MANIPULATION OF THE CUL4-DCAF1 UBIQUITIN LIGASE BY THE PARALOGOUS PRIMATE LENTIVIRAL ACCESSORY PROTEINS VPR AND VPX

by

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ABSTRACT

Upon infecting a host, viruses immediately face restriction by the host immune system, including innate and adaptive responses. To mediate efficient replication, viruses have evolved a number of mechanisms to subvert and bypass the host immune responses. Among the earliest immune protections encountered by viruses are a group of cell-intrinsic immunity proteins called “restriction factors.” Restriction factors have been identified that, if not counteracted, are capable of inhibiting viral replication throughout the viral life cycle. One of the primary mechanisms utilized to counteract these restrictions is the manipulation of the cellular ubiquitin ligase system to induce the directed and specific degradation of these cellular factors.

In the case of primate lentiviruses (HIVs and SIVs), four proteins (Vif, Vpu, Vpr, and Vpx) have been shown to alter the specificity of this cellular degradation machinery to target restriction factors. In this study, we explore the molecular interaction between the paralogous proteins Vpr (encoded by all primate lentiviruses) and Vpx (encoded by HIV-2 and some SIVs), the cellular ubiquitin ligase composed of Cul4-Roc1-DDB1-DCAF1 and the restriction factors they target for degradation (Mus81 in the case of Vpr and SAMHD1 in the case of Vpx). Through mutation of DCAF1, the substrate specificity factor for the ubiquitin ligase complex, to which Vpr and Vpx are known to directly interact, we show that although they share a high degree of homology, Vpr and Vpx interact with DCAF1 differently. In addition, through the generation of chimeric Vpr-

Vpx proteins, we explore the molecular determinants of Vpr and Vpx substrate specificity. To this end, we demonstrate that manipulation of Cul4-DCAF1 substrate specificity by Vpr and Vpx is mediated by nonlinear determinants within the respective proteins, in contrast to previously proposed models. Finally, we demonstrate that Vpr induces the degradation of Mus81 in a manner independent of the induction of G2 arrest, in contrast to recent reports.

Dedicated to those who came before

&

those who will come after.

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CHAPTER 1

INTRODUCTION

Cell Intrinsic Restriction of Viral Replication

An introduction to cellular restriction and viral countermeasures

Immediately following infection, pathogens begin to interact with immune effectors of the host, from the canonical cell-mediated immunity of the innate and adaptive immune cells, to the more recently identified cell intrinsic immunity mediated by cellular restriction factors. Restriction factors are a broad family of proteins involved in sensing and inhibiting the replication of intracellular pathogens. In the context of viral infections, restriction factors have been identified that act at every stage along replication cycles from uncoating (as exemplified by TRIM5 (reviewed in (Luban, 2012)) and limiting the supply of vital building blocks necessary for replication (such as the regulation of nucleotide availablity by SAMHD1 (reviewed in (Laguette and Benkirane, 2012; Schaller et al., 2012)), to recognition of genomes by cellular RNA and DNA sensors (reviewed in (Jensen and Thomsen, 2012; O'Neill, 2013)) and inhibiting release of new virions from infected cells (by BST-2/tetherin (reviewed in (Dubé et al., 2010)) (field reviewed in (Duggal and Emerman, 2012)).

Efficient replication in the face of this barrage of cellular restrictions has required viruses to evolve mechanisms to subvert these host factors. To this end, viruses utilize a

number of strategies, including mutating or hiding molecular motifs recognized by innate immune sensors. Perhaps the most ingenious strategy utilized to avoid cellular restriction is the manipulation of the cellular ubiquitin proteasome system (UPS) by viruses (reviewed in (Randow and Lehner, 2009)). The UPS functions as one of several pathways utilized by eukaryotes to regulate protein function through the conjugation of any of a highly structurally related class of ubiquitin-like proteins. The UPS serves as a dynamic and highly regulated network for modulation of cellular protein dynamics (Ravid and Hochstrasser, 2008).

To this end, a broad array of viruses have been identified to encode proteins that modulate the specificity and activity of the UPS in order to facilitate viral replication (Table 1.1). Thus far, viral manipulation has primarily been observed to redirect ubiquitin ligases towards the degradation of cellular factors that negatively affect viral replication (reviewed in (Randow and Lehner, 2009)); however, given the industrious nature of viral manipulation of cellular pathways, future studies will no doubt uncover examples of alternative affects of viral ubiquitin ligase hijacking. Primate lentiviruses (HIV/SIV) are no exception to this ubiquitin ligase modulation. Indeed, primate lentiviruses are known to encode four proteins which alter the substrate specificity of cellular ubiquitin ligases (Vif, Vpu, Vpr, and Vpx) (reviewed in (Gramberg et al., 2009; Harris et al., 2012). This chapter will focus on our understanding of the functions and differences between the paralogous lentiviral proteins Vpr and Vpx.

Vpr and Vpx: HIV/SIV's Fraternal Twins

Vpr the enigma

Of the primate lentiviral accessory proteins, the function of Vpr has remained the most elusive. Over the three decades since the identification of HIV as the causative agent of AIDS, a pleiotropic range of functions has been ascribed to Vpr. Initial reports indicated a slower rate of viral replication, *in vitro*, in viruses lacking Vpr. In addition, Vpr has been shown to enhance expression from the viral promoter. These results led to the early name: Viral protein regulatory (Vpr)(reviewed in (Guenzel et al., 2014)). Other early reports implicated Vpr in mediating nuclear import of the pre-integration complex (Heinzinger et al., 1994); however, these observations have been called into doubt and have been generally discredited in recent studies of Vpr activity.

Perhaps the most investigated aspect of Vpr function is its ability to induce cell cycle arrest at the G2/M transition, similar to that observed following DNA damage and/or replication stress (Re et al., 1995; Jowett et al., 1995; He et al., 1995; Rogel et al., 1995)(reviewed in (Andersen et al., 2008)). In 2003, our laboratory determined that Vpr induced the activation of the DNA damage sensor ATR and that this activation was necessary for Vpr-mediated G2 arrest (Roshal et al., 2003). Subsequent research from our lab and others demonstrated Vpr-induced ATR activation mirrored that of DNA damaging agents, such as UV irradiation, inducing the phosphorylation RPA32, a protein known to "mark" regions of single stranded DNA, as well as downstream mediators including Chk1 and Wee1, and the stabilization of Cdk1 (reviewed in (Andersen et al., 2008)). Interestingly, pulse field gel electrophoresis studies indicated Vpr was not inducing double-strand breaks (Lai et al., 2005). In agreement with this observation,

expression of Vpr failed to induce the activation of the closely related DNA damage sensor ATM or its immediate downstream signaling mediator, Chk2 (Zimmerman et al., 2004; Lai et al., 2005).

While numerous studies had investigated the downstream consequences of Vpr-induced G2 arrest, it took several years before significant insight into how Vpr was activating ATR signaling was achieved. In 1994, Vpr was shown to interact with a cellular protein of unknown function, originally named Vpr-Interacting-Protein (RIP or VprBP) (ZhaoS et al., 1994). Over a decade later, two groups identified RIP as a member of a family of proteins involved in dictating the substrate specificity of the Cullin 4-DDB1 ubiquitin ligase (DDB1-Cullin 4 Associated Factors, DCAFs) and renamed it DCAF1 (Jin et al., 2006; Angers et al., 2006). Soon thereafter, several groups demonstrated that induction of G2 arrest by Vpr is dependent on the activity of this ubiquitin ligase. Namely, knockdown of components of the ubiquitin ligase or expression of Vpr mutants that failed to interact with DCAF1 prevented Vpr induction of G2 arrest (Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Hrecka et al., 2007; Wen et al., 2007)(reviewed in (Dehart and Planelles, 2008)). Identifying the cellular protein(s) targeted for ubiquitination by Vpr has proven to be difficult.

Vpr and UNG2

Perhaps the most studied putative ubiquitination target of Vpr is the Uracil DNA Glycosylase (UNG2). In normal cellular physiology, UNG2 serves to repair mis-incorporated Uracil-deoxyribonuleotide through the catalytic removal of the base from the phosphate-sugar backbone, resulting in the generation of an abasic site, which is then

repaired via the potentially mutagenic base excision repair (BER) pathway (reviewed in (Planelles and Benichou, 2010)). Viral reverse transcription is known to result in the incorporation of dUTP into HIV DNA (Kennedy et al., 2011) and it is proposed that UNG2-generated abasic sites at these misincorporated uracils may induce hypermutation of the viral genome reminiscent of that generated by the APOBEC family of cellular restriction factors (reviewed in (Planelles and Benichou, 2010)).

Expression of Vpr in cells destabilizes UNG2 in a DCAF1-dependent manner (Schröfelbauer et al., 2005). However, this destabilization of UNG2 is not associated with the cell cycle phenotype observed during Vpr expression (Selig et al., 1997). Indeed, recent reports indicate UNG2 destruction may be a secondary effect of Vpr expression, as UNG2 appears to be expressed differentially throughout the cell cycle, with the lowest expression levels observed during the G2 phase of the cell cycle (Hagen et al., 2008). Intriguingly, the de Noronha lab recently reported UNG2 may itself be an endogenous substrate of DCAF1, raising the possibility that a consequence of Vpr manipulation of the DCAF1-containing ubiquitin ligase results in a general hyperactivation of the complex (Wen et al., 2012). In agreement with this hypothesis, Vpr was observed to induce an increased association of DCAF1 with activated Cul4, as measured by the increased degree of neddylation of Vpr-associated Cul4 (Hrecka et al., 2007).

Recent studies, however, have called into question the potential negative consequences of UNG2 to viral replication. Selig et al. demonstrated UNG2 is incorporated into budding virions in a Vpr-dependent manner (Selig et al., 1997). This encapsidation was observed to have a net positive effect on viral infectivity independent of UNG2 enzymatic activity (Guenzel et al., 2012). In support of the hypothesis that

UNG2 plays a positive role in HIV replication, a siRNA screen of DNA repair proteins demonstrated that proteins involved in BER, such as UNG2, but not those of the homologous or the nonhomologous end joining pathways, play an important role in viral replication (Espeseth et al., 2011). Interestingly, Yan et al. also reported a positive effect of incorporation of dUTP into viral reverse transcripts by reducing the rate of autointegration of the viral genomes, a replicative dead-end (Yan et al., 2011). Given the high concentrations of dUTP in macrophages (Kennedy et al., 2011), the beneficial role of UNG2 encapsidation was first observed in HIV infection of macrophages (Chen et al., 2004). While Chen et al. noted an increased mutation rate in viruses carrying the Vpr W54R mutant, which is unable to facilitate UNG2 encapsidation, these viruses were severely crippled in their ability to productively infect monocytes-derived macrophages (MDMs) from healthy donors (Chen et al., 2004). In addition, Jones et al. noted a HIV-1 tropism-dependent requirement for UNG2 in viral replication, in which infection by R5 tropic virus, capable of infecting macrophages, but not X4 tropic virus, was negatively affected by UNG2 depletion (Jones et al., 2010).

Vpr and Dicer

Though it has previously been shown that HIV infection alters the cellular RNA silencing pathways (Yeung et al., 2005), the mechanisms and benefits of this activity remained controversial. In 2010, Coley et al. reported a Vpr-dependent reduction of Dicer expression in MDMs (Coley et al., 2010); however, it remained unclear if this was a direct or indirect effect of Vpr expression in these cells. Subsequently, it was shown that this down regulation was mediated by a direct interaction between Vpr and Dicer and

dependent on the Cul4-DCAF1 ubiquitin ligase (Casey Klockow et al., 2013). In agreement with reports that Dicer depletion does not induce G2 arrest, Klockow et al. observe that Vpr-mediated Dicer depletion is not related to the Vpr G2 arrest phenotype (Bu et al., 2009; Casey Klockow et al., 2013). Instead, Dicer depletion appears to enhance HIV infection of macrophages, though the exact mechanism of this enhancement is unclear (Casey Klockow et al., 2013).

Vpr and the SLX4-Mus81 Complex

Though the induction of cell cycle arrest by Vpr is perhaps the most studied aspect of Vpr biology, it has remained the most poorly understood. Recently, Laguette et al. reported a putative mechanism for Vpr induction of G2 arrest. By analyzing the serial immunoprecipitation of a FLAG/HA-Vpr construct expressed in the monocyte THP-1 cell line, several members of the SLX4-containing endonuclease complex (SLX4, ERCC4, EME1, MUS81, TSPYL1, C20orf94, and ERCC1) were identified by mass spectrometry as Vpr interactors (Laguette et al., 2014). Previously, the SLX4 complex was identified as a structure-specific endonuclease involved in resolving Holiday junctions (reviewed in (Schwartz and Heyer, 2011)). Laguette et al. provide evidence that Vpr functions to activate this endonuclease, driving the degradation of HIV reverse transcripts. They further show that Vpr expression inhibits the induction of an interferon response and that this activity is dependent on SLX4 and Mus81 (Laguette et al., 2014). These observations led them to propose a model whereby Vpr activates this endonuclease complex to facilitate the degradation of reverse transcripts in order to prevent their recognition by cellular DNA sensors such as cGAS (Gao et al., 2013) or IFI-16

(Unterholzner et al., 2010). Interestingly, this model closely mirrors observations made in regards to the activity of the cytosolic endonuclease TREX1 (Yan et al., 2010). Further, Laguette et al. propose this activation to be the biases of the previously reported Vpr-induced G2 arrest through the inadvertent generation of genomic damage resulting in the activation of ATR (Laguette et al., 2014).

Several observations indicate this activation of the SLX4 complex by Vpr is dependent on the manipulation of the Cul4-DCAF1 ubiquitin ligase by Vpr. Specifically, wild type (WT) Vpr, but not the DCAF1-interaction-defective Vpr Q65R mutant, is able to co-immunoprecipitate SLX4 and Mus81. Additionally, while WT Vpr is able to induce poly-ubiquitination of exogenously expressed Mus81, the Vpr Q65R mutant fails to do so above background levels. The observations of Laguette et al. point to a unique mechanism of UPS manipulation by Vpr in comparison to other viral proteins known to modulate the UPS (Table 1). While a slight reduction of Mus81 protein level is observed following the expression of Vpr, the activation of the SLX4 does not appear to be mediated by the degradation of any of the identified subunits. In fact, depletion of many of the SLX4 complex subunits (SLX4, SLX1, EME1, or Mus81) by siRNA inhibit the ability of Vpr to induce G2 arrest (Laguette et al., 2014). This is in contrast to previously proposed models in which Vpr was hypothesized to induce the aberrant degradation of a cellular factor (reviewed in (Andersen et al., 2008)). This model postulates that down regulation of this factor would mirror Vpr activity, in contrast to what Laguette et al. observed. Additionally, while the manipulation of the UPS by other viral proteins seems to generally be directed at overcoming a factor involved in restricting viral replication, the Vpr activity reported by Laguette et al. is indirect, inducing the degradation of HIV

reverse transcripts to prevent downstream recognition by DNA sensors and the subsequent induction of interferon.

Similar to the recent observations by Wen et al. regarding Vpr-mediated UNG2 destabilization (Wen et al., 2012), Laguette and colleagues observed interaction of both SLX4 and Mus81 with DCAF1 in the absence of Vpr. In addition, Vpr appears to interact with the SLX4 complex only in the context of DCAF1 as the Vpr Q65R mutant does not interact with either SLX4 or Mus81. The dominant negative Vpr mutant R80A, which can interact with DCAF1 but fails to induce G2 arrest, has the ability to interact with SLX4 but not Mus81 (Laguette et al., 2014). Taken together, these results point to a model whereby Vpr activates the SLX4 complex by hyperactivating the Cul4-DCAF1 ligase towards an endogenous substrate.

The observations by Laguette and colleagues raise a number of unresolved questions. Vpr is known to be encapsidated within the incoming virion; however, it remains unclear if SLX4 complex activation is mediated by incoming or *de novo* Vpr. Although Vpr induced ubiquitination of Mus81, Vpr activation of the SLX4 complex activation was also dependent on Plk1 phosphorylation of EME1 (Laguette et al., 2014). How Vpr induces this phosphorylation remains unresolved. ATR activation, a hallmark of Vpr-induced cell cycle arrest (Roshal et al., 2003), has previously been shown to inhibit Plk1 activity (Deming et al., 2002). Additionally, it remains unclear how Vpr directs this activity, or if it does, toward unproductive reverse transcription products.

Vpx facilitates infection of noncycling cells

While several classes of noncycling cells, including resting CD4+ T-cells and myeloid lineage cells, are known to express the receptors necessary for HIV-1 infection, only modest levels of infection of these cells are observed *in vivo*. In contrast, HIV-2 and many SIVs are known to be capable of efficiently infecting these cells (Reviewed in (Yamashita and Emerman, 2006; Ayinde et al., 2010)). Initial studies demonstrated that this restriction was at the early stages of reverse transcription and was overcome by HIV-2/SIVs Vpx, present within the infecting virions. Indeed, providing Vpx *in trans* could dramatically increase the efficiency of HIV-1 infection of myeloid cells *in vitro* (Kaushik et al., 2009).

In 2011, two groups independently identified sterile alpha motif domain (SAM) and histidine/aspartic acid domain (HD) containing protein 1 (SAMHD1) as the cellular factor responsible for mediating this restriction (Hrecka et al., 2007; Laguette et al., 2011a). Utilizing mass spectrometry, both groups identified SAMHD1 as a myeloid cell-derived Vpx-specific interacting protein. Both groups were then able to show encapsidated Vpx was capable of inducing SAMHD1 degradation in target cells, facilitating infection with a lentiviral reporter (HIV-LUC-G in the case of Laguette et al. 2011, or HIV-GFP in the case of Hrecka et al. 2011). These studies were then able to demonstrate that SAMHD1 was sufficient for facilitating restriction of lentiviral infection of myeloid cells through siRNA-mediated SAMHD1 depletion.

Like its paralog, Vpx had previously been shown to manipulate the Cul4-DCAF1 ubiquitin ligase (Srivastava et al., 2008). In agreement with the hypothesis that Vpx facilitated the degradation of a myeloid restriction factor through manipulation of

DCAF1 specificity, Hrecka and colleagues observed Vpx-induced SAMHD1 degradation was inhibited following siRNA knockdown of DCAF1. In agreement with this observation, virus-like particle (VLP) treatment of restricted cells with Vpx Q76A, a mutant that has previously been shown to lack DCAF1 interaction (Srivastava et al., 2008), failed to facilitate HIV-1 GFP infection (Hrecka et al., 2011).

Mechanism of SAMHD1 restriction

Previously, SAMHD1 had been identified as one of seven genes (TREX1, RNASEH2A, RNASEH2B, RNASEH2C, and SAMHD1 (reviewed in (Crow and Livingston, 2008)) and ADAR1 (Rice et al., 2012) and MDA5 (Rice et al., 2014) in which polymorphisms have been linked to the rare hereditary condition Aicardi-Goutières Syndrome (AGS). AGS is a generally early onset inflammatory disorder characterized by elevated levels of IFN-α in cerebrospinal fluid in the absence of infection (Crow and Livingston, 2008), potentially due to elevated activity of transposable elements (reviewed in (Planelles, 2011)). The progression of disease is reminiscent of chronic viral infection, and its study has led to important insights into the genetic factors involved in control of congenital infection (Crow and Livingston, 2008).

While the mechanism of SAMHD1 restriction of HIV-1 infection was initially unclear, several pieces of evidence indicated an involvement in nucleotide metabolism. First, all other AGS loci are known to be involved in nucleotide metabolism (Crow and Livingston, 2008; Rice et al., 2012; 2014). Secondly, HD domain-containing proteins have largely been identified as phosphohydrolases that target nucleotides (reviewed in (Planelles, 2011)). Finally, SAMHD1 mediated restriction to HIV replication during

reverse transcription (Srivastava et al., 2008). In the years since the identification of SAMHD1 as the myeloid restriction factor, several groups have extensively examined the metabolic activities of SAMHD1. Soon after the initial reports on SAMHD1 restriction of HIV, Goldstone et al. demonstrated that SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase capable of hydrolyzing all dNTP species to deoxynucleoside and inorganic triphosphate (Goldstone et al., 2011). SAMHD1 triphosphohydrolase activity is tightly linked to cellular dNTP concentrations through an allosteric dGTP binding pocket (Goldstone et al., 2011; Amie et al., 2013b) that facilitates SAMHD1 oligmerization (Ji et al., 2013).

In agreement with the hypothesis that SAMHD1 restricts reverse transcription by regulating dNTP levels, Kim and colleagues observed that SAMHD1 expression strongly correlated with cellular dNTP concentrations in monocyte-derived macrophages (MDMs). Indeed, cell extracts from MDMs treated with Vpx containing VLPs, but not control VLPs, were able to facilitate an *in vitro* HIV RT-based primer extension assay (Kim et al., 2012). These observations are in agreement with early reports that the supply of exogenous dNTP to resting cell cultures was capable of overcoming their restriction to infection (Gao et al., 1993), (Amie et al., 2013a).

SAMHD1 expression has been shown to correlate well with the permissiveness to infection by HIV (Laguette et al., 2011a). In addition to myeloid lineage cells, SAMHD1 has recently been shown to mediate restriction of primary resting CD4+ T-cells (Baldauf et al., 2012). However, a number of nonrestrictive cell types including activated primary CD4+ T-cells and several cell lines including THP-1, 293FT, and HeLa are known to express measurable levels of SAMHD1. In addition, cycling U937 cells expressing

exogenous SAMHD1 were not restrictive to HIV infection. However, following differentiation to a noncycling state, following PMA treatment, both THP-1 and U937 cells became restrictive to infection (White et al., 2013b). These observations indicated that SAMHD1 restriction may be regulated posttranslationally. To this end, several groups analyzed SAMHD1 for posttranslational modifications by mass spectrometry. Phosphorylation of T592 by Cdk1 was determined to negatively regulate SAMHD1 restriction (White et al., 2013b; Cribier et al., 2013; Welbourn et al., 2013). Interestingly, while phosphorylation of T592 negatively affects SAMHD1 restrictive potential, no effect was observed on the triphosphohydrolase activity, indicating that enzymatic activity and restriction are separable characteristics of SAMHD1 (White et al., 2013b; Welbourn et al., 2013).

In addition to HIV, recent studies have also shown that SAMHD1 mediates restriction of a number of other viruses. SAMHD1 has been shown to restrict a number of other retroviruses (including FIV, BIV, EIAV, MLV, MPMV, and HTLV (White et al., 2013a; Gramberg et al., 2013; Taya et al., 2014)), as well as several DNA viruses (Hollenbaugh et al., 2013; Kim et al., 2013). However, depletion of SAMHD1 by treatment with Vpx containing VLPs was not able to relieve restriction for all tested retroviruses (Gramberg et al., 2013). This is likely due in part to differential [dNTP] requirements of the reverse transcriptase enzyme of retroviruses (reviewed in (Amie et al., 2013a)). Additional aspects of SAMHD1 biology may play a role in the differential restriction of these and other viruses. For example, while the SAM domain is believed to be involved in regulating protein-protein interactions (Rice et al., 2009), it is dispensable for HIV restriction (White et al., 2013a); however, it is necessary for the control of the

endogenous LINE-1 retroelements (Zhao et al., 2013).

