DISEASE PROGRESSION IN HIV-1 AND THE ROLE OF POLYMORPHISMS IN THE VPR GENE

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Disease progression in individuals infected with human immunodeficiency virus type 1 (HIV-1) normally consists of a decline of the host immunity into acquired immunodeficiency syndrome (AIDS); this is a topic of great public health significance with the rapidly increasing prevalence of HIV-1 infected individuals. However 5% of the infected population resist AIDS development and remain asymptomatic. These so called long term non progressors (LTNPs) control the virus and are able to mount an effective immunological response. The role of the HIV accessory gene, Vpr, in differential disease progression is addressed in this study. For this purpose sequences identified from LTNPs and progressors (RP) from the HIV database from Los Alamos National Laboratories were analyzed to find signature polymorphisms in the amino acid sequence of this protein. Several mutations in the coding sequence of Vpr were found to be associated with the LTNPs, in particular, the threonine at position 19 mutated to alanine (T19A) and arginine at position 90 mutated to asparagine (R90N). In contrast the following mutations were found to be associated with RPs, arginine at position 36 mutated to tryptophan (R36W), leucine at position 68 mutated to methionine (L68M), and arginine at position 85 mutated to tyrosine (R85Y). A series of *in vitro* assays show that mainly the RP-associated mutations exhibit changes in several canonical functions of Vpr, namely, its capacity to oligomerize, localize to the nucleus, and induce G2 cell cycle arrest. However, infecting peripheral blood mononuclear cells (PBMCs) with viruses harboring these Vpr mutations demonstrates no difference in the replication capacity

of the mutants compared to wild type virus. This study provides a basis to further delineate the mechanisms of Vpr function in disease progression.

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PREFACE

Instead of giving my acknowledgements like I've seen at the beginning of all the theses I've read, I would like to do something different. If you read the scientific portion in the latter portion of my thesis, you'll get to find out about the hypothesis, the experiments, and whatnot. My mentor would call it a story, and it is much like storytelling. It's the story of my project. What I think is missing from all of it though is that it doesn't say anything about the person who actually did the work. If you don't want to hear what I have to say about my story as I was working on this project, turn the page, but if you are interested, please read on.

When I first started this program I came in with a wide-eyed, gung-ho enthusiasm. I had the expectation that I would accomplish a bucket list of scientific achievements and I would be a hero. Unfortunately it didn't pan out that way. From day one, I learned that what it takes to be a scientist is far more than just good brains, although you do need that in abundance. In fact I would say that there is something even more important than just smarts to be a scientist. And that's a lot of grit, the "fight," as my mentor says. It's the willingness to do anything it takes to find the answers to the questions you ask. The strength to stay resilient even after continued failure and frustration. To invoke that oft-cited 80/20 rule, probably 20% of all efforts working in a lab will account for 80% of all successes. And that certainly applied to me. It takes a lot of guts to accept that, once you realize how few of your experiments actually yield usable results.

I found that all of my expectations of brilliant success were crushed when I realized that science requires more than just logic and knowing how to use a pipet. It has to become a way of life. I don't think I was completely ready to accept that challenge at the beginning. The first week I was here, I almost broke the centrifuge because of a silly mistake. There were times, when after retrying experiments for months, I felt I still had nothing to show. The mounting pressure and workload seemed to constantly exceed my capacity to meet the demand. Many times it seemed like my senior colleagues and superiors were always on a higher plane of scientific awareness, tossing around concepts and ideas in discussions that made my head spin. It was a humbling two years that I've spent working in this lab to say the least.

I realize the staggering amount of frustration that I had to deal with. It's a rite of passage that probably every science student has to pass to move on and a common experience among all scientists, from novice to Nobel winner. So with all this self-imposed adversity why haven't we all left science yet? It is because when we do succeed the successes are nothing short of spectacular. Even if it isn't on par with the discovery of $E=mc^2$, any scientific success is of huge magnitude because the odds can be so small. It is in appreciating the small victories that we can keep doing science everyday. It is like winning the lottery when things work.

Looking back now to when I started, even though I didn't win any prize for curing HIV or publish anything groundbreaking in *Nature* (yet), I did alright. I learned how to appreciate the answers to the questions we ask as scientists, whether or not they are what we expected or even if we asked the right question in the first place. In that I think I've found the grit to stay determined. And slowly but surely, I'm grasping how to think like a scientist. This is the reward that I got for my efforts and it is more valuable to me than prizes or papers (those are very nice to have though). These lessons are what made this project worth the effort.

To end, I would like to say thanks to many people. To Dr. Ayyavoo, my mentor, whose patience I am sure I've pushed all the way to its limit at times, who showed me that the work can be its own reward, who showed me the tremendous skill, vision, tenacity, and creativity it takes to be a scientist, who although will demand 100% of effort from you, but will give back 100%, and finally, who tolerated the constant pile of junk on my desk. To Courtney, whose scientific knowledge and skills go far beyond her few years of experience, who lent me a huge amount of help when I started working in the Ayyavoo lab, and who was great company during the late night lab hours. To Pruthvi who also helped me get comfortable working in the lab, whose enthusiasm I always admired, and whose lively personality is the kind that every lab could use. To Karolina whose efficiency and focus I wish I had and whose sense of humor is always appreciated. To Debjani, a true scientist whose incredible dependability and consistency I try to emulate, who is always willing to give help when you ask, who can always help me discuss a scientific problem, and who always lends an understanding ear when things go wrong. To Rajeev, whose great bench skills and vast source of knowledge I am always able to learn from and whose forward, very broad way of scientific thinking and planning I hope to develop one day. To Shalmali who can see crucial details that someone like me would easily miss, whose logic is constantly razor sharp, and who freely lends me a lot of advice. To Jess, who bravely (whether she knew it or not) took on a former project of mine that was giving me too much trouble, and who is much better at the bench than I was when I was a first-year. To Leah Walker, without whose preliminary work this project would not have been possible. To all of my fellow students, it is a pleasure having been classmates and friends with you. Finally, to Mom and Dad, whose contribution and support is more than I can describe here in a few sentences. To everyone

I've met here in IDM, I've learned so much from all of you, some things in ways that might not have been so obvious. It was a pleasure to work with and to get to know all of you.

1.0 INTRODUCTION

Following the breakthrough discovery of the human immunodeficiency virus type 1 (HIV-1) in 1983 [1, 2] as the cause of the Acquired Immunodeficiency Syndrome (AIDS), our understanding of the virus has progressed by leaps. Individuals inflicted with HIV-1 no longer are condemned to a death sentence due to the availability of anti-retroviral therapies since the early 1990s that have been steadily improving in their efficacy [3]. However even with such treatments the virus still evades eradication, especially in the developing world in which the prohibitive cost of antiretroviral drugs are a barrier to treatment [4]. While an effective vaccine would provide a solution to the problem of HIV, this has proven to be a daunting task with research for the last decade yielding no breakthroughs. Numerous challenges stand in the way to eradicating HIV by vaccine, which is the only feasible means to end the epidemic globally.

1.1 THE LIFE CYCLE OF HIV

1.1.1 The Initial Stage of Infection

The basic characteristics of the human immunodeficiency virus are its single-stranded RNA genome and the envelope that surrounds its capsid. The virus can be transmitted through exchange of bodily fluids, the primary means being sexual contact. Once the virus invades the

host, the virus first must enter its target cells. The main target cells express the CD4 antigen at their surfaces and one of two chemokine coreceptors, CCR5 or CXCR4. The virus binds to the CD4 antigen and subsequently the coreceptor, causing a conformational change in the viral ligand (the gp120/gp41 heterotrimeric complex) and release of the viral contents into the cell by fusion of the viral envelope with the cellular membrane (Figure 1) [5-8].

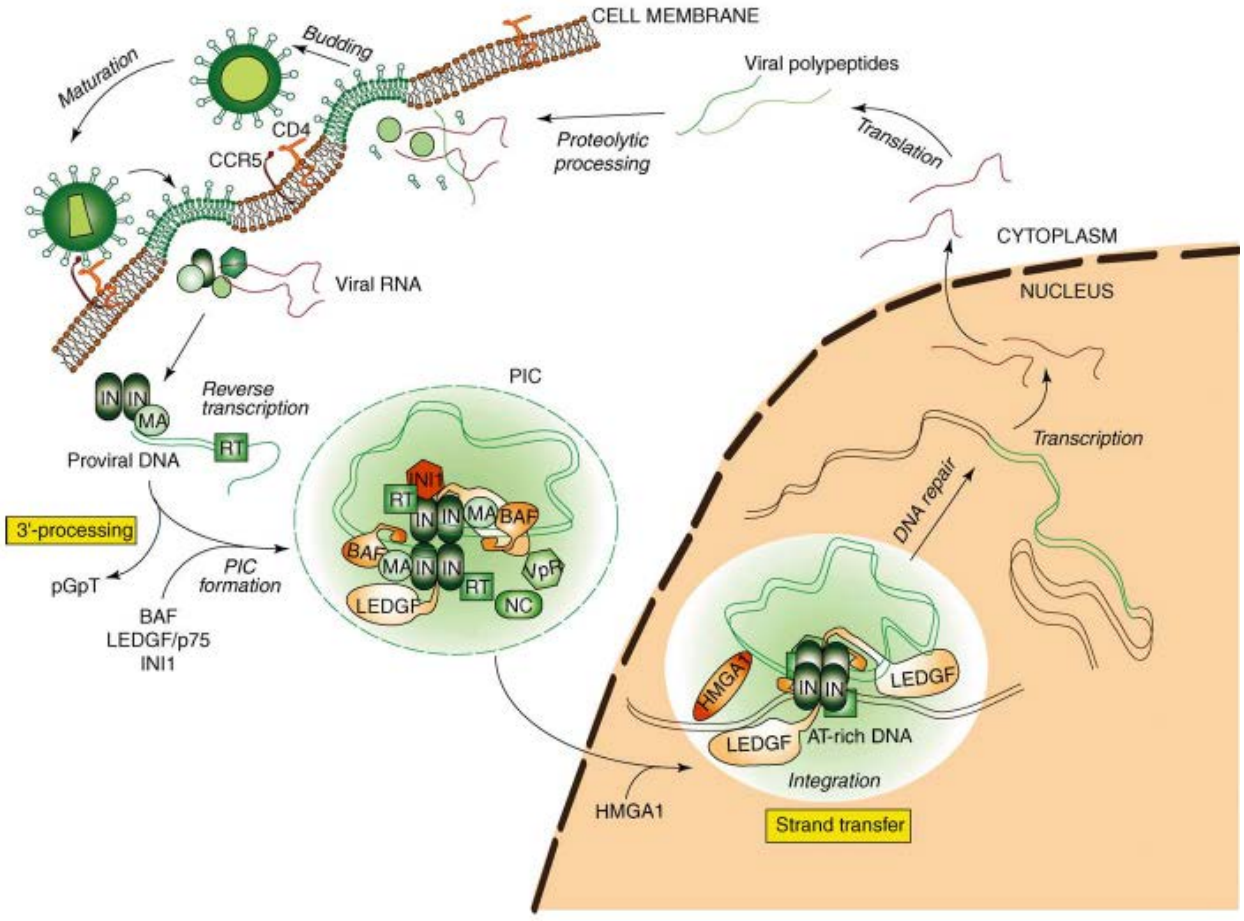


Figure 1. Viral Entry. From ref. [9] with permission.

Upon entry the remaining viral components—at this stage termed the preintegration complex (PIC)—enter the cytosol. The preintegration complex is composed of the viral capsid (mainly the p24 and matrix proteins), the accessory protein Viral Protein R (Vpr), integrase, reverse transcriptase (expressed as part of the capsid polyprotein), and the RNA genome [10]. The viral capsid uncoats allowing the reverse transcriptase to encode double-stranded DNA from

the RNA genome. Once the PIC reaches the nuclear membrane, it enters through the nuclear pores allowing integration of the viral cDNA into the host chromosome, completing the infection and initiating replication of the virus.

1.1.2 The HIV Genome

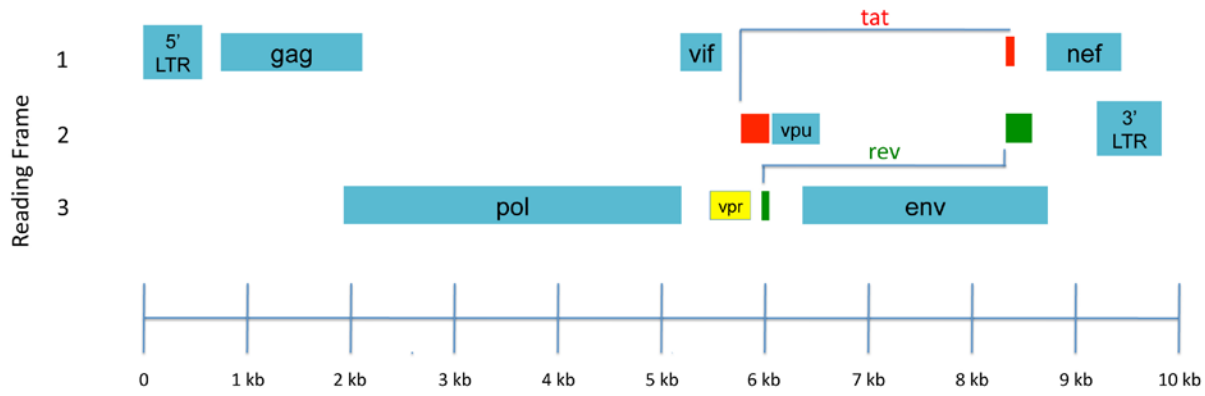


Figure 2. The Genetic Organization of HIV

The genome illustrated in Figure 2 [11] shows the organization and essential features of genetic sequence of HIV. The genes of HIV are distributed across three open reading frames. The long-terminal repeat (LTR) regions on the 5' and 3' ends of the sequence contain promoter sequences for numerous host transcription factors, notably NF-κB, Sp1, and GR (glucocorticoid receptor). As such the LTR regions are essential for initiating transcription of the proviral DNA.

The genes *gag*, *pol*, and *env* encode the structural proteins of HIV. The capsid protein is expressed by *gag*, the multifunctional enzyme with reverse transcriptase/protease/integrase activities by *pol*, and the surface antigen responsible for binding to the host CD4 receptor/coreceptor complex by *env*. Although Pol is not an essential part of the structural component of the virion, it is expressed as a polyprotein fusion with Gag.

The regulatory genes *tat* and *rev* are essential to HIV transcription. Tat enhances viral protein expression by a hundredfold [12]. It functions by binding to a stretch of sequences (TAR, transactivation response region) located in the 5' portion of the RNA transcript, enhancing processivity of RNA polymerase II. Rev functions as an adaptor between unspliced and partially spliced HIV transcripts which must be exported from the nucleus and the host nuclear shuttling machinery. It binds to Rev-response elements (RRE) in unspliced mRNAs partially spliced mRNAs and, in conjunction with the host nuclear shuttling machinery, exports them for expression of other viral proteins or packaging the whole unspliced genome into the virion [12, 13].

The so-called accessory genes, *vif*, *vpu*, *nef*, and *vpr* play various roles in enhancing infectivity and pathogenicity of the virus.

1.1.3 Diversity in the HIV Genome

The greatest obstacle in controlling HIV is its rapid mutation rate, a result of its error-prone replicative cycle. This feature enables the virus to produce quasispecies, a population of virus (in the case of HIV) composed of progeny genetically distinct from the parental virus. Like any other population composed of genetic variants, selection (natural or artifical) exerts pressures which enables only certain individuals to survive. In this case, selection is induced by the host immune response or a potential vaccine against the virus. However the ability of HIV to produce extensive diversity in its viral progeny randomly produces mutants that will eventually be able to escape not only the native host immune response but also a response generated by a vaccine against one or even multiple epitopes.

The HIV genome is contained in roughly 10,000 bases of RNA to be reverse transcribed into viral cDNA that is integrated into the host chromosome, completing the infection of a target cell. Mutations arise during this step of the replication cycle of HIV-1 as the reverse transcriptase does not have error-proofing activity. This process results in vast variation in the quasispecies that can arise from a single infection. The *env* gene is the most variable sequence in HIV-1. Because it is the main viral antigen which is responsible for binding to host CD4 receptors, the host immunity exerts the most pressure on this protein which causes escape mutations to arise so readily.

A number of studies have documented the variable nature of Env; in particular, Gaschen et al. (2002) found 20% variability in genes from isolates within a subtype, and up to 35% variability between subtypes [14]. To put this in perspective, Korber et al. (2001) compared HIV with variability in the influenza virus, which is reflected in the yearly update of flu vaccines [15]. Extrapolating from their phylogenetic analyses, the variability of Env in an HIV-infected individual is roughly 10%, which is much greater than the variability of the corresponding surface antigen (hemagluttinin) in influenza isolated from a whole population over the course of an entire year. Viral replication of HIV generates vast variation in the initial infection of a host that is amplified by transmission between individuals.

However, it is not only the *env* gene that exhibits large diversity. The conserved *gag* gene, coding for the capsid of HIV, shows 8% variability between isolates from within a subtype and 17% variability between subtypes; on the other end of the spectrum, *tat* shows 15% variability between isolates of the same subtype, and 35% variability between subtypes [15]. Gaschen et al. (2002) compared the % similarity of the nucleotide and amino acid sequences across the whole genome of HIV for a sample of subtype C isolates. They found that the

sequences coding for *gag* and *pol* (coding for the essential reverse transcriptase, protease, and integrase enzymes) exhibited roughly 90% similarity, the accessory proteins [14].

1.2 CLINICAL SIGNIFICANCE: LONG-TERM NON-PROGRESSORS (LTNP) AND RAPID PROGRESSORS

1.2.1 Disease Progression in HIV

Upon infection with HIV, the viral life cycle undergoes several stages. The first is an acute phase of infection marked by a sharp increase in the viral load of a recently infected individual followed by a reduction and in viral load that levels off, establishing the viral set point. This viral set point is maintained at a level between 11,000 to 50,000 RNA copies per ml of patient sera [16]. This reduction is generally explained as a result of the host's initial immune response against the virus.

The initial neutralizing antibody mounted by the host against the gp120 and gp41 moieties of the viral envelope and secretion of various cytokines [17, 18]. This viral set point coincides with asymptomatic infection which is maintained for ten years post-infection under most circumstances. Eventually HIV infection results in the hallmark loss of $CD4^+$ T-cells, an increase in viral load to an order of magnitude in millions of copies per ml [19], and severe immune dysregulation. The ultimate outcome is fatal by opportunistic pathogen associated infections.

A small subpopulation of infected individuals were found to control the infection and resist progression of health status to AIDS. This group was categorized as long-term non-

progressors. The normal progression to AIDS occurs ten years after seroconversion with patients exhibiting clinical signs of viral loads >50,000 RNA copies per ml and $CD4^+$ T cells dropping below 500 cells per μl of sera [17, 20, 21]. This contrasts with long-term non-progressors who comprise 3-5% of the HIV-infected population who are able to maintain $CD4^+$ counts above said threshold and have undetectable to very low viral loads (≤50 RNA copies per ml) [22].

While no comprehensive explanation of the phenomenon of long-term non-progressors in exists, there are numerous correlations between effective viral control in the absence of anti-retroviral therapy to host genetics and immunology. The well-known host polymorphism *CCR5-Δ32* encodes for a truncated form the chemokine receptor. This truncated protein lacks the signal which would enable it to be expressed at the membrane. Binding to the coreceptor CCR5 is an essential step in HIV viral entry and the absence of CCR5 at the membrane due to this deletion results in negated susceptibility to the virus. Homozygosity for *CCR5-Δ32* imparts complete resistance to HIV infection while heterozygosity is associated with slower progression to AIDS [23]. This polymorphism is found in higher proportions in LTNPs than in progressors [24-26]. However, both homozygous and heterozygous forms of this polymorphism are extremely rare and only occur in a small percentage of LTNPs.

The host genetic factor controlling the recognition of Gag epitopes that are targeted by $CD8^+$ T cells for lysis are the alleles that code for the MHC class I molecules. The host allele variant HLA-B57 is associated with viral control and non-progression in infected individuals. In addition to this correlation, Klein et al. (1998) demonstrated that the HLA-B57-restricted CTL responses exhibit log-fold increases in specific lysis against Gag epitope-expressing target cell compared to those of other HLA-restricted CTLs [27], which is likely one explanation for the occurrence of non-progression.

