UNDERSTANDING HOW VIRUSES MANIPULATE UBIQUITIN LIGASES TO AVOID INNATE IMMUNITY

by

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ABSTRACT

Soon after viruses enter the cell, they encounter host innate and adaptive immune responses that must be evaded. Restriction factors are proteins from the host innate immune response that impair the establishment of viral infection.

Primate lentiviruses encode “accessory” proteins, which are not required for viral replication but essential to counteract host restriction factors. The primate lentivirus accessory proteins are Nef, Vif, Vpu, Vpr, and Vpx. The main mechanism used by these proteins to counteract restriction factors is the manipulation of the ubiquitin proteasome system. While Vpu usurps Cul1, Vpr and Vpx hijack Cul4A by associating with DCAF1.

Through interaction with DCAF1, Vpx degrades SAMHD1, a protein that impairs viral reverse transcription in myeloid cell lineages. Vpr, on the other hand, induces poly-ubiquitination of Mus81 to activate the endonuclease complex SLX4com. SLX4com activation was suggested to induce cell cycle arrest in G2/M. SIVagm Vpr is homologous to HIV-1 Vpr in sequence and structure, but these two proteins have important functional differences. Unlike HIV-1 Vpr, SIVagm Vpr degrades SAMHD1 and induces G_2 arrest but in a species-specific manner. In this work, we generated chimeric proteins between HIV-1 Vpr and SIVagm Vpr, in order to understand the structure-function relationships in these proteins. We showed a *de novo* ability to arrest cell cycle in human cells when

the C-terminus of HIV-1 Vpr was grafted onto SIVagm Vpr. Using point mutants of HIV-1 Vpr, we were able to uncouple degradation of Mus81 from cell cycle arrest, suggesting that these two functions are independent.

To study the amino acid residues of DCAF1 that are responsible for interactions with Vpr and Vpx, we generated a large array of point mutants. We demonstrated that Vpr and Vpx interact with DCAF1 using a similar region on DCAF1, but establishing interactions with different residues.

We then used an inhibitor of the Neddylation pathway (MLN4924) to examine the role of CRUL in the abilities of accessory proteins to target host cell proteins. Our results demonstrate that inhibition of CRUL with MLN4924 did not affect downregulation of tetherin, suggesting that the trans-golgi entrapment is responsible for the observed effect.

Dedicated to everyone who was by my side during graduate school, especially to my parents, my brother and my husband, for their support and unconditional love!

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CHAPTER 1

INTRODUCTION

HIV/SIV Accessory Proteins and Cellular Restriction Factors

HIV-1 virus, the causative agent of AIDS (Barre-Sinoussi et al., 1983) a disease characterized by gradual suppression of the immune response, is an extremely pathogenic lentiviral responsible for the death of millions of people around the world. In 2013, 35 million people were living with HIV worldwide (WHO as of December, 2014). Although ART (Antiretroviral Therapy) is been used to control viral replication, slowing the progression to AIDS and decreasing the mortality rate, a cure is still not available. In 2013, around 1.5 million people died from AIDS (WHO as of December, 2014). Despite all the research on HIV-1, much remains to be learned.

HIV-1 is comprised of a small genome (around 10kb) and encodes for 9 genes. *gag*, *pol,* and *env* encode for structural proteins; *tat* and *rev* for regulatory proteins and *nef, vif, vpu, vpr,* and/or *vpx* (HIV-2/SIV) for accessory proteins. While structural and regulatory proteins are important for HIV-1 entry, reverse transcription, integration, and assembly, accessory proteins are required to evade antiviral immune response.

The viral infectivity factor (Vif) is a late gene product and is required for production of infectious particles. Its name came from observations where specific cell lines, were permissive for replication with Vif-deficient viruses, while in other cell lines the replication was blocked (Gabuzda et al., 1992). It was later reported that the nonpermissiveness effect was due to expression in these cell lines of a restriction factor called Apolipoprotein B mRNA-editing enzyme 3G (APOBEC3G) (Madani and Kabat, 1998; Sheehy et al., 2003). The host protein

APOBEC3G is a cytidine deaminase that functions during reverse transcription of the viral genome. APOBEC3G deaminates cytidine residues, converting them to uridine. When RT synthesizes the plus sense DNA strand, then the above chemical change results in an adenosine substitution in place of guanine. This, when repeated multiple times, will result in hypermutation and inactivation of the viral genome (reviewed in Feng et al., 2014). To inhibit this restriction, Vif functions as an adaptor molecule for the cullin-5 E3 ubiquitin ligase (Yu et al., 2003). By interacting with Cul5, via Elongin B/C (Elo B/C) subunits, Vif displaces the endogenous substrate and recruits APOBEC3G for ubiquitination and subsequent proteasomal degradation (Yu et al., 2003).

Viral protein-unique (Vpu) has this name because it is found only in HIV-1 but not in HIV-2. Vpu has numerous functions during progression of HIV-1 infection that range from downregulation of CD4 from the cellular membrane (Levesque et al., 2003), a process that involves the cullin RING ubiquitin ligase (CRUL) cullin-1 and the UPS (Margottin et al., 1998), to downmodulation of CCR7 (Ramirez et al., 2014), CD1d (Moll et al., 2010), BST-2/Tetherin (Van Damme et al., 2008), MHC-II (Hussain et al., 2008), and NTB-A (Shah et al., 2010). BST-2 is a transmembrane protein that inhibits nascent virus release from the infected cell. Vpu overcomes this restriction by downregulating and degrading BST-2/tetherin, a process that occurs independently of the CRULs. Because Vpu is absent in HIV-2 and SIV, downregulation of BST-2/tetherin occurs through the Env glycoprotein and Nef protein in these lentiviruses, respectively. The mechanism used by Vpu to downregulate BST-2/tetherin is still controversial.

While some studies suggest that this effect occurs through degradation by the UPS, our group (Ramirez et al. submitted for publication) and others suggest that downregulation of BST-2/tetherin from the cell surface is independent of the UPS. We showed that inhibition of CRULs function abrogates the ability of Vpu to downregulate CD4 from the cell surface but not BST-2/tetherin (Ramirez et al. manuscript submitted for publication), suggesting the effect of Vpu in BST-2/tetherin is independent of the CRULs.

While myeloid cell lineages and resting CD4+ T-cells are able to restrict HIV-1 infection, the same is not true for HIV-2 and SIV (Ayinde et al., 2010; Yamashita and Emerman, 2006). The underlying reason for this difference is that HIV-2 and SIV encode for a common protein called Viral protein X (Vpx) (Laguette et al., 2011). Myeloid cells and resting CD4+ T-cells express the sterile alpha motif (SAM) and histidine/aspartic acid domain (HD) containing protein 1 (SAMHD1), which is responsible for inhibition of HIV-1 infection (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that hydrolyzes dNTP to a deoxynucleoside and inorganic triphosphate (Goldstone et al., 2011). Through this function, SAMHD1 decreases the level of available intracellular pool of dNTPs, impairing viral reverse-transcription (Amie et al., 2013; Goldstone et al., 2011; Kim et al., 2012; Srivastava et al., 2008). Like other lentiviral accessory proteins, Vpx uses the $Cul4^{DDB1/DCAF1}$ubiquitin E3 ligase to overcome SAMHD1 restriction (Srivastava et al., 2008). Vpx manipulates this E3 ligase through direct interaction with the

adaptor protein DCAF1 (VprBP) (Hrecka et al., 2011; Srivastava et al., 2008) to induce SAMHD1 ubiquitination and degradation.

The viral protein R (Vpr) is a paralog of Vpx and also manipulates the UPS by hijacking the cellular $Cul4^{DDB1/DCAF1}$ ubiquitin E3 ligase. Vpr is known to induce, among other functions, cell cycle arrest in G_2-to-M transition, a process that requires interaction with DCAF1. Although several host proteins have been described to interact with Vpr, only in the beginning of 2014 was the SLX4 complex found to be targeted by Vpr to induce cell cycle arrest (Laguette et al., 2014). In contrast to what was expected, the SLX4comp was shown to be activated, instead of degraded, by Vpr. However, the exact mechanism by which this occurs and the reason why the virus induces cell cycle arrest remain to be understood. This chapter will focus on Vpr, its reported targets, and the most accepted molecular mechanism used by this accessory protein to arrest cell cycle. A detailed understanding of restriction factors and how HIV-1 evades their recognition will be crucial for novel drug development.

Vpr: The Enigmatic Multifunctional Protein

The accessory protein Vpr is a 14KDa protein that is conserved among primate lentiviruses, being found in HIV-1, HIV-2, and in SIV (Hattori et al., 1990; Ogawa et al 1989; Tristem et al., 1992). Because it is highly conserved through the evolution, it is believed that Vpr is extremely important for HIV pathogenesis (Emerman, 1996; Planelles et al., 1996; Stivahtis et al., 1997). NMR (nuclear magnetic resonance) studies of HIV-1 Vpr demonstrated that this protein is

comprised of a core of three alpha helices connected by flexible loops, flanked by amino- and carboxy-terminal unstructured regions (Figure 1.1) (Morellet et al., 2003). Initially, experiments in *rhesus* macaques demonstrated that, when the macaque was infected with SIVmac carrying a mutated Vpr, disease progression and viral replication were delayed, revealing the importance of Vpr *in vivo* (Hoch et al., 1995; Lang et al., 1993). Vpr is packaged into the virions, by direct interaction with the p6 region of Gag (Bachand et al., 1999; Paillart and Gottlinger, 1999; Selig et al., 1999) and is released upon viral entry into the cells, suggesting that Vpr is required for the early steps of viral infection (reviewed in Guenzel et al., 2014). Several functions were attributed to Vpr during the virus life cycle; among them are: induction of cell cycle arrest in G_2 phase, apoptosis, nuclear import of the preintegration complex, transactivation of the viral promoter, and increase in the fidelity of the reverse transcription (reviewed in Dehart and Planelles, 2008; Le Rouzic and Benichou, 2005). The most extensively studied characteristic of Vpr is the capacity to induce G_2 arrest, a function that is also conserved among Vpr from primate lentiviruses (Planelles et al., 1996).