Evolutionary Perspective on Vpr and Vpx Function

Evolution of Vpr and Vpx

Recently, several groups have analyzed the evolutionary history of Vpr and Vpx. Until recently, the evolutionary origin of Vpx function remained contentious. While Vpr is common to all primate lentiviruses, SAMHD1 antagonism, and the Vpx gene itself, have been acquired and lost over the course of HIV/SIV evolution. By analyzing Vpx and Vpr proteins from a broad array of primate lentiviruses for the ability to degrade SAMHD1 and overlaying this functionality on a phylogenic tree of the Vpr/Vpx genes, Lim et al. demonstrated that SAMHD1 antagonism was acquired by an ancestral Vpr preceding the "birth" of Vpx. This neofunctionality appears to have occurred only once, at the most recent common ancestor of SIVagm and SIVmus/mon/gsn/syk (Lim et al., 2011). Two key observations support this hypothesis: 1) the phylogenetically outgrouped SIVolc/wrc Vpr does not antagonize SAMHD1. 2) Vpr from SIVagm and SIVmus/mon/gsn/syk, which do not encode a Vpx gene, are able to facilitate SAMHD1 degradation (Lim et al., 2011), as well as mediate G2 arrest (Planelles et al., 1996) (Figure 1.1).

Analysis of SAMHD1 evolution provides further support to the evolutionary history of Vpr and Vpx. By examining the ratio of nonsynonymous to synonymous substitution within the SAMHD1 gene across a broad array of primates, three groups were able to observe strong, episodic, positive selection of the SAMHD1 gene (Lim et al., 2011; Laguette et al., 2011b; Zhang et al., 2012). Both Lim and Laguette

demonstrated SAMHD1 to be under strong selective pressure in Old World (Catarrhines) but not New World (Platyrrhines) Monkeys (Laguette et al., 2011b; Lim et al., 2011). Notably, Lim et al. observed positive selection beginning at the ancestral node giving rise to the subfamily *Cercopithecinae* (which are infected with primate lentiviruses known to encode for SAMHD1 antagonism) but not subfamily *Colobinae* (infected by SIVolc/wrc) or Hominoids (infected by SIVcpz/gor/HIV-1)(Lim et al., 2011). In contrast, Laguette et al. observed positive selection of SAMHD1 beginning after the split of Old World Monkeys from New World Monkeys, earlier than that predicted by Lim et al. and continuing through Hominoid evolution (Laguette et al., 2012). These differences are most likely due to different primate samplings, as well as different computational modeling. Regardless, although the Laguette et al. model would point to an earlier onset of SAMHD1 antagonism, it still supports a model whereby SIV acquired this activity prior to the "birth" of Vpx. Surprisingly, all three groups observed strong positive selection of SAMHD1 along the Hominoid lineage of orangutans. As orangutans are not known to be infected by any lentiviruses, and no other retroviruses tested so far are known to antagonize SAMHD1, the causative agent of this selection is unclear.

Interestingly, while HIV-1 and its zoonotic ancestor SIVcpz lack the ability to antagonize SAMHD1, the SIVs that gave rise to SIVcpz (SIVmus/mon/gsn and SIVrcm (Bailes et al., 2003)) both antagonize the SAMHD1 of their respective hosts, as well as human SAMHD1 in the case of SIVmus Vpr (Lim et al., 2011). Phylogenetic analysis indicates that SIVcpz lost SAMHD1 following recombination of SIVmus/mon/gsn and SIVrcm in which only the monofunctional SIVrcm Vpr was retained (Zhang et al., 2012; Etienne et al., 2013). This event was, at least in part, driven by the generation of a novel

Vif protein with enhanced hominid APOBEC3 antagonism (Etienne et al., 2013). It is tempting to speculate that the loss of SAMHD1 antagonism has, however, resulted in other profound changes in the pathology of lentiviral infection. For example, the reduced efficiency of myeloid cell infection by HIV-1 may result in reduced innate and subsequent adaptive immune activation, facilitating viral replication and pathogenesis. To this end, infection of myeloid cells by HIV-2 was recently shown to result in their activation through recognition of viral reverse transcripts by the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS); however, as HIV-1 is unable to complete reverse transcription, this activation was not observed (Lahaye et al., 2013; Puigdomenech et al., 2012). Furthermore, infection of humans and mandrills by SAMHD1-antagonizing viruses has been observed to result in slower disease progression compared to infection with non-SAMHD1 antagonizing viruses (SIVmnd2 and HIV-2 vs SIVmnd1 and HIV-1) (Nyamweya et al., 2013; Souquière et al., 2009). Finally, a recently completed 20-year longitudinal study of HIV-1/HIV-2 dually infected individuals showed a delayed onset of AIDS in dually infected individuals, compared to HIV-1 only infected individuals. Furthermore, they observed individuals first infected by HIV-2 showed the longest time to onset of AIDS (Esbjörnsson et al., 2012).

The Red Queen's court

Antagonism of host restriction factors by pathogens is hypothesized to induce an evolutionary arms race, the Red Queen hypothesis, whereby host evolution away from antagonism results in a corresponding evolution by the pathogen to regain the ability to antagonize the restriction factor (reviewed in (Duggal and Emerman, 2012)). While

several studies have examined molecular determinants of Vpr and Vpx function, the evolutionary events leading to the "birth" of Vpx from a bifunctional Vpr, as proposed by Lim et al. (Lim et al., 2011), remain unclear.

Following the identification of SAMHD1 as the Vpx-targeted myeloid restriction factor, several groups quickly demonstrated SAMHD1 was under strong evolutionary pressure (discussed above) (Lim et al., 2011; Laguette et al., 2011b; Zhang et al., 2012). The observation by Lim et al. that SAMHD1 is under positive selection in *Cercopithecinae*, but not New World Monkeys or Hominoids, strongly indicates that lentiviral infection has been the main driver of SAMHD1 evolution in primates.

While Lim and Laguette both computationally identified residues under positive selection, those identified by Lim et al. were largely N-terminal, while those identified by Laguette et al. localized in the C-terminus (Lim et al., 2011; Laguette et al., 2011b). Both groups were able to molecularly verify the importance of these respective regions for Vpx antagonism; however, the apparent discrepancy between these two studies is likely due to different computational methodologies and Vpx-SAMHD1 species pairs molecularly tested. In agreement with Laguette, Ahn and colleagues were able to demonstrate that the C-terminus of human SAMHD1 was sufficient to mediate interaction with SIVmac Vpx (Ahn et al., 2012). However, a more thorough investigation by Fregoso et al. resolved the differences by generating a number of SAMHD1 chimeras, in which the N and C-termini of different species' SAMHD1 were transposed, showing that the determinant of SAMHD1 interaction and degradation by Vpx has toggled back and forth between the N and C-terminus throughout the period of Vpx antagonism (Fregoso et al., 2013).

In contrast to the studies regarding Vpx-SAMHD1 co-evolution, little is currently known regarding the structural determinants of Vpr-mediated antagonism of the SLX4 complex. Early studies demonstrated a species-specific ability of Vpr to induce G2 arrest (Planelles et al., 1996), indicative of positive selection on the Vpr-targeted factor. It is tempting to speculate that it became too difficult for Vpr to maintain concurrent evolutionary arms races with two host proteins. In support of this hypothesis, Spragg and Emerman recently examined the evolution of the biofunctional SIVagm Vpr and SAMHD1 from phenotypically and genetically distinct AGM species. While the four AGM species are only evolutionarily separated by 3 million years, each population is infected by distinct SIVagm. Even within this relatively short period of evolutionary time, Vpr from different SIVagm appear to utilize different interfaces for SAMHD1 antagonism (Spragg and Emerman, 2013) .

Molecular Insights into the Manipulation of the Cul4-DCAF1 Ligase by Vpr and Vpx

Mechanisms of viral manipulation of ubiquitin ligases

Viral manipulation of the cellular ubiquitin ligase system is common across viruses from a diverse array of species (see Table 1.1). While the specific ligases manipulated and the restriction factors targeted are nearly as diverse as the viruses that manipulate them, three general strategies for altering the substrate specificity have been observed: 1) displacement of the substrate specificity receptor subunit of the ubiquitin ligase complex, as exemplified by the redirection of the Cul5-EloB/C complex towards APOBEC3 by Vif (Yu et al., 2003; 2004); 2) mimicry of endogenous substrate by a viral

protein, as seen in the case of Vpu manipulation of the Cul1-βTRCP (Margottin et al., 1998); 3) generation of a new substrate interface, involving interaction of the new substrate with both the viral protein and the endogenous substrate receptor, as seen in the redirection of the Cul4-DCAF1 ligase towards SAMHD1 by Vpx (Schwefel et al., 2014) (Figure 1.2). In addition, there is at least one known virally encoded ubiquitin ligase, the ICP0 protein of herpes simplex viruses (Boutell et al., 2002)(field reviewed in (Barry and Früh, 2006))

Differential manipulation of DCAF1 substrate specificity by Vpr and Vpx

Alignment of Vpr and Vpx proteins gave early hints to the difference in substrate recruitment by the respective proteins. Structural studies of Vpr showed that it formed a tight 3 α-helix bundle flanked by unstructured N and C-termini (Morellet et al., 2003). Primary sequence alignment of the monofunctional HIV-1 Vpr, bifunctional SIVagm Vpr, and SIVmac Vpx show that this helical core is highly conserved among these proteins (Figure 1.3, grey boxes). DCAF1 binding was mapped to a short, highly conserved leucine-rich motif located in the third α-helix (Figure 1.3, red box) (Le Rouzic et al., 2007). Virion encapsidation of Vpr and Vpx was mapped independently to helix 2 of the respective molecules (Yao et al., 1995; Mahalingam et al., 2001). These observations support the importance of this highly conserved helical core and its evolutionary conservation among primate lentiviral Vpr and Vpx.

In contrast, a high degree of divergence is seen between the unstructured termini of Vpr and Vpx. Mutational analysis of Vpr identified the C-terminal region to be important for Vpr-mediated G2 arrest, and presumably SLX4 complex activation

(reviewed in (Morellet et al., 2009)). In support of this hypothesis, expression of Vpr S79A or Vpr R80A mutants or a Vpr 1-78 truncation acted in a dominant negative manner towards the induction of Vpr G2 arrest. Mutations to the N-terminal unstructured region of Vpr generally showed no effect on G2 arrest (reviewed in (Morellet et al., 2009)); however, the homologous region within Vpx was shown to be necessary for Vpx to mediate myeloid cell infection and SAMHD1 degradation (Gramberg et al., 2010; Ahn et al., 2012). Moreover, while substrate interaction of Vpr and Vpx appear to be heavily mediated by the C and N-terminus, respectively, several studies indicated these interactions are not defined by linear determinants. To this end, Maudet et al. examined Vpr for mutants that would fit 3 criteria: 1) inactive for G2 arrest induction, 2) retained DCAF1 binding, and 3) located outside the C-terminal tail. Two residues were identified matching these requirements, K27, located in the first α-helix, and Y50, located in the linker region between α-helix 2 and 3 (Maudet et al., 2011); however, the molecular mechanism by which these residues are involved in the induction of G2 arrest will need to be elucidated in light of the identification of the SLX4 complex as the Vpr target. Interestingly, the linker between the second and third α-helices was previously shown to be important for Vpr-UNG2 interaction (Figure 3, yellow box)(Selig et al., 1997). In addition, the linker between the second and third α-helix of Vpx was recently shown to be involved in the Vpx-SAMHD1 interaction (Figure 3, orange boxes)(Schwefel et al., 2014).

Until recently, the exact mechanism by which Vpr and Vpx altered the substrate specificity of the Cul4-DCAF1 ubiquitin ligase remained unclear. Direct interaction between Vpr/Vpx and the substrate specificity subunit, DCAF1, of the ubiquitin ligase is

reminiscent of the endogenous substrate mimicry mechanism observed in the case of Vpu-mediated degradation of CD4 and Tetherin (Figure 1.2) (Margottin et al., 1998). DCAF1 is a large, 1507 amino acid, multiple domain protein comprised of a N-terminal armadillo domain (unknown function), a central LisH domain (involved in the dimerization of DCAF1 containing ubiquitin ligases (Ahn et al., 2011)), a WD40 domain (common to all DCAFs (Jin et al., 2006), involved in mediating substrate interaction), and a C-terminal acidic amino acid rich region (involved in the regulation of DCAF1 activity (Li et al., 2010))(reviewed in (Nakagawa et al., 2013)).

In agreement with the hypothesis that Vpr altered the specificity of DCAF1 by mimicking endogenous substrate, Le Rouzic et al. demonstrated that Vpr interacted directly with the DCAF1 WD40 domain. This interaction was shown to be mediated by the leucine rich motif within the third α-helix of Vpr (Figure 1.3, red box) (Le Rouzic et al., 2007). The high conservation of this motif in Vpx, as well as in the predicted tertiary structure of Vpr and Vpx supported the hypothesis that Vpr and Vpx altered DCAF1 specificity in a similar manner (reviewed in (Dehart and Planelles, 2008)).

Through identification of endogenous substrates and inhibitors of DCAF1, functional roles of several DCAF1 subdomains have been elucidated. However, direct interaction of Vpr and Vpx with the DCAF1 WD40 domain (Le Rouzic et al., 2007; Schwefel et al., 2014) has raised the question as to whether DCAF1 merely serves as a bridge linking Vpr and/or Vpx to the ubiquitin ligase complex to facilitate substrate degradation. Gerard et al. recently demonstrated that expression of the truncation minimally able to bind DDB1 (WD40 domain, plus a short region N-terminal to the WD40 domain known to mediate DCAF-DDB1 interaction (Li et al., 2009)) was unable

to facilitate induction of G2 arrest by Vpr in DCAF1-depleted cells (Gérard et al., 2014). In agreement with this observation, we have observed that truncation of any DCAF1 domain inhibits both Vpr and Vpx function in a dominant negative manner, indicating that DCAF1 does not merely serve as a bridge linking Vpr/Vpx to the Cul4 ligase (see Chapter 1).

In 2014, Schwefel et al. solved the crystal structure of a complex involving the DCAF1 (WD40 domain), SIVsm Vpx, and the SAMHD1 (C-terminal domain)(Schwefel et al., 2014). Similar to the interaction seen between other DCAFs and their substrates (Song and Kingston, 2008; Patel et al., 2008)(Reviewed in (Stirnimann et al., 2010)), Vpx was observed to interact with the DCAF1-WD40 domain primarily through interactions with a shallow groove on the top face of the WD40 domain. In agreement with previous observations that mutations to Vpx Q76 abrogate binding to DCAF1 (Srivastava et al., 2008), this residue was shown to hydrogen bond with DCAF1 N1135 and W1156. In addition to their role in coordinating Vpx Q76, DCAF1 N1135 and W1156 are involved in interactions with a number of residues in the first α-helix of Vpx (Schwefel et al., 2014). Through a mutagenic screen of DCAF1, we independently identified these residues as being involved in the Vpx interaction, as their mutation results in an inability of mutant (N1135A or W1156H) DCAF1 to co-immunoprecipitate Vpx (see Chapter 1). Additionally, both DCAF1 mutants acted in a trans dominant negative manner in regard to Vpx-mediated SAMHD1 degradation. While Vpr and Vpx share high homology in both the first and third α-helix, both DCAF1 N1135A and W1156H retain interaction with Vpr. Functional competition assays show Vpr is able to inhibit Vpx-mediated SAMHD1 degradation, while the reciprocal is not observed (see

Chapter 1). Two, nonmutually exclusive, models could explain the difference observed by us in regards to the interaction of DCAF1 with Vpr and Vpx, respectively: 1) higher affinity interaction between Vpr and DCAF1, than Vpx and DCAF1; 2) differential binding conformations of the Vpr-DCAF1 complex vs. Vpx-DCAF1 complex. Further biochemical studies will be necessary to fully distinguish between these two models.

Perhaps the most surprising observation from the Schwefel and colleagues structure is that SAMHD1 recruitment to the Vpx-DCAF1 complex involves direct interaction of SAMHD1 with DCAF1. This observation is in contrast to previous models of UPS manipulation by viral proteins in which restriction factor recruitment was dependent on interaction only with the viral protein (reviewed in (Barry and Früh, 2006)). In agreement with Schwefel et al., we observed that mutation of the DCAF1 residue D1092 inhibited Vpx-mediated SAMHD1 degradation in a dominant negative manner, but does not disrupt the Vpx-DCAF1 interaction (see Chapter 1). In further support of the differential modulation of DCAF1 specificity by Vpr and Vpx, while Vpx has been shown to interact with SAMHD1 independent of DCAF1 binding (Laguette et al., 2011a), Vpr Q65R (which is unable to bind DCAF1) did not co-immunoprecipitate with members of the recently described G2 arrest target (SLX4 or Mus81)(Laguette et al., 2014), supporting a model in which Vpr and Vpx modulate DCAF1 specificity by different mechanisms. Indeed, current studies show Vpr modulates the activity of DCAF1 towards endogenous substrates (Wen et al., 2012; Laguette et al., 2014). However, it remains unclear if this is a general function of Vpr manipulation of DCAF1, increasing the ubiquitination of all endogenous DCAF1 targets, or a more specific redirection of DCAF1 towards a limited subset of endogenous substrates. In contrast, Vpx appears to

exclude interaction of DCAF1 with at least some endogenous substrates, UNG2 and SLX4 complex (Laguette et al., 2014).

Consequences of lentiviral manipulation of the Cul4-DCAF1 ubiquitin ligase

Several studies have made intriguing observations regarding the broader effects of Vpr manipulation of the Cul4-DCAF1 ubiquitin ligase. Cullin-based ubiquitin ligase activity is dependent on auto-conjugation of a ubiquitin like modifier, NEDD8, to the Cullin backbone, resulting in conformational changes which facilitate E2 recruitment and ubiquitin transfer to substrate (Saha and Deshaies, 2008; Duda et al., 2008)(reviewed in (Soucy et al., 2010; Saifee and Zheng, 2008)). Intriguingly, Hrecka observed that Vpr preferentially associates with neddylated Cul4 (Hrecka et al., 2007). While the regulation of Cullin neddylation is still not fully understood, recruitment of substrate-bound receptor has been shown to, at least in part, promote conjugation of NEDD8 to Cullins (Chew and Hagen, 2007). These observations promote the hypothesis that Vpr binding of DCAF1 may drive assembly of active Cul4-DCAF1 complexes. Several additional lines of evidence support this hypothesis. Namely, other viral proteins are known to manipulate Cul4 ubiquitin ligase specificity by binding directly to DDB1 (see Table 1.1), resulting in the exclusion of DCAFs (Angers et al., 2006). We have observed that Vpr expression decreases the ability of one such viral protein, SV5 V-protein, to exclude DCAF1 from binding DDB1 (unpublished results). Furthermore, Vpr expression promotes the exclusion of other DCAFs (Schröfelbauer et al., 2007). Whether Vpx induces similar changes will require further investigation. However, preliminary evidence suggests at

least some differences between Vpr and Vpx in regards to Cul4 complex assembly. While Vpr has been observed to in result in an increased association of DCAF1 with DDB1, Vpx expression does not alter the DCAF1-DDB1 interaction (see Chapter 2).

The broader implications of viral manipulation of ubiquitin ligases have remained largely unstudied. Most studies into the manipulation of ubiquitin ligases by viral proteins have focused on the mechanism by which they interact with and redirect the specificity of their partner ligases. How these subversions alter the activity of these ligases towards their endogenous targets has been largely ignored. One recent study points to large alterations in the activity of the cellular ubiquitin ligase system by Vpr, but not other HIV-1 accessory proteins (Arora et al., 2014); however, the implications of this observation, especially in the context of *in vivo* infection, will require further investigation.

Table 1.1: Viral manipulators of the cellular ubiquitin proteasome system

Virus	Viral Protein	UPS Interaction	Target	Function	Reference
HPV	E6	HECT/E6-AP	p53	Dysregulation of cell cycle	(Scheffner et al., 1993)
Paramyxovirus SV5	V	Cullin4/DDB1	STAT1,2	Overcome type-1 interferon responses	(Horvath, 2004)
Hepatitis B	HBx	Cullin4/DDB1	Unknown	Apoptosis, DNA repair, cell cycle, and enhanced viral replication	(Sitterlin et al., 2000; Leupin et al., 2005)
	Vpu	Cullin1/β-TrCP1	CD4, Tetherin	Enhance viral release	(Bour et al., 2001; Margottin et al., 1998)
HIV-1	Vif	Cullin5/EloginB/C	APOBEC3G/F	Overcome restriction	(Yu et al., 2003; Mehle et al., 2004; Sheehy et al., 2003)
	Vpr	Cullin4/DDB1-DCAF1	SLX4 complex	Prevent recognition of viral reverse transcripts by DNA sensors	(Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Hrecka et al., 2007; Belzile et al., 2007; Wen et al., 2007)
HIV-2	Vpx	Cullin4/DDB1-DCAF1	SAMHD1	Overcome restriction to reverse transcription in myeloid cells	(Srivastava et al., 2008; Hrecka et al., 2011; Laguette et al., 2011a)
HCMV	UL35	Cullin4/DDB1-DCAF1	Unknown	Induce ATR-dependent cell cycle arrest in G2	(Salsman et al., 2012)
Herpes Viruses	ICP0	RING Finger E3	PML	Overcome antiviral activity associated with PML nuclear bodies	(Boutell et al., 2002)
Adenoviruses	E1B55k/E4 Orf6	Cullin5/Elongin B/C	MRE11 Complex, p53	Prevent recognition of viral DNA ends by the NHEJ machinery	(Harada et al., 2002; Querido et al., 2001)

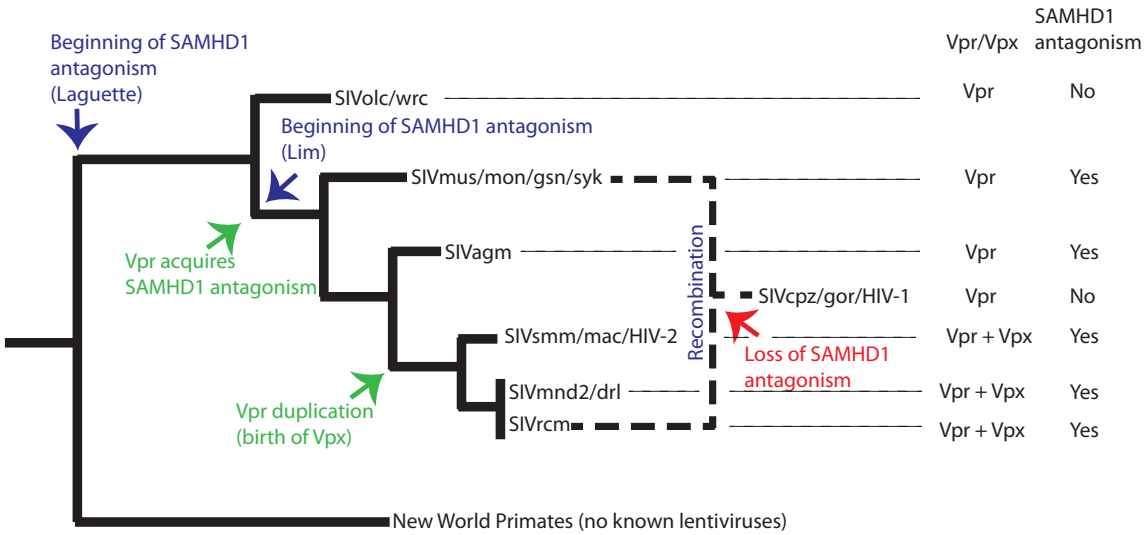

Figure 1.1: Overview of Vpr/Vpx phylogeny
Branch lengths are not depicted to scale. Adapted from (Lim et al., 2011; Laguette et al., 2011b; Planelles, 2012; Etienne et al., 2013).