These correlations between host immunologic and genotypic variation show a strong correlation to HIV control (reviewed in [28]); however such associations are not fully explanatory, given the presence of LTNPs who carry neither the *CCR5-Δ32,* HLA-B57 alleles nor other resistance-conferring genotypes. Another explanation for viral control is sought in the variation that arises in the viral genome. The rationale for the study into viral genetic variation lies in the high rates of mutation as discussed previously. Several studies have found interesting correlations between polymorphic variation in the genomes from viral isolates and the occurrence of LTNP status. Deacon et al. (1995) analyzed the viral genomes of a cohort of individuals that received blood transfusions from a donor later found to have given HIV-infected blood [29]. The cohort was found to have seroconverted after transfusion with the contaminated sample, but maintained the characteristic LTNP clinical features: ≥500 $CD4^+$ T cells per μl and undetectable viral loads after 10 years of seroconversion. The viruses characterized from three individuals in this cohort were found to show slower replication kinetics compared to NL4-3 and ADA laboratory strains. A genome analysis of these isolated virions showed deletions of varying length in the *nef*-U3 junction of the 3'-LTR regions, which explains the impaired replication, and also provides a likely relationship of viral genetic variation to the viral control associated with LTNPs.

Alexander et al. (2000) further corroborated the association between mutations in the viral genome and the slowed or non-progression of disease [30]. From the isolates of viral quasispecies from a small group of LTNPs, the authors found several polymorphisms in the *nef*-LTR junction, *env*, and *gag-pol* genes, which are all determinants of HIV infectivity and by extension viral pathogenesis. Infection of rhesus macaques with viral clones containing these

polymorphisms showed reduction of replication potential and stability of these mutations *in vivo*, lending support to the role of viral genetics in HIV disease progression.

1.2.2 Significance of the Accessory Protein, Vpr.

Viral Protein R (Vpr) is expressed by HIV and is highly conserved in HIV-1, HIV-2, and various SIV strains [31]. Early studies of Vpr in an SIV macaque model show that mutation of Vpr attenuates replication and can extend survival time, reducing the burden of disease pathogenesis on the animals under study [32, 33]. These studies underscore the role that Vpr plays in HIV pathogenesis.

1.2.2.1 The Structure and function of Vpr

Vpr is a 96 amino acid protein that is 14 kDa in size. Wecker et al. (2002) resolved the NMR structure of Vpr, revealing an N-terminal domain, three alpha helices, and a C-terminal domain [34]. However, these authors used trifluoroethanol which contains hydrophobic moieties that further studies discovered to disrupt folding of Vpr protein, which revealed a tertiary structure unlike what would be observed in natural conditions. Morellet et al. (2003) showed the structure of Vpr via NMR using a non-hydrophobic solvent, CD_3CN [35]. This allowed the closest possible approximation of the structure of Vpr as it is expressed in physiological conditions. The NMR structure displayed the secondary and tertiary structure aspects of Vpr structure. Residues 1-16 comprise the N-terminus, followed by the first alpha helix from 17-33, the second alpha helix from 38-50, the third alpha helix from 55-77, and the basic C-terminus from 78-96 (Figure 3). For the tertiary structure, the amphipathic alpha helices of Vpr fold in a manner which forces hydrophobic residues towards the external face of the protein, a thermodynamically unfavorable

state. To stabilize the tertiary structure, Vpr binds strongly to itself or to other cellular factors to shield these hydrophobic residues. The basic tenet that function follows structure becomes obvious with the numerous mutational analyses of Vpr.

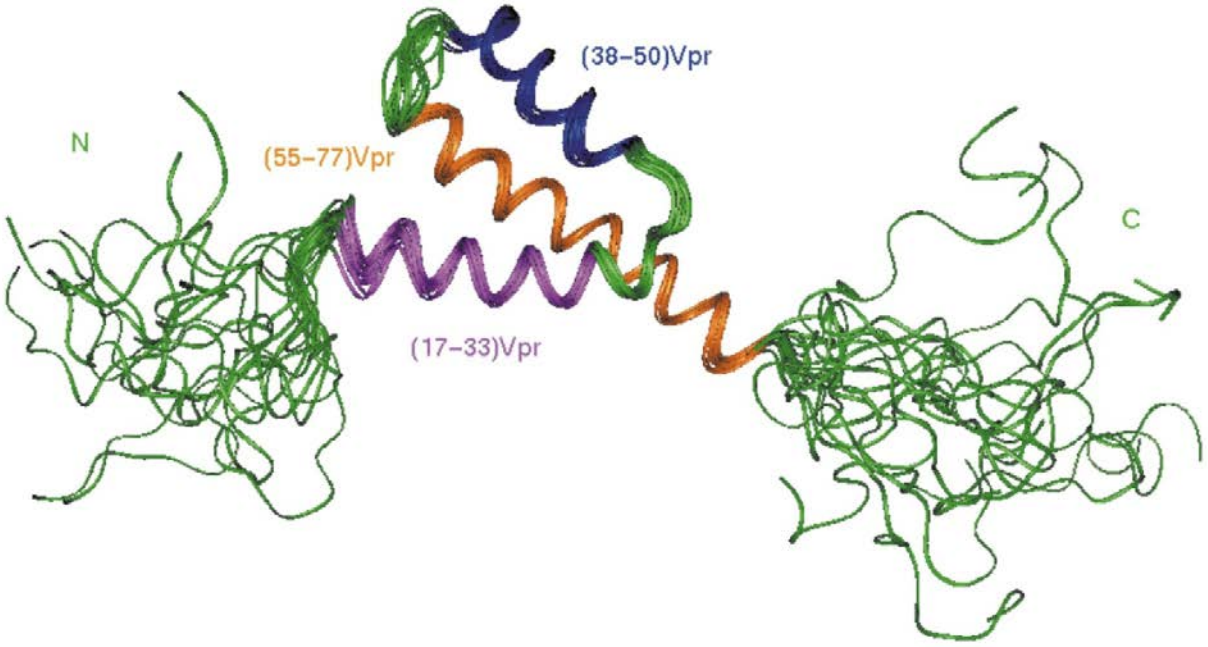


Figure 3. The Structure of Vpr (from ref. [35] with permission).

The structure-function relationship has been documented in numerous studies, which examined gain- and loss-of-function through mutational analysis. Point mutation of a number of residues among others in the sequence of Vpr (Y15, K27, Q44) disrupts the structure of Vpr [31]. The canonical features of Vpr discussed in this study have been extensively characterized. As mentioned previously, Vpr forms oligomers upon expression. Upon expression, it normally localizes to the nucleus, but it also interacts with the p6 region of the Gag capsid protein [36]. By this interaction, Vpr incorporates into the virion, inducing its function in the early phases of viral infection. Vpr induces arrest of cells in the G2 phase. It has cytopathic effects and allows replication of nondividing cells, particularly macrophages.

Oligomerization

Early in the field of research into Vpr, Zhao et al (1994) discovered that a leucine-isoleucine motif in the third helix of Vpr (^{60}LIRILQQLLFIHFR) plays a role in the capacity of Vpr to form oligomers [37]. By mutating each of the leucines and isoleucines individually to alanine in this motif, the group showed that these mutations abolish the ability of Vpr to oligomerize. This motif is thought to function as a leucine zipper which forms a binding domain endowing the oligomerizing feature to Vpr.

The previously mentioned Q44 residue in the second helix is shown to play an essential role in stabilizing the structure of Vpr. Fritz et al. (2008) studied the structural consequences of deleting this residue, finding through 3-D modeling of the Vpr ΔQ44 mutant abolished the integrity of the tertiary structure of Vpr [38]. This in turn disabled this mutant from oligomerizing. Venkatachari et al. (2010) showed a similar phenomenon with the residue A30. Mutation of the alanine to leucine abolished oligomerization of Vpr. The authors found that Vpr A30L falls at a critical interface of the first helix which was likely involved in protein-protein interaction of Vpr aka oligomerization [39]. Other studies have also concluded the same effect for this position [31].

Vpr Incorporation into the Virion

The interaction between Gag capsid protein and Vpr allows it to be packaged into the virion. This feature was elucidated by Venkatachari et al. (2010) [39]. The authors also correlated the previously discussed oligomerization function of Vpr as a necessary determinant of this interaction. The previously mentioned A30L and ΔQ44 mutants disrupt the Vpr from self-associating, which the authors found to abolish interaction with Gag.

Fritz et al. (2010) corroborated these results also finding that oligomerization is necessary for Gag interaction [36]. Using a construct of the p55 uncleaved protein and Vpr in an overexpression model, mutagenesis of several additional Vpr residues (L23F, L67A) abolished oligomerization and prevented interaction between Gag and Vpr confirming the necessity of oligomerization of Vpr to allow interaction with Gag to occur.

Nuclear Localization

The karyophilic property of Vpr has been strongly established. Vpr plays an important role in infecting the non-dividing macrophages, one of the two main infected cell types in the host. In non-dividing cells the nuclear membrane does not dissolve since no cell division occurs. The barrier of the nuclear membrane prevents the entry of other retroviruses in non-dividing cells such as the murine leukemia virus [10, 40]. Connor et al. (1995) established that Vpr enhances the infectivity of macrophages [41]; later Nie et al. (1998) and Popov et al. (1998) found that the nuclear localizing feature of Vpr seems to be the determinant of this feature, in which Vpr translocated the pre-integration complex across the nuclear membrane [42, 43]. The interaction of Vpr with the host importin-α is an essential part of the mechanism [44]. Although the enhancement of replication in macrophages is due to its non-dividing nature, another found that nuclear localization of Vpr also enhances replication in $CD4^+$ T-cells by the same mechanism [45].

Normally proteins that localize to the nucleus contain a nuclear localization signal (NLS) composed of a series of amino acids either in contiguous order or separated by a spacer sequence. Although the ability of Vpr to localize to the nucleus was already well established, there is no canonical nuclear localization signal in its sequence. Sherman et al. (2001) found that leucines in two LxxLL motifs (^{23}LLEEL^{26} and ^{64}LQQLL^{68}) as well as several arginine residues

in the C-terminal region in the sequence of Vpr contribute to the ability of Vpr to localize to the nucleus [46]. The mutations E21P, E24P; E24K; and A30P all disrupt nuclear localization [31]. The data indicate that the nuclear localizing signal may be attributed to the conformational structure of Vpr, as the mutations which disrupt structural integrity (A30P) and those that disrupt potential binding domains (L-A mutations in leucine zipper motifs) can abolish this feature. This implicates the necessity of Vpr to bind to cellular partners involved in the nuclear import mechanism.

G2 Cell Cycle Arrest

The process of the cell cycle is an event finely regulated by numerous kinases and phosphatases for the cyclin family proteins and several key patrolling factors, ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia mutated and Rad-3 related). During the junction between the G2/M phases of the cycle, ATM and ATR detect any present damage in the chromosome. If DNA damage is absent, then the cell cycle proceeds as normal and mitosis continues. However if the sentinel proteins detect DNA damage, then a massive signaling cascade occur and activate p53, Chk2, and BRCA1 among others. The pathways involved generally converge to activate p34cdc2, the p34 subunit of the cdc2 protein [31]. The cdc2 protein complexes with cyclin B1, and is regulated by a balance of kinases and opposing phosphatases. Net dephosphorylation activates the cdc2-cyclin B1 complex and halts the cell cycle in the G2/M phase to prevent division. At this point DNA repair mechanisms attempt to correct the mutations in the genome; in the event of irreparable damage, the cell goes into apoptosis.

Goh et al. (1998) demonstrated the property of Vpr to G2 cell cycle arrest [47]. The authors found that the rate of virus production is four times higher in the G2 phase than in the

G1-S phases combined. The G2 phase appears to enhance the activity of transcription from the LTR, leading to higher viral production. In their system they compared constructs that express Vpr and mutants that are Vpr-deleted in infected Jurkat T cells. The estimated time a cell infected with HIV-1 Vpr wt spends in each phase of the cell cycle is estimated to be 18 hours in the G1-S phases and 12-20 hours in G2. However, infection with HIV-1ΔVpr constructs shows that these cells only spend 2 hours in G2. The authors suggest that this is a mechanism *in vivo* for selection of viral quasispecies containing intact Vpr genes.

Gummuluru et al. (1999) further confirmed the enhancement of LTR activation in T cell lines as well as primary $CD4^+$ T cells in the presence of Vpr [48]. The authors found through serial deletion of the LTR region the promoter sequences required for Vpr-mediated LTR enhancement. In addition they used Vpr substitution mutants E24G and H71R. Vpr E24G is G2 cell cycle arrest competent while Vpr H71R is not. A single-round infection of primary $CD4^+$ T cells with HIV-1 ΔVpr and HIV-1 Vpr H71R results in significantly reduced production of p24 in supernatants compared to infections with HIV-1 Vpr wt and HIV-1 Vpr E24G.

The downstream signaling events that must occur for Vpr-induced G2 cell cycle arrest involve expression and activation of p21, a known inducer of the cell cycle blockade [49]. Expression of several mutations (Vpr A30L, R73A, and R80A) fail to induce G2 cell cycle arrest, and show significantly lower levels of p21 activation compared to expression of wild type Vpr.

Addressing the upstream signaling events, DeHart et al. (2007) found that Vpr activates the ubiquin proteasomal pathway, which leads to induction of cell cycle blockade [50]. Ubiquitination targets involve an ubiquitinating complex, a process analagous to phosphorylation by kinases. The complex contains adaptor proteins which bind to targets of ubiquitination, one of

which is the DCAF (DDB1 and CUL4-associating factors) (reviewed in [51]). DeHart and authors showed that Vpr associates with DCAF1 and inferred through an analysis using the two G2 cell cycle arrest defective mutants, Vpr R80A and Vpr Q65R, that it also binds to an unidentified target that regulates G2 cell cycle arrest [50]. This binding of Vpr to DCAF1 and a host cell cycle factor leads to the eventual induction of G2 arrest presumably through an ubiquitination pathway. The mutation Vpr R80A is able to bind DCAF1 but cannot induce G2 arrest. The model that these authors propose suggest that the R80A mutation is able to bind to DCAF1 but not the cell cycle associated partner. The authors found that Vpr R80A mutant act as a competitive inhibitor of wild type Vpr, reducing the levels of G2 cell cycle arrest in a dose-dependent manner. However, the Vpr Q65R mutant which is unable to bind DCAF1 does not show this inhibitive effect on wild type Vpr. These results suggest that Vpr initiates the cell cycle blockade by acting as an adaptor protein, binding to the ubiquitinationcomplex and a cell cycle associated cellular protein, targeting the latter for degradation leading to G2 cell cycle arrest.

Replication in Macrophages and CD4$^+$ T Cells

In monocytes and macrophages, although Vpr-defective virus is still infective and capable of replicating, wild type virus produces significantly higher titers of virus. As mentioned earlier Connor et al. (1995) demonstrated that titers of the p24 Gag subunit measured in supernatants of macrophages and monocytes infected with wild type HIV-1 across fourteen-day time points show a higher level replication compared to titers from monocytes/macrophages infected with HIV-1 Vpr-defective mutants [41]. This study and several others have demonstrated that this is due to the ability of Vpr to activate the LTR [41, 47, 48]. Ayyavoo et al. (1997) showed that treatment of PBMCs infected with a clinical HIV-1 isolate with recombinant Vpr greatly enhances viral replication [52]. The authors showed that this effect is at least partially due to the

interaction of Vpr with the glucocorticoid pathway. Several future publications established that this effect is due to interaction of Vpr with the glucocorticoid receptor [53, 54]. This leads to transactivation of glucocorticoid receptor elements in the HIV-1 LTR promoter region and enhances viral expression.

Nitahara-Kasahara et al. (2007) provided further insight into the mechanisms behind the enhancement of replication in macrophages and found a correlation to the previously discussed feature of nuclear localization. The authors confirmed that mutation of the leucine motif in the 1st α-helix (leucine residues 20, 22, 23, and 26 to alanine, αL-A) disrupted interaction of Vpr with importin-α, eliminating nuclear localization. The replication kinetics of primary differentiated macrophages infected with HIV-1 containing wild type Vpr, Vpr mutated at these leucine residues, or deletion of Vpr were compared. The authors found that wild type HIV produced high p24 titers across time points, p24 production from HIV-1ΔVpr and HIV-VprαL-A were drastically decreased. This corroborates the model that Vpr strongly enhances replication in non-dividing cells via the capacity of Vpr to traverse the nuclear membrane.

Although the enhancing effect of replication on $CD4^+$ T cells is more controversial [55], numerous reports have shown that Vpr does have an enhancing effect in systems using low multiplicities of infection [41, 56]. Iijima et al. (2004) showed that the nuclear localizing feature is also behind this. Viruses that contain mutations of several key residues that interfere with localization to the nucleus, in particular, L67A, H45W/I46A, and R87E/R88A, replicate at significantly reduced levels similar to HIV ΔVpr compared to wild type HIV.

1.3 RATIONALE

The above studies presented together suggest that Vpr function may play a role in disease progression. In addition to the circumstantial evidence provided by the mutagenesis analyses previously discussed, numerous studies correlate the presence of mutations in the Vpr gene to a lack of disease progression. An early study from Wang et al. (1996) documented the case of an HIV infected mother who had given birth to an uninfected child in 1983 who eventually seroconverted through breastfeeding [57]. At the time of the study, neither the mother nor the child progressed to AIDS and were classified with LTNP status. The authors isolated Vpr quasispecies present in the mother-child pair and analyzed the sequences. The results showed that numerous mutations in the C-terminal region of Vpr that altered the reading frame as well as the length of the amino acid sequence. As a comparison Vpr quasispecies isolated from known progressors did not contain such deletions and coded intact Vpr proteins.

Yamada and Iwamoto (2000) showed a similar finding in a comparison of the sequences of the accessory genes from LTNPs versus progressors [58]. A higher rate of mutations coding for defective accessory genes, including *vpr*, was found in the sequences isolated from LTNPs compared to the sequences from progressors. Interestingly the authors speculate that this array of mutations in the quasispecies from LTNPs attenuates the circulating viruses and leads to a stronger immune response due to the reduced cytopathicity; in other words these mutants possibly act as live attenuated vaccines allowing the host immunity to control HIV.

Yedavalli and Ahmad (2001) studied a cohort of mothers infected with HIV who were asymptomatic at the time of childbirth (although not yet considered LTNP by the 10 year definition) and did not transmit the virus to their children [59]. Again the Vpr quasispecies were isolated and analyzed by sequencing. Using the subtype B consensus sequence of Vpr as a

reference, the authors found numerous Vpr substitutions that were present in the Vpr quasispecies isolated from the patients.

A controversial association between a specific Vpr polymorphism was identified by Lum et al. (2003) [60]. The authors showed a correlation between LTNP status and the presence of a substitution of the 77^{th} residue of Vpr from an arginine to glutamine (R77Q). Their studies further demonstrated that Vpr containing R77Q is deficient in inducing apoptosis. However, another study by Fischer et al. (2004) contradicted these results, finding no association between R77Q and LTNP and further claiming that the R77Q mutation correlates more to subtype rather than disease progression [61]. A further study, though, in which only Vpr sequences were analyzed from only LTNP patients infected with HIV subtype B showed that the association between R77Q and lack of progression is present [62]. In addition to R77Q, several studies have identified Q3R and F72L as two Vpr polymorphisms from an LTNP patient which show altered Vpr function in the function of Vpr to induce apoptosis and its ability to localize to the nucleus, respectively [63, 64].

The preponderance of evidence indicates that Vpr has a significant involvement in the disease progression of HIV to AIDS. However, these studies make use of relatively small sample sizes and largely focus on one or two mutations to analyze in the functional analysis. These issues limit the conclusions that can be made on the specific effects of Vpr polymorphisms on function and disease progression. To address these issues, the present study makes use of the availability of patient-isolated Vpr sequences deposited in the HIV sequences database maintained by the Los Alamos National Laboratories, and the information on the disease status attached to a number of these sequences. The increased sample size enabled identification of signature polymorphisms in LTNP and progressors by frequency analysis.

1.4 HYPOTHESIS

Our hypothesis is that the Vpr sequences from LTNP and progressor groups will reveal mutations that should affect the canonical functions of Vpr discussed: oligomerization, subcellular localization, incorporation into the virion by interaction with Gag, and induction of G2 cell cycle arrest. Due to the functional differences these mutations should affect the capacity of HIV replication overall, indicating a change in the fitness and replication kinetics of the virus.

2.0 SPECIFIC AIMS

The intent of the aims below are to delineate the role of Vpr mutations in disease progression.

Aim 1: Characterize functional differences between LTNP- and progressor-derived Vpr sequences.