Vpr and Its Mechanism of Induction of G_2 Arrest

Induction of G_2 arrest by Vpr was first reported in 1995 (He et al., 1995; Jowett et al., 1995; Re et al., 1995; Rogel et al., 1995). The cell cycle arrest induced by Vpr is similar to that observed as consequence of DNA damage and replication stress (He et al., 1995; Jowett et al., 1995; Re et al., 1995; Rogel et al., 1995). In 2003, Roshal et al. showed that inhibition of ATR (ataxia

telangiectasia mutated and Rad3-related protein), a sensor of replication stress, but not ATM (ataxia telangiectasia mutated) abrogates the ability of Vpr to induce G_2 arrest (Roshal et al., 2003), suggesting that activation of ATR by Vpr was required for cell cycle arrest.

Cellular replication stress is a downstream consequence of stalled replication forks, which can be induced by UV-light induced DNA damage, depletion of deoxyribonucleotide, and inhibition of topoisomerase (McGowan and Russell, 2004). ATR is a serine/threonine kinase that, upon activation by single stranded DNA, phosphorylates the replication protein A 32KDa subunit (RPA32), checkpoint kinase 1 (Chk1), histone 2A variant X (H2AX), p53-binding protein 1(53AP1), and breast cancer-associated protein 1 (BRCA1) (Lai et al., 2005; Li et al., 2007; Roshal et al., 2003; Zimmerman et al., 2004), and induces formation of DNA-damage nuclear foci. Because Vpr does not induce DNA strand breaks to activate ATR, this observation leads to the hypothesis that Vpr is affecting an unknown upstream event that culminates in replication stress and ATR activation.

In 1994, VprBP or RIP (Vpr-interacting protein) was first reported as a Vpr binding partner with unknown function (Zhao et al., 1994). It was only in 2006 that several groups found VprBP as being associated with the DNA-damaged-specific biding protein 1 (DDB1), a molecule that functions as an adaptor molecule for cullin-4 ubiquitin E3 ligase, and VprBP was renamed DCAF1 (DDB1-cullin4 associated factor 1) (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006). Rapidly, our group and others were able to show that

the ability of Vpr to induce G_2 arrest is dependent on its interaction with DCAF1. siRNA depletion of DCAF1 inhibits G2/M arrest induced by Vpr by preventing Vpr from coimmunoprecipitating with DDB1, suggesting that DCAF1 is bridging Vpr onto Cul4 E3 ligase (DeHart et al., 2007; Hrecka et al., 2007; Wen et al., 2007). As a result of these observations, a model was developed suggesting that Vpr, through its interaction with DCAF1, binds to Cul4-DDB1 ubiquitin E3 ligase to trigger polyubiquitination and degradation of a specific cellular substrate (Figure 1.2). When this substrate is degraded, ATR is activated, inducing G_2 arrest. The 3rd alpha helix of Vpr contains a leucine-rich motif that was characterized as the DCAF1 interaction domain (Figure 1.1). Mutations within this region, i.e., Vpr(Q65R), results in abrogation of DCAF1 interaction and failure to induce G2/M arrest (Le Rouzic et al., 2007; Zhao et al., 1994). Another mutant, Vpr(R80A), where the mutation is within the carboxy-terminal unstructured region of Vpr, is still able to bind to DCAF1 but does not arrest the cell cycle (DeHart et al., 2007; Le Rouzic et al., 2007). All together, these observations demonstrated that binding of Vpr to DCAF1 occurs within Vpr's 3rd helix and it is required for induction of G_2 arrest. Because the mutant Vpr(R80A) does not induce G_2 arrest although binding to DCAF1 is preserved, it has been proposed that the carboxy-terminal region of Vpr is the binding motif for the G_2 arrest target protein (Dehart and Planelles, 2008; DeHart et al., 2007).

Vpr and Its Promiscuous Character

Over the years, extensive searches have been undertaken in an attempt to identify the cellular protein that interacts with Vpr and could be implicated in Vpr's induction of cell cycle arrest. Several groups were able to show, by distinct methods, that numerous host proteins interact with Vpr. Because of Vpr's "sticky" characteristics, a great amount of Vpr's binding partner seems to bind nonspecifically, while a few were characterized as true binding partners. The most studied binding partners of Vpr are described below.

Using Y2H, the DNA repair enzyme uracil DNA glycosylase (UNG2) was identified as a Vpr binding protein (Bouhamdan et al., 1996). Insertion of uracil into DNA may occur through cytosine deamination or misincorporation of dUMP during DNA synthesis, resulting in mutagenesis. UNG2 prevents mutation by removing uracil from DNA. In this process, the N-glycosylic bond is cleaved, generating an abasic site that is repaired by the base excision repair (BER) process (reviewed in Planelles and Benichou, 2009). It was observed that misincorporation of uracil into HIV-1 DNA occurs during reverse transcription (Kennedy et al., 2011) and generation of an abasic site by the enzyme UNG2 could induce lethal mutations in the viral genome.

Although several groups described effects of UNG2 in HIV-1 biology, the function of UNG2 during HIV-1 infection remains extremely controversial. UNG2 was described to be targeted by Vpr to the Cul4 E3 ubiquitin ligase, in a DCAF1-dependent manner (Ahn et al., 2010; Schrofelbauer et al., 2007; Schrofelbauer et al., 2005), resulting in UNG2 ubiquitination and degradation. However,

degradation of UNG2 by Vpr is not associated with Vpr induction of G_2 arrest (Selig et al., 1997). Interestingly, UNG2 was proposed to be a natural target of DCAF1 (Wen et al., 2012). Because Vpr was found to associate with hyper-neddylated Cul4A, which represents a hyperactivated form of this E3 ligase (Hrecka et al., 2007), it is believed that degradation of UNG2 is accelerated in the presence of Vpr. This result explains the decreased level of UNG2 in the presence of Vpr and suggests that UNG2 degradation is a side effect of Vpr's manipulation of the E3 ubiquitin ligase (Wen et al., 2012).

In contrast to the destabilization effect of Vpr on UNG2, it was observed that UNG2 is encapsidated into the virion in a Vpr-dependent fashion (Selig et al., 1997). Vpr binds to UNG2 through the residue Trp54, which is found in the loop between the second and third alpha-helices. Incorporation of UNG2 into the virion influences the accuracy of the reverse transcriptase, ensuring the maintenance of the integrity of the viral genome (Chen et al., 2004).

In 2014, Laguette et al. reported that the structure–specific endonuclease (SSE) regulator SLX4 complex (SLX4com) was targeted by Vpr to induce G_2 arrest. SLX4 was identified as a new Fanconi Anemia complex (FANCP or BTBD12) (Kim et al., 2013), which is involved in resolving Holiday junctions (HJ) during homologous recombination (HR) (Schwartz and Heyer, 2011). Fanconi Anemia is a disease characterized by a defect in DNA repair, which results in early onset of bone marrow failure and cancer. HR is a mechanism of high-fidelity, template-dependent DNA repair that functions in the repair of double strand break (DSB) and interstrand crosslinks (ICLs), restoration of DNA gaps

and recovery of stalled replication forks, and is responsible for accurate chromosome segregation (reviewed in Li and Heyer, 2008). Inappropriate repair of the DNA damage and the inability to resolve DNA replication stress can result in genomic instability, which might lead to chromosome loss, apoptosis, and cancer (Hoeijmakers, 2001). During DNA DSB repair or restoration of stalled replication forks, an intermediate form of a four-way DNA junction (HJ) is created. In order to complete the DNA repair, the HJ must be resolved (reviewed in Kim et al., 2013). SLX4 has been implicated in resolution of HJ by recruiting SSEs, such as Mus81-Eme1 Ercc1-Errc4 and SLX1, to the sites of DNA lesions (reviewed in Kim et al., 2013).

Laguette et al. (2014) observed that DCAF1 interacts with SLX4com in the absence of Vpr and this interaction is enhanced when Vpr is present. Furthermore, the kinase PLK1 is recruited to the complex triggering EME1 phosphorylation. Vpr-mediated remodeling of the SLX4com leads to Mus81 ubiquitination and activation of the endonuclease complex. Because of the high error rate of the viral reverse transcriptase enzyme, an accumulation of nonproductive DNA is produced after HIV-1 infection, which can activate intrinsic cellular DNA sensors, resulting in an antiviral type-I IFN response. To overcome this effect, Vpr activates the SLX4com to process HIV-1 DNA, avoiding cellular immune recognition by DNA sensors, such as IFI16 (Jakobsen et al., 2013) and cGAs (Gao et al., 2013). As a side effect of aberrant activation of SLX4com, replication forks are processed abnormally, resulting in G_2 arrest. SLX4com activation by Vpr was proposed to induce ATR activation, a hallmark of Vpr-

induced cell cycle arrest. Conversely, in *S. cerevisease*, SLX4 activation was shown to occur downstream of ATR activation (Ohouo et al., 2010).

It was observed that SLX4com interacts with Vpr in a DCAF1-dependent manner. WT Vpr but not Vpr(Q65R), the mutant that fails to bind to DCAF1, is still able to co-immunoprecipitate with SLX4 and Mus81. Interestingly, the mutant Vpr(R80A), which binds to DCAF1 but fails to induce G_2 arrest, also interacts with SLX4 (Laguette et al., 2014). Recently, another group has observed that the ability of Vpr to induce cell cycle arrest was dependent on Vpr's capacity to interact with SLX4 (Berger et al., 2014). Vpr was shown to induce modest degradation of Mus81 through manipulation of the $Cul4^{DDB1/DCAF1}$ E3 ubiquitin ligase (Laguette et al., 2014). In agreement, we also found that Vpr induces degradation of Mus81 but in a DCAF1- and G2 arrest-independent manner, since Vpr(Q65R) and Vpr(R80A) were able to degrade Mus81 (see Chapter 2). Nevertheless, siRNA against Mus81 or any of the components of the SLX4com (SLX4, EME1, and SLX1) inhibits G_2 arrest-induced by Vpr (Laguette et al., 2014). This result diverges from the proposed model of G_2 arrest, which implicates that Vpr induces ubiquitination and degradation of a cellular factor that culminates in cell cycle arrest (Andersen et al., 2008). To this end, down-regulation of the Vpr target would mimic the G_2 arrest induced by Vpr, the opposite result observed by Laguette et al. Furthermore, while all HIV/SIV accessory proteins that manipulate the UPS lead to degradation of a cellular protein, the effect observed by Laguette et al. is the opposite: upon ubiquitination, the complex is active instead of degraded. Because SLX4 binds to

DCAF1 constitutively, as an endogenous substrate, it seems that SLX4com activation by Vpr is a consequence of the hyperactivation of $Cul4^{DDB1/DCAF1}$ubiquitin E3 ligase by Vpr. The same mechanism was suggested for UNG2 degradation in the presence of Vpr (Wen et al., 2012).