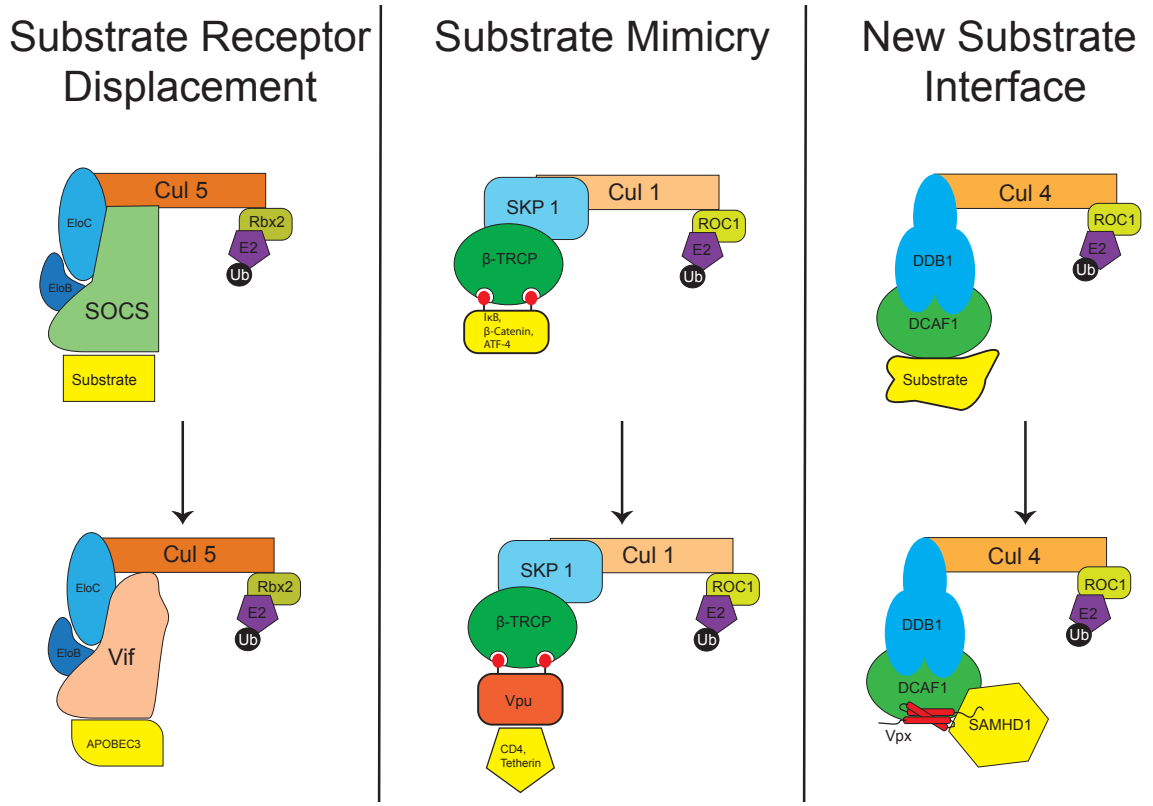

Figure 1.2: Mechanisms of viral manipulation of cellular ubiquitin ligases
Three mechanisms of viral manipulation of the UPS have been observed. 1) Through the displacement of the substrate specificity subunit of the ubiquitin ligase, the viral protein is able to recruit nonnative substrate. This mechanism is exemplified by the primate lentiviral proteins Vif manipulation of the Cul5 ubiquitin ligase. Vif has been shown to displace the native ligase receptor family, SOCS, through direct interaction with Cul5, as well as interactions with the adaptor proteins EloB/C (Yu et al., 2003; 2004). 2) Viral mimicry of endogenous ubiquitin ligase substrate. Cul1-SKP1-βTRCP is known to recruit substrates through interaction with a phospho-degron. Vpu is known to be phosphorylated on S52 and S56, generating a βTRCP phospho-degron motif. This substrate mimicry by Vpu facilitates the recruit by it of CD4 and Tetherin to the Cul1 ubiquitin ligase for ubiquitination and degradation (Margottin et al., 1998). 3) Generation of a novel substrate binding interface as recently described by Schwefel et al. Vpx was shown to interact with DCAF1, the substrate specificity subunit of the Cul4 ubiquitin ligase (Le Rouzic and Benichou, 2005; Srivastava et al., 2008), this binding results in the recruitment of SAMHD1 through interactions with both Vpx and DCAF1 (Schwefel et al., 2014).

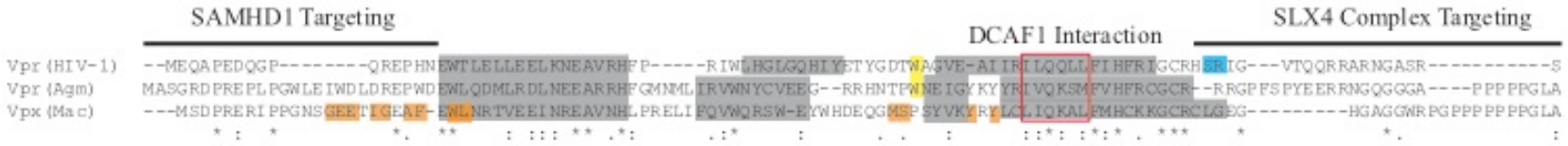

Figure 1.3: Alignment of Vpr from HIV-1 and SIVagm and Vpx of SIVmac
Structural alignment of HIV-1 Vpr, SIVagm Vpr, and SIVmac Vpx. Mutational analysis of Vpr has previously shown the unstructured C-terminus to be involved in determining G2 arrest functionality (reviewed in (Morellet et al., 2009)). Recently, the N-terminus of Vpx was determined to be necessary for Vpx interaction with SAMHD1 (Ahn et al., 2012). Key regions indicated: α-helices (grey boxes) as determined by NMR structure (Vpr (Morellet et al., 2003)) and in silico predictions for SIVagm Vpr and SIVmac Vpx. R80A residue of Vpr (blue box) have been shown to generate a G2 dominant negative molecule (Dehart et al., 2007). Recruitment of UNG2 is known to be dependent on W54 located in the linker between the second and third α-helix (yellow box)(reviewed in (Morellet et al., 2009)). A recently solved DCAF1(WD40)-Vpx-SAMHD(Ctd) co-crystal identified residues involved in SAMHD1 interaction (orange boxes) (Schwefel et al., 2014).

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CHAPTER 2

UNDERSTANDING THE MOLECULAR MANIPULATION OF THE CUL4-DDB1-DCAF1 UBIQUITIN LIGASE BY THE PARALOGOUS LENTIVRIAL ACCESSORY PROTEINS VPR AND VPX

Abstract

The primate lentiviral proteins Vpr and Vpx are both known to manipulate the cellular ubiquitin ligase comprised of Cul4-DDB1-DCAF1 to facilitate viral replication. While Vpr is common to all primate lentiviruses, Vpx is encoded only by a limited range of SIVs and HIV-2. Although Vpr and Vpx share a high degree of homology, they are known to mediate markedly different benefits for viral replication through the recruitment of different substrates to the ubiquitin ligase. Here we explore the interaction of Vpr and Vpx with the ubiquitin ligase substrate specificity receptor DCAF1. Through mutational analysis of DCAF1, we demonstrate that although Vpr and Vpx share a highly similar DCAF1 binding motif, they interact with DCAF1 differently. In addition, we observe that Vpr function is dominant to Vpx *in vitro.*

Introduction

Manipulation of the cellular ubiquitin proteasome system (UPS) to degrade cellular restriction factors is a common tactic employed across a broad array of virus classes. Human Immunodeficiency Virus (HIV) is no exception and encodes three such proteins (Vif, Vpu, and Vpr). In addition, some primate lentiviruses (including HIV-2) encode the Vpr paralog Vpx. While the mechanism of UPS manipulation and cellular targets of Vif and Vpu have been well characterized, the functions of Vpr and Vpx have been more difficult to elucidate (reviewed in (Malim and Emerman, 2008)).

Vpr is a short, 96aa protein that is highly conserved among primate lentiviruses (HIVs and SIVs). Vpr is expressed late during viral replication and is present in virions; however, its role within the virion is unknown (Cohen et al., 1990; Müller et al., 2000). Its function appears to be highly crucial for HIV infection as no primary isolates have been described which lack Vpr (reviewed in (Andersen et al., 2008)). Vpr has been shown to induced cell-cycle arrest at the G2/M transition through the activation of the DNA damage sensor ataxia telangiectasia and Rad3-related protein (ATR) (Roshal et al., 2003); however, the significance of cell cycle arrest for the virus is currently unclear (reviewed in (Andersen et al., 2008)).

In 1994, Vpr was shown to interact with a novel cellular protein, DDB1-Cullin4 Associated Factor 1 (DCAF1), previous known as RIP/VprBP (Zhao et al., 1994). The significance of this interaction remained unclear until several groups identified DCAF1 as a substrate specificity subunit for the Cullin4-DDB1-based ubiquitin ligase. Following this observation, our laboratory and several others proposed a model whereby Vpr hijacks the cellular ubiquitin ligase composed of Cullin4-DDB1-DCAF1 (hereafter referred to as

Cul4-DCAF1) recruiting an as of yet unidentified cellular target, resulting in its polyubiquitination and degradation (Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Hrecka et al., 2007; Belzile et al., 2007; Wen et al., 2007).

In addition to Vpr, some primate lentiviral lineages encode the Vpr paralog Vpx. Early studies identified a role for Vpx in the infection of myeloid lineage cells, dendritic cells, and macrophages, by overcoming a block present in these cells to viral reverse transcription (Yu et al., 1991). Following studies demonstrating that Vpr manipulated Cul4-DCAF1, the Skowronski lab demonstrated a similar requirement for Vpx to facilitate macrophage infection (Srivastava et al., 2008). In 2011, two groups identified SAMHD1 as the cellular protein targeted by Vpx, in the context of Cul4-DCAF1 (Laguette et al., 2012; Hrecka et al., 2011).

While much work has been done to understand the functional role of Vpr and Vpx in the context of viral replication, the mechanism by which they redirect the specificity of Cul4-DCAF1 has remained unresolved. Thus far, three mechanisms for manipulation of proteasomal degradation of cellular proteins have been observed: 1) the encoding of a viral E3 ubiquitin ligase, as is the case of the ICP0 protein of herpes viruses (Boutell et al., 2002), 2) the replacement of the substrate receptor of a cellular ubiquitin ligase by a virally encoded protein, as is the case of protein V from SV5 (Horvath, 2004) and Vif of primate lentiviruses (Yu et al., 2003; Mehle et al., 2004; Sheehy et al., 2003), and 3) mimicry of endogenous substrate by the viral protein, which ferries a cellular protein to be targeted for ubiquitination, as observed in the manipulation of Cul1-βTRCP by HIV-1 Vpu (Bour et al., 2001; Margottin et al., 1998).

In this study, we aim to investigate the manner by which Vpr and Vpx interact

with DCAF1, resulting in an alteration of substrate specificity. Using mutational analysis of the DCAF1 substrate-binding interface, we propose that although Vpr and Vpx share a highly homologous DCAF1 binding motif, Vpr and Vpx interact with the Cul4-DCAF1 ubiquitin ligase differently. In addition, we observe that Vpr and Vpx functions are dependent on DCAF1 WD40 domain residues not directly involved in binding Vpr or Vpx. Based on these observations and other recently published results, we propose a model whereby Vpr and Vpx recruit substrate in an unusual manner involving direct interaction between the virally defined substrate and DCAF1.

Materials and Methods

Cell culture

Exponentially growing 293FT and HeLa cells were cultured in Dulbecco minimal essential medium (DMEM)(Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (Invritogen). 293FT cells were transfected using the Calcium Phosphate method, as previously described (Zhu et al., 2001). 36 hr posttransfection cells were harvested, washed 2x with Phosphate Buffered Saline (PBS), and lysed as described below. HeLa cells were transfected with the FuGene HD (Promega, Madison, WI) per manufacturer's protocol.

Plasmids

Flag-DCAF1 was purchased from GeneCopeia (Germantown MD). DCAF1 truncations were made by generating unique restrictions sites where indicated (Figure 2.1) using Quikchange Lightning site-direct mutagenesis (Agilent Technologies, Santa

Clara, CA) per manufacturer's recommendations. DCAF1 WD40 point mutants were generated by site-generated mutagenesis using Quikchange Lightning according to manufacturer's recommendation. Primers were designed utilizing Agilent Technologies Quikchange online primer design tool. Myc-huSAMHD1 was purchase from OriGene (Rockville, MD). pcDNA3.1 was purchased from Invitrogen. HA-Vpr and HA-Vpx were subcloned from pHR-HA-Vpr/Vpx-ires-GFP (Dehart et al., 2007) into pFIN-EF1-GFP-2a-mCherH-WPRE (a kind gift of Dr. Semple-Rowland)(Verrier et al., 2011) in the place of mCherH.

Immunoprecipatations and Western blots

For immunoprecipation, cells were gentle detached by incubation in PBS and pelleted at 13,000rpm for 5 min in a tabletop microcentrifuge. Cells were lysed with Flag IP buffer (50 mM Tris HCI, pH 7.4, 15 0mM NaCl, 1 mM EDTA, and 1% TRITON X-100) in the presence of protease inhibitors (Complete EDTA free tablets; Roche, Indianapolis, IN). Lysate protein concentrations were determined by Pierce™ BCA (Thermo Scientific, Rockford, IL) and brought to equal protein concentration. Lysates were subject to immunoprecipitation using Anti-FLAG® M2 Magnetic Beads (SIGMA-ALDRICH, St. Louis, MO) in accordance with manufacturer recommendations. Briefly, lysates were incubated with beads for 2 hr (at RT) to overnight (at 4ºC). Beads were washed 5x with lysis buffer and proteins eluted with 3x-FLAG® Peptide, 100 µg/ml for 1 hr at RT. Cells used in degradations assays were lysed in SET Buffer (1% SDS, 50 mM Tris HCL, pH 7.4, 1 mM EDTA); lysates were thoroughly denatured by boiling for 5 min. Lysates and immunoprecipation samples were resolved by SDS-PAGE on 4-10%

Criteron™ TGX™ gels (Bio Rad, Hercules, CA) per manufacturer's recommendations and transferred to PVDF membrane (EMD Millipore, Billerica, MA). The following antibodies were used: FLAG® M2 and β-actin (Sigma Aldrich), HA.11 and 9E11(c-Myc)(Covance), DDB1 (Abcam).

Lentiviral vectors

pHR-Vpr-IRES-GFP, pHR-Vpr(R80A)-IRES-GFP, pHR-Vpx293-IRES-GFP, and pHR-GFP viruses were produced as previously described (Zimmerman et al., 2004; Roshal et al., 2003). Briefly, 293FT cells were transfected as described earlier with transfer vector, pCMV Δ8.2ΔVpr packaging plasmid, and pCMV-VSVG envelope plasmid at a ratio of 2.5:2.5:1. Supernatants were collected every 24 hr, until the cell monolayer died, and cleared of cell debris by centrifugation at ~825x-g for 5 min. Pooled supernatant was concentrated by ultracentrifugation at 25,000x-g for 2 hr. Concentrated virus was resuspended in DMEM with 10% FBS and 1% L-Glutamine and stored at -80ºC. HeLa cells were transduced in in presence of 10 μg/ml polybrene overnight. Viral titers were determined by GFP expression.

FACS and cell cycle analysis

HeLa cells were detached by trypsinization and washed in fluorescence-activated cell sorting (FACS) buffer (2% FBS and 0.02% Sodium Azide in PBS). For analysis of GFP, cells were immediately subject to flow cytometry. Cells for cell cycle analysis were pelleted at ~825x-g for 5 min and fixed with 70% ethanol at -20ºC overnight. Fixed cells were rehydrated by 2x wash in PBS. DNA content was determined by staining in 50

μg/ml propidium iodide in 0.1% Triton X-100 PBS with RNase A for 20 min. Cells were analyzed on a BD FACS Canto II flow cytometer using the FACSDiva software (Becton Dickinson, Mountain View, CA) and data analyzed using FlowJo (Tree Star Inc, Ashland, OR).

Results

DCAF1 interacts with the Cul4-DDB1 ubiquitin ligase backbone through a motif N-terminal to the WD40 domain

The modular nature of many cellular ubiquitin ligases allows for the regulated targeting and degradation of a diverse array of substrates (Nalepa et al., 2006). In the case of the Cul4-based ligases, the core ubiquitin ligase comprised of Cul4-Rbx1-DDB1 has been shown to interact with a large number of substrate receptors termed DDB1-Cullin4 Associated Factors (DCAFs) (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006). In 2007, our group and several others demonstrated that the HIV accessory protein Vpr interacted directly with DCAF1, resulting in a robust cell cycle arrest at the G2/M transition (Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Hrecka et al., 2007; Belzile et al., 2007; Wen et al., 2007). Subsequently, it was shown that the HIV-2/SIVagm Vpr paralog Vpx also utilized the same ubiquitin ligase (Srivastava et al., 2008).

Molecular analyses of DCAFs have revealed that most consist primarily of a WD40 domain (Higa et al., 2006), a highly conserved β-propeller structure involved in mediating protein-protein interactions (Stirnimann et al., 2010). In contrast, the complex domain architecture of DCAF1 (comprised of an N-terminal armadillo domain, a central

LisH domain, a C-terminal WD40 domain, and a highly acidic C-terminal tail) makes it unique among DCAFs (Jin et al., 2006) (Figure 2.1). In order to better determine the manner in which Vpr and Vpx manipulate the Cul4-DCAF1 ubiquitin ligase, we generated a number of Flag-tagged DCAF1 truncation mutants in which each domain has been removed individually or in conjunction with others. In addition, the annotated but undescribed DCAF1 isoform 3, which contains a large truncation in the armadillo domain generated via alternative splicing, was also tested (Figure 2.1). The ability of these Flag-DCAF1 truncations to interact with Vpr/Vpx and the Cul4-DDB1 ligase backbone was analyzed by co-immunoprecipitation (Co-IP).

Interaction of Vpr/Vpx with DCAF1 alone would not be expected to facilitate their function as ubiquitination of cellular proteins by DCAF1 requires interaction with the Cul4-DDB1 ubiquitin ligase backbone. To this end, Flag-DCAF1 truncations were analyzed for the presence of DDB1. As expected, full length DCAF1, both isoform 1 and isoform 3, were capable of interacting with DDB1 (Figure 2.2, lanes 2 and 3). In agreement with previous observations indicating a role of the WD40 domain in DDB1 interaction (Angers et al., 2006), truncations lacking the WD40 domain lost the ability to interact with DDB1 (Figure 2.2, lanes 4, 6-8). Surprisingly, the Flag-DCAF1 WD40 was unable to immunoprecipitate DDB1 (Figure 2.2, lane 11). This is in contrast to earlier reports in which “WD40” truncations were able to interact with DDB1 (Gérard et al., 2014; Le Rouzic et al., 2007). Most likely, this discrepancy is due to differences in truncation length (residues 1041-1377(Le Rouzic et al., 2007), 1041-1393(Gérard et al., 2014), and 1073-1396 in this manuscript). Recently, it was demonstrated that interaction between DCAFs and DDB1 is facilitated by a cryptic α-helix located N-terminal to the

WD40 domain (termed an Hbox) (Li et al., 2009). Gérard et al. recently determined the putative DCAF1 Hbox spans 1049-1062, which is absent from our WD40 truncation. In agreement with this hypothesis, the minimal truncation we generated of DCAF1 capable of interacting with DDB1 was that containing the LisH and WD40 domains (Figure 2.2, lane 9). As expected, truncations lacking this region were unable to interact with DDB1. Interestingly, the Δacidic truncation lost the ability to interact with DDB1 (Figure 2.2, lane 5). It has recently been shown that the acidic C-terminal tail is involved in regulatory function via interaction with the protein Merlin (Li et al., 2010). It is tempting to speculate that loss of DDB1 interaction by this truncation is due to regulatory activities mediated by the acidic tail.

Mutations of DCAF1 WD40 substrate binding interface disrupt Vpx-mediated SAMHD1 degradation

In order to determine the molecular mechanism by which the paralogous lentiviral accessory proteins Vpr and Vpx manipulate the substrate specificity of DCAF1, we generated a number of point mutations to the WD40 domain of DCAF1 (Table 2.1). Substrate interaction by DCAFs is believed to be mediated by interaction with this β-propeller structure. Previous reports have demonstrated that the "top" (by convention) of WD40 domains form a shallow groove that mediates interaction with proteins to be targeted for degradation (reviewed in (Trievel and Shilatifard, 2009; Stirnimann et al., 2010)). Using homology modeling between DCAF1 and the most closely related DCAF (WDR5) with a known structure (Figure 2.3A)(Schuetz et al., 2006; Song and Kingston, 2008; Patel et al., 2008), as well an *in silico* (ModBase) structural prediction of DCAF1's WD40 domain (Pieper et al., 2014)(Figure 2.3B), we generated a series of point

mutations which we predicted would fall around this substrate binding region.

293FT cells were transfected with DCAF1 truncations and point mutants, HA-Vpx and Myc-SAMHD1, and screened for the inhibition of Vpx-mediated degradation of SAMHD1, its recently described target (Laguette et al., 2011; Hrecka et al., 2011). Interestingly, DCAF1 truncations fail to mediate SAMHD1 degradation by Vpx (data not shown, and Figure 2.4). Specifically, the minimal truncation generated capable of binding DDB1, DCAF1 LisH-WD40, no longer mediated SAMHD1 degradation, indicating that binding of Vpx to the Cul4 ubiquitin ligase is not sufficient to mediate its activity. This observation indicates DCAF1 does not merely serve as a bridge by which Vpx brings SAMHD1 to the Cul4 ubiquitin ligase. In addition, several point mutants (D1092A, N1135A, and W1156H) were identified that failed to facilitate the degradation of SAMHD1 by Vpx (Figure 2.4, lanes 10, 12-13). Analysis of additional residues (H1134A and D1256A) to DCAF1 WD40 domain (Figure 2.4, lanes 11 and 14) as well as others (data summarized in Table 2.1) show no effect on the SAMHD1 degradation activity of Vpx, indicating that mutations to this region do not generally perturb Vpx function.

DCAF1 WD40 mutations disrupt Vpx binding

In order to better understand how these DCAF1 mutations disrupt Vpx function, they were analyzed for their ability to interact with Vpx by co-IP. As expected, immunoprecipitation of full length Flag-DCAF1 was able to bring down Vpx (Figure 2.5, lane 4). Interestingly, in contrast to previous reports regarding the highly related lentiviral protein Vpr ((Le Rouzic et al., 2007) and this report), the WD40 domain of DCAF1 was

not sufficient to co-IP Vpx (Figure 2.5, lane 5), while the longer truncation containing both the LisH and WD40 domains regained this function (Figure 2.5, lane 6), indicating that Vpx-DCAF1 interaction may involve residues outside of the WD40 region or may be dependent on assembly of the complete ubiquitin ligase machinery. Co-IP analysis of DCAF1 WD40 mutants revealed Vpx-mediated SAMHD1 degradation can be inhibited without disrupting Vpx binding. While DCAF1 mutations D1092A, N1135A, and W1156H all disrupted Vpx-mediated SAMHD1 degradation, only N1135A and W1156H (Figure 2.5, lanes 9 and 10) result in a loss of Vpx interaction, explaining their inability to mediate SAMHD1 degradation.