1. Identify patterns of phylogeny in LTNP- and progressor- derived Vpr sequences and compare to other accessory genes, Nef and Vif and regulatory gene, Tat.
2. Identify signature polymorphisms in LTNP-Vpr and progressor-Vpr sequences via frequency analysis.
3. Construct and verify expression of Vpr sequences in bimolecular fluorescence complementation reporter system.
4. Characterize the functions of the mutant Vpr genes in terms of the canonical functions of Vpr:
    a. Oligomerization
    b. Nuclear localization
    c. Incorporation into the virion (via interaction with Gag)
    d. Induction of G2 cell cycle arrest

Aim 2: Characterize the effects of LTNP- and progressor-associated Vpr polymorphisms on virus replication in primary PBMCs.

1. Clone the selected LTNP- and progressor-derived mutations identified in Aim 1 into proviral reporter construct.
2. Characterize mutant virus to confirm expression of essential viral proteins and virus production in producer cells.
3. Infect PBMCs with mutant virus and assess the replication kinetics of virus for changes in viral fitness compared to wild type virus control.

3.0 MATERIALS AND METHODS

3.1.1 Phylogenetic and Frequency Analysis of Vpr Sequences

Amino acid sequences of Vpr, Nef, Vif, Tat classified from long-term non-progressors, normal progressors, and rapid progressors were gathered from the HIV sequence database (http://www.hiv.lanl.gov/) (Los Alamos National Labs, NM, US). These sequences were then analyzed for phylogenetic grouping and frequencies of polymorphisms. To generate phylogenetic groupings, the sequences were manipulated using Geneious® and Galaxy® (http://g2.bx.psu.edu/) software. The sequences were then analyzed using MEGA software to generate the alignments using the MUSCLE algorithm and then the phylogenetic trees using the Neighbor-Joining method. The trees were then graphically manipulated using FigTree, and Inkscape softwares.

Frequency analysis for signature polymorphisms was generated via aligning Vpr, Nef, Vif, and Tat sequences from long-term non-progressors and normal progressors (as categorized from the sequence database). The alignment was compared with NL4-3 Vpr sequence as a reference and the frequencies of mutations were subjected to analysis yielding magnitude delta-p. Significance testing for the frequency analysis was performed using Bonferroni analysis.

3.1.2 Use of Bimolecular Fluorescence Complementation

The study described herein makes use of the bimolecular fluorescence complementation (BiFC) system for dual purposes: 1) to serve as a reporter for protein expression of DNA constructs and 2) to detect protein-protein interaction. In BiFC the N-terminal half and C-terminal halves of a fluorescent GFP derivative is tagged to the construct expressing the protein of interest (Vpr, in this study) via molecular cloning [65]. This generates two chimeras, one containing the N-terminal fragment of the Venus fluorescent protein tagged to a Vpr construct in this case and the other, the counterpart C-terminal tag (Figure 4) [39].

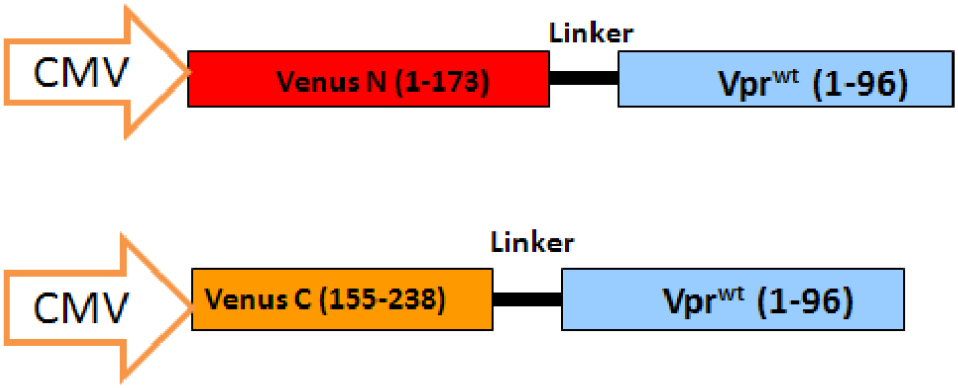


Figure 4. Schematic of BiFC Vpr Constructs.

Upon expression in targeted cells, interaction of the tagged proteins will cause the non fluorsenceing bystanding fluorescent tags to essentially reconstitute the full-length fluorophore, enabling detection by numerous means (Figure 5).

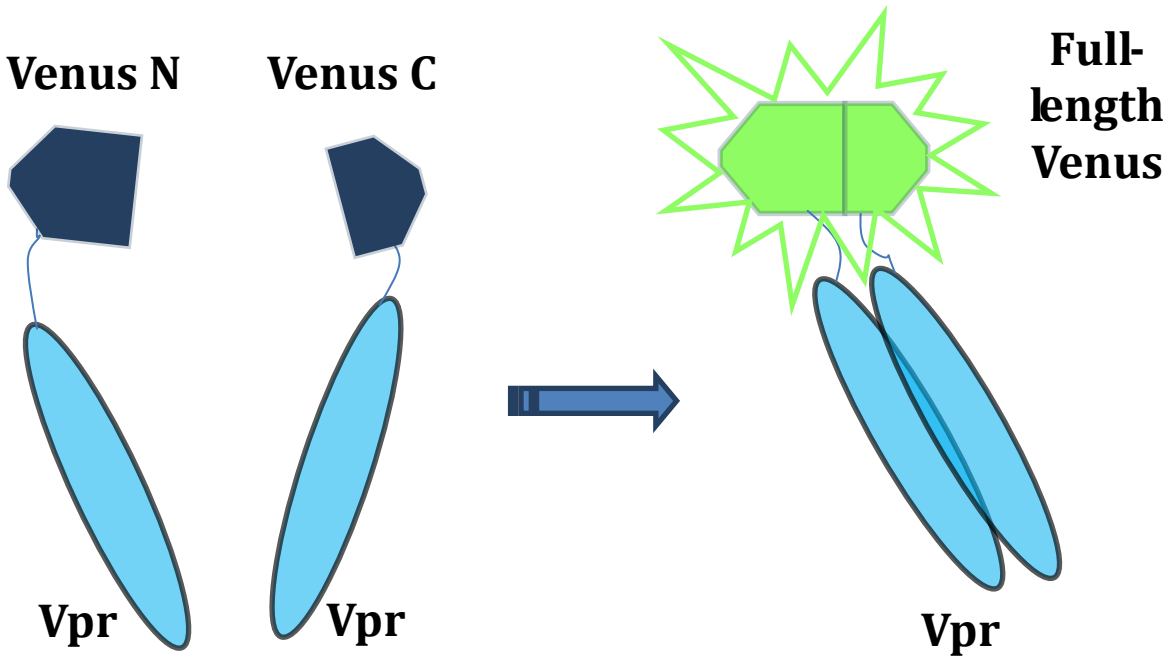


Figure 5. Schematic of BiFC System.

3.1.3 Generating Vpr Mutants in BiFC Constructs

VC-Vpr/VN-Vpr and VC-Gag/VN-Gag plasmids were constructed as previously described [39, 54]. The mutants were generated via site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Agilent, CA, US) as per manufacturer's protocols [66]. Oligonucleotides for the mutagenesis reactions were obtained from Integrated DNA Technologies (Coralville, IA, US). Upon obtaining mutants, each clone was then sequenced by the Sanger method for confirmation of the mutations. The clones containing only the specified mutation of interest were selected for further use.

3.1.4 Cells

HEK293T and TZM-bl cells were maintained at 37°C, 5% CO_2 in DMEM (Mediatech, VA, US) supplemented with 10% Fetal Bovine Serum, 1% glutamine, and 1% penicillin/streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from three healthy donors. Blood samples were gently layered over equivalent volumes of lymphocyte separation medium (Mediatech, VA, US) and centrifuged at 1800×g to separate PBMCs through the gradient. PBMCs were counted, collected, and maintained in RPMI (Mediatech, VA, US) supplemented with 10% Fetal Bovine Serum, 1% glutamine, and 1% penicillin/streptomycin. PBMCs were activated for three days prior to infection with PHA (5 ng/ml) and then incubated with IL-2 (1 U/ml) throughout the infection phase.

3.1.5 Expression of Mutant Vpr Constructs and Immunoblotting

HEK293T cells were seeded in 6 well plates at ~7×10^5 cells per well. To express each construct, the cells were cotransfected with 0.5 μg each (1 μg total) of VC-Vpr and VN-Vpr mutant constructs using Polyjet (Signagen, MD, US) reagent per manufacturer's protocols. Cells were collected via mechanical dislodging followed by centrifugation (450 g, 5 min.) and washing with PBS 48 hours post transfection. Cells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X-100, 1% NP-40, 0.1% SDS, and SIGMAFAST™ protease inhibitor from Sigma). Samples were electrophoresed by SDS-PAGE under reducing conditions, and transferred to a PVDF membrane (Millipore, MA, US). Total protein content of samples was determined via bicinchoninic acid assay (Pierce, IL, US). Fifty μg of protein was run via SDS-PAGE under reducing conditions and then transferred to a PVDF membrane. The membrane was then blocked in a 5% BSA solution in PBS-T (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween-20) for 1 hour at room temperature with agitation. This was followed with incubation in primary antibody (anti-HA, anti-Gag, anti-actin, anti-Vpr; 1:4000, 1:500, 1:4000, 1:5000) diluted in a 2% BSA solution PBS-T at 4°C overnight with agitation. After three washings in PBS-T for 5 min. at room temperature with agitation, the membranes were incubated in goat-anti-mouse conjugated to HRP (1:3000) diluted in a 2% BSA solution in PBS-T. Following PBS-T washings blots were developed using WesternBright ECL HRP substrate (Advansta, CA, US). Anti-HA and anti-actin antibodies were obtained from Sigma (MO, US), anti-Gag from the NIH AIDS Research and Reference Reagent Program (MD, US), anti-mouse from Cell Signaling (MA, US).

3.1.6 Flow Cytometry

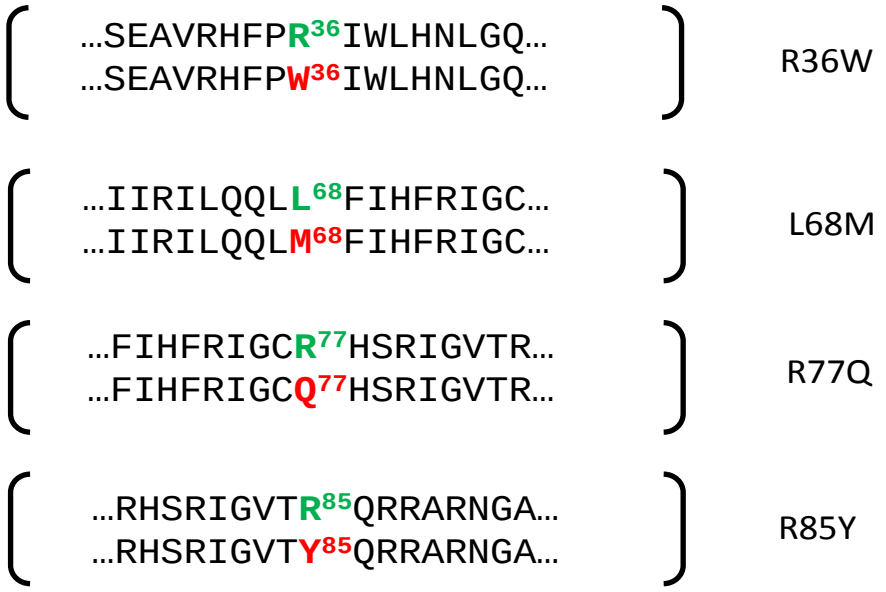
BiFC interaction and cell cycle analysis was assessed via flow cytometry. HEK293T cells transfected with BiFC-Vpr constructs were collected 48 hours post-transfection by aspirating medium, followed by washing with PBS, and trypsinization. Cells were counted for flow cytometry (1×10^6 cells). The cells were then washed in PBS, fixed with 4% paraformaldehyde for 20 minutes at room temperature, and either analyzed immediately for BiFC interaction or further stained for cell cycle analysis. For cell cycle analysis, fixed HEK293T cells were then washed, and further fixed with 70% ethanol for 1 hour at room temperature or at 4° C overnight, and then stained with cell cycle solution (propidium iodide, 50 µg/ml; RNase A, 50-100 µg/ml) at 37° C for 30-40 minutes. Cells were then run through the flow cytometer (BDFACSCanto®) and analyzed with BDFACSDiva® software or Weasel software (obtained from http://www.wehi.edu.au/, website of the Walter and Eliza Hall Institute of Medical Research (Victoria, Australia)).

3.1.7 Microscopy

HEK293T cells were seeded on coverslips in a 24-well plate (~4×10^4 cells per well) and transfected with BiFC-Vpr plasmids. Following 24 hours, cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 20 minutes. After fixation, cells were then washed with PBS and stained with DAPI for 15 minutes for visualization of nuclei. The cells were then washed with PBS twice and mounted on glass slides using gelvatol mounting media. The coverslips were allowed to adhere and harden to slide. The slides were viewed under the

Olympus Fluoview 500 upright confocal microscope at 100x magnification. Images were adjusted and merged using ImageJ® software.

3.1.8 Cloning Mutants into Proviral Reporter Constructs



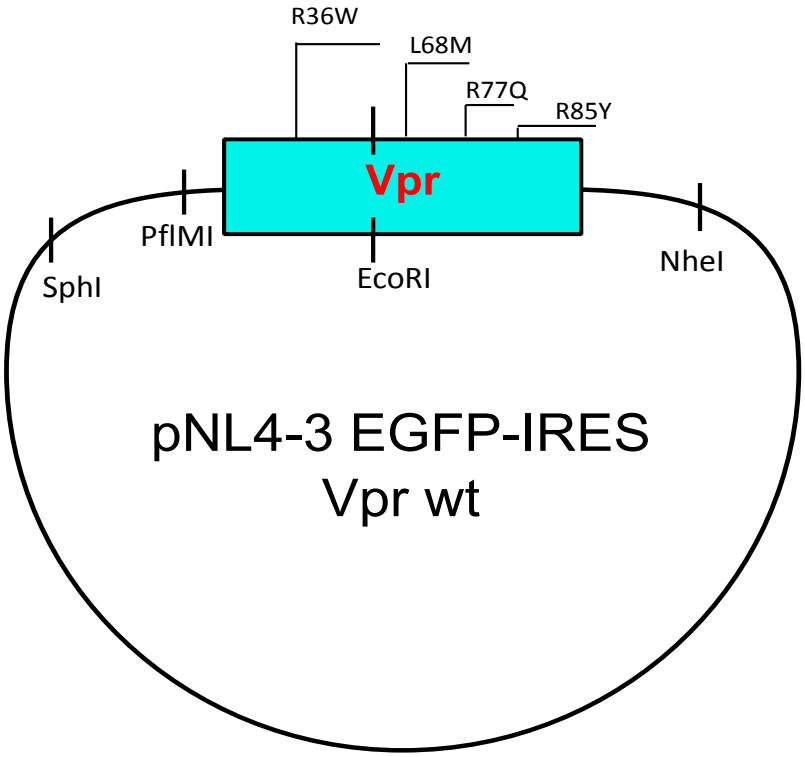


Figure 6. (A) Primer pairs used (B) Schematic representation of cloning sites used to introduce Mutations into Proviral Reporter Construct

For subsequent infection studies, the proviral construct expressing the NL4-3 strain of HIV-1 which also contains an EGFP-IRES reporter element was used in which to introduce the selected Vpr mutations. The overall strategy of introducing the selected Vpr mutations into the proviral construct is illustrated in Figure 6; the amino acid sequences with the original residue in Vpr (highlighted in green) are represented in the top lines of each bracket while the mutations (highlighted in red) are represented in the bottom lines. The mutations Vpr R36W, L68M, R77Q, and R85Y were introduced into the proviral construct on a shuttle vector containing the Vpr fragment of interest. This required cloning of the 5' half of Vpr and the 3' half from the pNL4-3 EGFP-IRES template into separate shuttle vectors.

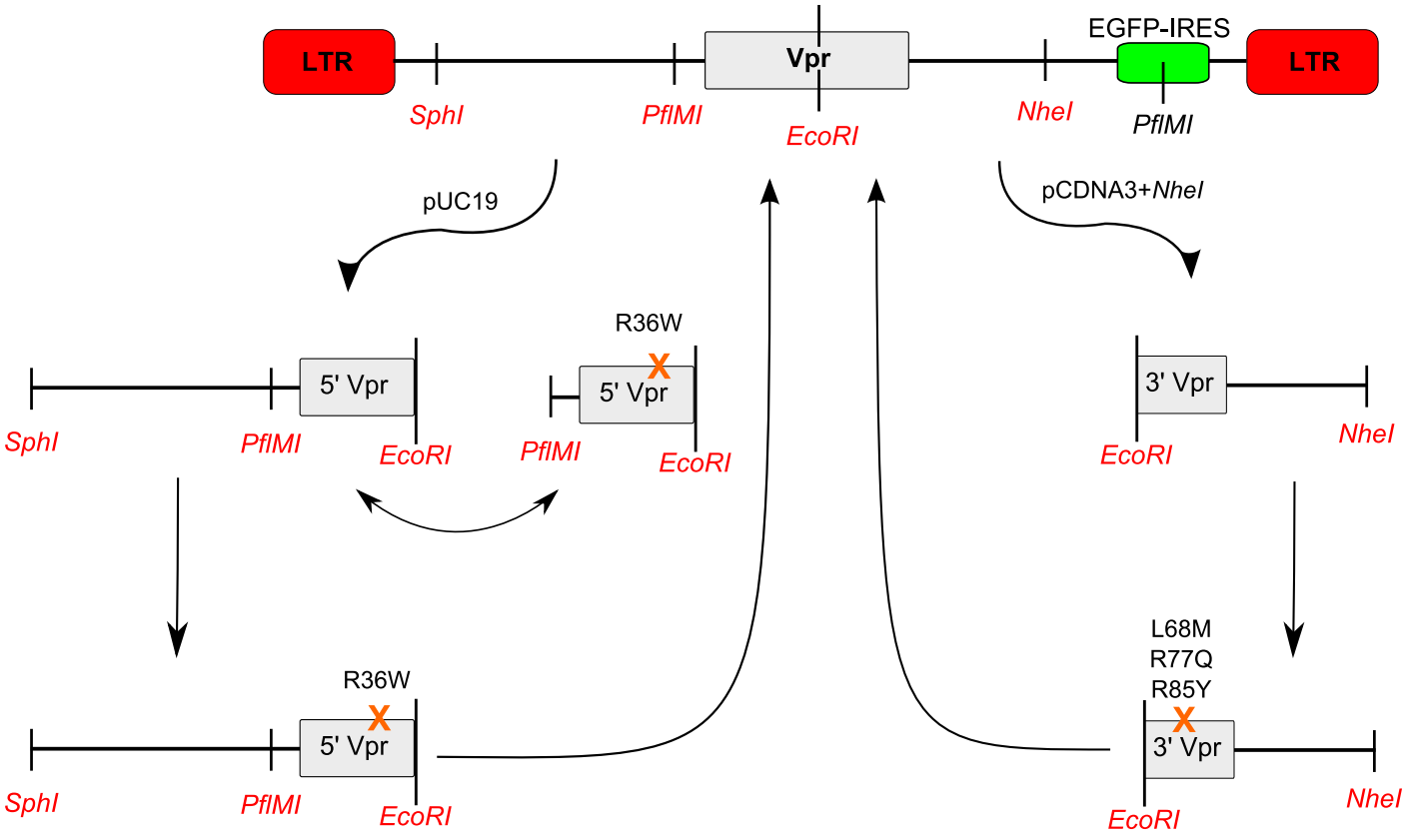


Figure 7. Cloning Strategy to Introduce Mutations into Proviral Reporter Construct.

To generate Vpr R36W, the 5' half of Vpr was excised using *SphI* and *EcoRI* sites (Figure 7). This fragment, *SphI*-5'Vpr-*EcoRI* was ligated into pUC19 with the cognate restriction sites. A previous vector containing the Vpr R36W mutation was substituted into the pUC19 *SphI*-5'Vpr-*EcoRI* fragment using the *PflMI* and *EcoRI* restriction sites. Once the mutated fragment, *SphI*-5'Vpr R36W-*EcoRI*, was generated, it was then reinserted into the pNL4-3 EGFP-IRES to reform the full-length construct.

To generate the mutations Vpr L68M, R77Q, and R85Y, the 3' fragment of Vpr was excised from pNL4-3 EGFP IRES using restriction sites *EcoRI* and *NheI* and ligated into the shuttle vector pCDNA3 containing an additional *NheI* site that was previously added by PCR (Figure 7). The pCDNA3 *EcoRI*-3'Vpr-*NheI* fragment was then mutagenized using the Quikchange II Site Directed Mutagenesis Kit as described above to generate the L68M, R77Q, and R85Y mutants. The mutated *EcoRI*-3'Vpr-*NheI* fragments were then reinserted into the pNL4-3 EGFP IRES to reconstitute the full length construct.