Viral Manipulation of Host Ubiquitin E3 Ligase

Viral infection and replication relies on the host cellular machinery. Due to the fact that the UPS controls protein functionality by posttranslational modification, it is not a surprise that viruses have evolved to co-opt the UPS to their own advantage.

The UPS controls protein degradation, trafficking, signaling, cell cycle, and transcription by ubiquitination of targets. Ubiquitin is a small protein (76 amino acid) that is attached to target molecules to modify its function. Ubiquitination requires three sequential steps performed by three different enzymes: 1) ubiquitin activating enzyme (E1), through ATP-dependent reaction, activates the ubiquitin molecule by formation of a thiol ester bond between cysteine in the E1 enzyme and the C-terminal glycine of ubiquitin; 2) activated ubiquitin is transferred, by trans-esterification, to a ubiquitin conjugating enzyme (E2); 3) The E2 then transfers the ubiquitin to the ε-amino group of a substrate lysine, resulting in an isopeptide bond (reviewed in Randow and Lehner, 2009). The mammalian genome encodes two E1 enzymes, approximately 40 E2 enzymes, and more than 400 E3 ubiquitin ligases. There are three families of E3 ubiquitin ligases: RING (really interesting new gene) HECT (homologous to E6AP carboxy

terminus) and U-box-containing proteins like CHIP (carboxyl terminus of Hsc70-interacting protein) (Ardley and Robinson, 2005; Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009). The largest family of E3 ubiquitin ligases is the RING family and the complexes formed by cullin-RING are the most versatile class. Transfer of the ubiquitin to the substrate can occur in two different ways, depending on the type of E3 ubiquitin ligase. In the HECT type, the ubiquitin is transiently transferred first to the E3 ubiquitin ligase and then to the substrate; however, in the RING and U-box type, E3 ubiquitin ligase binds to the E2 and facilitates the transfer of the ubiquitin from the E2 conjugating enzyme directly to the substrate (reviewed in Randow and Lehner, 2009; van Wijk and Timmers, 2010). While the E3 enzyme is responsible for the substrate specificity, the E2 conjugating enzymes coordinate the ubiquitin linkage type, which dictates the fate of the target. The two most studied linkage types are Lys63 and Lys48 chains. One example is the Lys63 chain induced by the E2 conjugating enzyme, ubc13. This type of linkage is involved in endocytosis, cellular trafficking, and DNA repair (Pan and Schmidt, 2014). The Lys48 linkage, in contrast, labels proteins for degradation by the proteasome system.

Conjugation of ubiquitin to a target molecule is not permanent. Deubiquitinases (DUBs) and ubiquitin-like proteases function by removing the ubiquitin molecule from the targeted protein (Love et al., 2007).

Viruses manipulate the E3 ubiquitin ligase to control cellular machinery and there are at least three different strategies adopted by viruses to change the E3 ligase substrate specificity: a) viral mimicry of endogenous substrate, such as

the mechanism used by HIV-1 Vpu to degrade CD4 by the Cul1-βTRCP (Margottin et al., 1998); b) displacement of the substrate specificity receptor of the E3 ubiquitin ligase, as seen in the case of degradation of APOBEC3G by Vif using Cul5-EloB/C (Yu et al., 2003); and c) the viral protein creates a new substrate interface within the ubiquitin E3 ligase, where the binding of the new target protein requires interaction with the viral protein and with the cellular substrate receptor. This last mechanism is employed by Vpx towards SAMHD1 degradation by the $Cul4^{DDB1/DCAF1}$ E3 ubiquitin ligase (Cassiday et al., 2014; Schwefel et al., 2014).

Vpr and its paralog Vpx are approximately 50% identical in amino acid sequence and it is believed that Vpx was acquired by duplication of Vpr during the evolution of primate lentiviruses (Tristem et al., 1992). While Vpr is present in all primate lentiviruses, Vpx is only encoded by HIV-2, SIV of rhesus macaque (SIVmac), and SIV of sooty mangabeys (SIVsm). Despite the sequence similarity and the fact that both proteins utilize the same ubiquitin E3 ligase, they have very different functions. Vpx overcomes SAMHD1 restriction by inducing SAMHD1 ubiquitination and degradation (Hrecka et al., 2011; Laguette et al., 2011). The N-terminal unstructured region of Vpx is required for this function (Ahn et al., 2012; DeLucia et al., 2013). On the other hand, HIV-1 Vpr does not degrade SAMHD1 but induces G2 arrest through the $Cul4^{DDB1/DCAF1}$ E3 ubiquitin ligase, a mechanism that requires HIV-1 Vpr C-terminal unstructured region (DeHart et al., 2007; Di Marzio et al., 1995; Le Rouzic et al., 2007). Both Vpr and Vpx require a

leucine-rich domain, within their 3rd alpha helix, to interact with the cellular substrate receptor DCAF1 (Le Rouzic et al., 2007; Srivastava et al., 2008).

The cellular substrate receptor DCAF1 is a large protein composed of several domains. The N-terminal domain is an armadillo domain, with unknown function. The LisH domain is found in the central part of DCAF1 and is proposed to be involved in dimerization of the molecule; the WD40 domain, which is common to all DCAFs, is also found in the central part of the molecule and it is involved in substrate interaction. The carboxy-terminal region comprises an acidic region that regulates DCAF1 activity (Nakagawa et al., 2013). Because Vpr/Vpx interacts with the WD40 domain of DCAF, it was suggested that DCAF1 functions as a bridge, linking Vpr/Vpx to the ubiquitin E3 ligase (Le Rouzic et al., 2007; Schwefel et al., 2014). However, overexpression of a minimal domain of WD40 necessary to bind to DDB1 failed to induce G2 arrest by Vpr (Gerard et al., 2014), suggesting that DCAF1 is not simply functioning as a bridge. In agreement with that we found that overexpression of truncated domains of DCAF1 inhibit cell cycle arrest (unpublished data) and SAMHD1 degradation (Chapter 2).

Recently, the solved crystal structure of a complex comprising the WD40 domain of DCAF1, SIVsm Vpx, and the carboxy-terminal domain of smSAMHD1 (Schwefel et al., 2014) demonstrated that the recruitment of SAMHD1 to the E3 ubiquitin ligase occurs through direct interaction between SAMHD1, Vpx, and DCAF1, in a ternary complex. In agreement with this finding, we demonstrated that mutation at the residue D1092 of DCAF1, which was shown to mediate a

direct ionic interaction with the lysine 622 of SAMHD1 (Schwefel et al., 2014), although able to bind to Vpx is unable to interact with SAMHD1 (Chapter 2). This result suggests that a ternary complex between Vpx, DCAF1, and SAMHD1 must exist in order for Vpx degrade SAMHD1.

Using mutants of DCAF1, we observed that Vpr and Vpx, although sharing highly homology sequence and requiring the same conserved motif to interact with DCAF1, which is found in the 3rd alpha helix, use distinct residues in DCAF1 for the interaction (Chapter 2)

Its is known that the activity of cullins is controlled by neddylation (Saifee and Zheng, 2008). Neddylation is the covalent attachment of the ubiquitin-like molecule NEDD8 by specifics E2 conjugating enzymes (Ube2M and Ube2F) to cullins (Huang et al., 2009). Neddylation of cullins occurs on their C-terminal domain and induces a conformational change that shortens the distance between the substrate to the E3 ligase, facilitating the transfer of the ubiquitin molecule to the target protein (Saifee and Zheng, 2008). It was observed that Vpr associates with hyper neddylated Cul4A (Hrecka et al., 2007), suggesting that Vpr might enhance the interaction between the natural substrates with DCAF1 (Wen et al., 2012), causing its premature ubiquitination (Hrecka et al., 2007). Recently, a new small molecule inhibitor called MLN4924 was shown to inhibit the NEDD8-activating enzyme (NAE), blocking cullin neddylation, and inactivating Cullin Ring Ubiquitin Ligases (CRULs). MLN4924 was initially shown to block the capacity of HIV-1 Vif to degrade APOBEC3G (Stanley et al., 2012). Later, it was reported that MLN4924 also inhibited Vpx's ability to degrade SAMHD1 (Hofmann et al.,

2013; Wei et al., 2014). Our group found that this inhibitor also blocks the ability of Vpu to downregulate CD4, but it has no effect on BST2/Tetherin (see Chapter 3). As expected, the NAE inhibitor impairs the induction of G_2 arrest by Vpr (Ramirez et al. submitted for publication), confirming the requirement of the Cul4 ubiquitin E3 ligase function for Vpr induction of cell cycle arrest.

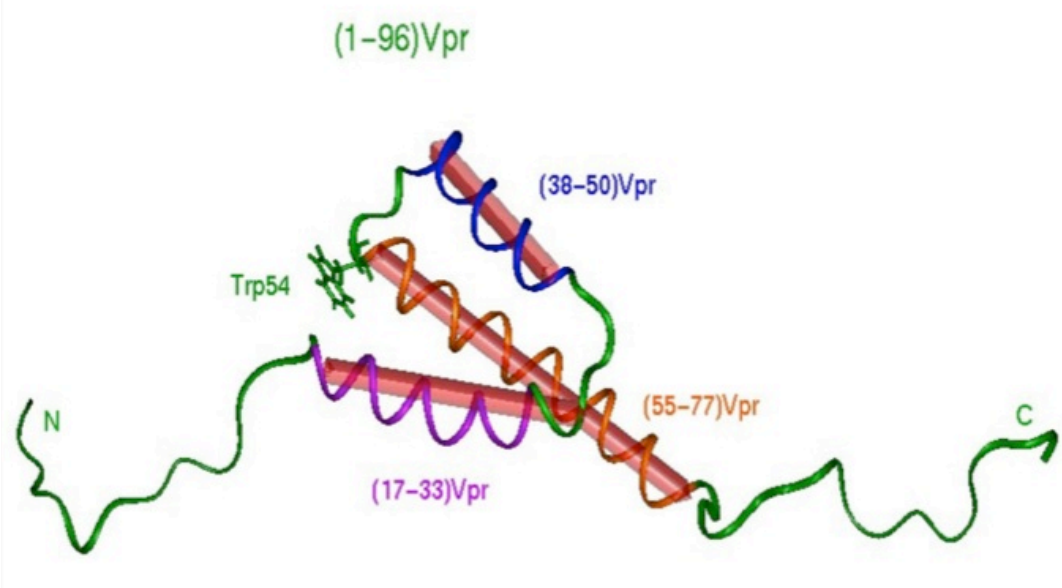

Figure 1.1 Three-dimension structure of the HIV-1 Vpr protein based on the NMR (Morellet et al., 2003). Vpr is comprised of three alpha helices (red) that are connected and flanked by flexible loops (green). The residue Trp54 is required to interact with UNG2. The third alpha helice (residues 55-77) contains the region necessary for interaction with DCAF1 (Q65). The carboxy-terminal unstructured region (C) is the predicted binding site for the G2 arrest putative target. Reproduced with permission from (Le Rouzic and Benichou, 2005).