Interestingly, DCAF1 D1092A, which failed to facilitate SAMHD1 degradation, retained binding to Vpx (Figure 2.5, lane 7). Based on these results and a recently published co-crystal of DCAF1 (WD40, residues 1058-1296)-Vpx-SAMHD1 (C-term) (Schwefel et al., 2014) we propose a novel mechanism by which Vpx alters the substrate specificity of the DCAF1-containing ubiquitin ligase. In this model, Vpx generates a novel substrate binding surface involving Vpx and DCAF1 in the recruitment of SAMHD1.

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Vpr interacts with DCAF1 differently than Vpx

While a recently published crystallographic study determined the Vpx-DCAF1 binding interface, no structural data exist regarding the Vpr-DCAF1 interaction (Schwefel et al., 2014). A high degree of homology between Vpr and Vpx led us to hypothesize they would interact with DCAF1 in a similar manner. Analogous mutations in the third helix of both Vpr (Q65R) and Vpx (Q76R) have been shown to abrogate their ability to interact with DCAF1 (Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Belzile et al., 2007; Srivastava et al., 2008). However, several studies point towards different mechanisms of DCAF1 manipulation by Vpr and Vpx. Most striking perhaps are the observations regarding interaction of Vpr and Vpx with their respective targets. While Vpx and SAMHD1 have been observed to form stable binary complexes independent of DCAF1 (Hrecka et al., 2011), Vpr mutants unable to bind DCAF1 were not able to interact with the newly identified G2 arrest target(s), SLX4 and Mus81 (Laguette et al., 2014).

In order to examine the molecular interaction between DCAF1 and Vpr, DCAF1 mutations were tested for their ability to interact with Vpr. As expected, Vpr was able to co-IP with Flag-DCAF1 (Figure 2.4, lane 4). In agreement with previously published observations (Le Rouzic et al., 2007), the DCAF1 WD40 domain was sufficient to

mediate Vpr binding (Figure 2.6, lane 5). The longer LisH-WD40 truncation also retained Vpr binding (Figure 2.6, lane 6). In contrast to Vpx, both the N1135A and W1156H mutants retained Vpr binding (Figure 2.6, lanes 9 and 10).

Recently, several groups demonstrated Vpr alters Cul4-DCAF1 function by enhancing ubiquitination of specific endogenous targets, UNG2 and SLX4 complex (Wen et al., 2012; Laguette et al., 2014), raising the possibility that mutations to DCAF1 WD40 may inhibit Vpr-mediated G2 arrest without abrogating Vpr binding. In order to assess the effect of DCAF1 truncations and WD40 mutants on Vpr-mediated G2 arrest, HeLa cells were transfect with Vpr in the presence or absence of DCAF1 mutants. 36 hr posttransfection cells were harvested and analyzed for G2 arrest by FACS. As expected, G2 arrest was observed in cells transfected with Vpr alone or with WT DCAF1. Expression of either DCAF1 WD40 or LisH-WD40 was observed to inhibit Vpr-mediated G2 arrest in a dominant negative manner (Figure 2.7). One possible explanation for this effect is that these truncations may serve as molecular sinks for Vpr (capable of binding Vpr or Vpr and DDB1, respectively, but not the G2 substrate) reminiscent of the previously described effect of DDB1 overexpression (Wen et al., 2007). Surprisingly, several DCAF1 WD40 mutants (D1092A, N1135A, W1156H, and D1256A) also inhibited the induction of G2 arrest by Vpr. These observations demonstrate Vpr activity is more sensitive to DCAF1 WD40 mutations than Vpx.

Vpr Function is dominant to Vpx

Discrepancies with regard to DCAF1-mutant binding to Vpr and Vpx may be explained by two, nonmutually exclusive, hypotheses: 1) differential binding affinities of

Vpr and Vpx to DCAF1 and/or 2) differential binding interfaces in the Vpr-DCAF1 vs Vpx-DCAF1 interaction. In order to distinguish between these to hypotheses, we examined the functional competition between Vpr and Vpx. To this end, HeLa cells were transduced with virus expressing Vpr and either a control virus (GFP), Vpx, or the dominant negative Vpr mutant R80A (Dehart et al., 2007). 36 hr posttransduction cell cycle profiles were determined for each cell population. As expected, Vpr transduced cells arrested in G2 (Figure 2.8). Co-expression of Vpr R80A reduces the percentage of cells in the G2 phase in a dose-dependent manner (Figure 2.8C-E). In contrast, expression of Vpx had no effect on Vpr-mediated G2 arrest (Figure 2.8F-G).

In order to determine if Vpr acted in a similar manner, 293FT cells were transfected with an HA-Vpx expressing plasmid alone or in the context of HA-Vpr at 2:1 or 1:1. As expected, Vpx expression resulted in the depletion of SAMHD1 (Figure 2.9, lane 1 vs lane 2). In contrast to the inability of Vpx to relieve Vpr-mediated G2 arrest, co-expression of Vpr robustly inhibited Vpx-induced SAMHD1 degradation (Figure 2.9, lanes 3 and 4 vs lane 2).

These observations, taken together, support a model in which Vpr and Vpx bind DCAF1 in a competitive manner. These observations cannot distinguish whether this competition is for a shared binding site, to which Vpr binds more tightly, or binding to an allosteric site. However, we favor a model in which Vpr and Vpx utilize a similar binding interface on DCAF1 based on the high homology between their third α-helix, known to be the major determinant of DCAF1 binding (Le Rouzic et al., 2007; Srivastava et al., 2008; Schwefel et al., 2014)(reviewed in (Morellet et al., 2009)). In agreement with the hypothesis that Vpr has higher affinity for this binding domain, no mutations to the

DCAF1 WD40 domain were identified which abrogated Vpr binding (Table 2.1, data not shown, and Figure 2.6).

Vpr but not Vpx enhances the DCAF1 interaction with DDB1

It has recently been reported that co-IP of some DCAF1 mutants with DDB1 is enhanced in the presence of Vpr (Gérard et al., 2014). Examining our own co-IPs, we observed a similar phenomenon, whereby Vpr enhanced interaction between WT DCAF1 and DDB1 (Figure 2.10a, lanes 3 and 4). Based on the observed difference between Vpr and Vpx with respect to their interaction with DCAF1, we were curious as to the effect of Vpx on the DCAF1-DDB1 interaction. Expression of Vpx had no apparent effect on the DCAF1-DDB1 interaction (Figure 2.10b lanes 2 and 4).

Discussion

While several studies have examined the molecular determinants on Vpr and Vpx for DCAF1 interaction, little is known in regards to the residues in DCAF1 that mediate these interactions. In this study, we generated a series of point mutations to the previously described Vpr minimal binding domain of DCAF1 (the WD40 domain) (Le Rouzic et al., 2007) and screened them for their ability to mediate Vpr and Vpx interaction and function. Surprisingly, although Vpx and Vpr share a high degree of homology in their DCAF1 binding motifs, we observed differential effect of these mutations on their ability to facilitate Vpr and Vpx interaction. Two DCAF1 WD40 mutations, N1135A and W1156H, were identified which failed to co-IP with Vpx. These residues were recently identified as being involved in the coordination of the Vpx Q76 residue, the mutation of

which is known to abrogate DCAF1 binding (Srivastava et al., 2008), in a recently resolved Vpx-DCAF1(WD40)-SAMHD1 co-crystal (Schwefel et al., 2014). While we would expect these residues to coordinate the analogous Vpr Q65, their mutation does not abrogate binding with Vpr.

This difference may be due in part to different binding affinities of Vpr and Vpx for DCAF1. To address this hypothesis, we conducted a functional competition assay between Vpr and Vpx. In support of the hypothesis that Vpr binds DCAF1 with higher affinity than Vpx, expression of Vpr was able to inhibit the degradation of SAMHD1 by Vpx, though the reciprocal relationship was not observed. Furthermore, expression of Vpr, but not Vpx, was seen to enhance the interaction between DCAF1 and DDB1. While we favor a model in which Vpr binds more tightly to DCAF1 than Vpx, our observations do not exclude the possibility that Vpr and Vpx utilize different binding interfaces on DCAF1. More thorough biochemical analysis will be necessary to fully distinguish between these two possibilities.

Recent studies have highlighted the therapeutic potential of targeting HIV-1 accessory gene function (Stanley et al., 2012; Wei et al., 2014; Nekorchuk et al., 2013; Hofmann et al., 2013). While these studies are promising proofs of concept, a more thorough understanding of the molecular architecture of the ubiquitin ligases in the context of viral manipulation will be necessary to develop more specific pharmacological inhibitors. Disruption of Vpr activity is a compelling avenue for future drug development. The presence of Vpr among all primate lentiviruses indicate a strong selective pressure for Vpr function of viral pathogenesis. Indeed, *in vivo* studies in a rhesus macaque model of AIDS indicated a delayed onset of disease in monkeys infected with Vpr-deficient

viruses (Lang et al., 1993; Hoch et al., 1995). In addition, retrospective analysis of a vaccine study conducted in chimpanzees in which two animals were challenged with a Vpr-deficient HIV revealed reversion of the defective Vpr reading frame in both animals (Goh et al., 1998)(Reviewed in (Andersen et al., 2008)). The highly conserved natures of the Vpr/Vpx DCAF1 binding motif (Srivastava et al., 2008), as well as that of DCAF1 (Zhang et al., 2008) present a novel target for future drug development, for which the evolutionary cost of resistance mutations by the virus may be too great.

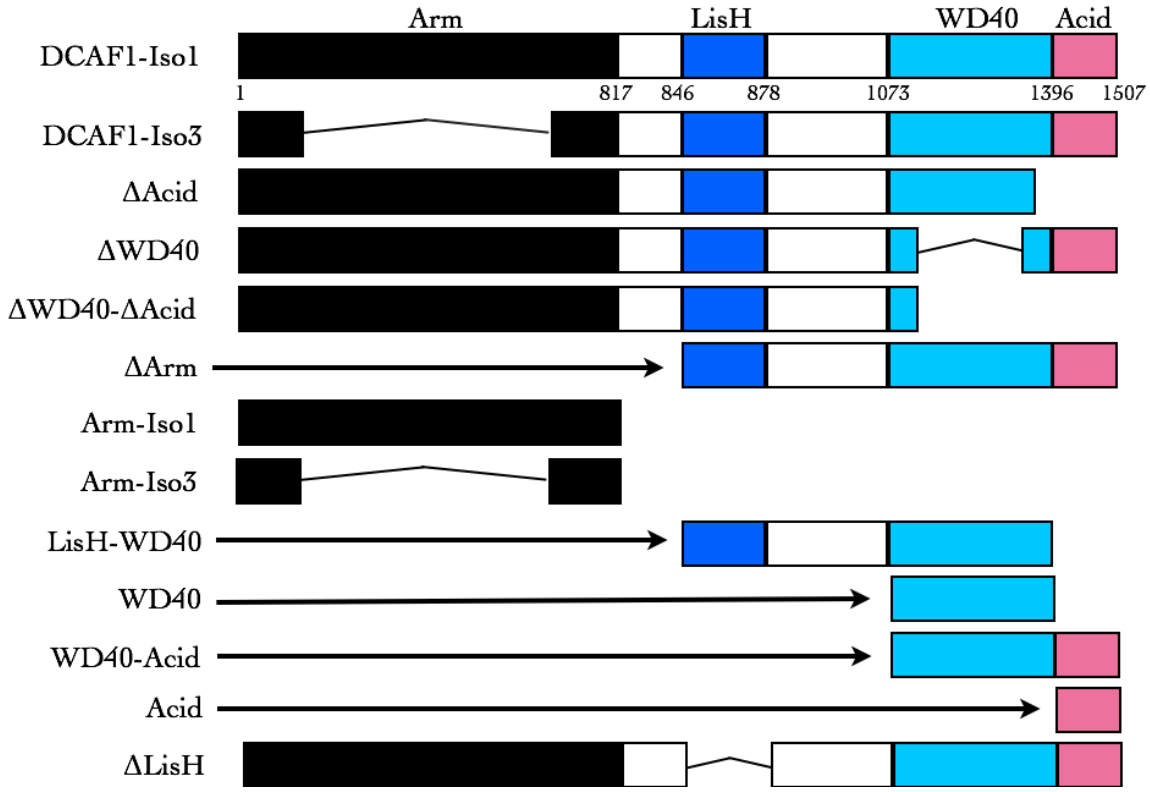

Figure 2.1 DCAF1 domain architecture
Domain architecture of WT DCAF1 (Iso1) and truncations.

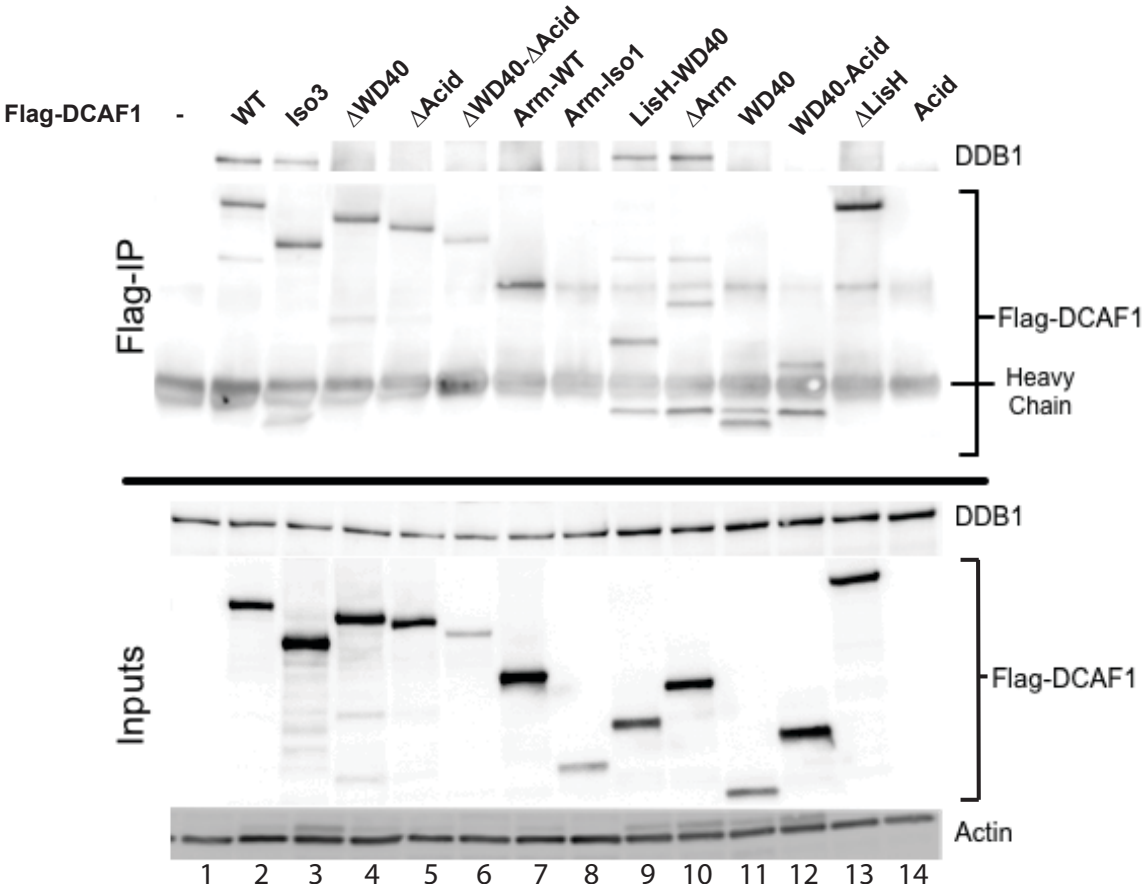

Figure 2.2: DCAF1 truncations DDB1 co-IP
293FT cells were transfected with plasmids expressing Flag-DCAF1 or the truncations described. Cell lysates were subject to Flag-immunoprecipation and analyzed for the presences of DDB1 by Western blot.

Table 2.1: DCAF1 mutations summary
DCAF1 Mutations were screened for their ability to facilitate Vpx-mediated SAMHD1 degradation. Truncation and select point mutants were screened for Vpr function and interaction with Vpx/Vpr/DDB1. Blanks represent mutant not tested for that particular interaction/function.

	Function		Interaction		
	SAMHD1 Deg	G2 Arrest	Vpr	Vpx	DDB1
Iso1	Yes	Yes	Yes	Yes	Yes
Iso3	No		Yes	Yes	Yes
dWD40	No		No	Yes	No
dAcid	No		No	Yes	No
dWD40-dAcid	No		No	No	No
Arm-Iso1	No		No	Yes	No
Arm-Iso3	No		No	No	No
Lish-WD40	No	No	Yes	Yes	Yes
dArm	No	No	Yes	No	Yes
WD40	No	No	Yes	No	No
WD40-Acid	No	No	Yes	No	No
Acid		No			
dLish	No	No	No	No	No
D1092A	No	No	Yes	Yes	Yes
H1134A	Yes	Yes	Yes	Yes	Yes
N1135A	No	No	Yes	No	Yes
T1141A	Yes		Yes		
T1155A	Yes		Yes		
W1156H	No	No	Yes	No	Yes
E1178A-D1179A	Yes		Yes		
H1180A	Yes		Yes		
K1196A	Yes		Yes		
N1221A-N1222A	Yes		Yes		
R1225A	Yes		Yes		
N1226A	Yes		Yes		
D1256A	Yes	No	Yes	Yes	Yes
K1257A	Yes		Yes		
S1263A	Yes		Yes		
D1295A	Yes		Yes		
Q1296A	Yes		Yes		
Q1316A	Yes		Yes		
D1320A	Yes		Yes		
F1334Y	Yes		Yes		
V1350T	Yes		Yes		
N1379A	Yes		Yes		

A.

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DCAF1          ---------EDESGFTCCAFSARERFLMLGTCTGQLKLYNVFSGQEEASYNCHNSAITHL 1141
WDR5           NYALKFTLAGHTKAVSSVKFSPNGEWLASSSADKLIKIWGAYDGKFEKTISGHKLGISDV 93
                        ...:.  **..  :*  .:.   :*::..:.*: * : . *: .*: :

DCAF1          EPSRDGSLLLTSATWSQPLSALWGMKSVFDMKHSFTEDHYV---EFSKHSQDRVIGTKGD 1197
WDR5           AWSSDSNLLVSAS--DDKTLKIWDVSSGKCLKTLKGHSNYVFCCNFNPQSNLIVSGSFDE 151
                 * *..**::::  .:    :*.:.*   :*     .:**   :*. :*:  * *: .:

DCAF1          IAHIYDIQTGNKLLTLFNPDLANNYKRNCATFNPTDDLVLN---DGV--LWDVRSAQAIH 1253
WDR5           SVRIWDVKTGKCLKTL----PAHSDPVSAVHFNRDGSLIVSSSYDGLCRIWDTASGQCLK 207
                 ..*:*::**: * **     *:.   ... **  ..*::.   **:  :**. *.*.::

DCAF1          KF-DKFNMNISGV-FHPNGLEVIINT-----EIWDLRTFHLLHTVPALDQCRVVFNHTGT 1306
WDR5           TLIDDDNPPVSFVKFSPNGKYILAATLDNTLKLWDYSKGKCLKTYTGHKNEKYCIFANFS 267
               .: *. *  :* * * ***  ::  *     ::**  . : *:* .. .: .  :  . :

DCAF1          VMYGAMLQADDEDDLMEERMKSPFGSSFRTFNATDYKPIATIDVKRNIF--DLCTDTKDC 1364
WDR5           VTGGKWIVSGSEDNLV------------YIWNLQTKEIVQKLQGHTDVVISTACHPTENI 315
               *  *  : :..**:*:              :*    : : .:: : ::.    *  *::