3.1.9 Virus production, packing and Titration of Mutant Virus

HEK293Ts were seeded in 10 cm^2 tissue culture dishes (3×10^6 cells). After 24 hours, the cells were transfected using 5 μg of viral DNA construct with 15 μl of Polyjet® reagent for a single plate. Following 12 hours of incubation, the media was changed (7 ml) and incubated further for 48-60 hours. At 60-72 hours post-transfection, the supernatants were harvested, cleared by centrifugation at 830×*g* for 5 minutes, and then filtered through a 0.22 μm membrane (Millipore, MA, US).

To titer the infectivity of the virus supernatants, TZM-bl cells were seeded in triplicate (~2×10^4 cells per well) on a 96-well plate for 24 hours. Cells were then treated with serial

dilutions of viral supernatants and incubated for another 48 hours. The cells were then stained with β-gal substrate, counted across the triplicate samples, and averaged to calculate the infectious particles per ml.

3.1.10 Infection of PBMCs and Replication Kinetics

PBMCs isolated as described above were activated with PHA (5 ng/ml) for 72 hours. Following activation, 1×10^7 cells were infected for 16 hours (overnight) at an MOI of 0.1 for each virus sample in IL-2 (1 U per ml) containing media. Supernatants were harvested at day 3 and the infectivity of the supernatants was measured by luciferase assay in TZM-bl cells after a 36 hour incubation at 37°. At day 6, 5×10^5 PBMCs were collected, fixed in 4% paraformaldehyde at room temperature for 20 minutes, and analyzed through flow cytometry for expression of EGFP.

3.1.11 Statistical Analysis

Statistics were performed using the Graphpad Prism® software suite. Comparisons of samples to control were done separately using the two-tailed Student's t-test using a significance level of $p<0.05$.

4.0 RESULTS

4.1 AIM 1: CHARACTERIZE THE FUNCTIONAL DIFFERENCES BETWEEN LTNP- AND PROGRESSOR-DERIVED VPR SEQUENCES

4.1.1 Phylogenetic Analysis of Vpr, Vif, Tat, and Nef Sequences

Amino acid sequences of four of the protein sequences were collected from the HIV Sequence Database (www.hiv.lanl.gov) from the Los Alamos Laboratories repository. As a preliminary study, the protein sequences of Vpr derived from patients classified as long-term non-progressors, rapid progressors, and normal progressors were gathered. The other accessory proteins of HIV (Vif, Tat, and Nef) all of which have a role in disease pathogenesis were also included as a comparison.

Protein and not nucleotide sequences were used because of the rationale that only non-synonymous mutations would affect the function of these proteins. Altering the coding sequence could change the structure and hence, function, of these proteins. The phylogenetic analysis will determine if there is a correlation between the patterns of disease progression and functional sequence variation.

The progression of disease for each sequence was previously indicated in the database. After collecting the marked sequences for Vpr, Vif, Tat, and Nef, each sequence was tagged as

"LTNP" (long-term non-progressor), "NP" (normal progressor), or "RP" (rapid progressor) in Geneious® software to facilitate further analysis. The tagged sequences for each accessory protein were combined and aligned using the MUSCLE algorithm. The phylogenetic analysis was then performed using the neighbor-joining method.

The phylogenetic analysis for Vpr was performed using 177 sequences derived from long-term non-progressors, 92 sequences from normal progressors, and 10 sequences from rapid progressors (Figure 8). The phylogeny of LTNP sequences indicates that the LTNP sequences are highly similar to one another as shown by the extensive clustering. The normal progressor sequences also cluster together; however, the sequences exhibit somewhat more diversity than LTNPs. The rapid progressor sequences do not cluster together and are highly unrelated.

The phylogenetic analysis of Tat shows a similar clustering pattern for LTNP sequences (Figure 9). These sequences appear to be more similar to one another than to NP or RP sequences. The NP and RP sequences tend to cluster together in the phylogeny of Tat.

The LTNP sequences for Vif are also highly similar to one another as shown in Figure 10. Although the NP and RP sequences are less abundant, the NP sequences do show a pattern of clustering, though the greater dispersion of clusters in the tree indicates that they are less similar to one another, relative to LTNP sequences. The RP sequences, once again are interspersed throughout the tree, indicating high diversity.

Interestingly, the clustering pattern differs in the phylogeny of Nef sequences compared to the above. The NP sequences for Nef are highly similar to one another (Figure 11). The LTNP sequences also cluster together but relatively less so. The NP sequences show some clustering, but are interspersed throughout the phylogeny.

These results indicate that status of disease progression and the sequence variation do relate, for the accessory proteins in a similar fashion, with LTNP sequences showing lower sequence diversity than NP sequences. The LTNP and NP clusters tend to separate from one another indicating that the sequences from the LTNP group tend to be more related to each other than to NP sequences and vice versa. In Vpr the similarity of LTNP sequences and the separate similarity of NP sequences indicate a pattern in the sequences themselves which are correlated to disease progression. Subsequently a frequency analysis was done to further identify any specific polymorphisms in the sequences of Vpr that can be correlated to long-term non-progressors and progressor phenotypes.

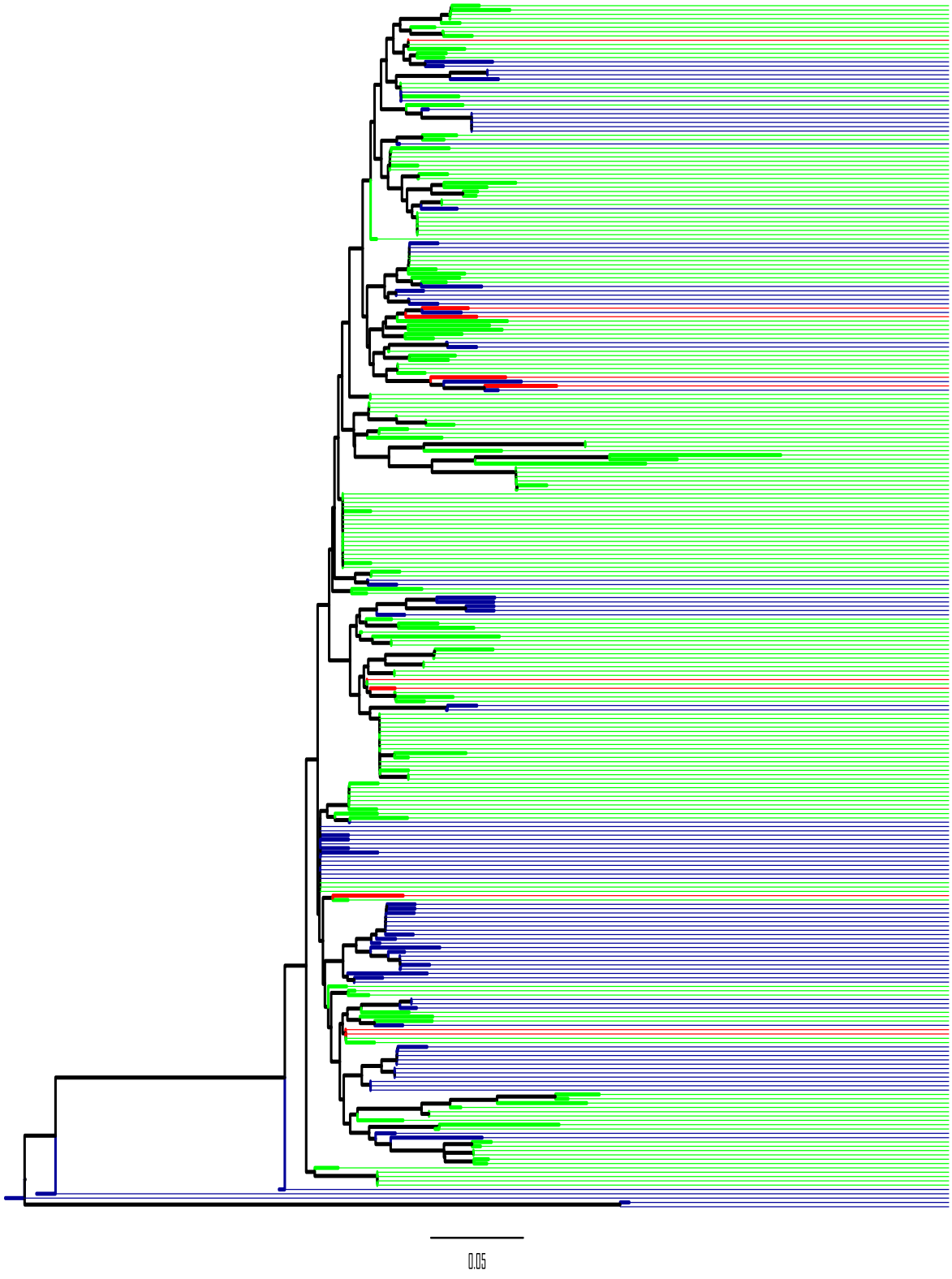


Figure 8. Phylogenetic Analysis of LTNP, NP, and RP-derived Vpr Sequences. LTNP, n=177; NP, n=92; RP, n=10

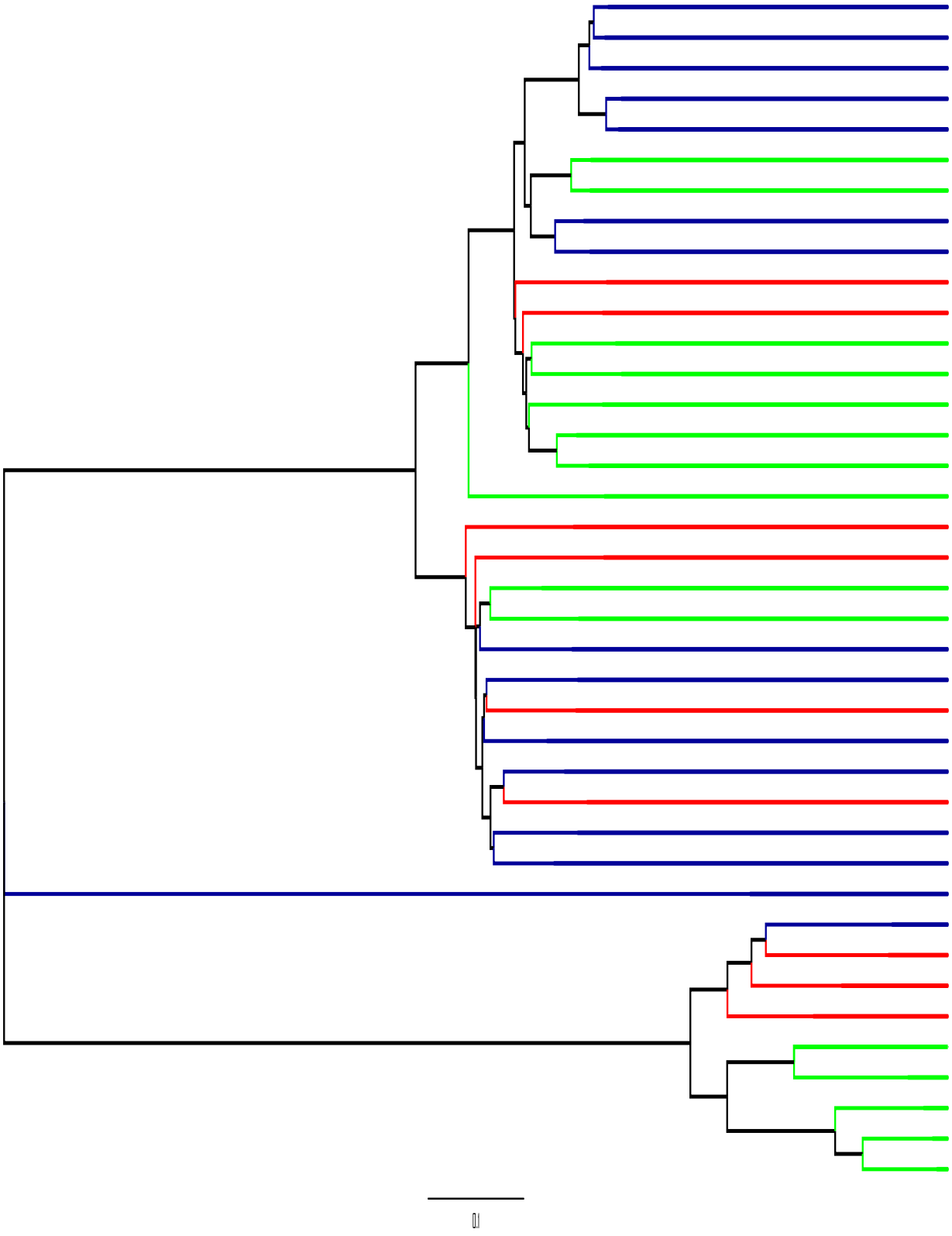


Figure 9. Phylogenetic Analysis of LTNP, NP, and RP-derived Tat Sequences. LTNP, n=51; NP, n=21; RP, n=9

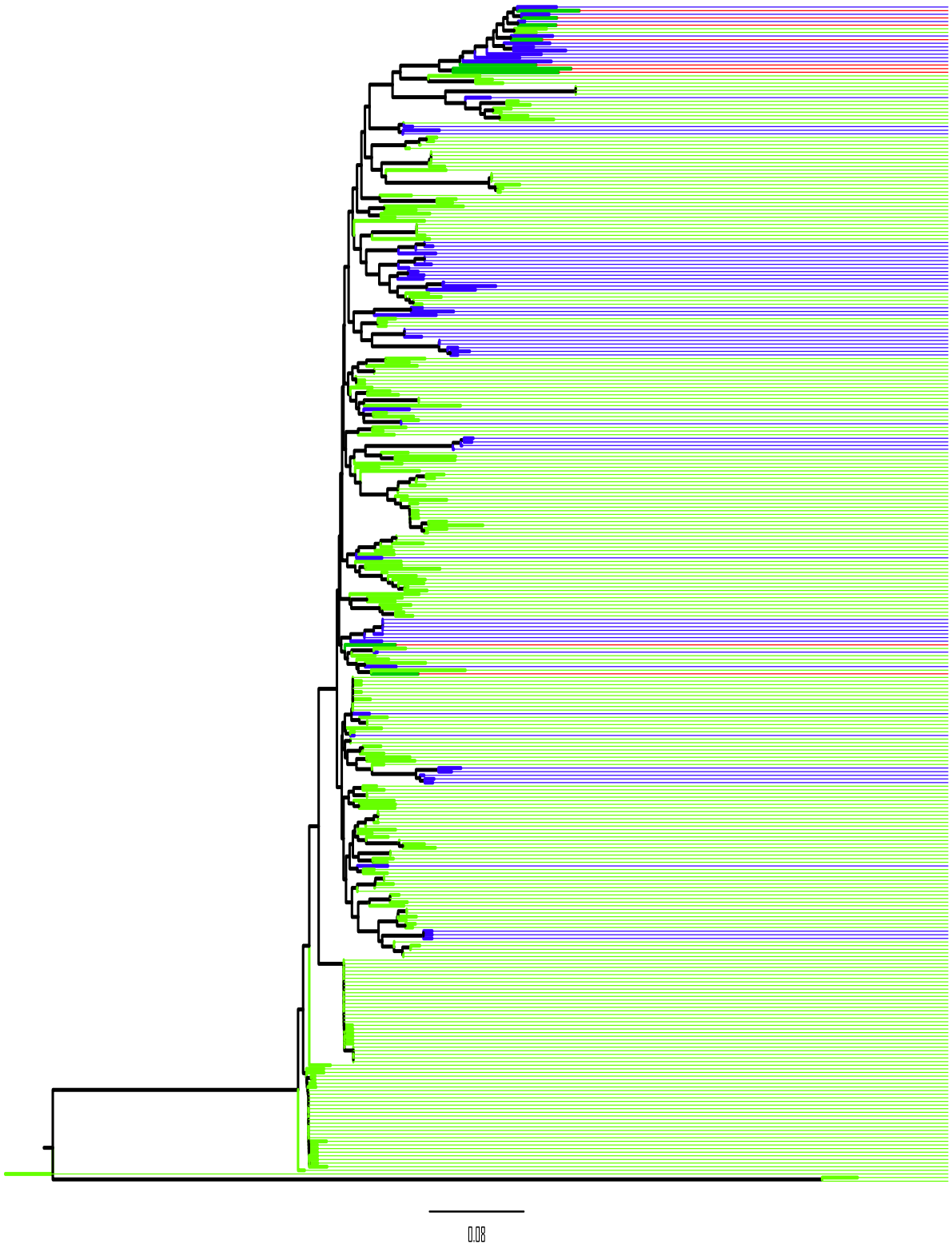


Figure 10. Phylogenetic Analysis of LTNP, NP, and RP-derived Vif Sequences. LTNP, n=250; NP, n=66; RP, n=9

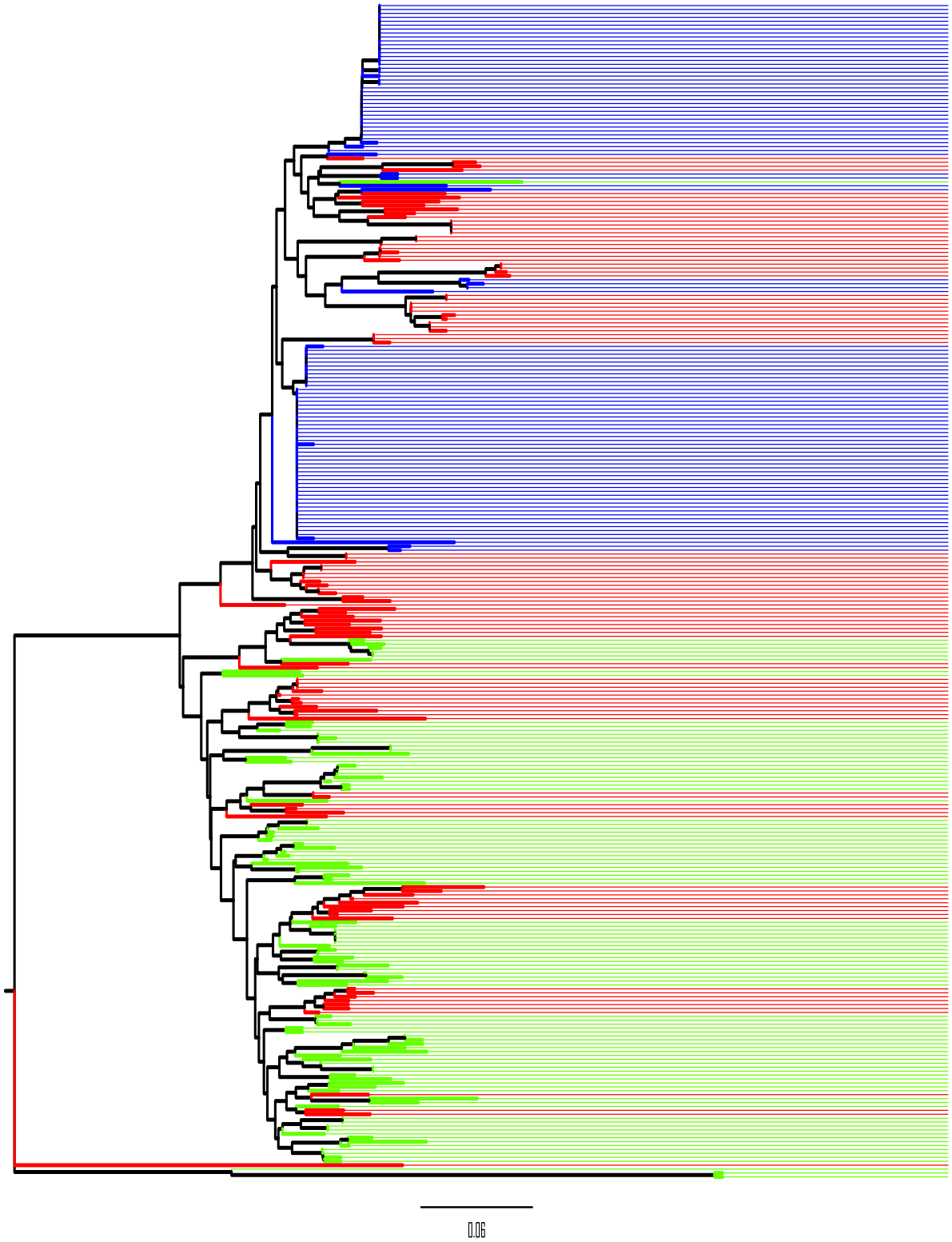


Figure 11. Phylogenetic Analysis of LTNP, NP, and RP-derived Nef Sequences. LTNP, n=100; NP, n=100; RP, n=100

4.1.2 Frequency Analysis of Vpr Sequences

Although the Vpr sequence data gathered for the phylogenetic analyses shows an association of LTNP and RP groups with the protein sequences isolated from the respective clinical cohorts and although a majority of these sequences originate from subtype B, the subtype information is unavailable for a number of these sequences or come from subtype C or D. In addition, the status of disease progression from the use of the categorical search is not available for most sequences, even though they may contain this information within the accession tags. As such this crude analysis of Vpr protein sequences serves only as an initial study. The variability of Vpr across subtypes is a confounding factor in mutational analyses of this protein which is evident in two studies which contradict the association with polymorphism in Vpr and disease progression [61, 67]. As a result of these problems we chose to focus on sequences originating from subtype B with several further criteria and manually culled LTNP and RP sequences as defined from the accession descriptions rather than the categorical search.