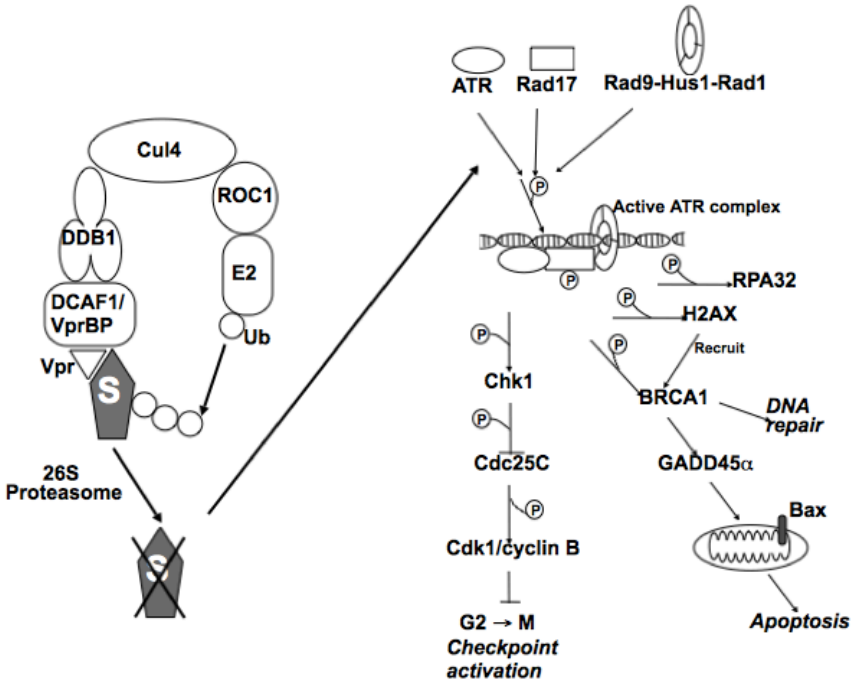

Figure 1.2**.** Overview of the proposed mechanism used by HIV-1 Vpr to induce G_2 arrest.
Vpr interacts with the Cul4A-DDB1 E3 ligase via direct binding to DCAF1, leading to the recruitment of a host restriction factor for ubiquitination and degradation. Degradation of this putative protein activates ATR, which phosphorylates H2AX, BRCA1, p53, and Chk1. Chk1 phosphorylation phosphorylates CDC25c activating Cdk1 resulting in G_2 arrest. Reproduced with permission from (Andersen et al., 2006).

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CHAPTER 2

DETERMINANTS FOR DEGRADATION OF SAMHD1, MUS81, AND INDUCTION OF G_2 ARREST IN HIV-1 VPR AND SIVAGM VPR

Abstract

Vpr and Vpx are a group of highly related accessory proteins from primate lentiviruses. Despite the high degree of amino acid homology within this group, these proteins can be highly divergent in their functions. In this work, we constructed chimeric and mutant proteins between HIV-1 and SIVagm Vpr in order to better understand the structure-function relationships. We tested these constructs for their abilities to induce G_2 arrest in human cells and to degrade agmSAMHD1 and Mus81. We found that the C-terminus of HIV-1 Vpr, when transferred onto SIVagm Vpr, provides the latter with the *de novo* ability to induce G_2 arrest in human cells. We confirmed that HIV-1 Vpr induces degradation of Mus81 although, surprisingly, degradation is independent and genetically separable from Vpr's ability to induce G_2 arrest.

Introduction

The HIV-1 genome encodes structural proteins (Gag, Pol, and Env), regulatory proteins (Tat and Rev), and accessory proteins such as Vif, Vpr, Vpu, and Nef. HIV-2, SIVmac, and SIVsmm encode Vpr, Vpx, Vif, and Nef as accessory proteins. Vpr and Vpx from HIV-2, SIVmac, and SIVsmm are highly related to each other and are thought to have arisen through gene duplication (Tristem et al., 1992). SIVagm encodes Vif, Vpr, and Nef. A Vpu homolog is not found in the HIV-2/SIVmac/SIVsmm or the SIVagm phylogenetic groups.

From the entry step to the time of release from the host cell, lentiviruses encounter several restriction factors that function to inhibit viral infection and are

considered innate immune effector mechanisms. TRIM5α, APOBEC3G, sterile alpha motif (SAM) and HD domain-containing protein 1 (SAMHD1), and tetherin are examples of host restriction factors. These restriction factors are typically overcome by the accessory proteins encoded by lentiviruses (reviewed in Strebel, 2013).

Three of the four accessory proteins in each HIV-1 (Vif, Vpr, and Vpu) and HIV-2 (Vif, Vpr, and Vpx) antagonize innate immunity by a common mechanism, the ubiquitin-proteasome system (UPS). These proteins modify the specificity of cullin-RING ubiquitin ligases (CRUL) such that noncognate proteins are modified and later degraded.

Vpr has been associated with induction of cell cycle arrest in G_2 and apoptosis (He et al., 1995; Jowett et al., 1995; Stewart et al., 1997). Vpr induces these effects via activation of the ATR kinase (Roshal et al., 2003), a result of manipulation of the ubiquitin ligase $CRUL4^{DDB1/DCAF1}$ (Belzile et al., 2007; DeHart et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Schrofelbauer et al., 2007; Wen et al., 2007). Recently, the ubiquitination target for the Vpr/CRUL4 complex has been identified (Laguette et al., 2014). Vpr induces premature activation of the SLX4 complex (SLX4com) (Laguette et al., 2014). Vpr increases the binding of DCAF1 to the scaffold protein SLX4 and, together with the polo-like kinase-1 (PLK1), promotes SLX4com remodeling, which results in Mus81 degradation (Laguette et al., 2014). As a consequence of the untimely SLX4com activation, replication forks are processed incorrectly, causing cell cycle arrest in G_2 (Laguette et al., 2014). The authors showed that by activating SLX4, Vpr

prevents the viral DNA from stimulating cellular DNA sensors, which would normally trigger a type-I interferon response (Laguette et al., 2014)

Nuclear magnetic resonance (NMR) studies indicate that HIV-1 Vpr is comprised of three bundled alpha helices connected by short flexible loops and flanked by flexible amino - and carboxy-terminal unstructured regions (Morellet et al., 2003). The region on Vpr that binds to DCAF1 was mapped within the third α-helix, involving a leucine-rich motif (DeHart et al., 2007; Le Rouzic et al., 2007). The Vpr Q65R amino acid substitution within this region disrupts the interaction between Vpr and DCAF1, resulting in inability to induce G_2 arrest (DeHart et al., 2007; Le Rouzic et al., 2007). The C-terminal unstructured region of Vpr is predicted to be required for interaction with the target, since the mutant R80A within that region is capable of interacting with DCAF1 but is unable to cause G_2 arrest (DeHart et al., 2007). Furthermore, Vpr R80A acts as a dominant-negative protein because it binds to DCAF1 and blocks the Vpr-binding site (DeHart et al., 2007; Le Rouzic et al., 2007).

Vpx is encapsidated in HIV-2 and SIVmac virions and antagonizes SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011a). SAMHD1 interferes with the ability of the virus to efficiently synthesize viral cDNA during reverse transcription because it reduces the available cellular pools of dNTPs (Goldstone et al., 2011). Consequently, SAMHD1 diminishes the capacity of the virus to infect macrophages, dendritic cells, and quiescent T-cells wherein dNTP levels are normally very low (Hrecka et al., 2011; Laguette et al., 2011b). Vpx triggers degradation of SAMHD1 by associating with DCAF1 and recruiting SAMHD1 to

the $CRUL4^{DDB1/DCAF1}$ E3 ligase. SAMHD1 is subsequently ubiquitinated and degraded by the proteasome. Inhibition of the interaction between Vpx and DCAF1 by the mutant Vpx(Q76A) results in failure to degrade SAMHD1. HIV-1 Vpr and SIVmac Vpx appear to bind to similar or overlapping regions on DCAF1, although the amino acid residues in DCAF1 involved in interaction with either accessory protein are not identical (Cassiday et al., 2014).

African green monkeys (AGM) are endemically infected with several strains of simian immunodeficiency viruses collectively known as SIVagm (Jin et al., 1994). This group of primate lentiviruses encode three accessory genes: Vpr, Vif, and Nef. SIVagm Vpr is bifunctional because it can induce G_2 arrest and also antagonize SAMHD1. Unlike HIV-1 Vpr, SIVagm Vpr is highly species-specific, as it induces G_2 arrest in AGM cells but not in human ones (Fletcher et al., 1996; Planelles et al., 1996), and is able to degrade agmSAMHD1 but not human SAMHD1 (hSAMHD1) (Lim et al., 2012).

In order to understand the structure-function relationships for HIV-1 Vpr and SIVagm Vpr, we constructed chimeric proteins by exchanging homologous domains of $HIV\text{-}1_{NL4\text{-}3}$ Vpr and SIVagm.gri Vpr. We then investigated the ability of the different chimeras to cause cell cycle arrest in G_2 and to induce degradation of SAMHD1 and Mus81. We also investigated whether the structural requirements toward degradation of Mus81 are the same as those required for induction of G_2 arrest by HIV-1 Vpr.

Results

To study the structure-function relationships in HIV-1 Vpr and SIVagm Vpr, a set of 4 chimeras and 2 truncations were constructed, as shown in Figure 2.1A. The exchange points for the chimeras were designed based on the published nuclear magnetic resonance (NMR) structure of HIV-1 Vpr and the high degree of amino acid homology with SIVagm Vpr (Figure 2.1B).

Determinants Required for Induction of G_2 Arrest

It was previously suggested that the C-terminal unstructured region of HIV-1 Vpr was required to interact with a putative G_2 arrest-related cellular factor (DeHart et al., 2007; Di Marzio et al., 1995; Le Rouzic et al., 2007). In support of the previous notion, the point mutant Vpr(R80A), although capable of interacting with DCAF1, was unable to induce G_2 arrest, and behaved as a dominant-negative protein by competing with wild-type Vpr for DCAF1 binding (DeHart et al., 2007).