DCAF1          YLAVIENQGSMDALNMDTVCRLYEVGRQRLAEDEDE                        1400
WDR5           IASA--------ALENDKTIKLWK---------SDC                        334
                 :.        **: *.. .*::         .*
```

B.

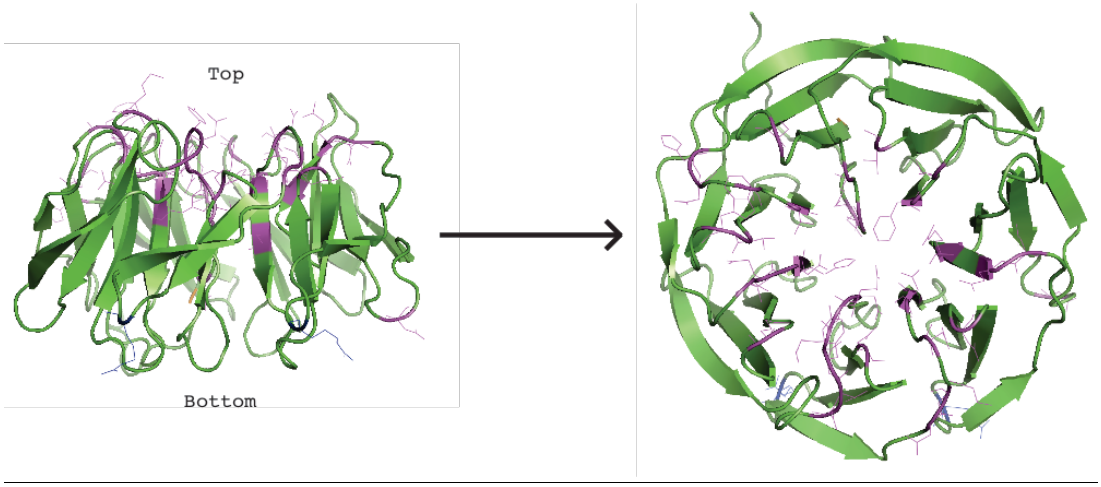

Figure 2.3: DCAF1 mutation strategy
A) DCAF1 WD40 domain and WDR5 protein sequences were aligned using ClustW2 (Larkin et al., 2007). WDR5 tertiary structure is indicated below (bold lines – β-sheets, narrow lines connect β-sheets which form respective "blades" of the β-propeller. Homology is indicated below, asterisks (*) indicate identity, colons (:) and periods (.) similarity. Purple arrowheads indicate residues mutated in DCAF1. B) ModBase (Pieper et al., 2014) predicted structure of the DCAF1 WD40 domain. Residues mutated in our screen are highlighted in purple.

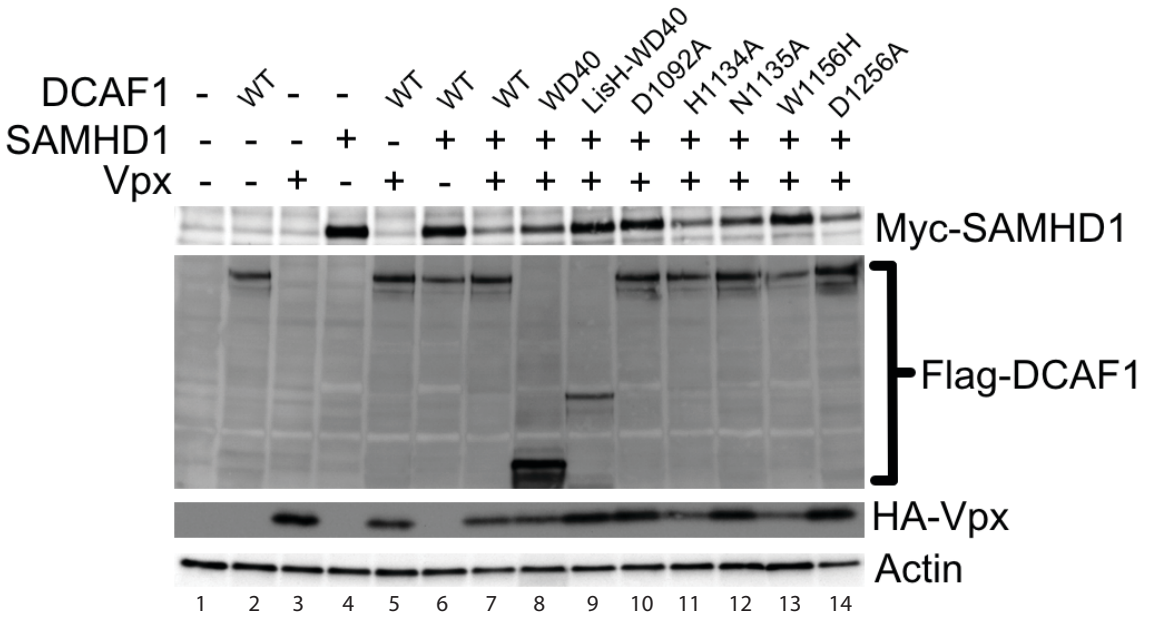

Figure 2.4: Analysis of DCAF1 mutations for their ability to facilitate Vpx-mediated SAMHD1 degradation

Cells were transfected with human Myc-SAMHD1, HA-Vpx, and/or Flag-DCAF1 mutant expression plasmids as indicated. 36 hr posttransfection cells were lysed and analyzed for the presence of SAMHD1 by Western blot.

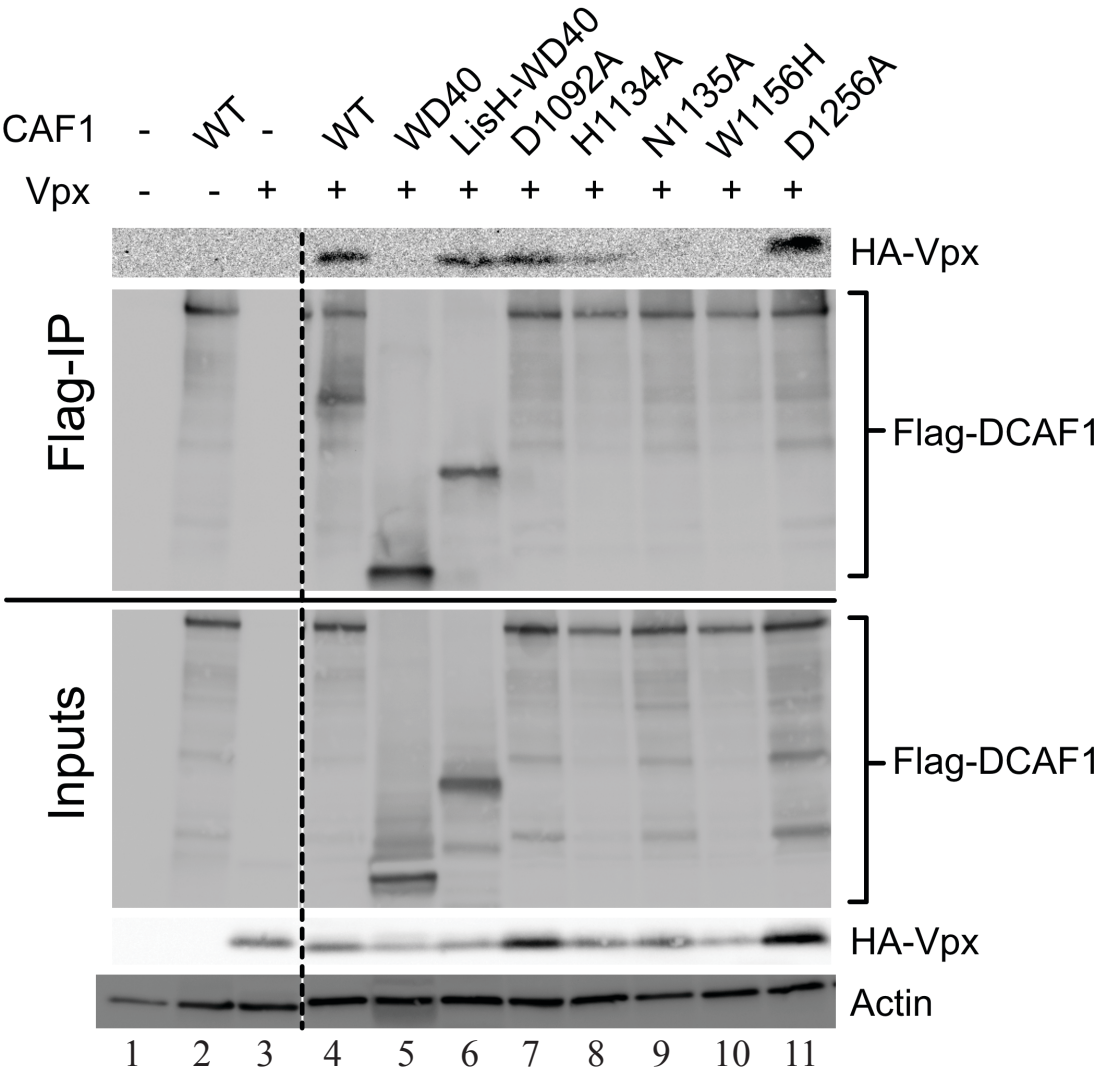

Figure 2.5: Analysis of DCAF1 truncations and point mutant ability to interact with Vpx
293FT cells were transfected with plasmids expressing Flag-DCAF1 mutations and HA-Vpx as indicated. Cell lysates were subject to Flag-immunoprecipation and analyzed for the presences of HA-Vpx by Western blot.

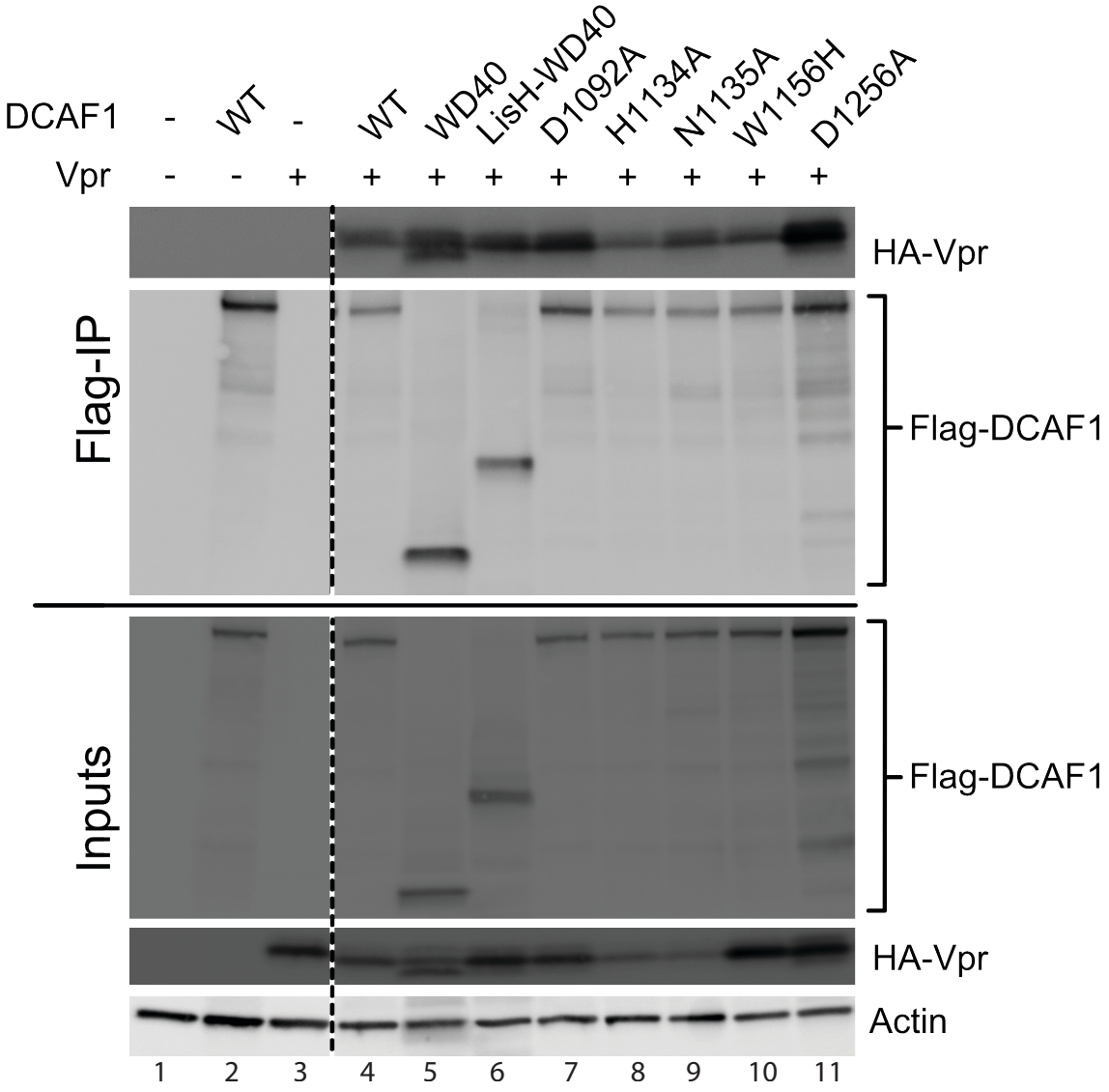

Figure 2.6: DCAF1 determinants of Vpr interaction are different than those of Vpx
293FT cells were transfected with plasmids expressing Flag-DCAF1 mutations and HA-Vpr as indicated. Cell lysates were subject to Flag-immunoprecipation and analyzed for the presences of HA-Vpr by Western blot.

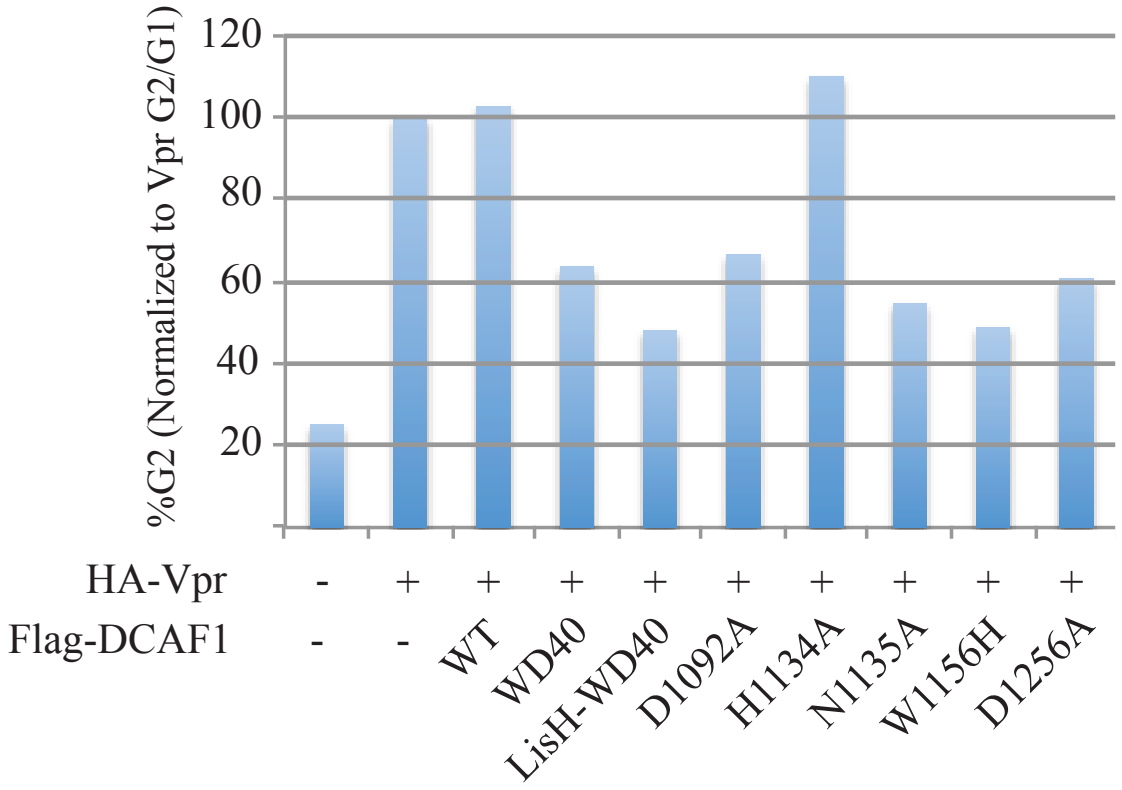

Figure 2.7: DCAF1 WD40 mutants inhibit Vpr induced G2 arrest
HeLa cells were transfected with DCAF1 mutants or empty vector control plasmids and Vpr as indicated. 36 hr posttransfection cells were analyzed for cell cycle arrest by FACS.

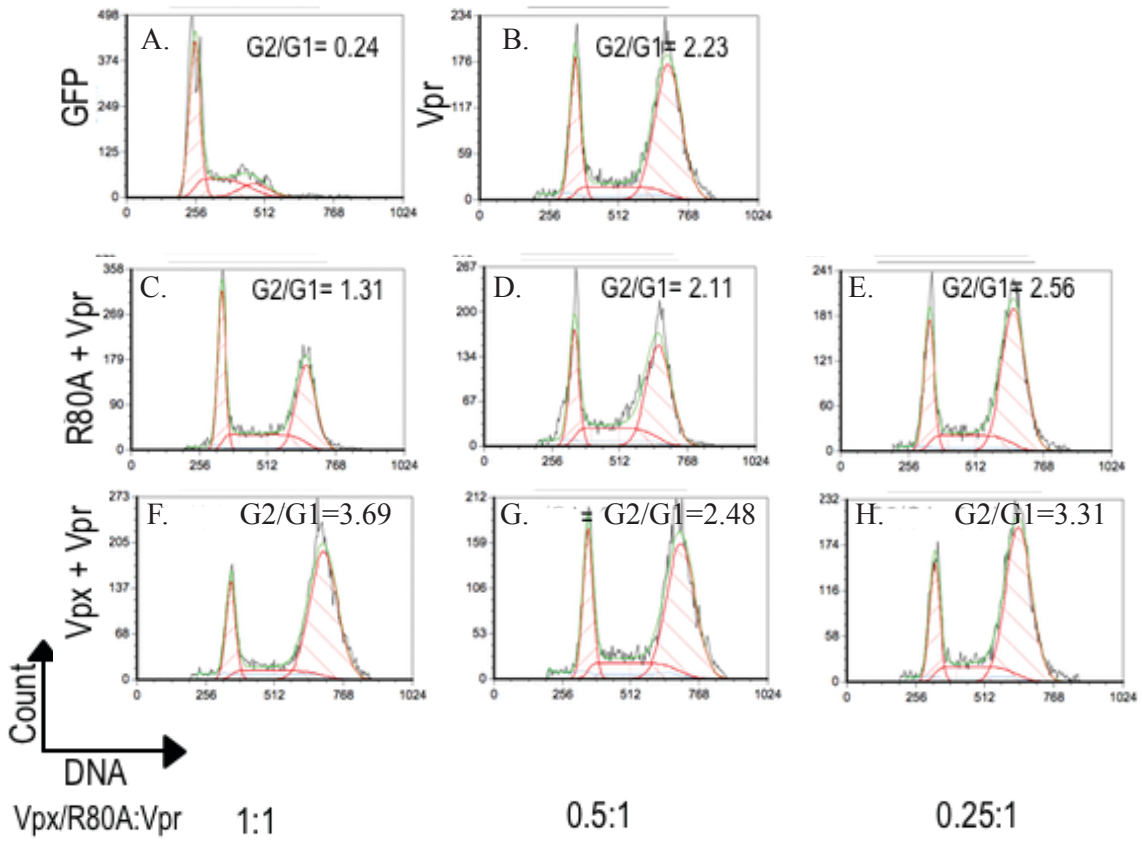

Figure 2.8: Vpx fails to compete Vpr mediated G2 arrest
Cells were transduced with control (GFP) or Vpr expressing virus alone or with Vpx or the dominant negative Vpr R80A and analyzed for G2 arrest by FACS. Infection ratios were determined by the percent GFP+ cells of Vpr, Vpx, or R80A singly infected cells.

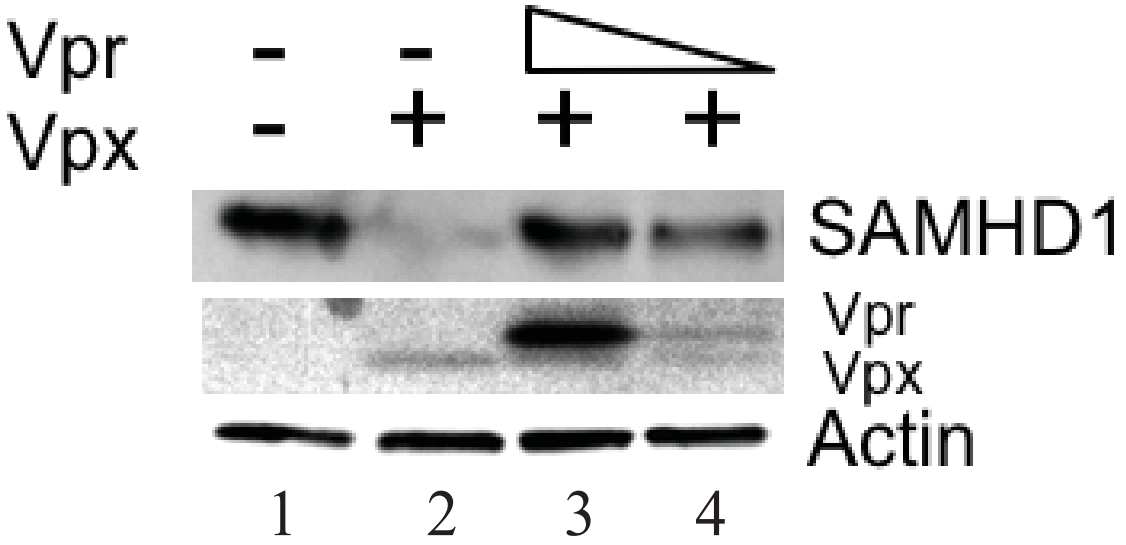

Figure 2.9: Vpr functions dominantly to Vpx
293FT cells were transfected with a HA-Vpx plasmid in the presence or absence of a HA-Vpr expression plasmid. All cells were transfected with equal amounts of total plasmid DNA by transfection of pcDNA3.1. Cells were lysed 36 hr posttransfection and endogenous SAMHD1 levels were analyzed by Western blot.

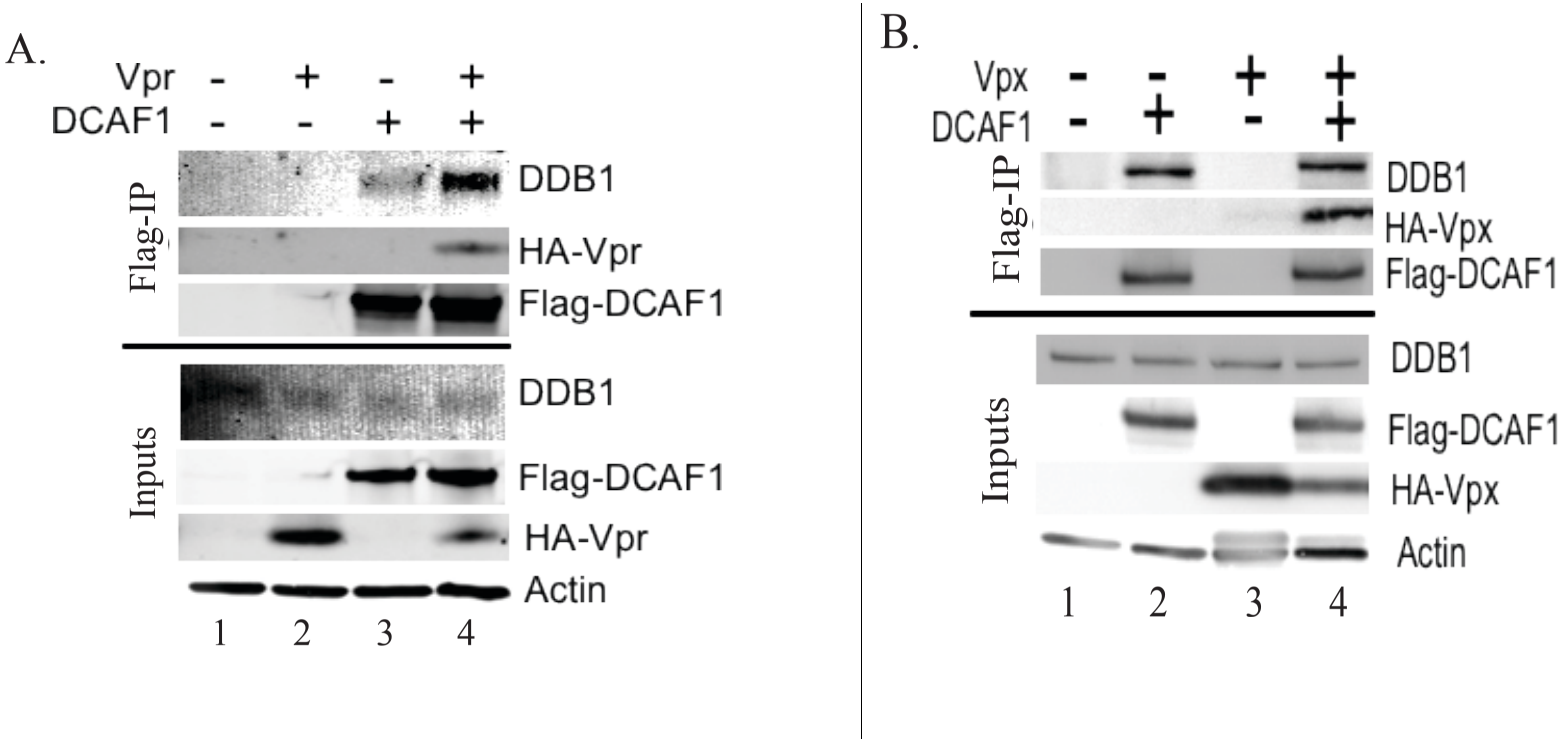

Figure 2.10: Effect of Vpr and Vpx expression of the DCAF1-DDB1 interaction
Flag-DCAF1 was immunoprecipated in the presence or absence of Vpr (A) or Vpx (B) and analyzed for the DDB1 by Western blot.

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CHAPTER 3

ANALYSIS OF THE STRUCTURAL DETERMINANTS OF G2 ARREST AND SAMHD1 DEGRADATION BY THE SIV(AGM) VPR

My co-author on this work was Ana Beatriz DePaula-Silva

Abstract

The HIV-1 accessory protein Vpr is known to induce G2 arrest by manipulating the Cul4-DDB1-DCAF1 ubiquitin ligase, inducing the ubiquitination of Mus81. Induction of cell cycle arrest by Vpr is conserved among primate lentiviruses. In addition to Vpr, a subset of primate lentiviruses encode the Vpr paralog Vpx, which functions to enhance myeloid cell infection by inducing the degradation of the restriction factor SAMHD1. Interestingly, some Vpr homologs, namely SIVagm Vpr, are bifunctional, able to induce G2 arrest and the degradation of SAMHD1. To better understand the structure-function relationship between HIV-1 Vpr and SIVagm Vpr, we generated several HIV-1 Vpr truncations and chimeras between these two proteins. We tested the ability of these chimeric proteins to induce G2 arrest in human cells and degrade SAMHD1. From our analyses, we conclude that substitution of the C-terminal unstructured region of SIVagm Vpr by the same region of HIV-1 Vpr confers to this

chimera the *de novo* ability to arrest human cells in G2. In addition, while the N-terminal domain of SIV Vpx was shown to be important for degradation of SAMHD1, we show additional determinants are necessary for this activity. Finally, the ubiquitination target for HIV-1 Vpr was recently reported to be Mus81. While we confirm that HIV-1 Vpr induces degradation of Mus81 in a proteasome- and Cullin-dependent manner, our results indicate that this function is independent of the ability of Vpr to induce G2 arrest.

Introduction

The primate lentiviral protein Vpr is conserved among all primate lentiviruses (HIV/SIV). Studies of other mammalian lentiviruses have identified Vpr-like genes in several evolutionarily distant lentiviruses pointing to an ancient origin for Vpr function (R\reviewed in (Gifford, 2012)). Vpr expression of has been shown to induce a pleotropic number of effects including increased infection of nondividing cells, involvement in pre-integration complex nuclear import (Heinzinger et al., 1994), and the manipulation of the Cul4-DCAF1 ubiquitin ligase facilitating degradation of UNG2 (Schröfelbauer et al., 2005) (reviewed in (Andersen et al., 2008)) and induction of G2 cell cycle arrest (Dehart et al., 2007; Le Rouzic et al., 2007; Wen et al., 2007; Hrecka et al., 2007; Belzile et al., 2007; Schröfelbauer et al., 2007) through the activation of the endonuclease SLX4 complex (Laguette et al., 2014).

In addition to Vpr, a subset of primate lentiviruses encode the highly related protein, Vpx (Gifford, 2012). Evolutionary studies indicate it arose following a gene duplication event in the most recent common ancestor of the SIVs infecting the *Cercopithecinae* tribe Popionini (SIVmnd/mac/smm) (Lim et al., 2011)(reviewed in

(Planelles, 2011)). Although Vpr and Vpx share a high degree of structural homology (Tristem et al., 1990) they exert functionally distinct roles in viral replication, suppression of interferon response (Laguette et al., 2014), and infection of myeloid cells by inducing the degradation of SAMHD1 (Laguette et al., 2011; Hrecka et al., 2011) (Reviewed in (Laguette and Benkirane, 2012)), respectively. Interestingly, the SIVs which infect the five genetically distinct African Green Monkey (AGM) species, collectively referred to as SIVagm, encode a single Vpr gene capable of inducing both G2 arrest and SAMHD1 degradation (reviewed in (Planelles, 2011)).

In addition to their distinct functionality, Vpr and Vpx have undergone evolutionary arms races (reviewed in (Duggal and Emerman, 2012)), resulting in a species-specific ability to antagonize their respective restriction factors. While HIV-1 Vpr is able to induce cell cycle arrest in cells from several primate species, SIVagm Vpr activities (both the induction of cell cycle arrest and the degradation of SAMHD1) are known to be restricted to antagonism of the AGM restriction factor homologs (Planelles et al., 1996; Lim et al., 2011).

In this study, we aim to understand the molecular determinants of G2 arrest and SAMHD1 degradation, respectively. To this end, we generated a number of HIV-1 Vpr-SIVmac Vpx and HIV-1 Vpr – SIVagm Vpr chimeras. While early studies have demonstrated the necessity of the unstructured N and C-terminus for SAMHD1 degradation (Gramberg et al., 2010) and G2 arrest induction (Le Rouzic et al., 2007; Morellet et al., 2009), respectively, it remains unclear if additional determinants are necessary to mediate these functions.

Here we demonstrate that simple transposition of Vpr or Vpx unstructured termini

onto the reciprocal paralog is not sufficient to confer neofunctionality to the chimeric proteins, indicating Vpr and Vpx function are not defined by simple linear determinants. In further support of this hypothesis, we observe that the transposition of the SIVagm Vpr C-terminus onto HIV-1 Vpr retained the ability to induce G2 arrest in human cells. Finally, we explore the link between Vpr G2 arrest and the degradation of the newly identified Vpr target Mus81, a subcomponent of the SLX4 complex, recently described as being ubiquitinated in a Vpr-dependent manner (Laguette et al., 2014). In contrast, we observe Mus81 to be degraded by Vpr in a G2 arrest and DCAF1-independent manner.

Materials and Methods

Cell culture

Exponentially growing 293FT and HeLa cells were cultured in Dulbecco minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (Invitrogen). 293FT cells were transfected using the Calcium Phosphate method, as previously described (Zhu et al., 2001). 36 hr posttransfection, cells were harvested, washed 2x with Phosphate Buffered Saline (PBS), and lysed as described below. HeLa cells were transfected with the FuGene HD (Promega, Madison, WI) per manufacturer's protocol. Cells were treated, when indicated, with 0.5 μM Epoxomicin (SIGMA-ALDRICH) or 100 nM MLN4924 (MedChem Express, Princeton, NJ) for 18 hr.

Plasmids

pFIN-EF1-GFP-2A-mCherry-WPRE vector was kindly provided by Susan L. Semple-Rowland. MluI and EcoRV restriction sites were added to this vector by site-directed mutagenesis using the QuikChange Lightning (Agilent Technologies). HIV-1 Vpr, SIVagm Vpr, and SIVmac Vpx were PCR amplified with MluI and EcoRV restriction sites added to the primer, and cloned into pFIN-EF1-GFP-2A-mCherry-WPRE in place of mCherry. Chimeras were generated by PCR and and cloned into pFIN-EF1-GFP-2A-mCherry-WPRE using MluI and EcoRV restriction sites. cDNA for Mus81 was purchased from DNASU (Tempe, AZ). Mus81 was PCR amplified and N-terminus tagged with V5 epitope. V5-Mus81 was cloned into pFIN-EF1-GFP-2A-mCherry-WPRE in place of GFP using NheI and BspeI restriction sites. A stop codon was added before the 2A generating into pFIN-EF1-V5-Mus81. HA-AGM-SAMHD1 was kindly provided by M. Emerman.

Immunoprecipitation and Western blots

For immunoprecipitation, cells were gently detached by incubation in PBS and pelleted at 13,000 rpm for 5 min in a tabletop microcentrifuge. Cells were lysed with Flag IP buffer (50 mM Tris HCI, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100) in the presence of protease inhibitors (Complete EDTA free tablets; Roche, Indianapolis, IN). Lysate protein concentrations were determined by Pierce™ BCA (Thermo Scientific, Rockford, IL) and brought to equal protein concentration. Lysates were subject to immunoprecipitation using Anti-FLAG® M2 Magnetic Beads (SIGMA-ALDRICH, St. Louis, MO) in accordance to manufacturer recommendations. Briefly,

lysates were incubated with beads for 2 hr (at RT) to overnight (at 4ºC). Beads were washed 5x with lysis buffer and proteins eluted with 3x-FLAG® Peptide, 100 µg/ml for 1 hr at RT. Cells for degradations assays were lysed in SET Buffer (1% SDS, 50mM Tris HCL, pH 7.4, 1mM EDTA); lysates were thoroughly denatured by boiling for 5 min. Lysates and immunoprecipitation samples were resolved by SDS-PAGE on 4-10% Criteron™ TGX™ gels (Bio Rad, Hercules, CA) per manufacturer's recommendations and transferred to PVDF membrane (EMD Millipore, Billerica, MA). The following antibodies were used: FLAG® M2 and β-actin (Sigma Aldrich), HA.11 and 9E11(c-Myc)(Covance), DDB1 (Abcam) and V5 (Life Technologies, Carlsbad, CA)

Lentiviral vectors

pFIN GFP-2A-HIV-1 Vpr, pFIN GFP-2A-SIVmac Vpx, pFIN GFP-2A-SIVagm Vpr, and pFIN GFP viruses were produced as previously described (Zimmerman et al., 2004; Roshal et al., 2003). Briefly, 293FT cells were transfected as described earlier with transfer vector, pCMV Δ8.2ΔVpr packaging plasmid, and pCMV-VSVG envelope plasmid at a ratio of 2.5:2.5:1. Supernatants were collected every 24 hr, until the cell monolayer died. Cell debris was cleared by centrifugation at ~825x-g for 5 min. Pooled supernatant was concentrated by ultracentrifugation at 25,000x-g for 2 hr. Concentrated virus was resuspended in DMEM with 10% FBS and 1% L-Glutamine and stored at -80ºC. HeLa cells were transduced in the presence of 10µg/ml polybrene overnight. Viruses were tittered by GFP expression.