The amino acid sequences of Vpr from LTNPs and progressors (normal- and rapid-progressor sequences combined) were aligned and compared to the reference NL4-3 Vpr sequence. All mutations away from the NL4-3 Vpr sequence were tabulated for each position. The frequencies of mutations at each amino acid of Vpr were obtained as a percentage. The analysis included 192 sequences from long-term non-progressors and 102 sequences from progressors. The percentage of mutations at each position associated with the two disease groups is indicated in

Table 1. A label of “absent” indicates that there is no change from the NL4-3 reference at the specific residue.

The results yielded five mutations of interest. These were chosen on the basis of meeting both of two criteria:

1. having only one or two mutations at a position associated with the LTNP group and no change of residue in the progressor (NP/RP) group, or vice versa.
2. having a magnitude delta-p greater than 0.1.

Although multiple mutations at a specific residue may be associated with either disease status, the potential for complicating the conclusions of this study precluded selecting such mutants. The magnitude delta is the increase in probability that a mutation results in either disease phenotype, and significance is associated with a magnitude delta-p$\geq$0.1. For example, at residue 19, the residue A is mutated from the original T residue at 24.48% frequency with a magnitude delta-p of 0.281. This indicates that there is a 28.1% higher chance of an alanine resulting in the long-term non-progressor phenotype, or conversely, no change of amino acid resulting in a progressor phenotype. The mutations selected for further study are highlighted in Table 1 and are as follows: T19A, R36W, L68M, R85Y, and R90N. The only exception to the criteria of the magnitude delta-p is R85Y, which was selected on the basis of a separate analysis which did yield a much higher proportion of progressor sequences exhibiting this polymorphism (data not shown).

Table 1. Frequency Analysis of Vpr Mutants from LTNP and NP/RP Groups

Residue	Aminoacid in NL43 (Wild Type)	Percent frequency		Magnitude Δ-p
		LTNP (n=192)	NP/RP (n=102)	$\lvert p_{LTNP}-p_{NP/RP} \rvert$
3	Q	H (2.54) R (4.57)	Absent Absent	0.073
16	N	H (5.58)	Absent	0.055
19	**T**	**A (24.48)**	**Absent**	**0.281***
28	S	T (7.65)	Absent	0.204*
32	R	K (18.87)	K (2.96)	0.149*
36	**R**	**Absent**	**W (17.64)**	**0.156***
37	I	L (15.3) V (12.2)	Absent V (41.16)	0.02
42	L	Absent	F (2.94)	0.024
55	A	Absent T (32.64)	V (4.9) T (20.58)	0.065
63	I	Absent T (3.06)	S (6.86) T (31.36)	0.265*
68	**L**	**Absent**	**M (15.68)**	**0.157***
84	T	S (0.51)	S (12.74)	0.326*
85	**R**	**Absent**	**Y (6.36)**	**0.083**
86	Q	R (33.66) Absent	R (5.88) P (9.8)	0.183*
87	R	G (4.59)	Absent	0.08
88	R	P (14.28) T (4.98) G (0.51)	Absent Absent G (4.9)	0.149*
89	A	R (23.97)	R (4.9)	0.222
90	**R**	**N (18.36)** A (4.59)	**Absent** Absent	**0.266***
91	N	R (6.59) G (13.77)	R (34.3) Absent	0.202*
94	S	R (22.44) A (7.68)	Absent A (34.3)	0.014
96	S	R (4.59)	R (35.28)	0.264*

4.1.3 Expression of Vpr Mutant Constructs

Once the mutants were selected from the frequency analysis, they were then generated in BiFC Vpr constructs via site-directed mutagenesis. The panel of mutants spans the length of the amino acid sequence of Vpr from 1st, 2nd, and 3rd helix to the C-terminal domain (Figure 12, top panel). Evidence has established these three domains as containing residues which are important for various Vpr functions [31, 68, 69]. The selection of mutants served as a starting point to identify potentially essential residues to Vpr function in the context of disease progression.

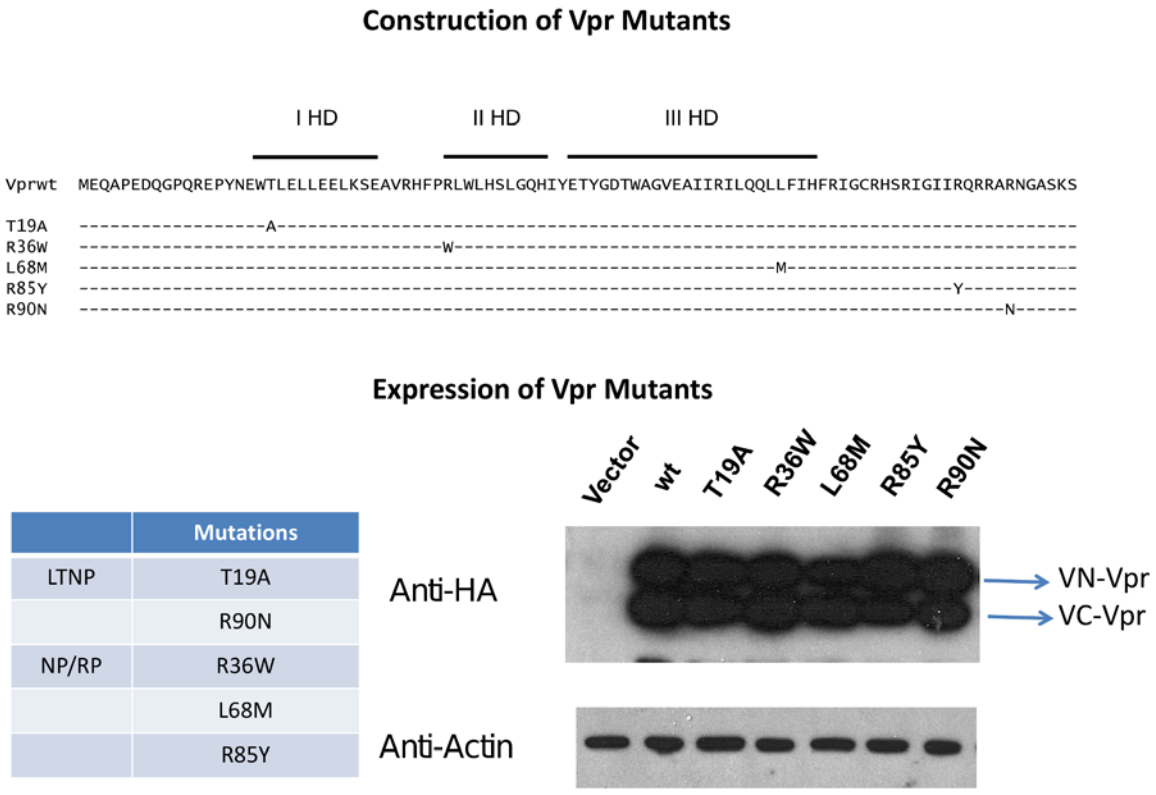


Figure 12. Construction and Expression of Vpr Mutants. The coding sequence of Vpr is represented in the top panel. The wild-type sequence derives from the NL4-3 strain of HIV-1. Expression levels of each BiFC construct in HEK293Ts were measured by western blot (bottom panel).

As the mutants selected have not been previously identified in the literature, it is not known whether these mutants disrupt the expression of Vpr. Altered levels of expression of Vpr could partially explain the disease progression; this would affect the induction of various Vpr functions, which could confound the subsequent studies. Previous work from our lab has established that Vpr expression is stable at 48 hours post-transfection [70]. Each pair of BiFC Vpr mutants was transfected in HEK293T cells and harvested at this time point to assess the expression levels of the mutants as compared to wild type. Each mutant maintains a high level of expression in each of the constructs similar to wild type Vpr (Figure 12, bottom panel), excluding the possibility that these mutants affect the functions of Vpr due to expression level.

4.1.4 Vpr Oligomerization

A hallmark feature of Vpr is the formation of oligomers upon expression. This phenomenon has been characterized in previous studies [36, 39, 70]. Protein oligomerization enhances stability by covering exposed hydrophobic residues from the hydrophilic environment by the protein-protein interactions. The LTNP and progressor mutants selected for this study span the three α-helices and the C-terminal region of Vpr, all of which, as discussed previously, have structural importance; given the necessity of structural integrity for oligomerization to occur, it is likely that these mutations could alter this function. It is likely that the RP-associated mutations should show higher levels of oligomerization which could result in higher levels of virion incorporation.

To analyze this, HEK293T cells were cotransfected with VC-Vpr and VN-Vpr plasmids containing the LTNP- and progressor-associated mutations. Thirty-six to forty-eight hours posttransfection, the cells were fixed and collected for analysis through flow cytometry. This was done in three independent experiments. To analyze the oligomerization of the Vpr mutants, the

mean fluorescent intensities were compared between each individual mutant and wild type Vpr. In order to reduce interexperimental variability, the values were normalized within each experiment. As shown in Figure 13, the normalized MFI for progressor-associated mutant Vpr R36W exhibits a 45.64% increase compared to wild type Vpr; this difference is significant with a p-value of 0.0287. None of the other mutants showed a difference in their ability to oligomerize compared to wild type.

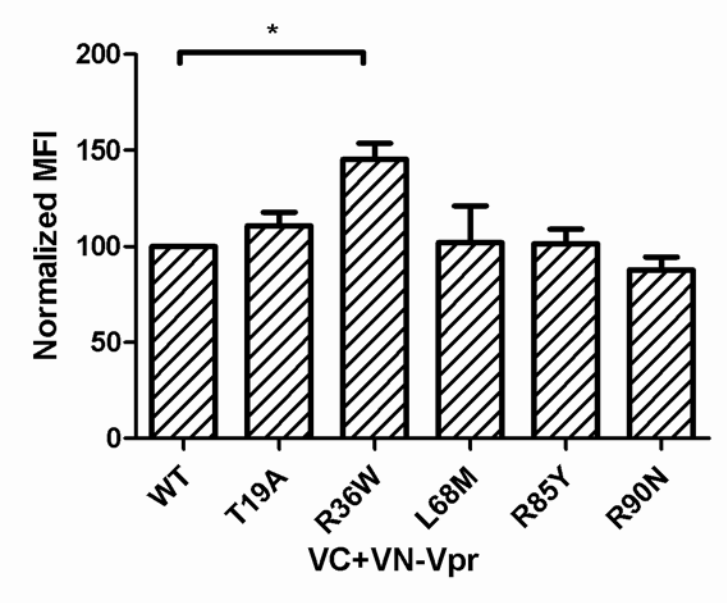


Figure 13. Oligomerization of Vpr Mutants. The oligomerization of Vpr mutants was measured by flow cytometry. The MFI values were normalized to WT as a percentage in each of three independent experiments (n=3). Error bars represent SEM. * indicates $p<0.05$.

As transfection efficiency is a possible explanation for the increased oligomerization for Vpr R36W, the percentages of cells transfected was analyzed. As with the MFI, the percentages were normalized to the wild type Vpr positive control. A comparison of each sample to wild type Vpr showed no significant difference in the percentage of FITC-positive cells, showing that there is no difference in the transfection efficiency of the mutants as compared to wild type.

As Vpr R36W associates with the RP group, it is expected to observe such an increase in the level of oligomerization of this mutant. As oligomerization is necessary for packaging of Vpr into the virion,this function may increase the levels of Vpr packaged into the particle.

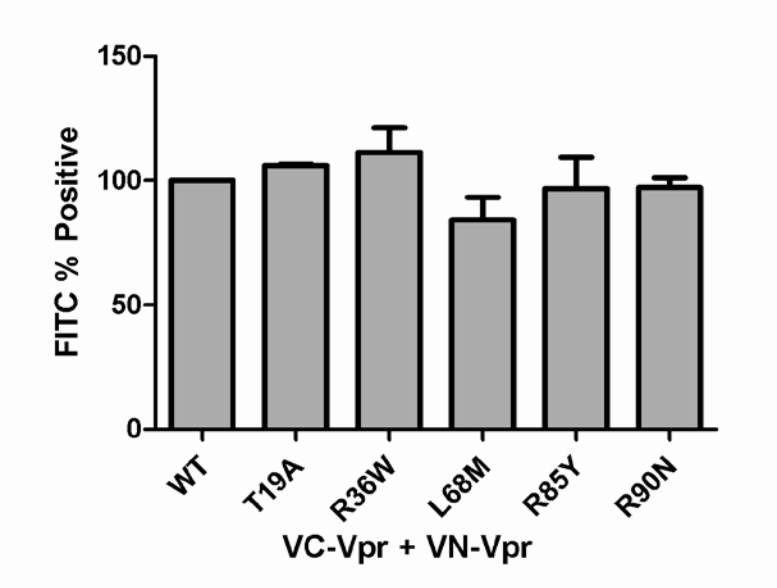


Figure 14. Percentage of Transfected Cells with VC-Vpr and VN-Vpr Mutants Does Not Change across Samples. All samples are normalized to the wt control, which is considered as 100%. Values are averages of three independent experiments (n=3). Error bars represent SEM.

The values in Table 2 represent the non-normalized FITC-positive percentages and MFIs. Note that the negative vector control showed 0% positive cells and a much lower MFI than the mutants, indicating that this assay was specific for VC-Vpr and VN-Vpr interaction.

Table 2. Oligomerization of Vpr Mutants Expressed as Non-Normalized FITC % and MFI

Cotransfection of Vpr Mutants		FITC % Positive Mean ± SEM	Mean Fluorescence Intensity
Vector		0 ± 0	229 ± 111.6
VC	**VN**		
wt	wt	41.83 ± 8.81	18805 ± 616.9
T19A	T19A	44.27 ± 9.24	20923 ± 1934
R36W	R36W	45 ± 6.29	27362 ± 1561
L68M	L68M	36.63 ± 9.96	19309 ± 3852
R85Y	R85Y	42.73 ± 12.7	19045 ± 1158
R90N	R90N	41.37 ± 10	16573 ± 1531

4.1.5 Subcellular Localization

The ability of Vpr to localize to the nucleus enhances infection of macrophages and T cells, presumably by enhancing translocation of the preintegration complex through the nuclear membrane [42, 44, 45, 71]. A previous study found a polymorphism in a Vpr isolate from an LTNP patient in the third helical domain, F72, mutated to leucine, which is unable to localize to the nucleus [63].

However, the small sample sizes (n=1-7) of this data limits the conclusions that can be made about the correlation between nuclear localization of Vpr and disease progression. The study herein compares both RP-associated polymorphisms and LTNP-associated polymorphisms in Vpr, to circumvent this problem. The selected BiFC-Vpr mutants were transfected in HEK293T cells and which were then fixed and stained with DAPI for visualization of nuclei before viewing with microscopy. The results corroborate previous findings of wild type Vpr localizing to the nucleus (Figure 15). The LTNP-associated mutations T19A, and R90N, showed no change in the pattern of distribution in the cell from wild type Vpr, also localizing to the nucleus. In contrast, the RP mutations Vpr R36W, and R85Y did show differences from wild type Vpr. Vpr R36W localized throughout the nuclei and cytoplasm, and formed puncta as opposed to the diffuse pattern as seen in wild type Vpr (Figure 16). While Vpr R85Y mainly localized to the nucleus, the FITC signal also showed a fainter distribution into the cytoplasm, indicating an increased shuttling outside of the nucleus. As expected given the conservative change of the leucine to a methionine, Vpr L68M did not show any changes in subcellular localization.

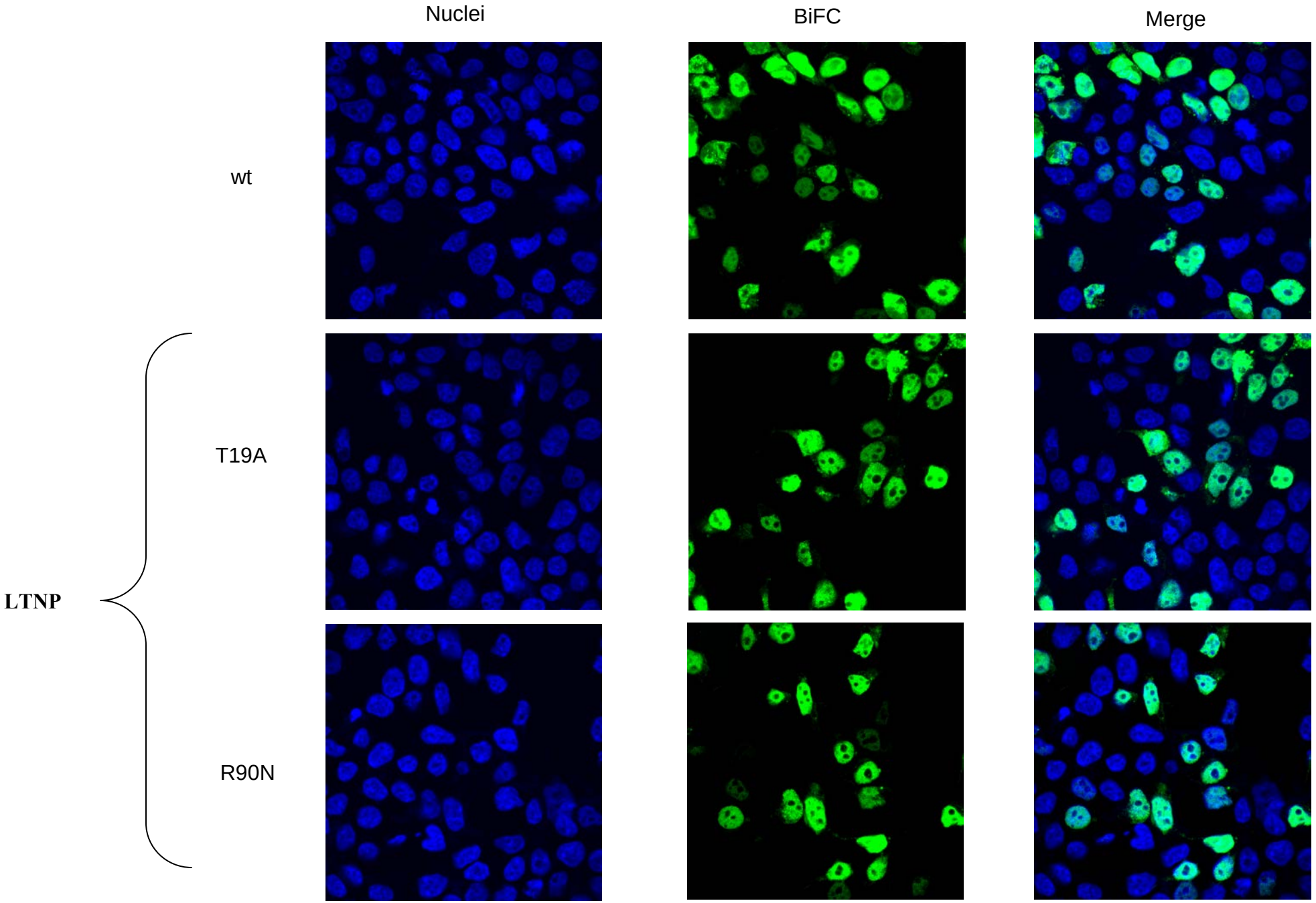


Figure 15. Subcellular Localization of wild type Vpr and LTNP Vpr mutants. HEK293T cells transfected with the indicated BiFC Vpr mutants were visualized at 100X magnification. Blue, DAPI; green, FITC.