We first constructed two truncations in HIV-1 Vpr (Vpr1-80 and Vpr1-84; Figure 2.1 A. encoded by lentiviral vectors (Verrier et al., 2011). HeLa cells were then transduced with VSV-G-pseudotyped lentivirus vectors (Supplemental Figure 2.1) encoding HIV-1 Vpr, HIV-1 Vpr(R80A), or each of the indicated truncations. As shown in Figure 2.2A and 2.2B, HIV-1 Vpr, but not HIV-1 Vpr(R80A), HIV-1 Vpr(1-80), or HIV-1 Vpr(1-84) induced cell cycle arrest. The percentages of cells transduced with the corresponding lentivirus vectors are shown in Supplemental Figure 2.2A.

While HIV-1 Vpr is able to induce cell cycle arrest in human and non-human primate cells, SIVagm Vpr arrests AGM, but not human cells (Planelles et al., 1996). We asked whether transposition of the C-terminal domain (HIV-1 Vpr residues 78-96) onto SIVagm Vpr (Ch1) would confer upon SIVagm Vpr a *de novo* ability to induce G_2 arrest in human cells. As shown in Figure 2.2C and 2.2D, Ch1 was able to induce G_2 arrest in human cells. Therefore, the inability of SIVagm Vpr to function in human cells can be overcome by a determinant within the C-terminal domain of HIV-1 Vpr.

Using the inactive truncation HIV-1 Vpr(1-80) as the recipient, we asked whether grafting the C-terminus of SIVagm Vpr (Ch2) would enable induction of G_2 arrest in human cells. HeLa cells transduced with the Ch2 also underwent cell cycle arrest (Figure 2.2C and 2.2D). The C-terminal domain of SIVagm Vpr is intrinsically capable of recruiting the target, leading to G_2 arrest in human cells when in the context of HIV-1 Vpr. One possible explanation for the previous observation is that, while DCAF1 is highly conserved across primate lentiviruses (Berger et al., 2014), the target protein may be variable. In addition, these observations suggest that the interaction of Vpr with the target may be dependent, in part, on determinants that lie upstream of the C-terminal domain of Vpr.

In contrast with the above findings, transposition of the N-terminal unstructured region of HIV-1 Vpr to SIVagm Vpr (Ch3) did not confer upon this chimera the ability to induce G_2 arrest in human cells (Figure 2.2C and 2.2D). The reciprocal exchange (Ch4), which contained most of HIV-1 Vpr with the N-

terminus of SIVagm Vpr, was still capable of inducing arrest in human cells (Figure 2.2C and 2.2D). The above results indicate that the N-terminal unstructured region of HIV-1 Vpr is not required to induce cell cycle arrest.

In an effort to test whether the chimeras maintained the capacity to fold correctly, we verified their abilities to interact with DCAF1. HIV-1 Vpr, SIVagm Vpr, and chimeras Ch1 to Ch4 were able to Co-IP with DCAF1 (Figure 2.3, lanes 1 and 4). In contrast, the mutant HIV-1 Vpr(Q65R), did not efficiently co-IP with DCAF1 (Figure 2.3)

The Amino Terminal Domain of SIVagm Vpr Is Required for Degradation of AgmSAMHD1

SIVagm Vpr is bi-functional in AGM cells, where it can arrest cells in G_2 and induce degradation of agmSAMHD1. However, SIVagm Vpr, when expressed in human cells, is unable to perform either function (Lim and al., 2012). We wished to analyze which domain(s) in SIVagm Vpr may be important for targeting agmSAMHD1 for degradation. To that end, we tested the chimeras between SIVagm Vpr and HIV-1 Vpr. AgmSAMHD1 was degraded by SIVmac Vpx and SIVagm Vpr (Figure 2.4A, lanes 5 and 6, respectively) but not by HIV-1 Vpr (Figure 2.4, lane 4) as previously reported (Hrecka et al., 2011). Addition of epoxomycin to cells transfected with SIVagm Vpr prevented the degradation of agmSAMHD1 (compare lanes 6 and 11), confirming the involvement of the UPS.

To examine the potential role of the C-terminal domain of SIVagm Vpr in degradation of agmSAMHD1, we tested Ch1. As shown in Figure 2.4A, lane 7,

Ch1 remained capable of inducing degradation of agmSAMHD1. Therefore, the C-terminal domain of SIVagm Vpr is dispensable for the degradation of SAMHD1. The reciprocal chimera (Ch2) in which the C-terminus of SIVagm Vpr was transferred onto HIV-1 Vpr (lane 8) failed to degrade agmSAMHD1. These results suggest that the C-terminal unstructured region of SIVagm Vpr is not sufficient to confer upon HIV-1 Vpr the ability to degrade agmSAMHD1.

The N-terminal domain of SIVmac Vpx was previously shown to be required to overcome SAMHD1 restriction in myeloid cells (Ahn et al., 2012; DeLucia et al., 2013). To confirm this notion, we tested Ch3. As shown in Figure 2.4A, lane 9, Ch3 failed to induce degradation of agmSAMHD1. We interpret these data to mean that the loss of the native N-terminal domain of SIVagm Vpr in Ch3 ablated the ability to target agmSAMHD1 for degradation. Previous findings for the related protein, SIVmac Vpx, demonstrated that its amino-terminal domain is required to induce hSAMHD1 degradation (Ahn et al., 2012; DeLucia et al., 2013). Therefore, we speculate, by analogy, that the amino-terminal domain of SIVagm Vpr is also required for degradation of agmSAMHD1.

The Ch4, which contains the N-terminus of SIVagm Vpr (residues 1-26) followed by HIV-1 Vpr (residues 17-96), also failed to trigger degradation of agmSAMHD1 (Figure 2.4A, lane 10). Therefore, we conclude that the N-terminal domain of SIVagm Vpr, although required (see above), is not sufficient for this function. Recently, a crystal structure of a complex of DCAF1/SIVsmVpx/smSAMHD1 demonstrated that residues between the 2nd and 3rd alpha helices of SIVsm Vpx interact with smSAMHD1 (Schwefel et al., 2014)

Therefore, by analogy, it is possible that a second determinant required for degradation of agmSAMHD1 might lie in the homologous location of SIVagm Vpr. Schwefel et al. identified two residues in SIVsm Vpx, Met62, and Ser63, which establish a hydrophobic interaction and a hydrogen bond, respectively, with Lys622 and Phe621 in SAMHD1. SIVagm Vpr does not have significant conservation in this area and, specifically, equivalent residues to Met62 and Ser63 are not present in SIVagm Vpr.

Degradation of Mus81 Is Independent of Vpr's Ability to Induce G_2 Arrest

The SLX4 complex was recently proposed as a target for HIV-1 Vpr (Laguette et al., 2014). In the presence of HIV-1 Vpr, the Mus81 protein, a constituent of the SLX4 complex, is ubiquitinated, contributing to the activation of the complex and leading to G_2 arrest (Laguette et al., 2014). We observed that HIV-1 Vpr induces degradation of Mus81 as reported by Laguette et al. (Laguette et al., 2014), although to a modest degree (Figure 2.5A).

We then asked whether the degradation of Mus81 correlated with the ability of known HIV-1 Vpr mutants and homologues to induce G_2 arrest. We conducted these experiments in the presence or absence of the neddylation inhibitor that blocks CRULs, MLN4924 (Soucy et al., 2009), or the proteasome inhibitor, Epoxomicin (Meng et al., 1999). Surprisingly, HIV-1 Vpr(R80A) (Figure 2.5B, compare lanes 3 and 11,) and HIV-1 Vpr(Q65R) (compare lane 3 and 15 and Supplemental Figure 2.3), two mutants that are deficient in G_2 arrest

induction, degraded Mus81 to a similar degree as did wild-type HIV-1 Vpr. Degradation of Mus81 for the above experiment was quantified by densitometry (Figure 2.5C). Vpr(Q65R) is deficient in binding to DCAF1 and, therefore, to degrade Mus81, it may utilize a different DCAF than that involved in induction of G_2 arrest. Vpr(R80A) is thought to be unable to bind the putative G_2 arrest-related target, but is still able to induce degradation of Mus81 (Figure 2.5B lane 11). Therefore, it appears that the molecular determinants of Mus81 degradation induced by Vpr are different from those required for induction of G_2 arrest.

We then asked whether V5-Mus81 was degraded in the presence of chimeras 1-4 and also in the presence of Vpx and SIVagm Vpr. As depicted in Figure 2.5D, SIVmac Vpx and SIVagm Vpr did not induce degradation of Mus81 (lanes 5 and 6). Ch1, a construct that induces G_2 arrest, did not target Mus81 for degradation (lane 7). Ch2, which also induced G_2 arrest, was able to induce degradation of Mus81 (lane 8). Therefore, as we showed above with the Vpr point mutants Q65R and R80A, the ability to induce G_2 arrest does not correlate with degradation of Mus81 when using our chimeric constructs. Furthermore, Ch2 is able to induce Mus81 degradation in the absence of HIV-1 Vpr's unstructured C-terminal domain, pointing at another important difference between the requirements for G_2 arrest and degradation of Mus81.

Transpositon of the N-terminus of HIV-1 Vpr into SIVagm Vpr (Ch3) did not lead to Mus81 degradation (Figure 2.5D, lane 9). Ch4, which contains the N-terminus of SIVagm Vpr (residues 1-26) followed by HIV-1 Vpr (residues 17-96), induced degradation of Mus81 (Figure 2.5D, lane 10). These results suggest that

the N-terminal unstructured region of HIV-1 Vpr is also not critical for Mus81 degradation.