FACS and cell cycle analysis

HeLa cells were detached by trypsinization and washed in fluorescence-activated cell sorting (FACS) buffer (2% FBS and 0.02% Sodium Azide in PBS). For analysis of GFP+, cells were immediately subject to flow cytometry. Cells for cell cycle analysis were pelleted at ~825x-g for 5 min and fixed with 70% ethanol at -20ºC overnight. Fixed cells were rehydrated by two washes in PBS. DNA content was determined by staining in 50µg/ml propidium iodide in 0.1% Triton X-100 PBS with RNase A for 20 min. Cells were analyzed on a BD FACS Canto II flow cytometer using the FACSDiva software (Becton Dickinson, Mountain View, CA) and data analyzed using FlowJo (Tree Star Inc, Ashland, OR).

Results

HIV-1 Vpr unstructured C-terminus is necessary for the induction of G2 arrest

It was previously proposed that the C-terminus unstructured region of HIV-1 Vpr is required to recruit the G2 arrest targeted protein (Di Marzio et al., 1995)(Reviewed in (Morellet et al., 2009)). Furthermore, the point mutant Vpr R80A, although able to interact with DCAF1, is unable to induce G2 arrest, resulting in a dominant negative phenotype by competing for DCAF1 binding (Dehart et al., 2007; Le Rouzic et al., 2007). To directly test for the presence of an essential G2 arrest determinant within the C-terminus unstructured region of HIV-1 Vpr, we made two truncations in HIV-1 Vpr (Vpr1-80 and Vpr1-84) and tested for their ability to induce G2 arrest in human cells. HeLa cells were transfected with empty vector, HIV-1 Vpr, HIV-1 Vpr R80A, HIV-1 Vpr (1-80), or HIV-1 Vpr (1-84). 48 hr posttransfection cell cycle was analyzed by flow

cytometry. As shown in Figure 3.1, HIV-1 Vpr, but not HIV-1 Vpr R80A, HIV-1 Vpr (1-80), nor HIV-1 Vpr (1-84), was able to arrest the cell cycle in G2.

Unstructured termini are not sufficient to facilitate neofunctionality of monofuctional Vpr or Vpx

A NMR structure of Vpr revealed that it is comprised of a tight three α-helical bundle flanked by unstructured N- and C-termini (Morellet et al., 2003). Alignment of Vpr and Vpx show high homology between the two proteins within these α-helices (Figure 3.2, grey boxes), while the termini are divergent. These observations have led to the hypothesis that substrate specificity of Vpr and Vpx is defined by these unstructured termini. In agreement with this hypothesis, previous studies indicate that mutations disrupt the Vpr-induced G2 arrest cluster in the C-terminal unstructured region of Vpr (Morellet et al., 2009). The N-terminus of Vpx has been shown to be necessary for recruitment of SAMHD1 for degradation (Gramberg et al., 2010). Interestingly, alignment of the bifunctional SIVagm Vpr reveals it to have high homology with Vpx in the N-terminus, while its C-terminus (with the exception of a poly-proline stretch shared with Vpx) is homologous to that of Vpr. These observations led us to hypothesize that transposition of these domains would result in the generation of chimeric proteins capable of mediating both SAMHD1 degradation and inducing G2 arrest. In order to test this hypothesis, we generated a series of HIV-1 Vpr/SIVagm Vpx chimeras in which the unstructured N- and C-termini were transposed between the paralogous proteins (Figure 3.3).

In order to determine if the monofunctional proteins had acquired an additional function confeered by grafting the corresponding N or C terminal domains from a heterologous protein, resulting in a bifunctional protein capable of both inducing G2 cell cycle arrest and SAMHD1 degradation (reminiscent of the bifunctionality observed in SIVagm Vpr), HeLa cells were transduced with lentiviruses expressing full length HIV-1 Vpr, SIVmac Vpx, or chimeric proteins. Transduced cells were analyzed for G2 arrest by flow cytometry (Figure 3.4A). As expected, expression of HIV-1 Vpr induced a robust accumulation of cells in the G2 phase, while SIVmac Vpx had no effect on the cell cycle profile compared to uninfected cells. Transposition of SIVmac Vpx N-terminus onto HIV-1 Vpr (X_{1-22} R_{16-96}) had only modest, if any, effect on the induction of G2 arrest, indicating that the Vpr G2 arrest determinants are either not present within the N-terminal region, or these residues are conserved within the N-terminus of SIVmac Vpx. Transposition of the C-terminus of HIV-1 Vpr onto SIVmac Vpx ($X_{1-88}R_{78-96}$) did not confer G2 arrest functionality to this chimera. These observations indicate that Vpr G2 arrest determinants are not linear and localized only within the unstructured C-terminal region. This is in agreement with previous observations in which mutations to the linker region between the second and third α-helix were able to abrogate Vpr-induced G2 arrest (reviewed in (Morellet et al., 2009)). Finally, lysates of transduced cells were analyzed for the ability of WT or chimeric proteins to induce the degradation of SAMHD1. Transposition of the putative functional determinant domain (SIVmac Vpx N-terminus) failed to confer SAMHD1 degradation to X_{1-22} R_{16-96}. In contrast, both WT SIVmac Vpx and $X_{1-88}R_{78-96}$ were able to induce SAMHD1 degradation (Figure 3.4B).

Understanding the molecular determinants of species-specific G2 arrest

To study the structure-function relationships in HIV-1 Vpr and SIVagm Vpr, a set of 6 chimeras were constructed as shown in Figure 3.5. The exchange points for these chimeras were designed based on the published NMR structure of HIV-1 Vpr (Morellet et al., 2009) and were intended to maintain the predicted secondary structure of the parental proteins. In order to address the contribution of the poly-proline domain (PPD), which is present at the C-terminus of SIVagm Vpr but not HIV-1 Vpr, we generated a SIVagm Vpr truncation without the PRD (ΔP).

To test whether the chimeras maintained the capacity to fold correctly, we verified their ability to interact with the well-established cellular partner, DCAF1. 293FT cells were transfected with pCDNA (empty vector control), HIV-1 Vpr, SIVagm Vpr, chimeras, or HIV-1 Vpr Q65R that is unable to interact with DCAF1 (Dehart et al., 2007; Le Rouzic et al., 2007). 48 hr posttransfection cells were harvested, lysed, and immunoprecipitated (IP) with anti-HA antibody. As expected, HIV-1 Vpr and SIVagm Vpr were able to Co-IP with DCAF1, while Vpr Q65R was not able to interact with DCAF1, as previously reported. All chimeras were able to interact with DCAF1 (data not shown), indicating they were capable of properly folding.

The unstructured C-terminal region of HIV-1 Vpr is sufficient to confer upon SIVagm Vpr the ability to induce G2 arrest in human cells

While HIV-1 Vpr is able to induce cell cycle arrest in human and nonhuman primate cells, SIVagm Vpr was observed to only arrest AGM cells (Planelles et al., 1996). We hypothesized that grafting the C-terminus unstructured region of HIV-1 Vpr

onto SIVagm Vpr would confer upon SIVagm Vpr the *de novo* ability to induce G2 arrest in human cells. HeLa cells were transduced with HA-HIV-1 Vpr, HA-SIVagm Vpr, or chimera expressing lentiviral vectors. In agreement with this prediction, transposition of the C-terminus unstructured region of HIV-1 Vpr onto SIVagm Vpr (chimera 1) conferred the ability to induce G2 arrest in human cells (Figure 3.6). Surprisingly, transposition of the C-terminus unstructured region of SIVagm onto Vpr HIV-1 Vpr (chimera 2) resulted in a chimera that retained the ability to induce cell cycle arrest in human cells, although to a lesser degree when compared with HIV-1 Vpr. These results indicate that recruitment of the G2 arrest substrate is dependent on residues outside the unstructured C-terminus. This is in agreement with previous results which identified several residues within the linker region between the second and third α-helices (reviewed in (Morellet et al., 2009)). Alignment of HIV-1 Vpr and SIVagm Vpr show a high degree of homology between these proteins within this region, as well as within the 3 α-helices, indicating additional determinants may be present elsewhere within HIV-1 Vpr and SIVagm Vpr. Surprisingly, transposition of the entire third α-helix alone (chimera 6) or with C-terminus of HIV-1 Vpr onto SIVagm Vpr (chimera 5) abrogated the ability of this chimera to induce G2 arrest. Whether this is due to an inability to recruit G2 arrest substrate or improper folding will require further analysis.

Degradation of agmSAMHD1 by SIVagm Vpr requires the N and C-terminal domains of SIVagm Vpr

In order to determine the domains in SIVagm Vpr that are important to target agmSAMHD1 for degradation, we tested whether the chimeras between SIVagm Vpr and

HIV-1 Vpr could degrade agmSAMHD1. 293FT cells were co-transfected with HA-agmSAMHD1 and vectors encoding HA-HIV-1 Vpr, HA-SIVagm Vpr, or chimeras. 36 hr posttransfection cells were harvested and the expression of exogenous agmSAMHD1 was analyzed by Western blot. In agreement with previous reports (Lim et al., 2011), agmSAMHD1 was degraded by SIVagm Vpr (Figure 3.7, lanes 5) but not by HIV-1 Vpr (Figure 3.7 lane 4). The N-terminal domain of SIVmac Vpx was previously suggested to be required to overcome SAMHD1 restriction in myeloid cells (Gramberg et al., 2010). Therefore, we expected that transfer of the HIV-1 Vpr C-terminal unstructured region onto SIVagm Vpr would not affect SAMHD1 degradation. Surprisingly, this chimera was unable to facilitate the degradation of agmSAMHD1 (Figure 3.7, lane 5). This result indicates that the SIVagm Vpr C-terminus is necessary for the degradation of SAMHD1; however, transposition of this domain onto HIV-1 Vpr (chimera 2) did not confer the ability to degrade SAMHD1. This suggests that in the context of HIV-1 Vpr, SIVagm Vpr C-terminus is not sufficient to induce SAMHD1 degradation (Figure 3.7, lane 6). In agreement with our earlier observations of HIV-1 Vpr/SIVmac Vpx chimeras, the N-terminus of SIVagm Vpr was necessary, but not sufficient for the degradation of agmSAMHD1 (Figure 3.7, Lane 4 vs 7 and 8).

HIV-1 Vpr-induced degradation of Mus81 is independent of the induction of G2 arrest

The SLX4 complex was recently identified as the G2 arrest target of HIV-1 Vpr (Laguette et al., 2014). In the presence of Vpr, Mus81 is ubiquitinated, contributing to the activation of this complex, leading to G2/M arrest. Although Laguette *et al.* observed a

Vpr-dependent ubiquitination of Mus81, they propose a model in which this promotes the activation of the SLX4 complex (Laguette et al., 2014)(reviewed in (Cohen, 2014)). This is in contrast to earlier models that proposed Vpr-mediated G2 arrest was induced through the degradation of a cellular factor (Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Hrecka et al., 2007; Belzile et al., 2007; Wen et al., 2007)(reviewed in (Andersen et al., 2006). Based on this difference, we wished to further explore the effect of Vpr on Mus81. In agreement with Laguette and colleagues, we observe a Vpr-dependent destabilization of Mus 81 (Figure 3.8).

We then asked whether degradation of Mus81 correlated with the ability of Vpr induce G2 arrest. 293FT cells were co-transfected with V5-Mus81 and HIV-1 Vpr, HIV-1 Vpr R80A, or HIV-1 Vpr Q65R. 48 hr posttransfection cells were lysed and levels of Mus81 were analyzed by Western blot. As shown in Figure 3.9, HIV-1 Vpr induced degradation of Mus81 (Figure 3.9 compare lanes 3 and 4). Surprisingly, HIV-1 Vpr R80A (Figure 3.9 compare lanes 3 and 11) and HIV-1 Vpr Q65R (compare lane 3 and 15), two mutants that fail to induce G2 arrest, also degrade Mus81. This observation indicates that the destabilization of Mus81 is independent of the ability of Vpr to induce G2 arrest or manipulate the Cul4-DCAF1 ubiquitin ligase. Interestingly, inhibition of the proteasome with epoxomicin, or the more specific inhibition of Cullin-based ubiquitin ligases with MLN4924 (Soucy et al., 2009), resulted in the stabilization of Mus81 in the presence of HIV-1 Vpr (Figure 3.9, lane 4 vs lanes 7 and 8). These observations demonstrate that the destabilization of Mus81 by Vpr is an ubiquitination and Cullin family ubiquitin ligase-dependent activity.