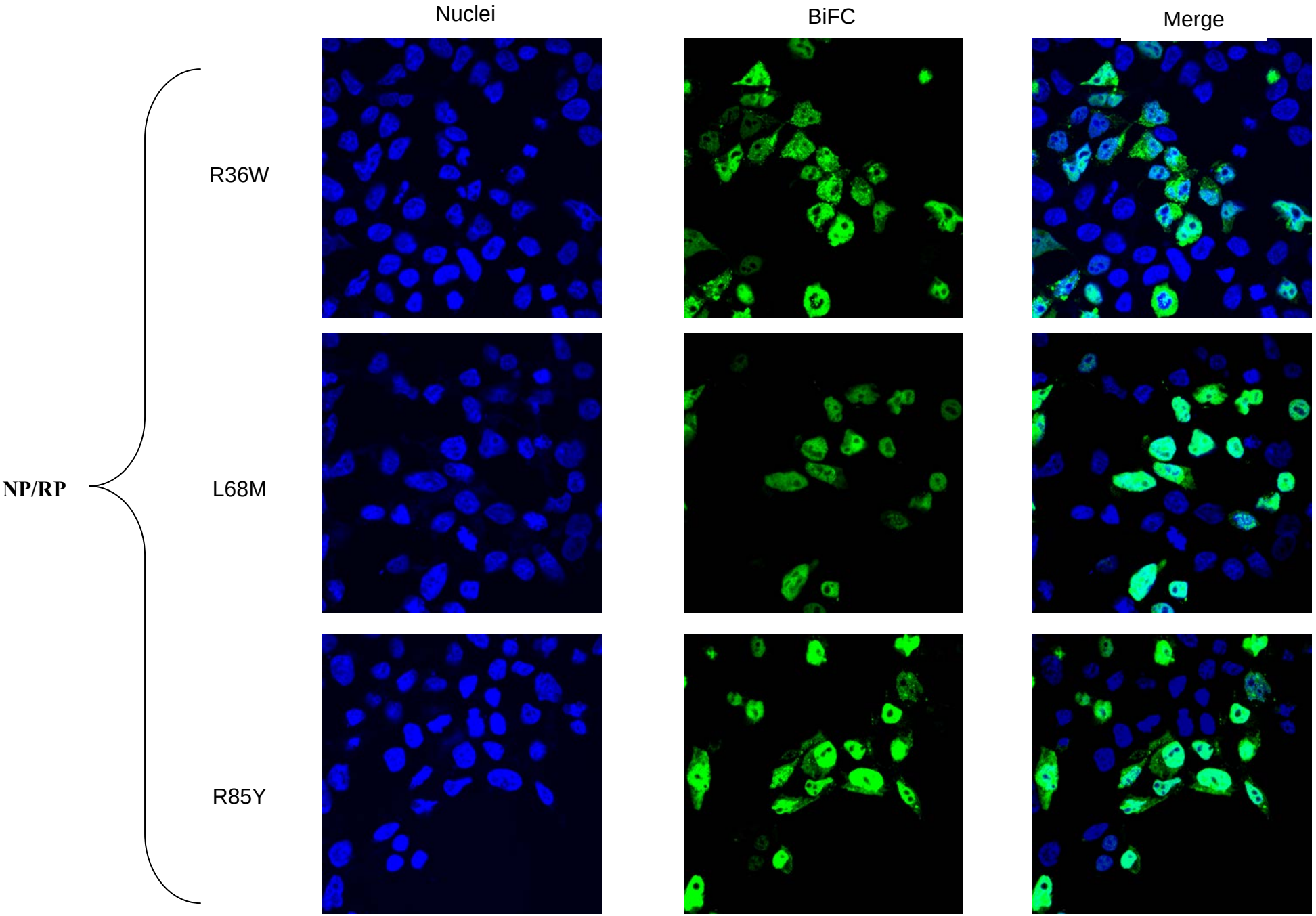


Figure 16. Subcellular localization of progressor Vpr mutants. HEK293T cells transfected with the indicated BiFC Vpr mutants visualized at 100X magnification. Blue, DAPI; green, FITC

4.1.6 Interaction between Gag and Vpr

Given previous findings demonstrating that oligomerization of Vpr is essential to interaction with the Gag capsid for incorporation of the former into the virion [36, 39] and the possibility that altered nucleocytoplasmic shuttling would affect the interaction with Gag as the Gag protein mainly localizes to the cellular membrane, the interaction of the Vpr mutants and Gag was analyzed using BiFC. The VC-Gag and VN-Gag constructs were cotransfected with the complementary VN-Vpr and VC-Vpr mutants and analyzed by flow cytometry for the interaction with Gag. As with the previous analysis, the MFI values and percentages of FITC-positive cells for each transfection were normalized to the wild type Vpr positive control to reduce the variability between the experiments. There was no significant change observed in the interaction between any of the mutant Vpr and Gag (Figure 17).

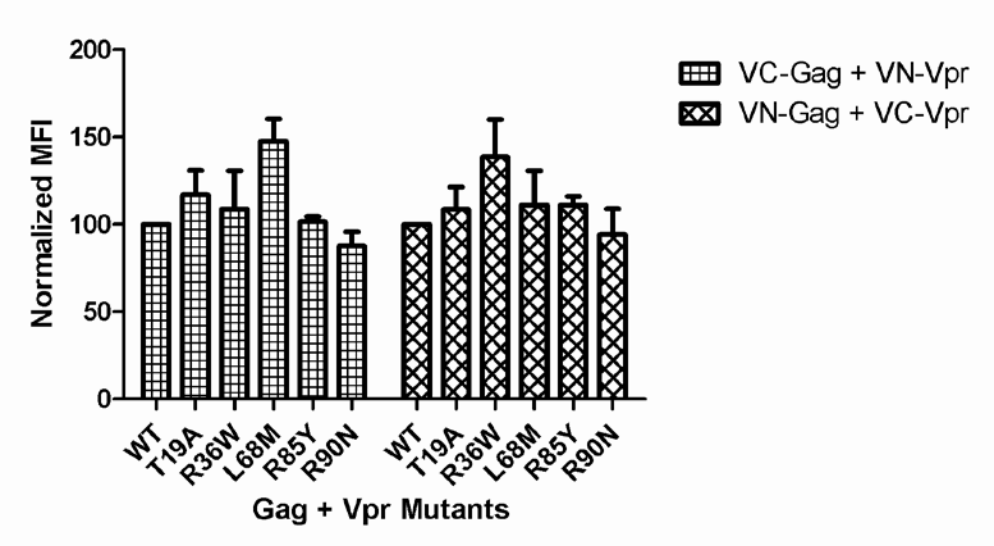


Figure 17. Interaction between Gag and Vpr. The interaction between Gag and Vpr was assessed by BiFC via flow cytometry. MFI values were normalized to wild type and expressed as a percentage. The data represent three independent experiments (n=3). Error bars represent SEM.

In Figure 17 indicates that when VN-Vpr L68M is cotransfected with VC-Gag the normalized MFI is 47.63% higher than with VN-Vpr wild type with VC-Gag, and this comparison reached p=0.0651. However, this difference was not observed in the complementary cotransfection of VC-Vpr L68M with VC-Gag. The same observation is made with cotransfection of VN-Vpr R36W and VC-Gag. Given the results overall, these mutants do not affect the interaction between Vpr and Gag.

To ascertain whether the lack of difference is due to variable transfection efficiency masking any changes, transfection efficiency was assessed. The normalized percentages of FITC-positive cells were analyzed across all samples and compared to the Vpr wild type control for each complementary set of cotransfections. There was also no significant difference in the percentages of FITC-positive cells indicating equal transfection efficiencies (Figure 18).

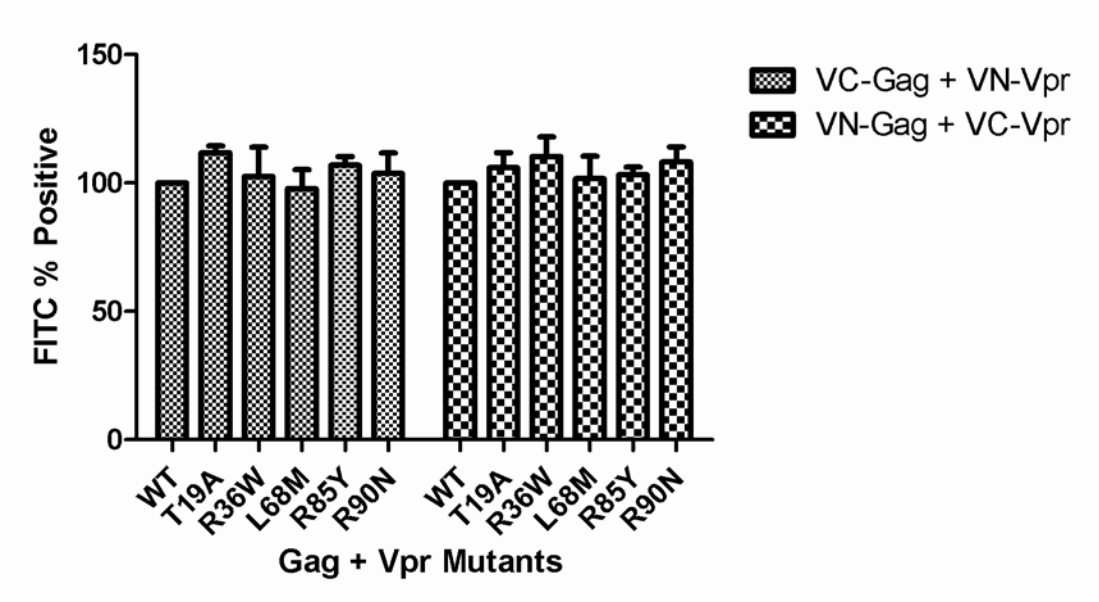


Figure 18. Percentage of Cells Transfected with Gag and Vpr Mutants Does Not Change across Samples. All samples for were normalized to the WT control (considered as 100%) for their respective groups. The data represent three independent experiments (n=3). Error bars represent SEM.

The non-normalized values are reported in Table 3. The negative vector control showed no FITC-positive cells and the MFI value was at 200, an order of magnitude lower than the

samples. This indicated the specificity of the BiFC system to detect the interaction between the Vpr mutants and Gag.

Table 3. Interaction of Gag and Vpr Expressed in Non-Normalized FITC % and MFI

Cotransfection of Vpr Mut.+Gag		FITC % Positive Mean ± SEM	Mean Fluorescence Intensity
Negative control		0 ± 0	200 ± 60.17
VC	**VN-Vpr**		
Gag	wt	41.6 ± 4.45	4805 ± 1354
Gag	T19A	46.4 ± 4.42	5795 ± 1851
Gag	R36W	43.27 ± 8.46	5798 ± 2576
Gag	L68M	40.73 ± 5.31	7181 ± 2088
Gag	R85Y	44.23 ± 3.5	4825 ± 1278
Gag	R90N	38.93 ± 6.97	4281 ± 1279
VC-Vpr	**VN**		
wt	Gag	46.03 ± 5.502	22618 ± 4496
T19A	Gag	48.23 ± 4.288	25172 ± 6423
R36W	Gag	50.37 ± 5.668	33224 ± 10139
L68M	Gag	46.1 ± 3.974	26395 ± 7929
R85Y	Gag	47.27 ± 4.987	25220 ± 5221
R90N	Gag	49.27 ± 4.099	21498 ± 5607

4.1.7 G2 Cell Cycle Arrest

As previously discussed, the function of Vpr to induce G2 cell cycle arrest has been well-characterized. Vpr acts through the ATR-p21 pathway, presumably using the DCAF1-ubiquitin complex as an initiator of the signaling events necessary to halt the cell cycle at G2/M phase. This effect enhances the transcription of the viral genes from the long terminal repeat promoters [47].

It is unclear what effect these LTNP- and RP-associated mutations in Vpr would have on the induction of G2 cell cycle arrest. To analyze this, HEK293T cells were transfected with the VC-Vpr and VN-Vpr mutants, fixed, and stained with propidium iodide to assess the cell cycling

profiles of the samples. The gating strategy is illustrated in Figure 19. Using FSC-H and FSC-A, the single-celled populations were analyzed in isolation as the presence of aggregated cells would prevent distinguishing the cell cycle profile in subsequent steps. Out of the singlets, the highly FITC-positive cell population was selected for analysis as the presence of G2 cell cycle arrest was most evident in that gate (Figure 19, right panel).

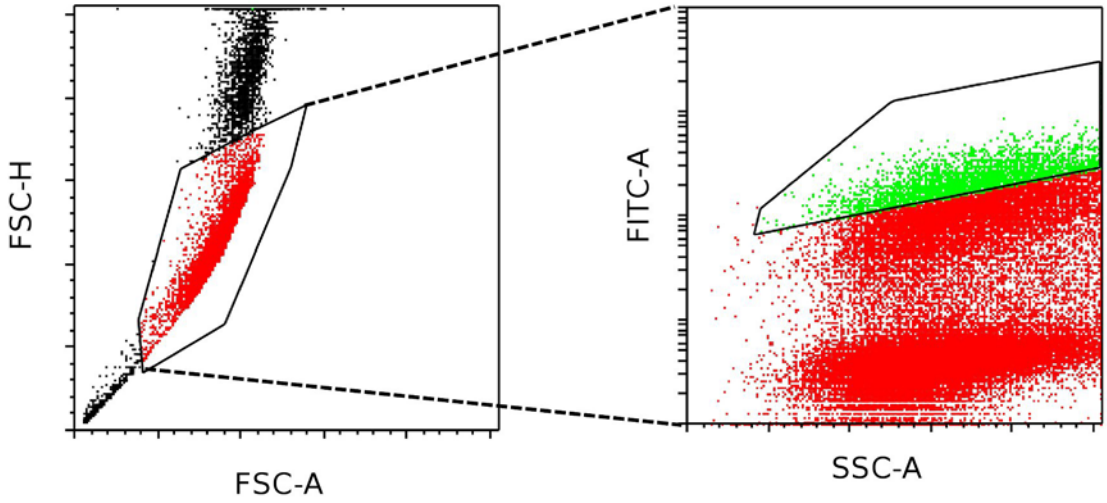


Figure 19. Gating Strategy for Cell Cycle Analysis. HEK293T cells transfected with BiFC wild type Vpr and Vpr mutants were analyzed by flow cytometry and gated for singlets (left panel). Subsequently, a subpopulation of these cells that were highly FITC positive were selected for cell cycle analysis (right panel), except for the vector control.

Following the gating operations, the gated populations were then analyzed for their cell cycle profiles. The data were analyzed using a Gaussian algorithm to fit the cell cycle peaks to their proper distributions (Figure 20). In the cells transfected with the negative vector control, a relatively low proportion of cells is present in the G2 phase; however, when transfected with wild type Vpr, the cells show a much higher level of cells in the G2 phase. This is indicative of the induction of G2 cell cycle arrest.

The results in Figure 20 clearly show a defect in the induction of G2 cell cycle arrest in two of the three RP-associated mutations, R36W and L68M, which appear to have a low

proportion of cells present in the G2 phase, similar to the negative vector control. Although these qualitative results do suggest a drastic decrease in the G2 arresting capability of R36W and L68M, the differences in the LTNP-associated mutations are less clear.

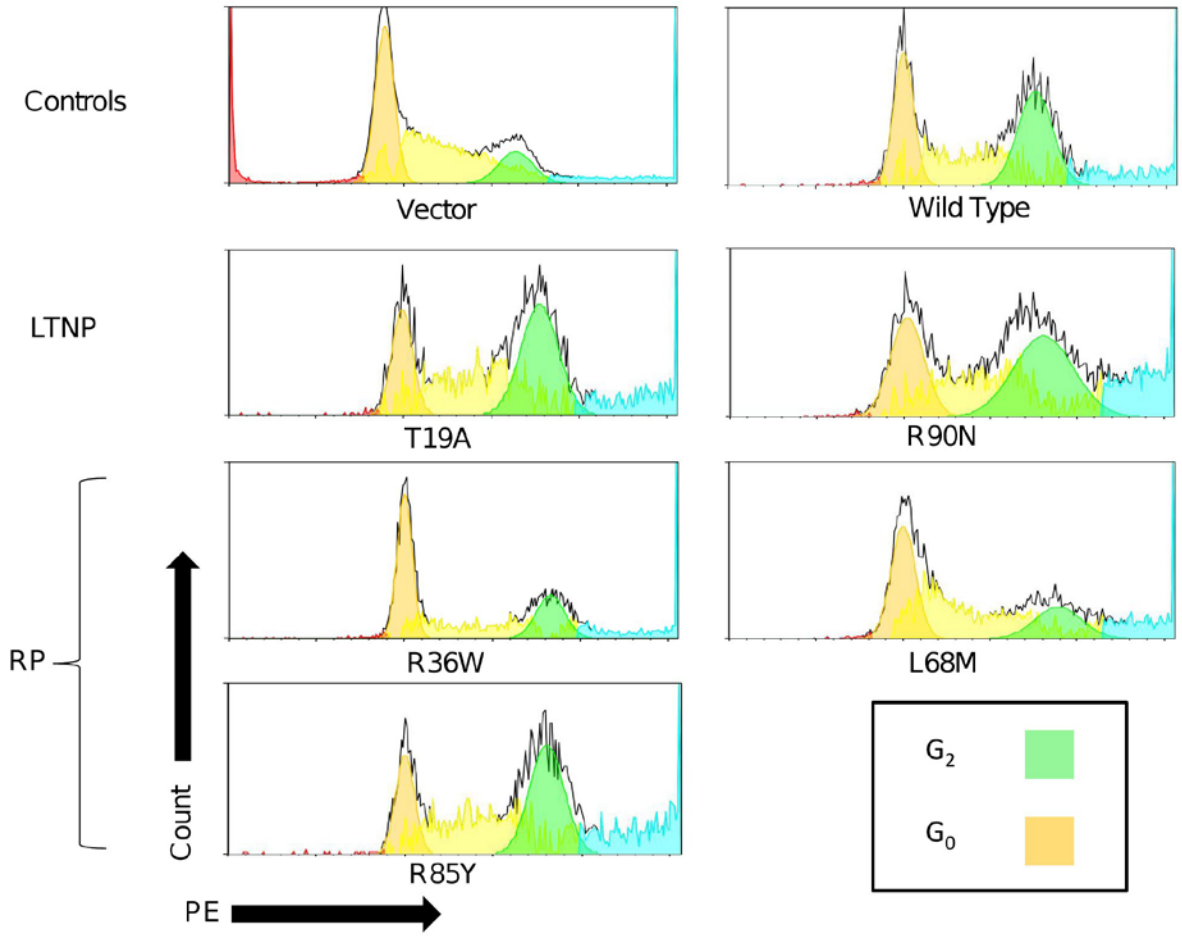


Figure 20. Cell Cycle Analysis of Single Representative Experiment. The histograms represent the results of one single experiment of three independent experiments overall.

To quantify the data, the ratio of the percentage of cells in the G2 phase to the percentage of cells in the G0 phase for each sample was compared to wild type. Across three independent experiments, the G2/G0 ratio for the cells transfected with the RP-associated mutants R36W and L68M both showed a highly significant decrease in the G2/G0 ratio to levels less than half that

of wild type Vpr and similar to the negative vector control (p=0.0012 for Vpr R36W and p=0.0028 for Vpr L68M). There was no significant difference in the G2/G0 ratio for Vpr R85Y from wild type.

For the LTNP-associated mutants, Vpr T19A and R90N both showed modest increases in the G2/G0 ratios. The difference between wild type Vpr and mutant T19A reached significance with p=0.459. The increase observed in Vpr R90N compared to wild type approached significance with with p=0.0989.

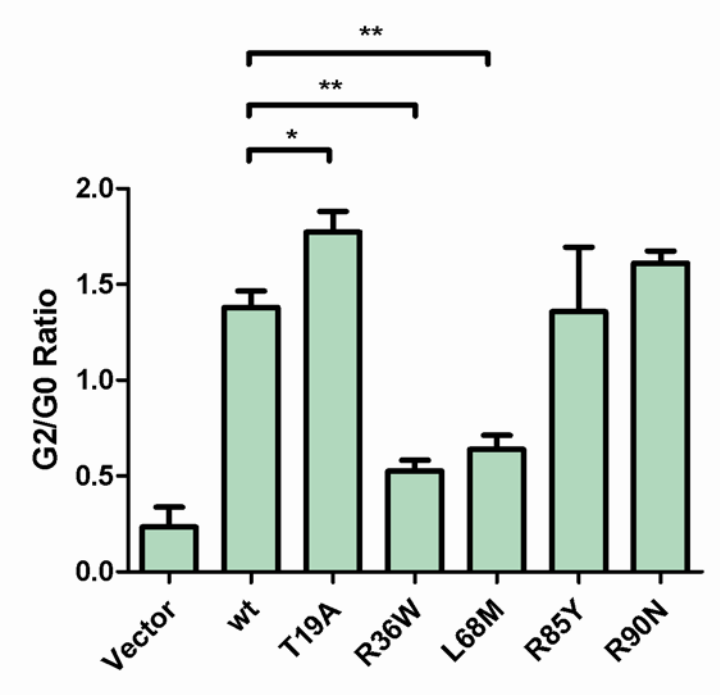


Figure 21. Vpr Mutations Alter G2 Cell Cycle Arrest. The ratio of the percentage of cells in the G2 phase to the percentage in the G0 phase are represented and indicate the level of G2 cell cycle arrest. The data represent three independent experiments (n=3). Error bars represent SEM. * indicates $p<0.05$; ** indicates $p<0.01$.