Inhibition of the proteasome with Epoxomicin caused stabilization of Mus81 in the presence of HIV-1 Vpr or HIV-1 Vpr(R80A) (Figure 2.5B, compare lanes 4 to 7 and 11 to 12, respectively) or HIV-1 Vpr(Q65R) (Supplemental Figure 2.3). Because Epoxomicin targets the proteasome, degradation of ubiquitinated proteins is inhibited (Meng et al., 1999). Blockade of Cullin activity by the neddylation inhibitor MLN4924 (Soucy et al., 2009) also stabilized Mus81 in the presence of HIV-1 Vpr, HIV-1 Vpr(R80A) (Figure 2.5B; compare lanes 4 to 8 and 11 to 13, respectively), and HIV-1 Vpr(Q65R) (Supplemental Figure 2.3). Interestingly, treatment with Epoxomicin, but not with MLN4924, led to the stabilization of Mus81 (Figure 2.5B, compare lane 3 with 5 and 6). This result suggests that Mus81 is controlled naturally by the ubiquitin proteasome system but independently of a CRUL. The above data, taken together with the observation that Vpr(Q65R) is active at inducing degradation of Mus81, suggests that Vpr manipulates a CRUL not containing DCAF1, for targeting Mus81. We also observed that SIVagm Vpr, which causes G_2 arrest in AGM cells, was unable to degrade agmMus81 (Supplemental Figure 2.4), supporting that also for SIVagm Vpr, degradation of Mus81 is independent of the induction of G_2 arrest.

Discussion

The main conclusions from this study are as follows: (i) the C-terminal unstructured region of HIV-1 Vpr contains a determinant required for induction of

G_2 arrest; transfer of the C-terminus of HIV-1 Vpr onto SIVagm Vpr is sufficient to confer upon this chimera a *de novo* ability to induce G_2 arrest in human cells; (ii) the carboxy-terminal unstructured region of SIVagm Vpr is intrinsically capable of recruiting the G_2 arrest-related target in human cells when transferred onto HIV-1 Vpr, but not in its native configuration within SIVagm Vpr; (iii) the amino-terminal domain of SIVagm Vpr is required for agmSAMHD1 degradation, although it is not sufficient; (iv) targeting Mus81 for degradation and induction of G_2 arrest are independent, separable functions of HIV-1 Vpr; and (v) degradation of Mus81 by HIV-1 Vpr is proteasome- and cullin-dependent, although it is likely that DCAF1 is not involved in this activity.

The amino-terminal domain of SIVagm.gri Vpr, like the homologous regions in HIV-2 and SIVmac (Ahn et al., 2012; DeLucia et al., 2013), was required for degradation of agmSAMHD1. The crystallographic data for SIVsm Vpx (Schwefel et al., 2014) showed that Glu residues 15 and 16 establish ionic interactions with Arg residues 609 and 617, respectively, in SAMHD1. While residues Glu 15 and 16 are conserved in HIV-2, SIVmac, SIVsm, and SIVrcm Vpx, SIVagm Vpr only contains one Glu residue in the corresponding region (Glu 16). Therefore, it is tempting to speculate that Glu 16 from SIVagm Vpr may interact with either Arg 609 or 617 in SAMHD1.

Our observations suggest that the inability of SIVagm Vpr to induce G_2 arrest in human cells is not intrinsic to this protein, because its C-terminal domain, when grafted onto HIV-1 Vpr, generates a chimera that is active. Because SIVagm Vpr is able to bind hDCAF1, the species-specificity of this

protein likely can be explained based on the different ability to interact with SLX4 complex in the different species. Recently, it has been reported that interaction between HIV-1 Vpr or SIV Vpr and SLX4 correlates with induction of G_2 arrest (Berger et al., 2014). Since Mus81 is targeted for ubiquitination by Vpr (Laguette et al., 2014), we decided to focus on this protein to investigate whether the requirements for induction of G_2/M arrest and Mus81 degradation were the same. We found that HIV-1 Vpr induced degradation of Mus81 through the proteasome system, by manipulating CRUL, since addition of Epoxomicin or MLN4924 relieved this effect. Interestingly, HIV-1 Vpr(R80A) and HIV-1 Vpr(Q65R) degraded Mus81 in our hands. Therefore, our findings, at first sight, appear to be at odds with those of Berger et al., who reported that knockdown of SLX4 (of which Mus81 is an integral component) abolished the cell cycle arrest by the lentiviral proteins (Berger et al., 2014). One possible explanation that might reconcile our findings with those of Berger et al. (Berger et al., 2014) could be if the modest degradation of Mus81 that we and Laguette et al. (Laguette et al., 2014) observe is simply not essential for induction of G_2 arrest. It would still remain to be explained why Vpr(R80A) is competent for interaction with the SLX4 complex (Berger et al., 2014; Laguette et al., 2014), but incapable of inducing G_2 arrest.

Materials and Methods

Cell Lines and Transfections

HeLa cells and HEK293FT (Invitrogen) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% of fetal bovine serum (FBS) and 2mM of L-glutamine (Invitrogen). HEK293FT cells were transfected by Calcium Phosphate method, as previously described (Zhu et al., 2001). HeLa cells were transfected using FuGENE HD (Promega) according to manufacturer's instructions

Plasmids

HA-HIV-1Vpr, HA-SIVagm.gri Vpr (from Grivet monkeys), HA-SIVmac Vpx, and each of the chimeras were PCR amplified and cloned into pFIN-EF1-GFP-2A-mCherry-HA-WPRE, a kind gift of Dr. Susan Semple-Rowland (Verrier et al., 2011) in place of mCherry-HA. The truncations HIV-1 Vpr(1-80) and HIV-1 Vpr(1-84) were generated on HIV-1Vpr using QuickChange Lightning (Agilent Technologies). Mammalian expressing vector encoding HA-agmSAMHD1 was a kindly provided by Dr. Michael Emerman. Mus81 cDNA was purchased from DNASU, PCR amplified, N-terminus tagged with V5 epitope cloned into pFIN vector (without mCherry) in place of GFP.

Immunoprecipitation and Western Blot

Cells were washed in PBS and lysed in NETN buffer, in the presence of phosphatase (PhosSTOP; Roche) and protease (Complete EDTA free tablets;

Roche, Indianapolis, IN) inhibitors. The concentration of proteins were determined by PierceTM BCA (Thermo Scientific, Rockford, IL). Magnetic Beads (SIGMA-ALDRICH, St Louis, MO) were coated with anti-HA antibody (HA1.1, Covance) for 30 minutes at room temperature (RT). For immunoprecipitation, cell lysates were incubated with the beads coated with anti-HA for 1h at 4°C. Beads were washed 3 times with NETN buffer and proteins were eluted in Lamelli buffer and boiled for 10 minutes. Samples were subjected to SDS-PAGE on 4-12% acrylamide gel Criteron^{TM} TGX gel (Bio-Rad, Hercules, CA) and transferred to PVDF membrane (EMD Millipore, Billerica, MA). For degradation assay, cells were lysed using SET buffer (1% SDS, 50mM tris-HCl, pH 7.4, 1mM EDTA) and boiled for 20 minutes. Samples were resolved by SDS-PAGE as described above. V5 antibody (Sigma Aldrich), β-actin (Sigma Aldrich), and rabbit polyclonal DCAF1 antibody were kindly provided by Dr. Ling-Jun Zhao (Saint Louis University).

Cell Cycle Analysis

HeLa cells were treated with trypsin, washed with PBS, and stained with Propidium Iodide (PI) using Hypotonic PI buffer (Sodium Citrate, Triton X-100, Propidium Iodide, RNAse). Samples were analyzed by flow cytometry for DNA content. FlowJo was then used to analyze the cell cycle.

Lentiviral Vectors

Lentiviral vectors were produced in HEK293FT cells. Briefly, transfer plasmid (12.5µg), packaging plasmid (12.5µg), and envelope (5µg) plasmid were co-transfected in HEK293FT cells by calcium phosphate. Supernatants were collected every 12h until the monolayer died. Lentiviruses were concentrated by ultracentrifugation at 25,000rpm for 2h at 4°C. Viruses were titrated in HeLa cells.

Drugs

Epoxomicin (Calbiochem) was solubilized in DMSO and used at 1µM. MLN4924 (MedChem Express) was diluted in DMSO and used at 1µM.

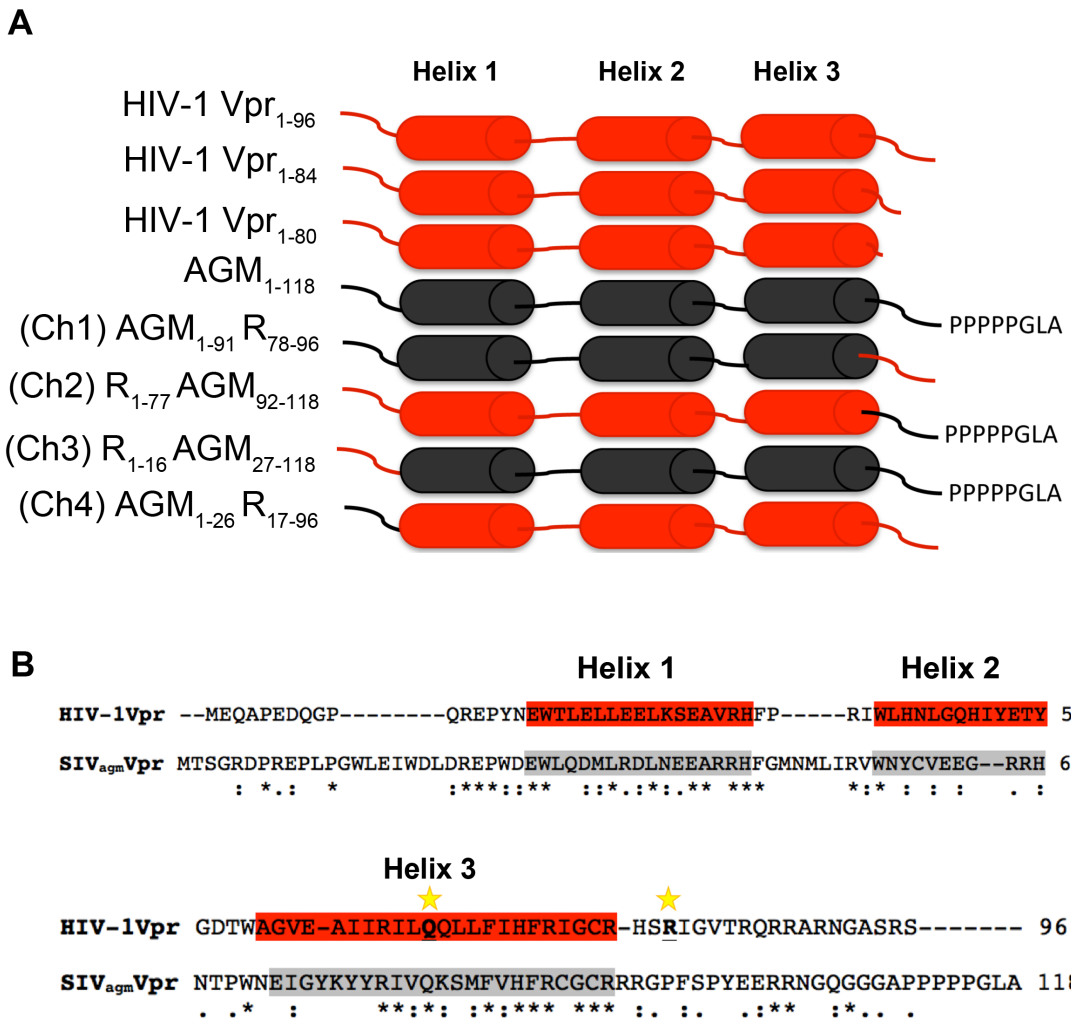


Figure 2.1. Construction of chimeras between HIV-1 Vpr and SIVagm Vpr. A. Schematic representation of the chimeras between HIV-1 Vpr and SIVagm Vpr. HIV-1 Vpr is comprised of three alpha helices, symbolized as barrels, flanked by amino-and carboxy-terminal unstructured regions that are connected by short flexible loops. R represents HIV-1Vpr and AGM represents SIVagm.gri Vpr. The subscript numbers denote the amino acid residues. B. Alignment between HIV-1Vpr and SIVagm.gri Vpr, illustrating the amino acid conservation between these two strains. An asterisk indicates fully conserved residues; colon represents amino acid conservation with strongly similar properties; period designates amino acid conservation with weakly similar properties. Highlighted residues mark alpha helices and yellow stars mark residues Q65 and R80.

