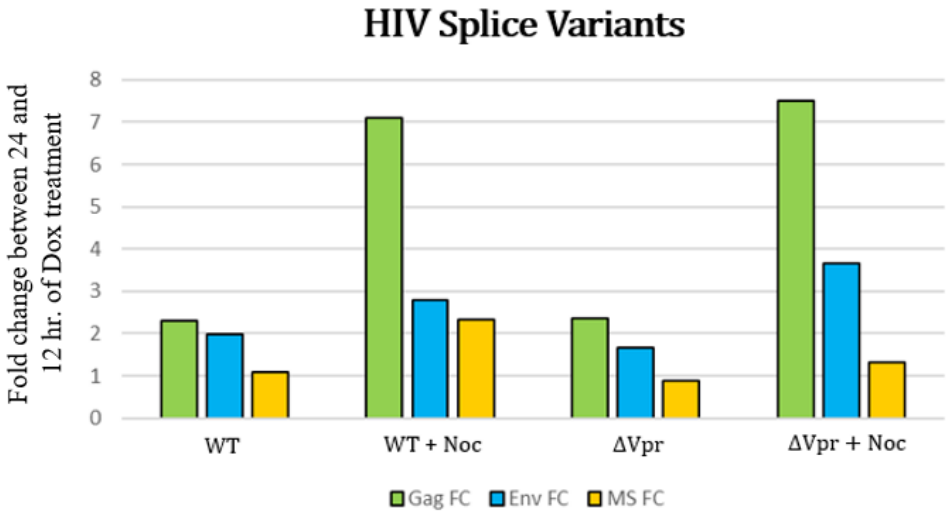

Figure 20: Fold change of HIV splice variants in doxycycline-treated cells with or without Vpr and Noc treatment between the 12 and 24 hr. time points. RNA from doxycycline-treated 293 cells with tet-inducible HIV wild-type or ΔVpr treated with or without nocodazole were quantitated by TaqMan real-time PCR. Results from 24 hr. samples were normalized to collected samples from hour 12, and fold-change values were calculated using ΔΔCt analysis of wild-type or ΔVpr +/- nocodazole.

Our analysis of selected HIV splice variants by TaqMan did not reveal any Vpr- or cell cycle-dependent changes in Env mRNA that could account for the observed increase in Env protein expression in Vpr-expressing cells. However, a limitation of this assay is that there are several other Env-mRNA splice variants that are not detected by this assay, which is specific for the most abundant singly spliced Env mRNA. Therefore, we began exploring other assays that prime for numerous viral spliced transcripts. One of these is known as the fluorescent HIV splicing assay (Takata et al., 2018). We have begun to optimize this assay for our experimental system and hope this can give a more complete picture of any changes in minor HIV splice variants that could be caused by Vpr.

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