Interestingly, the results overall indicate that the level of G2 arrest is slightly increased for the two LTNP-associated mutations studied, T19A and R90N. In contrast, two of the three RP-associated mutations are almost completely defective in their capacity to induce cell cycle blockade. The results suggest an opposite association from the expectation that the RP-associated

mutations would be more likely to result in higher levels of G2 arrest. A higher level of G2-cell cycle arrest would be expected to allow the virus to replicate to a higher extent while a lower level would stall virus replication as shown by previously discussed studies. This implies that the level of G2 arrest must be regulated for the virus to replicate at optimal levels while evading immune responses, or that this feature may have different roles at the various stages of disease progression.

4.1.8 Aim 1 Conclusions

Several signature polymorphisms in the amino acid sequence of Vpr were identified from a set of LTNP and RP sequences obtained from the HIV database. From these sequences, the effect of these mutations on the canonical functions of Vpr were studied through an *in vitro* overexpression system that made use of bimolecular fluorescence complementation as a reporter system. From this system, while there were interestingly no changes observed in Vpr interaction with Gag, one or more of these polymorphisms alter the functions of Vpr studied herein: oligomerization, subcellular localization, and G2 cell cycle arrest.

The RP-associated Vpr R36W shows substantially higher levels of oligomerization compared to wild type Vpr. The localization of this mutation is found throughout the cell as opposed to the localization of wild type Vpr, which is located mainly in the nucleus. It also is deficient in the induction of G2 cell cycle arrest. The other RP-associated mutations selected in this analysis, L68M and R85Y showed changes in one of these functions. The former was also defective in G2 cell cycle arrest while the latter showed nuclear localization but with a presence in the cytoplasm, in contrast to wild type Vpr which is entirely nuclear.

The LTNP-associated mutations did not show any difference from the NL4-3 wild type Vpr in oligomerization or subcellular localization as the RP-associated mutations did. However, both T19A and R90N showed slight increases in their induction of G2 cell cycle arrest over wild type. These changes indicate that there are possible functional correlates of Vpr to disease progression in LTNP and RP groups.

4.2 AIM 2: CHARACTERIZE THE EFFECTS OF LTNP- AND PROGRESSOR-ASSOCIATED VPR POLYMORPHISMS ON VIRAL REPLICATION.

In the previous aim, the RP-associated polymorphisms alter Vpr function in several of its established aspects. In contrast the LTNP-associated polymorphisms, show only slight changes in these functional assays. Although the RP-associated mutants functionally alter Vpr in an *in vitro* overexpression system, their effect on overall fitness of the virus is unknown. To analyze the effect of these mutants in a more relevant system, the current aim is to introduce these mutants into the proviral genome and study the effect on viral replication in PBMCs.

The previous aim has identified RP-associated mutations R36W, L68M, and R85Y significantly changing Vpr function. The R36W mutant shows higher levels of oligomerization, distribution throughout the cell as opposed to nuclear localization as seen in wild type Vpr, and its capacity to induce G2 cell cycle arrest is abolished. The L68M mutant is also unable to induce G2 cell cycle arrest to a similar degree as R36W. The final mutation, R85Y, is shown to be altered in its nucleocytoplasmic shuttling, showing higher levels of Vpr outside of the nucleus. These functions potentially have major effects on the fitness of the virus, as previous studies have shown (see sect. 1.2.2.1). The previous evidence indicates all three of these mutants more

likely to affect overall fitness of the virus; thus these mutations were selected for the subsequent assays.

On the other hand, the LTNP-associated mutations, T19A and R90N only exhibit slight changes in the induction of G2 cell cycle arrest in which the levels are shown to be somewhat higher than wild type Vpr. This increase only reached significance for the T19A mutant. Neither of these mutations are altered in the other functions of Vpr from wild type. Because these mutants show very little change from wild type in the canonical functions of Vpr, the T19A and R90N polymorphisms were excluded from further analysis.

To avoid eliminating the study of LTNP mutations altogether, another Vpr polymorphism established in the literature as likely associated with LTNP status was selected in place of T19A and R90N. The polymorphism Vpr R77Q has been associated with LTNPs in two studies, and has shown significantly lower cytopathicity compared to wild type in an *in vitro* system [60, 62]. However this mutation, although it has been demonstrated to have reduced cytopathicity compared to wild type Vpr, has not been studied in the context of a more relevant primary cell model, so this polymorphism was selected for the subsequent assays.

4.2.1 Cloning Vpr mutants into Proviral Constructs for Virus Packaging

Study of the RP-associated mutants Vpr R36W, L68M, and R85Y and the LTNP-associated Vpr R77Q required insertion into a proviral construct expressing the NL4-3 genome and an EGFP reporter. Constructing the mutant proviral reporter construct, pNL4-3 EGFP IRES, uses a multi-step cloning strategy, first inserting the fragment containing the 5' and 3' halves of Vpr into separate shuttle vectors, introducing the mutations into the shuttle vectors, and then inserting the mutated fragments into the original construct (see Figure 7 for illustration).

To generate Vpr R36W into the proviral construct, the isolated *SphI-EcoRI* fragment from pNL4-3 containing the 5' half of Vpr (Figure 22, lane 1) was inserted into shuttle vector pUC19 (Figure 22, lane 2). This insertion was confirmed by restriction digest with *EcoRI* to linearize the fragment and measure the size of the band (~6.4 kilobasepairs) (Figure 22, lanes 3 & 4). The mutation Vpr R36W was introduced by restriction digest using a previously mutated vector with the restriction sites *PflMI* and *EcoRI*. Subsequently the mutated fragment was inserted into the pNL4-3 EGFP IRES vector using *SphI* and *EcoRI*. Upon restriction digest with *PflMI* and *EcoRI*, four out of the five clones exhibited the correct banding pattern, confirming the success of the ligation (Figure 22, right panel). As a final verification, the constructs were sequenced to confirm the presence of the mutation and the specificity of the mutagenesis reaction. No mutations other than the specified Vpr R36W occurred in any of the clones.

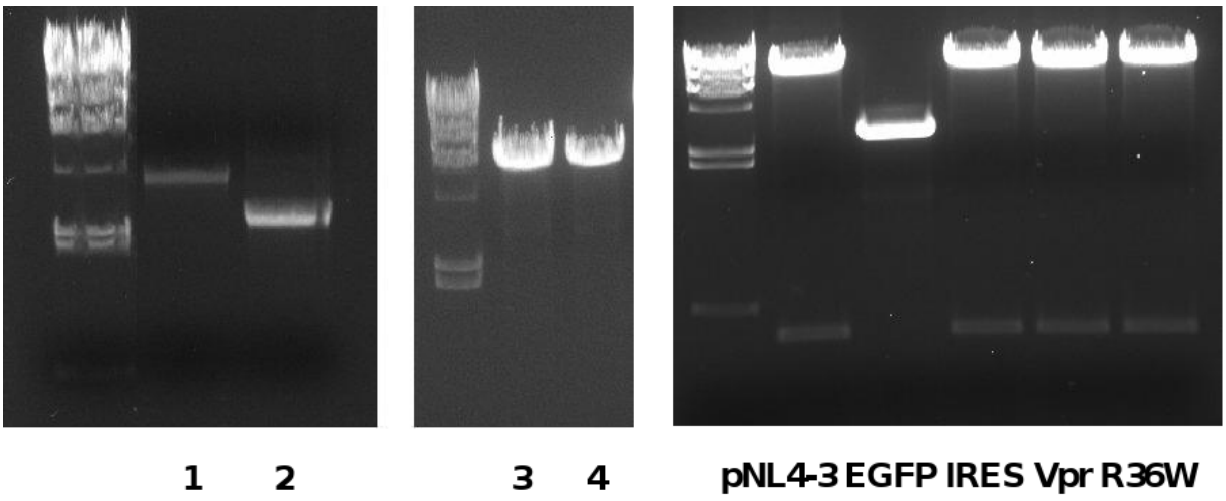


Figure 22. Cloning Vpr R36W into pNL4-3 EGFP IRES. Lane 1, *SphI-EcoRI* fragment isolated from pNL4-3 EGFP-IRES; lane 2, pUC19 linearized with *SphI* and *EcoRI*; lanes 3 & 4, *SphI-EcoRI* fragment from pNL4-3 inserted into pUC19 and linearized by *EcoRI*. Right panel, confirmation of four out of five clones by restriction digest of pNL4-3 EGFP IRES Vpr R36W clones using *PflMI* and *EcoRI*.

Generating Vpr L68M, R77Q, and R85Y required the insertion of the 3' half of Vpr into the pCDNA3 shuttle vector which contained a unique *NheI* site added by PCR using sites *EcoRI-*

NheI. The pCDNA3+*NheI* shuttle vector (Figure 23, lane 1) was linearized by digestion with *EcoRI-NheI* and the *EcoRI-NheI* fragment from pNL4-3 EGFP IRES was isolated (Figure 23, lane 2). The latter was then inserted into pCDNA3+*NheI* and each mutation, Vpr L68M, R77Q, and R85Y was generated via site-directed mutagenesis. The clones were then sequenced to verify the specificity of mutation. The correct mutations were confirmed and the mutated fragments were inserted into the pNL4-3 EGFP IRES vector using *EcoRI-NheI* sites. This insertion was restriction digested by *EcoRI* and *NheI*, yielding the proper-sized fragment for all mutants (Figure 23, lanes 3-8). These reconstructed mutants were then sequenced to verify the integrity of DNA. No extraneous mutations were found outside of the specified Vpr L68M, R77Q, and R85Y mutations.

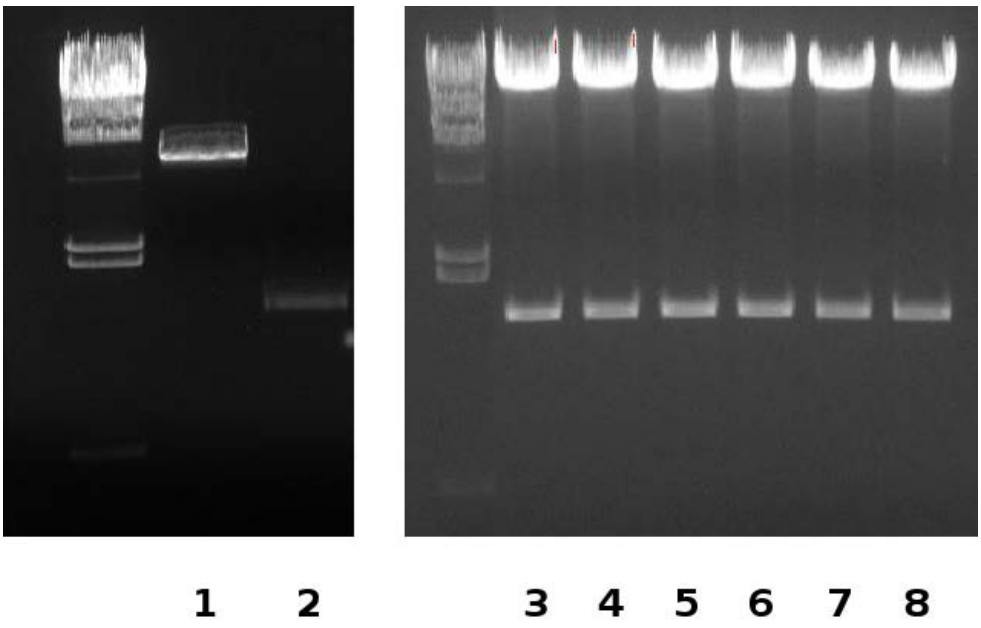


Figure 23. Cloning Vpr L68M, R77Q, R85Y into pNL4-3 EGFP-IRES. Lane 1, pCDNA3+*NheI* linearized with *EcoRI* and *NheI*; lane 2, *EcoRI-NheI* fragment isolated from pNL4-3 EGFP-IRES; lanes 3-8, pNL4-3 EGFP-IRES mutants restriction digested with *EcoRI* and *NheI* for confirmation; lanes 3 & 4, pNL4-3 EGFP IRES Vpr L68M; lanes 5 & 6, pNL4-3 EGFP IRES Vpr R77Q; lanes 7 & 8, pNL4-3 EGFP IRES Vpr R85Y.

4.2.2 Characterizing Mutant Proviral Constructs

The first set of assays on the viral genomes is to determine the viral titers in terms of p24 and the number of infectious particles and also ensure the expression of the viral proteins in the mutants generated. This is analagous to the expression analysis on Vpr mutations in Aim 1 (sect. 4.1.3). In the case that the subsequent assays show altered viral fitness, these assays can provide an explanation if the virus produced is defective or enhanced.

Each mutant proviral construct (pNL4-3 EGFP-IRES Vpr R36W, L68M, R77Q, R85Y) was packaged in HEK293T cells using transfection to express the virus. The supernatants were subjected to p24 ELISA, and the infectious particles were titrated by a TZM-bl ß-gal activity assay. The p24 and infectivity titers indicate a trend of a reduced ratio of infectious particles per ng p24 for all the mutants compared to wild type virus (Table 4). Interestingly, this reduction of infectious units is similar to the ΔVpr mutant virus which does not express Vpr (included in subsequent analysis as a control), which shows a lower ratio compared to wild type virus as well.

Table 4. p24 and Infectious Titers of Mutant Virus.

NL4-3	p24 (ng/ml)	TZM-bl titer (Infectious Particles per ml)	Infectious Particles per ng p24
Vpr wt	368.7	2.6×10^6	7051.80
ΔVpr	747.6	2.9×10^6	3879.08
Vpr R36W	907.3	2.7×10^6	2975.86
Vpr L68M	926.8	2.7×10^6	2913.25
Vpr R77Q	1330	1.5×10^6	1127.82
Vpr R85Y	615	1.15×10^6	1869.92

The expression of viral proteins was measured by immunoblot on HEK293T supernatants from cells transfected with the proviral mutant constructs for packaging virus (Figure 24).

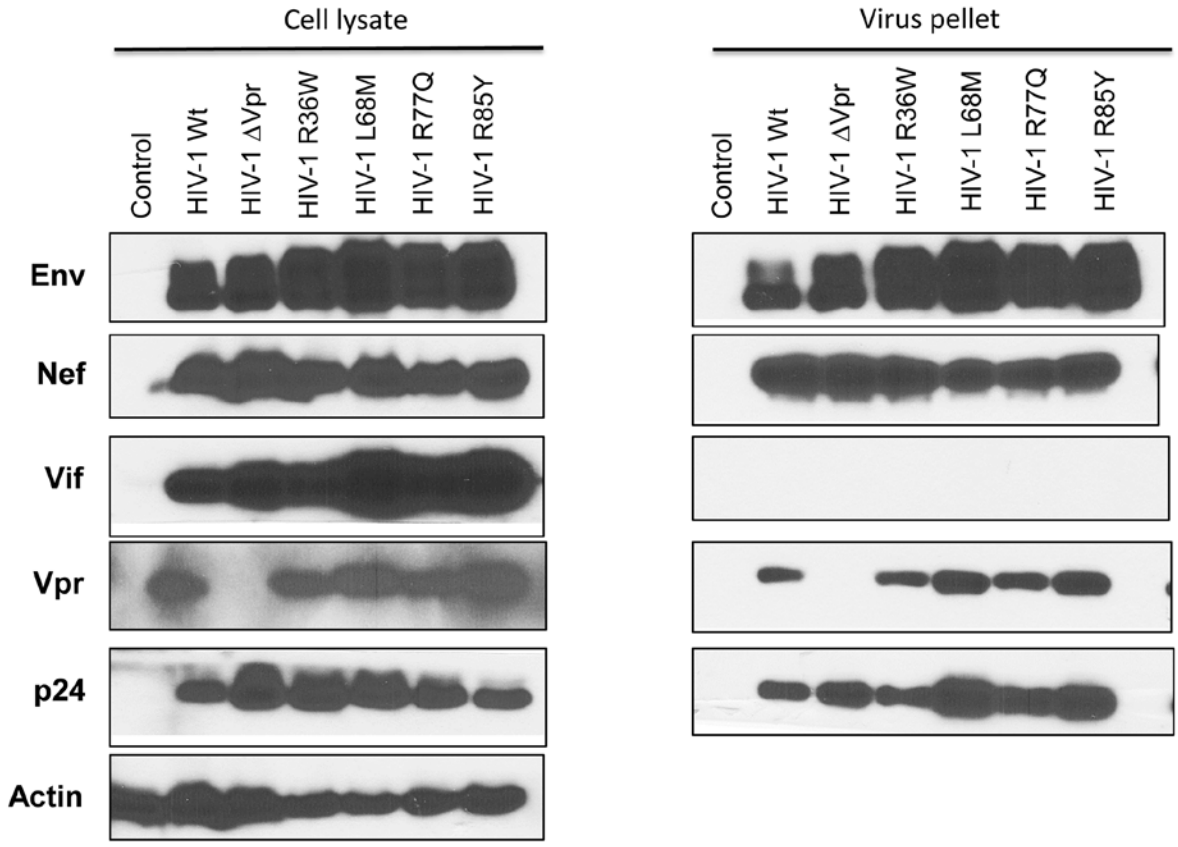


Figure 24. Expression of Viral Proteins in HEK293T Cells.

The results indicate that the virus containing Vpr mutants are not defective for expression of the essential proteins, indicating the success of the cloning strategy to introduce polymorphisms into Vpr and the intact infectivity of the virus necessary for the subsequent assay.

4.2.3 Assessing the Infectivity of Mutant Virus in PBMCs

To assess the overall viral growth of the mutants, PBMCs isolated from three healthy donors were infected with mutant virus at an MOI of 0.1 using the TZM infectivity titers in Table 4. The

supernatants were harvested at days 0, 3, 6, and 9 and were subjected to p24 ELISA to test for replication kinetics.

The ELISA results indicate that there are differences in the replication kinetics between the mutants in the individual donors. Mutant virus carrying the LTNP-associated Vpr R77Q mutation showed slower replication kinetics in two of the three donors compared to wild type virus. Interestingly, the RP-associated mutant virus HIV-1 Vpr R85Y also showed slower replication kinetics compared to wild type in these same two donors. However, when the data were combined across all three donors, there were no significant differences in replication kinetics between the wild type virus and the mutant viruses (Figure 25). This is mainly due to the large error due to the variability between donors, as the replication kinetics follow different time courses for each individual's PBMCs. For example, the values Donor 63 at day 9 range from 20-60 ng/ml while Donor 64b ranges from 10-30 and Donor LW ranges from 12-20.