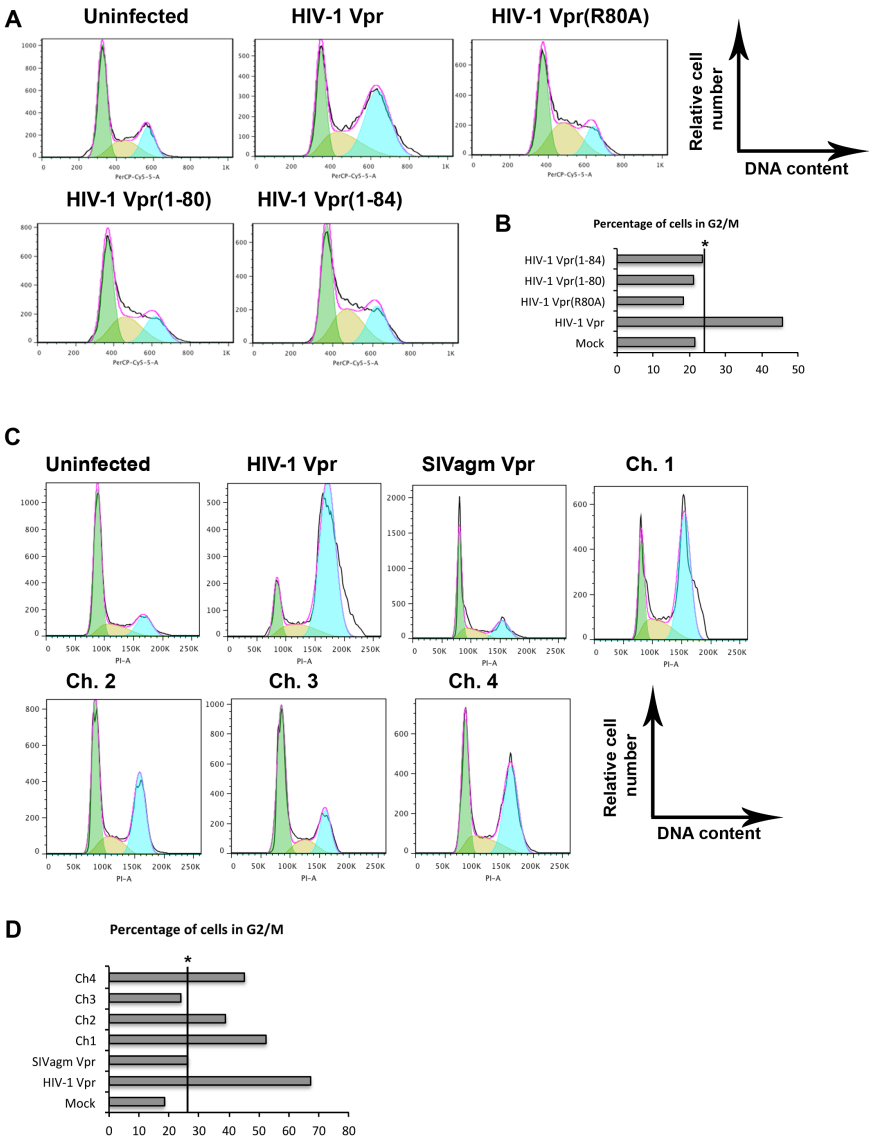


Figure 2.2. G_2 arrest determinants are found in the C-terminal domains of HIV-1 Vpr and SIVagm Vpr.
A Cell cycle analysis of HeLa cells transduced with VSV-G-pseudotyped lentiviral vectors for HIV-1 Vpr, HIV-1 Vpr(R80A), HIV-1 Vpr(1-80), or HIV-1 Vpr(1-84). 48h post transduction, DNA staining (propidium iodide) was used to quantify the cell cycle by flow cytometry. B. Positive values are above the mean percent of cells in G_2 from 3 negative control repeats plus 3 times the standard deviation (line marked with an asterisk) corresponding to a 99.7% interval of confidence. C. Cell cycle analysis of HeLa cells transduced with VSV-G-pseudotyped lentiviral vectors for HIV-1 Vpr, SIVagm Vpr, and each of the chimeras. These experiments were performed at least three time.

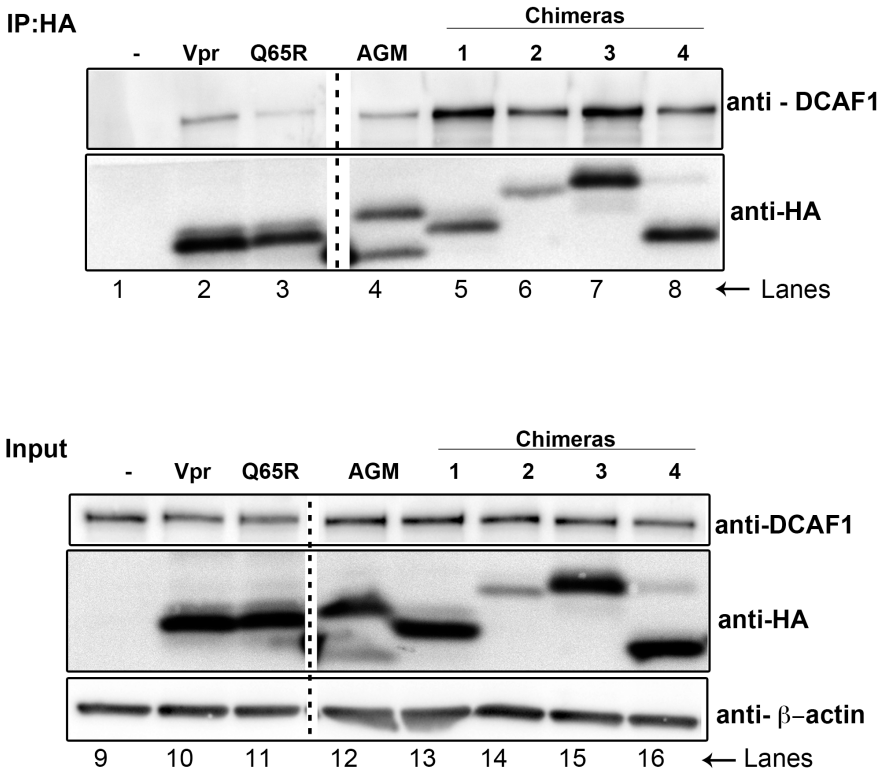


Figure 2.3. Chimeras Co-IP with endogenous DCAF1.
HEK293FT cells were transfected with HIV-1 Vpr, HIV-1 Vpr(Q65R), SIVagm.gri Vpr, or each of the chimeras. 48h post-transfection cells were lysed and subjected to immunoprecipitation using magnetic beads coated with anti-HA antibody.

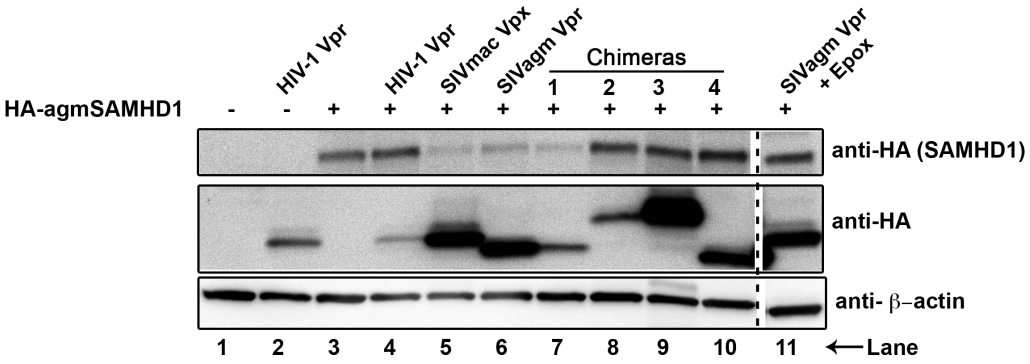


Figure 2.4. The N-Terminal unstructured domain of SIVagm Vpr is required for agmSAMHD1 degradation.
293FT cells were co-transfected with HA-agmSAMHD1 and with vectors encoding HA-HIV-1 Vpr, HA-SIVagm Vpr, HA-SIVmac Vpx, or each of the chimeras. 48h post-transfection cells were harvested and the level of ectopic agmSAMHD1 was analyzed by Western blot using anti-HA. Results are representative of three independent experiments.