Recently, expression of Vpr has been shown to result in large changes to the

cellular ubiquitin ligase pathway (Arora et al., 2014). In order to determine if Mus81 destabilization was merely due to modulation of the UPS by Vpr, we examined the effect of the highly related SIVmac Vpx and SIVagm Vpr on Mus81 stability. To this end, cells were transfected with V5-Mus81 and SIVmac Vpx or SIVagm Vpr. In contrast to HIV-1 Vpr (Figure 3.10, lane 3 vs 4), neither SIVmac Vpx or SIVagm Vpr induced degradation of V5-Mus81 (Figure 3.10, lanes 6 and 12, respectively). Taken together, these results point to a model whereby Vpr specifically induces the degradation of Mus81 in a Cullin ubiquitin ligase-dependent, G2 arrest-independent manner.

Discussion

Vpr is a highly conserved accessory protein encoded by all primate lentiviruses and found in all primary isolates of HIV-1 (reviewed in (Andersen et al., 2006)). Although the exact function of Vpr remains unclear, expression of Vpr is known to induce cell cycle arrest at the G2 phase, putatively through the activation of the SLX4 complex, in order to inhibit induction of an interferon response to viral reverse tanscripts (Laguette et al., 2014). In addition to Vpr, some primate lentiviruses are known to encode the paralogous Vpx, involved in mediating infection of myeloid cells through the degradation of SAMHD1 (Laguette et al., 2011; Hrecka et al., 2011)(Reviewed in (Planelles, 2011)). Interestingly, the SIV species that infect AGM encode a Vpr that is able to induce G2 arrest and degradation SAMHD1, in a species-specific manner. This bifunctional Vpr, as well as evolutionary studies (Lim et al., 2011; Laguette et al., 2012), support a model whereby Vpr acquired SAMHD1 antagonism before the “birth” of the monofunctional Vpx. While the exact evolutionary event leading to the “birth” of Vpx

remains controversial, sustaining antagonism against two cellular proteins may have been too evolutionarily difficult (Planelles, 2012). In this study, we examine the structural determinants of G2 arrest and SAMHD1 degradation by Vpr and its homologs.

Previous studies have demonstrated the necessity of the unstructured N and C-termini of Vpx and Vpr for their function, respectively (Le Rouzic et al., 2007; Ahn et al., 2012). While we had hypothesized that transfer of these domains between HIV-1 Vpr and SIVmac Vpx would confer neofunctionality on the respective chimeric proteins, we observe that the molecular determinants of Vpr G2 arrest and Vpx SAMHD1 degradation involve residues outside of these regions. In agreement with this observation, a recently resolved crystal structure of DCAF1(WD40)-Vpx-SAMHD1(C-terminal domain) demonstrated that residues located within the linker between the second and third α-helix of Vpx are involved in the recruitment of SAMHD1 (Schwefel et al., 2014).

In order to gain a better understanding of the molecular determinants of G2 arrest and SAMHD1 degradation, we generated a second set of chimeras between HIV-1 Vpr and the more closely related SIVagm Vpr. In contrast to SIVmac Vpx, SAMHD1 degradation by SIVagm Vpr was abrogated by transposition of either HIV-1 Vpr N or C-terminus. This differential requirement of SIVagm Vpr C-terminus for SAMHD1 degradation may be in part explained by the recently described toggling of the determinant on SAMHD1 for lentiviral antagonism (Fregoso et al., 2013). Even within the closely related AGM species, the residues on SAMHD1 and SIVagm Vpr that mediate their interaction are under strong evolutionary pressure (Spragg and Emerman, 2013).

In contrast to SAMHD1 degradation, the species-specific induction of G2 arrest

by SIVagm Vpr was more amenable to modulation. Indeed, while WT SIVagm Vpr is unable to induce cell cycle arrest in human cells (Planelles et al., 1996), transposition of the HIV-1 Vpr C-terminus onto SIVagm Vpr conferred the ability to induce G2 arrest in human cells. While the C-terminus of Vpr is known to be necessary for the induction of G2 arrest (this work and (Le Rouzic et al., 2007)), the SIVagm Vpr C-terminus is able to restore G2 arrest activity of these HIV-1 Vpr truncations. These observations support a hypothesis whereby Vpr-mediated G2 arrest is dependent on a nonlinear determinant within the protein. This observation indicates that the determinants leading to species-specific induction of G2 arrest by Vpr are located outside of this region. To fully resolve the molecular mechanisms of Vpr activity will require further biochemical analysis.

Finally, we examine the connection of G2 arrest with the recently described Vpr ubiquitination target, Mus81 (Laguette et al., 2014). In contrast to what Laguette and colleagues report, we observe Mus81 degradation to be independent of Vpr induction of G2 arrest. Indeed, the G2 arrest defective Vpr mutant, R80A, is able to induce the degradation of Mus81. Additionally, this activity appears to be independent of Vpr manipulation of the Cul4-DCAF1 ubiquitin ligase as Vpr Q65R, which is unable to interact with DCAF1, also induces degradation of Mus81. Finally, we observed that the degradation of Mus81 by Vpr is dependent on a Cullin ubiquitin ligase, as the neddylation inhibitor MLN4924 inhibits Mus81 degradation by Vpr. Further study will be necessary to elucidate the exact mechanism by which Vpr exerts this function. We have previously observed Vpr to interact with Cul1 (unpublished results), whether Vpr modulates the activity of an additional ubiquitin ligase towards the destabilization of Mus81 will be an area of active interest.

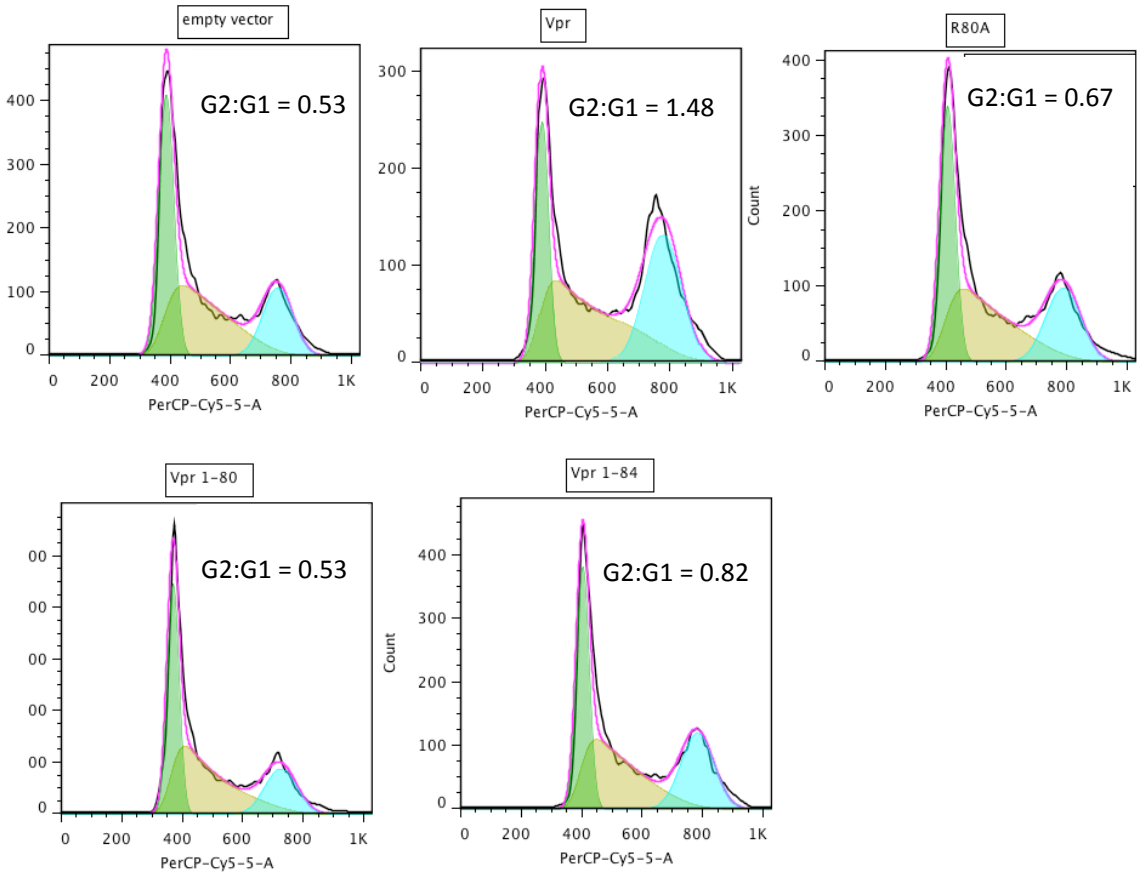


Figure 3.1 HIV-1 Vpr C-terminal unstructured terminus is necessary for the induction of G2 arrest

HeLa cells were transfected with empty control vector, Vpr or truncations as indicated. 48hr posttransfection cells were harvested and cell cycle profiles were analyzed by FACS.

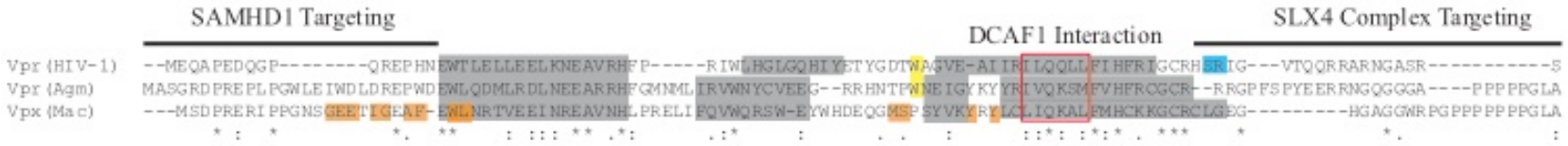


Figure 3.2: Alignment of Vpr from HIV-1 and SIVagm and Vpx of SIVmac
Structural alignment of HIV-1 Vpr, SIVagm Vpr, and SIVmac Vpx. Mutational analysis of Vpr has previously shown the unstructured C-terminus to be involved in determining G2 arrest functionality (reviewed in (Morellet et al., 2009)). Recently, the N-terminus of Vpx was determined to be necessary for Vpx interaction with SAMHD1 (Ahn et al., 2012). Key regions indicated: α-helices (grey boxes) as determined by NMR structure (Vpr (Morellet et al., 2003)) and in silico predictions for SIVagm Vpr and SIVmac Vpx. R80A residue of Vpr (blue box) has been shown to generate a G2 dominant negative molecule (Dehart et al., 2007). Recruitment of UNG2 dependent on W54 located in the linker between the second and third α-helix (yellow box) (reviewed in (Morellet et al., 2009)). A recently solved DCAF1(WD40)-Vpx-SAMHD(Ctd) co-crystal identified residues involved in SAMHD1 interaction (orange boxes) (Schwefel et al., 2014).

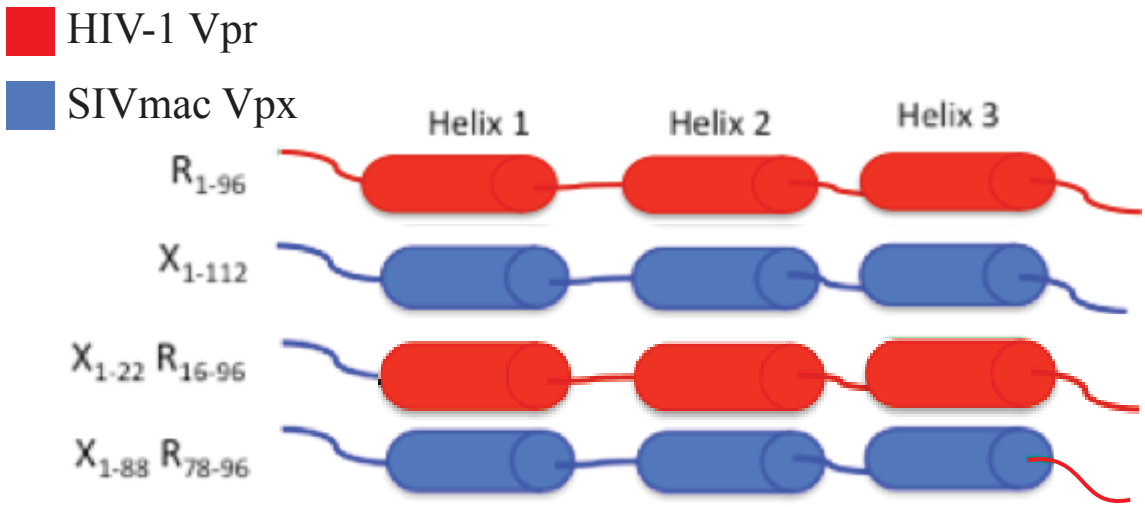
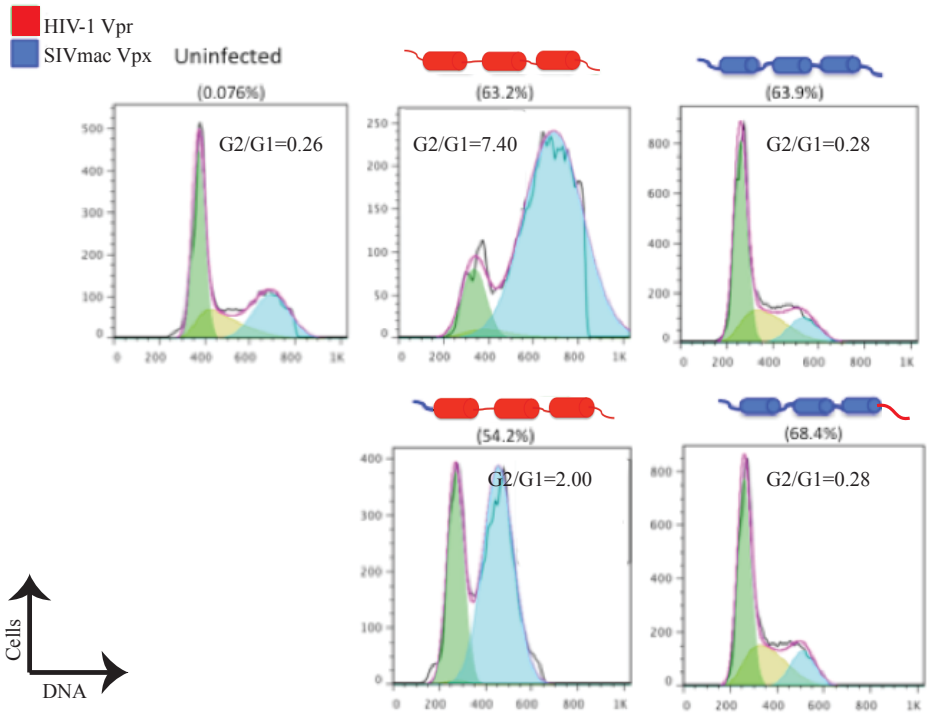


Figure 3.3 Schematic of HIV-1 Vpr/SIVmac Vpx chimeras
Chimeric Vpr/Vpx proteins were generated through the transposition of Vpr C-terminus and Vpx N-terminus as indicated. Proteins are comprised of residues of Vpr or Vpx, respectively, as indicated.

A.



B.

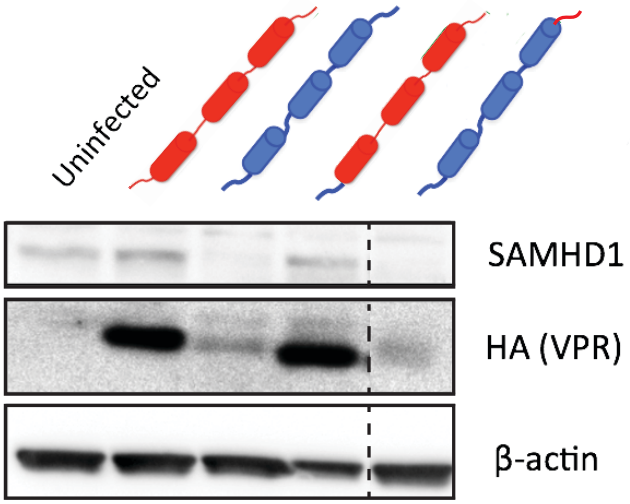


Figure 3.4 Cell cycle analysis of HIV-1 Vpr/SIVmac Vpx chimeras
HeLa cells were transduced with lentiviral vectors expressing WT Vpr/Vpx or chimeras as indicated. Viruses were tittered by expression of the reporter protein GFP, percentages indicated in parenthesis above histograms. A) G2 arrest compared by ratio of G2/G1 as indicated. B) SAMHD1 degradation by HIV-1 Vpr, SIVmac Vpx, and chimeras was analyzed by Western blot.

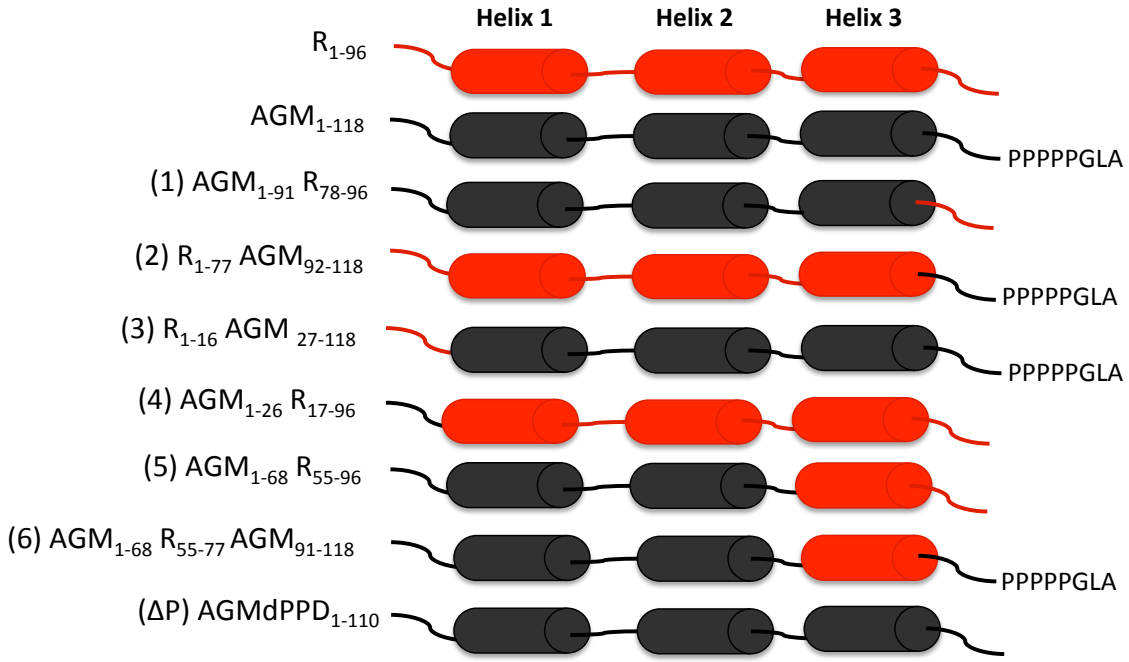


Figure 3.5 Schematic of HIV-1 Vpr/SIVagm Vpr chimeras
Chimeric HIV-1 Vpr/SIVagm Vpr proteins were generated through the transposition of regions as indicated. Subscript numbers represent residues of originating protein. Barrels represent α-helices based on the Vpr NMR structure (Morellet et al., 2009), and SIVagm Vpx sequence homology to Vpr. Lines represent unstructured regions.

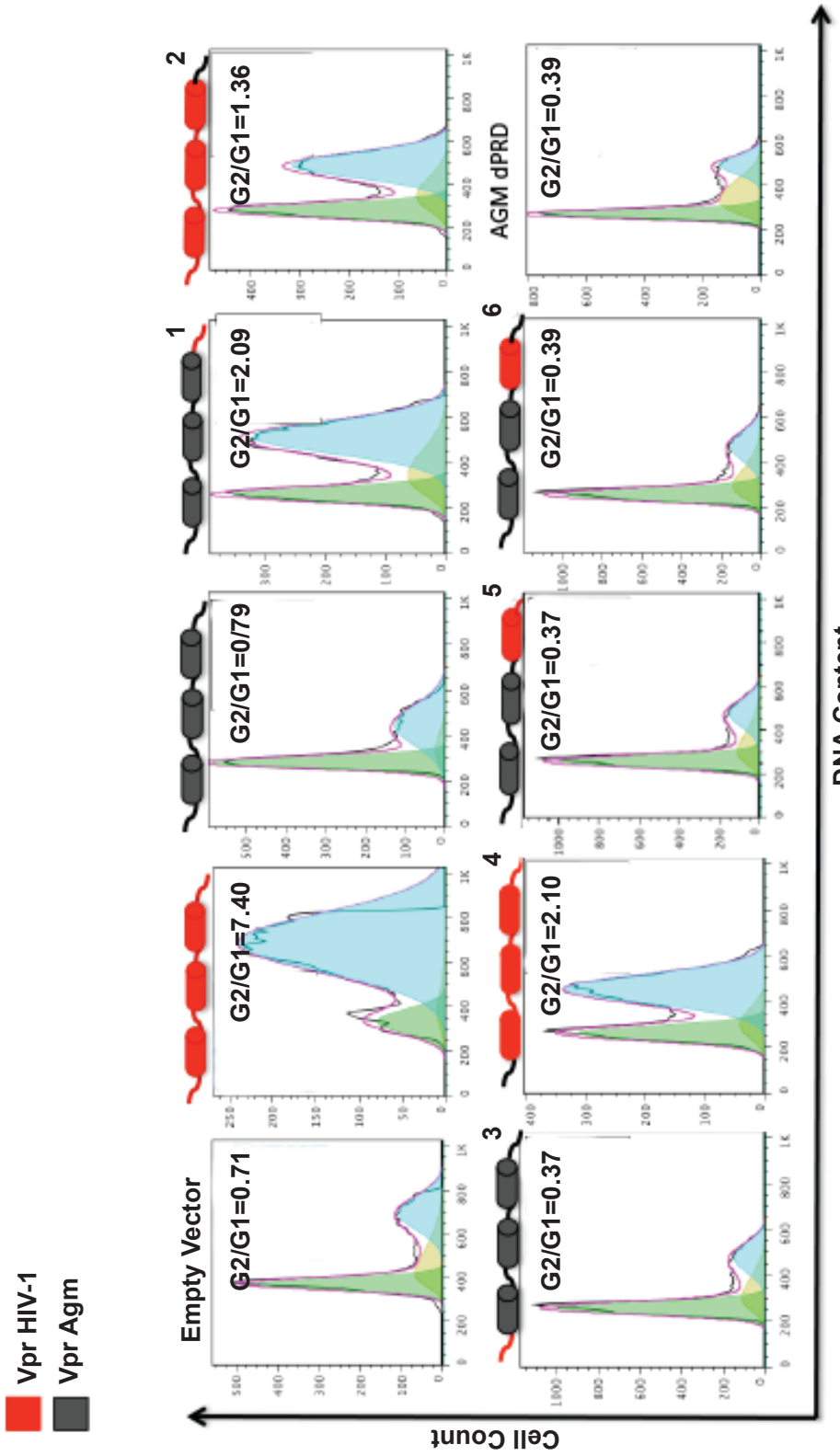


Figure 3.6 Cell cycle analysis of HIV-1 Vpr/SIVagm Vpr chimeras
HeLa cells were transduced with control virus, HIV-1 Vpr, SIVagm Vpr, or chimeric proteins as indicated. 36 hr posttransduction cells were harvested and cell cycle profiles were determined by FACS analysis.

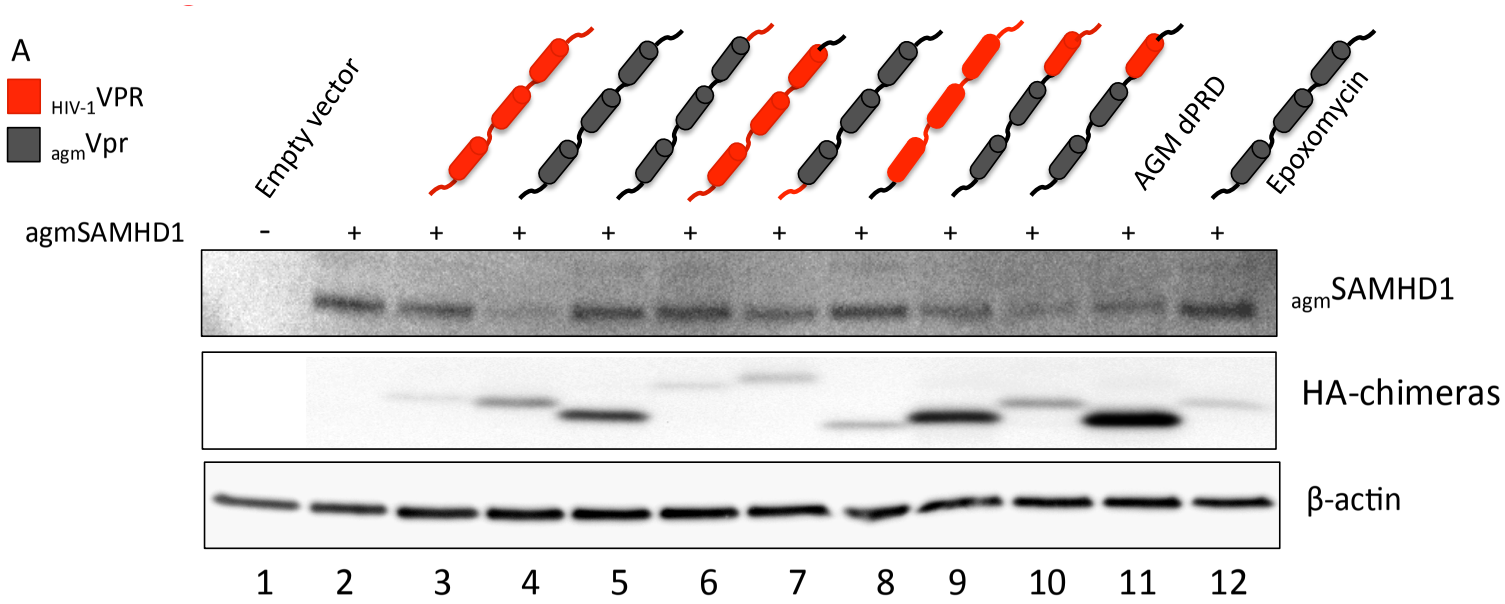


Figure 3.7 Analysis of SAMHD1 degradative ability of HIV-1 Vpr/SIVagm Vpr chimeras
293FT cells were transfected with HA-agmSAMHD1 expressing vector and WT HIV-1 Vpr, WT SIVagm Vpr or chimeras as indicated. 36 hr posttransfected cells were lysed and SAMHD1 stability was analyzed by Western blot.

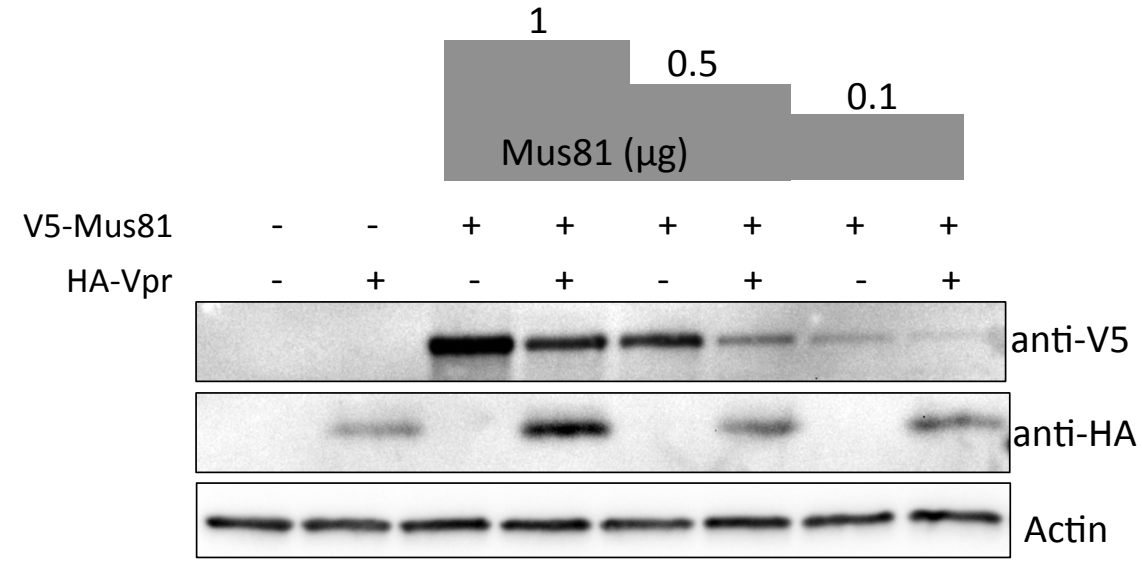


Figure 3.8 Vpr induces the degradation of Mus81
293FT cells were transfected with 1.0µg, 0.5µg or 0.1µg of V5-Mus81 and HA-Vpr as indicated. 48 hr posttransfection cells were lysed and analyzed for the degradation of Mus81 by Western blot.

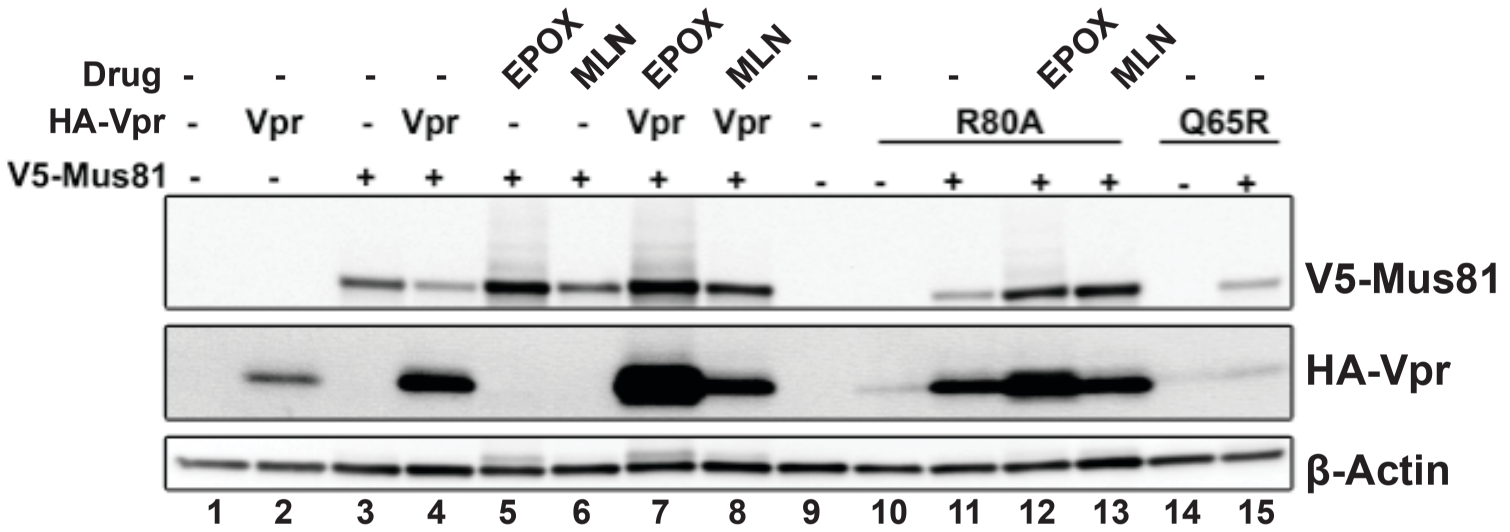


Figure 3.9 The G2 arrest defective Vpr mutants, R80A and Q65R, retain Mus81 degradation activity
293FT cells were transfected with V5-Mus81 and Vpr constructs as indicated. 48 hr posttransfection cells were lysed and analyzed for V5-Mus81 by Western blot. Proteasome and Cullin ubiquitin ligase-dependent nature of V5-Mus81 degradation was determined by treatment with 0.5 μM epoxomicin or 100 nM MLN4924, respectively, for 18 hr before cell lysis.

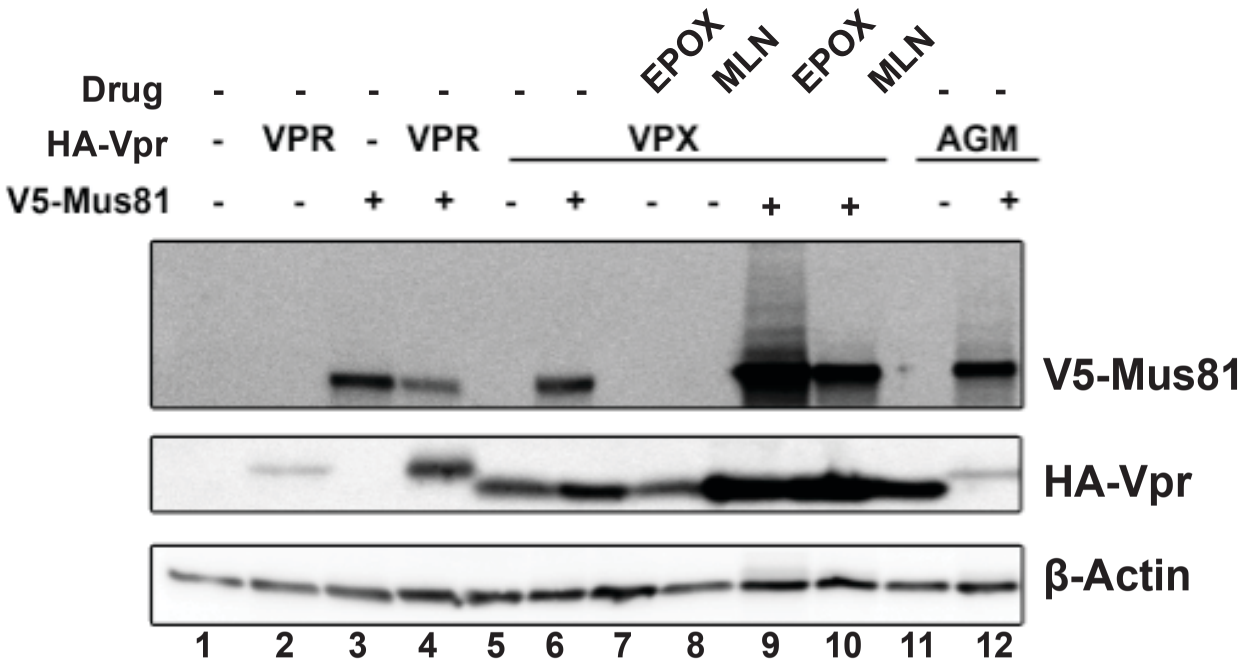


Figure 3.10 SIVmac Vpx and SIVagm Vpr fail to induce the degradation of human Mus81
293FT cells were transfected with human V5-Mus81 and HIV-1 Vpr, SIVmac Vpx, or SIVagm Vpr as indicated. 48 hr posttransfection cells were lysed and V5-Mus81 levels were analyzed by Western blot.

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CHAPTER 4

DISCUSSION

Current Views on Vpr and Vpx Biology

In the thirty years since the discovery of HIV as the causative agent of AIDS, researchers have intensely investigated the mechanisms of pathogenesis. The ~9kb HIV genome encodes 9 genes and 15 proteins. While the major function of nearly all of these proteins have been well elucidated over the ensuing decades, the small 96-aa accessory protein, Vpr, has remained largely enigmatic (Guenzel et al., 2014). Early studies revealed Vpr to be a virion-associated protein involved in infection of nondividing cells and enhancement of transcription from the viral promoter (reviewed in (Emerman, 1996)). Vpr was shown to induce cell cycle arrest at the G2/M transition through the activation of the ssDNA damage sensor ATR (Roshal et al., 2003); however, the importance of this biology has remained unclear.

Perhaps the first real clue to the role of Vpr in HIV-1 replication came following the determination that it modified the activity of a cellular ubiquitin ligase comprised of Cul4-DDB1-DCAF1 (Cul4-DCAF1) (Dehart et al., 2007; Le Rouzic et al., 2007; Wen et al., 2007; Hrecka et al., 2007; Schröfelbauer et al., 2007) (reviewed in (Andersen et al., 2008; Guenzel et al., 2014)). Manipulation of cellular ubiquitin ligases is a mechanism common to numerous viruses to induce the degradation of cellular restriction factors

(Randow and Lehner, 2009). Indeed, 2 additional HIV accessory proteins, Vif and Vpu are known to alter the specificity of the UPS (Collins and Collins, 2014). These observations lead to the hypothesis that Vpr was subverting the Cul4-DCAF1 ubiquitin ligase to induce the degradation of an as of yet unidentified host protein involved in inhibiting HIV replications. Recently, Laguette et al. reported Vpr induced the ubiquitination of Mus81, triggering the activation of the SLX4 complex, as the long sought after Vpr G2 arrest target (Laguette et al., 2014); however, our results call this observation into question. In addition to Vpr, a subset of primate lentiviruses (including SIVmac and HIV-2), are known to encode Vpx, a Vpr paralog, thought to have arisen following a gene duplication event, necessary for the efficient infection of myeloid cells through the Cul4-DCAF1-mediated degradation of SAMHD1 (Laguette et al., 2011; Hrecka et al., 2011)(Reviewed in (Planelles, 2011)).