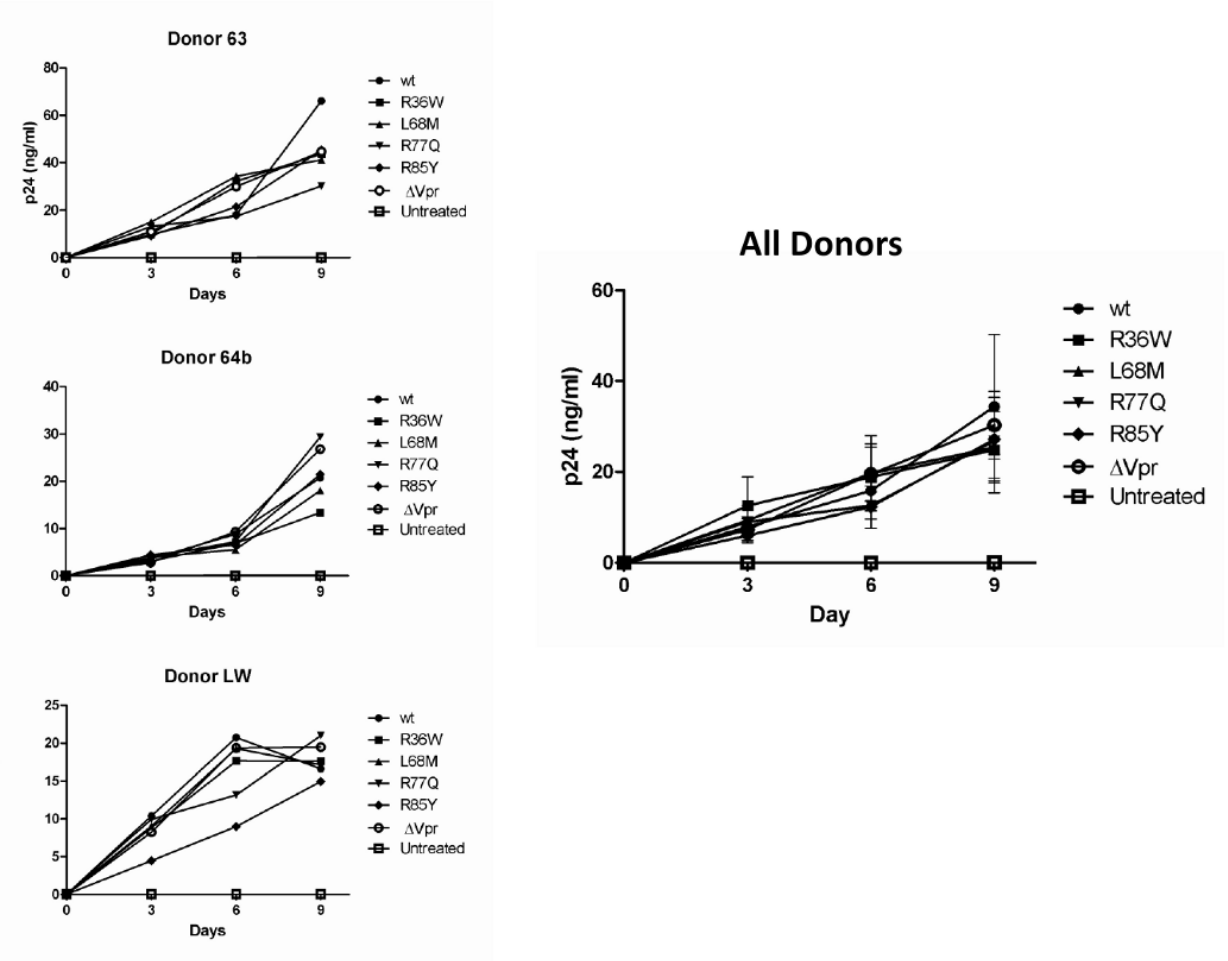


Figure 25. Replication Kinetics of Mutant Virus Assayed by p24 ELISA. Supernatants collected at 3 day intervals from infected PBMCs across three donors were subjected to p24 ELISA. Data from each donor are represented individually in the left panel. The data were concatenated and the SEM was calculated for all three donors. Note that the large SEM is mainly due to the values on the Y-axis (p24) for each individual donor graphing at a different scale.

The previous studies from this lab have generally shown peak infection of PBMCs at day 6 which was the endpoint of this assay. To test for differences in the population of cells infected with virus, the PBMCs from a representative donor was analyzed through flow cytometry to measure the level of EGFP-expression, the reporter for successful infection of a cell. At day 6 PBMCs of one representative donor of the three were harvested and analyzed through flow cytometry to measure the percentage of infected cells in the PBMC population. At day 6 there

were no large differences in the percentage of PBMCs infected with the viral Vpr mutants compared to PBMCs infected with wild type virus (Figure 26), indicating that the infected population of PBMCs is relatively stable at the peak of infection that time point and that mutation in Vpr does not change the proportion of HIV-1 producer cells.

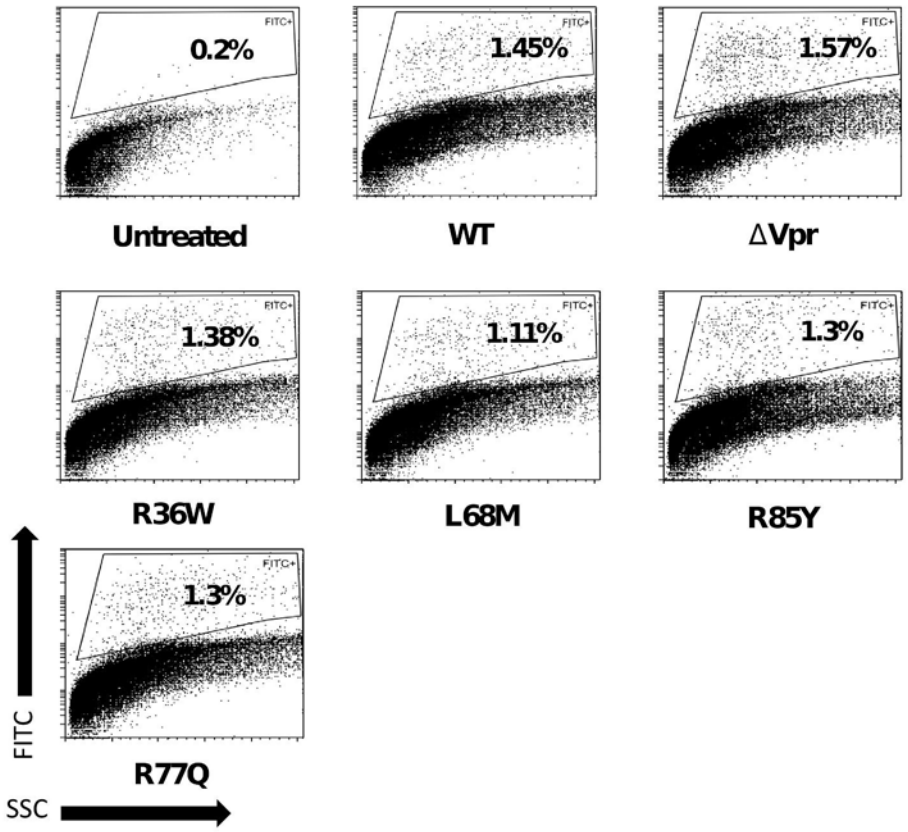


Figure 26. Percentage of Infected PBMCs in Representative Donor on Day 6 of Infection.

4.2.4 Aim 2 Conclusions

The results in Aim 1 demonstrated the effect of LTNP- and RP-associated mutations on Vpr function. The objectives of Aim 2 are to establish any effect these Vpr mutations have on the virus itself. After cloning each mutation into an NL4-3-based proviral construct containing an EGFP reporter, there is evidence of a change in the replication potential of LTNP-mutant virus Vpr R77Q and RP-mutant virus Vpr R85Y in PBMCs from two of the three donors. Interestingly, both of these viruses showed lower replication potential compared to wild type Vpr.

5.0 DISCUSSION

The HIV protein Vpr exhibits diverse sequence variation characteristic of all proteins expressed by the lentivirus. It has a major role in the viral life cycle, enhancing infection in macrophages, inducing G2-cell cycle arrest and cytopathicity in T-cells. These features of Vpr indicate the functions of this protein play a role in pathogenesis *in vivo*. The presence of the different patterns of disease progression in the HIV-infected population, as seen in long-term non-progressors rapid progressors, indicate a possible correlation between the sequence variation of Vpr and AIDS.

Polymorphic variation in *nef*, another accessory gene of HIV-1, is associated with LTNPs in a number of studies [72, 73]. An interesting study of the *env* gene showed that a distinct polymorphism in the amino acid sequence is associated with changes in viral infectivity and can result in the shift from acute to chronic infection [74]. These associations between these two genes and disease progression prompted the hypothesis that the presence of a signature polymorphisms in the Vpr molecule. As described above, Vpr itself has a role in pathogenesis, and the presence of such disease-progression associated mutations may lead to functional changes of Vpr itself. This in turn would likely affect virus replication in target cells and pathogenesis of the virus overall.

The numbers of sequences used in the mentioned association studies of Vpr (see sect. 1.3) generally use a limited sample size (n=1-10) which makes statistical analysis difficult for the

identification of signature sequences linked to disease progression. The present study circumvents this issue by use of the HIV-1 Vpr sequences available through the Los Alamos National Labs HIV sequences database. A preliminary phylogenetic analysis of Vpr sequences categorized as originating from LTNPs or progressors (normal and rapid) showed that the LTNP sequences mostly clustered separately from the progressor sequences. To compare Vpr with Nef, both accessory proteins which are determinants of viral pathogenesis, a preliminary phylogenetic analysis of Vpr and Nef sequences revealed a clear clustering of LTNP and RP sequences into their respective groups. Such a clustering pattern of LTNP and RP was also observed with Vif and Tat protein sequences. However, there are several limitations from the phylogenetic analysis. Although this data shows an association of LTNP and RP groups with the protein sequences isolated from the respective clinical cohorts and although a majority of these sequences originate from subtype B, the subtype information is unavailable for a number of these sequences or come from subtype C or D. In addition, the status of disease progression from the use of the categorical search is not available for most sequences, even though they may contain this information within the accession tags. As such this crude analysis of Nef and Vpr protein sequences serves as an initial study. As a result we chose to focus on sequences originating from subtype B with several further criteria and manually culled LTNP and RP sequences as defined from the accession descriptions rather than the categorical search. Choosing only Vpr alleles from subtype B removes the confounding differences that can occur with analysis of sequences from multiple subtypes [61] which serves to bolster any identified associations.

Several of the characterized functions of Vpr served as a starting point to study these mutations, namely, the oligomerization of Vpr, the localization of Vpr to the nucleus, and the

induction of G2 cell cycle arrest. These functions should show changes between the LTNP and RP mutants.

The RP-associated mutation Vpr R36W shows a higher level of oligomerization compared to wild type. Its location in the turn (residues 34-47) between helical domains I and II of Vpr indicate that it is likely a critical residue to maintain structure of the protein. The arginine in the wild type sequence is a positively charged basic residue. The mutation to tryptophan results in a substantial change the side chain of the amino acid, introducing a benzene structure. Given the critical location of R36 and the large change of amino acid, it is expected to observe changes in structure and function. It likely results in exposure of the hydrophobic faces of the first two helices which would lead to stronger binding of the Vpr monomers to shield the hydrophobic faces from the environment. Interestingly none of the other mutations studied showed any differences in oligomerization from the wild type Vpr. Although it is the only RP-associated mutation that showed higher levels of oligomerization out of the three studied (L68M, R85Y), it does indicate the possibility that oligomerization may have a role in pathogenesis. It was not found to interact more or less strongly with Gag; however, this functional study does not show whether the levels of packaged Vpr in the virion increase. This would need to be tested via quantitative western blot.

Although Vpr does not contain canonical nuclear localizing signal motifs in its amino acid sequence, it has been shown to contain various residues across its three alpha helices and the C-terminus which enable Vpr to translocate the nuclear membrane [46]. Wild type Vpr generally localizes to the nucleus as corroborated in our study. Comparing the mutations to this pattern, we have found differences with the RP-associated Vpr R36W and Vpr R85Y. Vpr R36W localizes across the whole cell, and exhibited the formation of puncta, as expected with the higher levels

of oligomerization. This indicates the possibility of an alteration in the nucleocytoplasmic shuttling mechanism of Vpr and the formation of higher-level oligomers. The mutation Vpr R85Y also showed diffuse localization in the cytoplasm, although it mostly remained in the nucleus. The literature reports that the nuclear localization feature of Vpr seems to enhance virus replication for both macrophages and lymphocytes, although through two different means; in the former, the localization function of Vpr itself enhances nuclear entry of the preintegration complex, and in the latter, the localization function of Vpr seems to enable Vpr to act as a transactivator [43-45] The mechanism of nuclear import of Vpr acts through importin-α, a host shuttling protein. The study raises questions on the effect that these mutations have on nuclear localization in the context of viral replication in primary macrophages and T-cells. The difference in the pattern of localization of Vpr R36W and R85Y implies that perhaps two separate mechanisms are involved.

The ability of Vpr to induce G2 cell cycle arrest has been characterized throughout the literature extensively, although many details of the mechanism remain unknown. Blockade of the cell cycle in the G2 phase enhances viral transcription from the LTR promoters [48, 75] in a number of cell types, including primary CD4+ T cells. It has been noted that the subcellular localization of Vpr to the nucleus is a necessary, but not sufficient, criterion of G2 arrest [76]. Our data has corroborated this claim, as the mutants Vpr R36W and L68M are deficient in this function. Although Vpr R36W is distributed throughout the whole cell and is unable to induce G2 cell cycle arrest, the nuclear localizing Vpr L68M exhibits the same loss of cell cycle blockade. Vpr R85Y does show some diffuse localization into the cytoplasm, but the majority of the Vpr signal remained in the nucleus, which can explain the intact G2 arresting function. An interesting detail is the mutation of the leucine at residue 68 to methionine. The drastic loss of

G2 cell cycle arrest is a surprising phenomenon because this is a conservative change that was not expected to show much of a difference. It is likely that this particular residue which falls in the 3rd helix leucine motif (LxxLL[68]) is a binding determinant to a cellular factor involved in the G2 arrest pathway through which Vpr acts. This motif is known to play a role in the interaction between glucocorticoid receptor (GR) and Vpr [54, 77]. However our previous unpublished data suggests that Vpr L68M does not have a role in Vpr-GR interaction, our data here imply that this mutation does play a role in the G2 cell cycle arrest pathway. Although these particular residues have not been studied for signaling pathways, helical domains I and III as well as the C-terminus contain determinants that can alter G2 cell cycle arrest, presumably through interaction of Vpr with the DCAF1-ubiquitin complex [55].

Another interesting observation is the pattern in the G2 arrest results. Both Vpr T19A and Vpr R90N, two LTNP-associated mutations, result in modestly increased levels of G2 arrest, although this trend was only approaching significance with the latter. In contrast, two of the three RP-associated mutations almost completely abolish G2 cell cycle arrest. These correlations imply that the LTNP mutations seem to be associated with higher levels of G2 arrest and RP mutations with reduced levels of G2 arrest. While these results are counterintuitive given that G2 arrest enhances viral replication and would be expected to be increased in RP mutations rather than abolished, it seems that there is a possible explanation in immune evasion. A relatively recent data suggests that the induction of G2 cell cycle arrest upregulates the natural killer cell ligand, NKG2D, on infected cells targeting them for cytolysis, and that Vpr mutants that are unable to induce G2 blockade are able to evade detection by NK cells [78]. However given the small sample size (two LTNP mutations vs. three RP mutations), a more comprehensive analysis of Vpr sequences is needed.

While the residues chosen do show significant differences between LTNP and RP, their frequencies are all <30% in either group. Perhaps it is not a single residue or a domain, but rather the changes that work collectively or compensate each other within Vpr as well as other genes that influence pathogenesis. A study into the residues that show multiple polymorphisms and significantly different frequency would be a first step in delineating the role of compensatory mutations. Furthermore, host genetics and HLA polymorphism also play a significant role in disease progression, thus disease progression *in vivo* is determined by multiple factors including Vpr polymorphism and functions. Longitudinal isolates of virus collected from LTNP and RP patients may be necessary to compare viral evolution between those who do not progress to AIDS and those who do.

Although the studies of Aim 1 do show substantial changes in the functions of Vpr, it is unclear how these functional changes correlate to the differences seen in Aim 2. The lower replication potential of the LTNP-associated Vpr R77Q and RP-associated Vpr R85Y perhaps may be correlated with changes in the individual functions of Vpr, as these two mutations seem to affect induction of apoptosis and subcellular localization, respectively. The characterized functions of Vpr indicate that these mutations act in tandem with one another. These mutations were studied in isolation, and it may be necessary to identify new methods to study compensatory mutations. As an apt example the functional phenotype observed for the Vpr R36W mutant shows that many changes result compared to wild type Vpr: greater oligomerization, localization throughout the cell, and abolition of G2 cell cycle arrest. While G2 cell cycle arrest alone enhances the production of virus and disruption of G2 arrest would more likely be associated LTNP mutations rather than RP mutation, this function may not act alone, but in concert with the other functions of Vpr. This entails that the overall phenotype of Vpr

R36W may be indicative of progression to AIDS rather than any of its functions individually. This is most likely true of all of the other mutations studied. The evolution of viral sequences longitudinally may be necessary to identify functional association with the characteristics of the virus and disease pathogenesis.

In order to more clearly delineate differences in the replication potential of these Vpr-mutated viruses, the MOI may need to be optimized. Early studies on the effect of Vpr mutation on viral replication demonstrated that differences in replication potential between mutants were only observable at a low MOI whereas, a tenfold increase in MOI resulted in no differences between wild type virus and mutants [41, 56]. The results in these studies indicate that the MOI used herein is masking a difference that these Vpr mutants may exhibit. An early report shows that a very low proportion of all viral particles produced in an infected individual is replication competent [79], indicating that a very low MOI, perhaps lower than the MOI used here, may reveal not only such differences in Vpr but also a more relevant result to overall disease pathogenesis.

A possibility is that viral replication is less affected by Vpr mutations than the cytokine production of these PBMCs. A recent study published from our lab has shown that Vpr has an effect on production of the inflammatory cytokines IL-1β and IL-8 in monocyte derived macrophages, contributing to cytotoxicity of the virus [80]. These results indicate the possibility that these LTNP- and progressor-associated mutations can differentially regulate the immune response.

The present study also only analyzed the effect of these Vpr mutations on the replication of virus in whole PBMCs, but not in the separate cell populations that are the main targets of HIV-1, macrophages and $CD4^+$ T cells. The function of Vpr is clearly different in these two cell

types as the role of Vpr in macrophages appears to be as an anchor for the preintegration complex to traverse the nuclear membrane and enable infection of the non-dividing cell. In $CD4^+$ T cells, Vpr potentiates replication by causing G2 cell cycle arrest and can result in apoptosis; however, in this cell type Vpr is not necessary for production of virus. Our study does not address the question of the changes of these LTNP- and RP-associated mutations in the context of cell specificity. In addition, the strain of virus used, NL4-3, is an X4-tropic strain, mainly infecting the T cell population, which may indicate that the potential effect of these Vpr mutations on viral replication may be a macrophage-specific phenomenon.

5.1 CONCLUSIONS

The findings of this study show that Vpr mutations alter its functions and can alter the replication potential. This provides credence to the speculation that differential viral evolution can lead to attenuation of the virus. This evidence may provide a rationale for designing drug targets or vaccines against the previously ignored "accessory proteins" of HIV, a category in which Vpr is included. Interfering with the functions of these accessory proteins would eliminate much of the pathogenicity of HIV and this would lead to improved immune responses against the virus in an individual. Such novel treatments would be necessary in the future to control the HIV epidemic.

6.0 FUTURE DIRECTIONS

In particular, the results of aim 1 reveal that structural change of Vpr causes functional differences. In particular, the RP-associated Vpr R36W, L68M, and R85Y show some substantial differences. The mechanisms of these differences provide many questions to be answered in the context of the models of the molecular interactions of Vpr in the cell. The interaction of Vpr with importin-α enabling nuclear localization is an established phenomenon that needs to be addressed for these three mutations which show a large difference in their ability to translocate into the nucleus. The mechanisms can be delineated by continuation of the BiFC analysis using a tagged VC/VN importin-α. It is also unclear what effect these mutations have on the initial steps fo the infection after entry. These Vpr mutations could likely affect the integration of proviral DNA as a result of interference with nuclear translocation. This would be evident by quantifying the 2-LTR circles in the nucleus via PCR.

The LTNP polymorphism Vpr T19A enhances G2 cell cycle arrest while the RP mutants Vpr R36W and L68M abolish cell cycle blockade. The recent work establishing that the mechanism involves the DCAF1-ubiquitin ligase complex indicates that these mutations likely affect some aspect of this interaction [50, 55]. The ability of these Vpr mutants to interact with DCAF1 can be delineated again with an interaction study using BiFC or coimmunoprecipitation.

These Vpr-interacting partners are already established in the literature, and the structural domains of such interactions have already been delineated. However, these newly identified

LTNP- and RP-associated mutations in Vpr have not been structurally examined. The protein structure of Vpr needs to be visualized and must be analyzed via computational modeling to find the potential folding patterns of each of these mutant proteins. This will enable identifying any structural changes that can affect interaction of Vpr with its cellular partners.

Aim 2 serves as a pilot for future studies. While no differences in replication in PBMCs were observed, the experiment may require a further reduction of MOI to observe any changes imparted by Vpr mutation in the proviral DNA. In the case that these changes are still unobserved, the replication kinetics of the mutations must be tested in macrophages and $CD4^+$ T cells separately as Vpr acts differently in each cell type. The cytokine production of PBMCs, $CD4^+$ T cells, and macrophages infected with these mutants needs to be analyzed. This necessitates the use of macrophage-tropic virus in addition to the currently used NL4-3 strain.

The results of this study yield many more leads to study the role of the LTNP-associated and RP-associated Vpr mutations *in vitro* to delineate the mechanisms of altered Vpr function and and their role in infection of a primary cell system which has more relevance to disease pathogenesis.

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