New insights into the differential manipulation of Cul4-DCAF1 by Vpr and Vpx

In this work, we explore the molecular mechanisms by which Vpr and Vpx manipulate the Cul4-DCAF1 ubiquitin ligase and exert their function. Vpr and Vpx are both known to form a tight 3 α-helical bundle flanked by unstructured N and C-termini (Morellet et al., 2009; Schwefel et al., 2014). While a highly conserved motif located in the third α-helix has been shown to be necessary for the interaction of Vpr and Vpx with DCAF1, little is know about the molecular determinants within DCAF1 that facilitate this interaction (Le Rouzic et al., 2007). To this end, we generated a number of DCAF1 truncations and point mutants in order to define this interaction. Surprisingly, although

the residues of Vpr and Vpx involved in DCAF1 are highly homologous we observe differential interaction of these viral proteins with DCAF1. Most notably, mutations to DCAF1 N1135 and W1156 abrogate Vpx binding, while they had no effect on the interaction with Vpr. A recently solved DCAF1(WD40)-Vpx-SAMHD1(Ctd) co-crystal identified these residues as being involved in coordinating Vpx Q76, mutation of which was previously shown to abrogate Vpx-DCAF1 interaction (Le Rouzic et al., 2007; Schwefel et al., 2014). While the exact cause of these differences will require more thorough biochemical analysis, based on the high homology and conservation of the Vpr/Vpx DCAF1 binding motif, we favor a model in which Vpr binds DCAF1 with higher affinity than Vpx.

To examine this hypothesis, we tested the effect of Vpr expression on Vpx-mediated SAMHD1 degradation. In agreement with a model in which Vpr binds DCAF1 more tightly than Vpx, we observed that Vpr was able to robustly compete Vpx function. In contrast, while the dominant negative Vpr R80A mutant was able to inhibit Vpr induction of G2 arrest, expression of Vpx had no effect.

In addition, we explore the molecular determinants within Vpr and Vpx that facilitate the recruitment of their respective targets. Previous studies have identified the N and C-terminal unstructured regions of Vpx and Vpr, respectively, for the degradation of SAMHD1 and induction of G2 arrest (Ahn et al., 2012; Le Rouzic et al., 2007); however, these observations were made in the context of truncations. Interestingly, while Vpr and Vpx are known to be “monofunctional” proteins, inducing cell cycle arrest or mediating SAMHD1 degradation, a subset of primate lentiviruses (SIVagm) encode a bifunctional Vpr capable of facilitating both functions. To further elucidate the molecular

determinants of Vpr and Vpx function, we generated a series of chimeric proteins by transposing the unstructured regions of HIV-1 Vpr and SIVmac Vpx as wells as between HIV-1 Vpr and the bifunctional SIVagm Vpr. These studies revealed additional residues outside of the unstructured N and C-termini mediate Vpr and Vpx function. This is in agreement with a recently published crystal structure of DCAF1(WD40)-Vpx-SAMHD1(Ctd), in which residues within the linker region mediate interaction between Vpx and SAMHD1 (Schwefel et al., 2014). This observation was further confirmed by the loss of agmSAMHD1 degradation ability of all HIV-1 Vpr/SIVagm Vpr chimeras. Surprisingly, the species-specific ability to induce G2 arrest appears to have arisen by compensatory mutations in both the Vpr α-helical bundle as well as the unstructured C-terminus. More refined study of the evolution and structure of Vpr will be necessary to identify the specific residues involved in mediating Vpr interaction with the G2 arrest target.

Finally, Laguette and colleagues recently reported the activation of the SLX4 endonuclease complex, through the ubiquitination of Mus81, by Vpr as the causative mechanism of Vpr G2 arrest (Laguette et al., 2014). In contrast to their observations, we demonstrate that the G2 arrest-defective Vpr mutants Vpr Q65R and Vpr R80A are both capable of inducing degradation of Mus81. To verify that Mus81 degradation is not simply an indirect effect of DCAF1 manipulation, we examined the ability of SIVmac Vpx and SIVagm Vpr, both capable of binding human DCAF1 but unable to induce G2 arrest in human cells. Unlike HIV-1 Vpr, neither of these homologs were able to induce Mus81 degradation. The exact mechanism by which Vpr causes the destabilization of Mus81 remains unclear; however, pharmacological inhibition of the Cullin ubiquitin

ligase family with MLN4924 inhibited Vpr-induced Mus81 degradation. The discrepancy between the observations of Laguette et al. and our own concerning Vpr-mediated Mus81 will require further investigation. These studies raise important questions regarding the mechanism utilized by HIV to avoid recognition of nucleic acids by cytosolic DNA and RNA sensors.

Future Perspectives

Cul4 ubiquitin ligases and manipulation by other viral proteins

In addition to HIV/SIV, several other viruses have been observed to manipulate Cul4-based ubiquitin ligases (Table 1.1). While the effect of SV5 V-protein manipulation of the Cul4-DDB1 complex has been thoroughly studied (reviewed in (Barry and Früh, 2006)), the functional role in viral replication of other viral Cul4 manipulators remains largely unclear. Perhaps most interesting is the recent observation that the UL35 protein of cytomegalovirus triggers G2 arrest by interaction with Cul4-DCAF1, reminiscent of Vpr activity. Interestingly, several members of the SLX4 complex (SLX4, ERCC1 and ERCC4, but not MUS81 or EME1) were identified as UL35 interactors by mass spectrometry (Salsman et al., 2012). Whether UL35 activates the SLX4 complex as Vpr does is yet to be resolved; however, this observation lends credence to the hypothesis that SLX4 activation may be a strategy utilized by multiple viruses to avoid recognition of exogenous nucleic acids and the subsequent induction of an interferon response.

In addition to primates, lentiviruses have been identified as infectious agents in a large number of mammals including rabbits, horses, cows, and wild and domestic cats. Genetic studies have identified Vpr-like genes in a large number of these lentiviruses

(reviewed in (Gifford, 2012)). While the study of many of these viruses has generally been quite limited, studies in FIV indicate viral-mediated SLX4 complex activation may be quite old evolutionarily. While the exact function of FIV Vpr ortholog, OrfA, remains unresolved, expression of OrfA has been shown to induce G2 cell cycle arrest (among other phenotypes). OrfA protein sequence alignment with Vpr shows a high degree of homology between the two proteins. Of particular note, the DCAF1 binding motif of Vpr is nearly identical (Gemeniano et al., 2004). Although it is currently unknown if OrfA manipulates the UPS in general, or Cul4-DCAF1 specifically, the high conservation of DCAF1 (particularly of the WD40 domain) from humans to *Arabidopsis* (Zhang et al., 2008) supports the hypothesis that Cul4-DCAF1 manipulation may be common throughout lentiviruses.

Targeting accessory gene function as a therapeutic

Current antiretroviral therapies have focused on directly targeting viral enzymatic function. To date, these strategies have proven to be effective; however, they suffer from several drawbacks, even in comparison to antibiotics. Namely, antivirals currently in use have suffered from two major drawbacks: 1) modern antivirals are generally effectively against very limited ranges of virus ("one drug/one bug") and 2) high rates of mutations, especially in RNA viruses, has been observed to lead to rapid resistance to antivirals (reviewed in (Volberding and Deeks, 2010; Lou et al., 2014)). To this end, it has been proposed that targeting cellular factors necessary for viral replication may provide an avenue for the development of drugs with a broader range and a higher evolutionary barrier to escape (reviewed in (Coley et al., 2009; Linero et al., 2012)).

While accessory genes are not required for viral replication in some *in vitro* systems, viruses lacking these genes are severely hindered during *in vivo* infection (reviewed in (Malim and Emerman, 2008; Malim and Bieniasz, 2012)). Manipulation of Cullin-based ubiquitin ligases by a large number of viruses (table 1) make identification of pharmacological modulators of Cullin activity a compelling putative antiviral target. A recently identified small molecule inhibitor of Cullin activation currently in phase I clinical trials as a potential cancer therapy, MLN4924 (Soucy et al., 2009; 2010), is a compelling proof of concept for the antiviral potential of inhibiting accessory protein function.

Cullin activity is dependent on the autoconjugation of the ubiquitin like modifier NEDD8 to the Cullin, facilitating later substrate ubiquitination by inducing conformational changes within the Cullin. Cullin neddylation follows an analogous pathway to cellular protein ubiquitination, in which NEDD8 is activated in an ATP-dependent manner by the NEDD8 activating enzyme (NAE) followed by transfer to an E2 and finally conjugation to the Cullin (Saha and Deshaies, 2008; Duda et al., 2008)(reviewed in (Soucy et al., 2010; Saifee and Zheng, 2008)). MLN4924 functions as an AMP analogue with high specificity for NAE, inhibiting NEDD8 activation, and subsequently specifically inhibits Cullin activity (Soucy et al., 2009). Indeed, the clinical effectiveness of UPS inhibition in cancer treatment has already been demonstrated following the recent approval of proteasome inhibitor bortezomib (VELCADE) (Soucy et al., 2010).

To this end, several groups have demonstrated the antiviral potential of Cullin inhibition by MLN4924. In 2012, Stanley et al. demonstrated that MLN4924 treatment

inhibited Vif-mediated APOBEC3 degradation, mimicking infection of ΔVif virus, allowing for APOBEC3 encapsidation into budding virions and induction of hypermutation of the HIV-genome (Stanley et al., 2012b). In addition, MLN4924 has been shown to inhibit Vpx-mediated SAMDH1 degradation, resulting in the inhibition of myeloid cell infection even in the presence of Vpx (Wei et al., 2013; Hofmann et al., 2013; Nekorchuk et al., 2013). Finally, inhibition of neddylation appears to inhibit Vpu-mediated CD4 degradation and Vpr-mediated Mus81 degradation (Chapter 3). It will be of great interest to determine if similar effects are observed in the context of viral infections *in vivo*.

While MLN4924 serves as a strong proof of concept for the antiviral effects of directly targeting cellular proteins, resulting in the inhibition of accessory protein function, further research will be necessary before similar therapeutic strategies will be effective clinically. While MLN4924 specially targets Cullin based ubiquitin ligases, without broadly effecting cellular protein turnover, it has been shown to exert robust cytotoxic effects (Soucy et al., 2009). While such cytopathies may be acceptable in cancer treatments, for which MLN4924 is in clinical trials, they may be less well tolerated in the treatment pathogens, such as HIV. Further insight into the molecular architecture of virally hijacked ubiquitin ligases may provide even more specific targets for future chemotherapeutic targeting. Indeed, manipulation of Cul5 by Vif provides one such example. While two unique NEDD8 E2s have been identified, UBE2F and UBE2M (UBC12), Cul5 has been shown to be uniquely, among Cullins, neddylated by UBE2F (reviewed in(Soucy et al., 2010)). To this end, Stanley and collogues observed Vif-mediated APOBEC3 degradation to be UBE2F dependent (Stanley et al., 2012a).

Understanding the specific architectural features of ubiquitin ligases manipulated by viral proteins holds great promise for the development of future broad range antivirals.

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