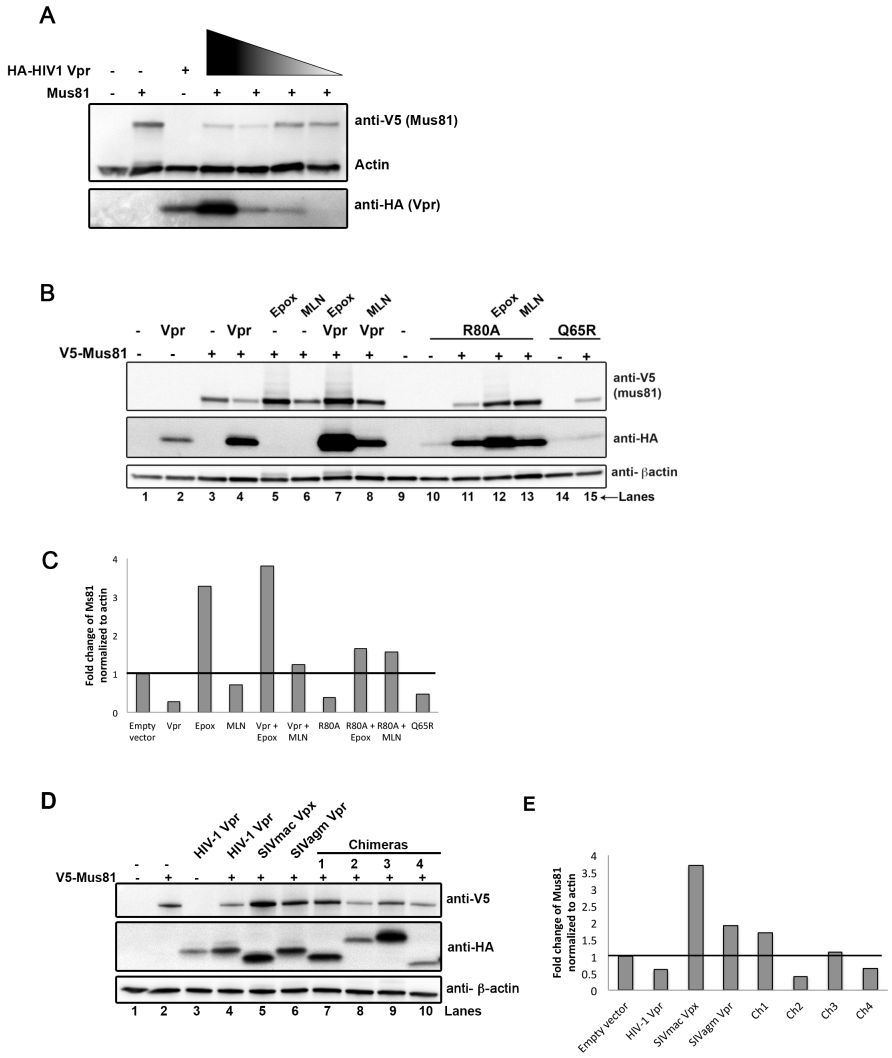
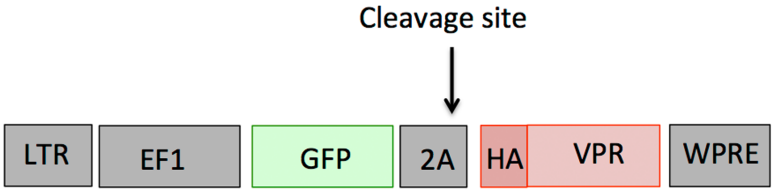
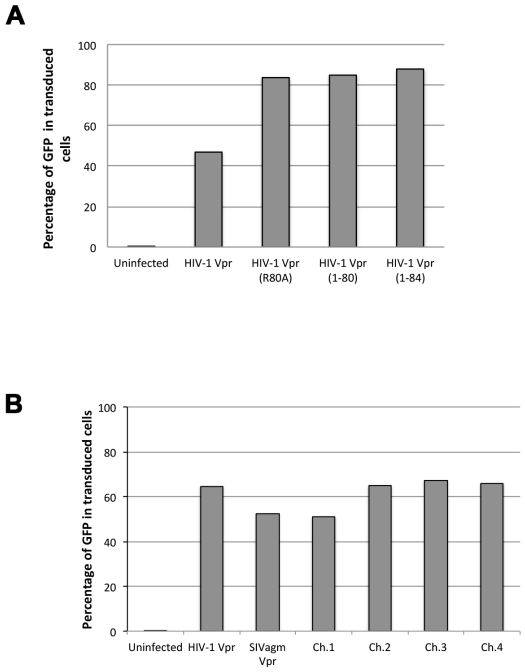


Figure 2.5. Degradation of Mus81 by HIV-1 Vpr is independent of its ability to induce G_2 arrest.

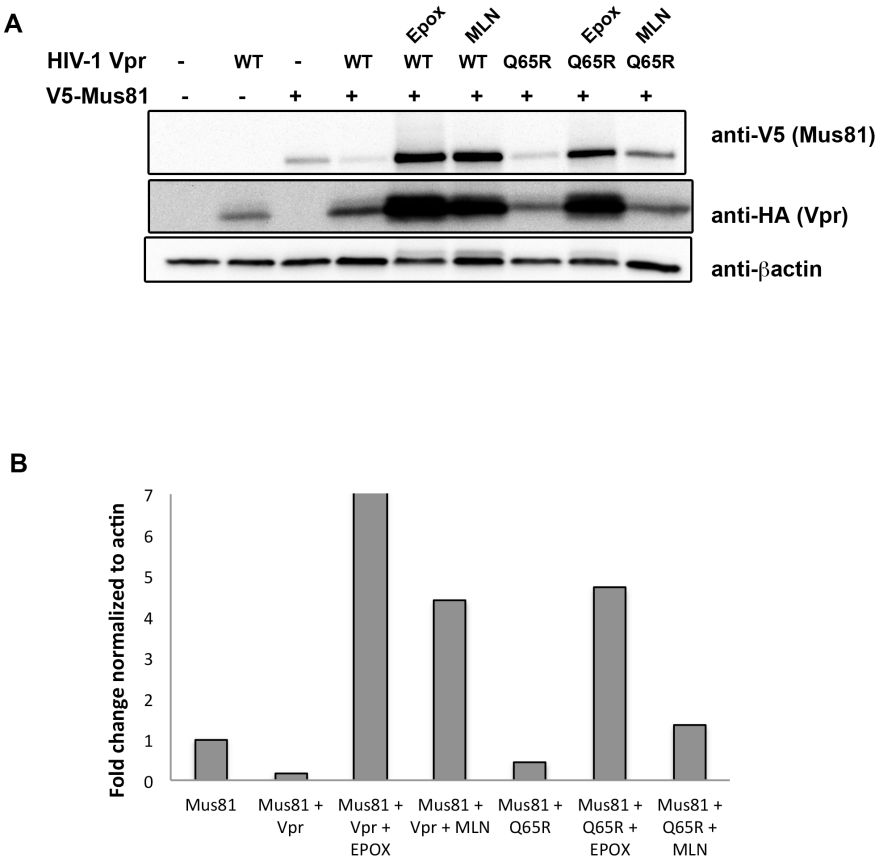
A. HEK293FT cells were cotransfected with HA-HIV-1 Vpr (from 1μg to 0.05 μg) and constant amount of V5-Mus81. 48h after transfection, cells were lysed and the level of Mus81 was analyzed by Western blot using anti-V5 antibody. B. HEK293FT cells were cotransfected with V5-Mus81 and HIV-1Vpr, or HIV-1 Vpr(R80A), or HIV-1 Vpr(Q65R). 18h prior to cell harvesting, Epoxomicin or MLN4924 were added to cells. Levels of exogenous Mus81 were measured by Western blot using anti-V5 antibody. C. Quantification of Mus81 normalized to β-actin from panel B. The line indicates the basal level of Mus81 in the presence of empty vector. Protein level below this line represents degradation of Mus81. D. HEK293FT cells were cotransfected with V5-Mus81 and HIV-1 Vpr or SIVagm Vpr or SIVmac Vpx or each of the chimeras. E. Quantification of Mus81 normalized to β-actin from panel D.



S2.1. Schematic representation of the pFIN-EF1-GFP-2A-HA-Vpr-WPRE vector. The EF1 promoter drives the expression of GFP and HA-Vpr as a single polyprotein. Co-translational cleavage within the 2A site (between amino acid residues 18 and 19) results in equimolar amounts of both proteins. LTR (Long Term Repeat); EF-1 elongation factor 1 alpha promoter; GFP, green fluorescent protein; 2A-like cleavage peptide from porcine *Teschovirus*; WPRE (woodchuck hepatitis virus posttranscriptional regulatory element). Bacterial sequences for this plasmid are not shown.

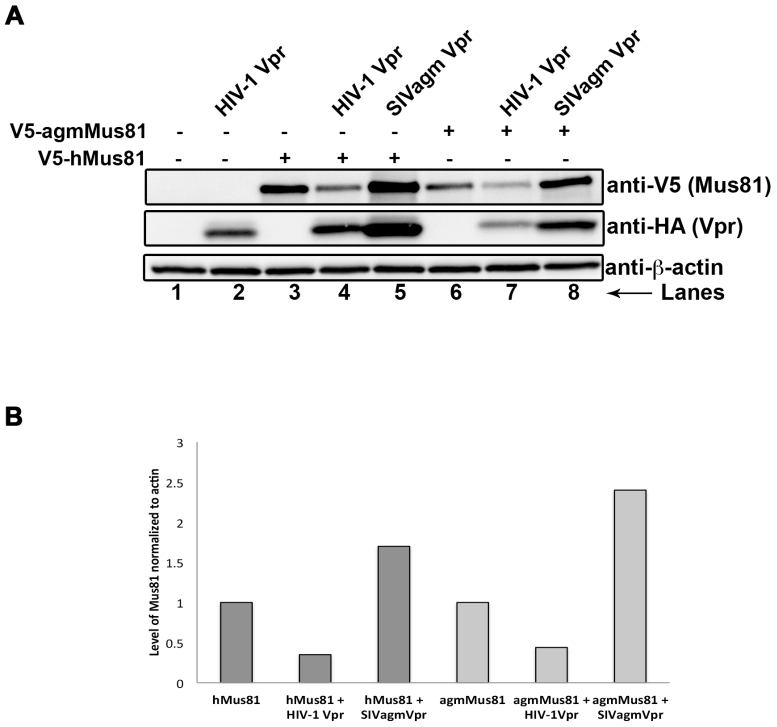


S2.2. Transduction efficiency of HeLa cells.
The efficiency transduction was measured by GFP expression from the lentiviral vector. Further cell cycle experiments were only performed when the efficiency of transduction was at least 45%. Shown is a representative experiment of 3 independent repeats.



S2.3. HIV-1 Vpr(Q65R) degrades Mus81 in a proteasome and Cullin–dependent manner.

A. 293FT cells were transfected with human V5-Mus81 in the presence of HIV-1 Vpr(WT) or HIV-1 Vpr(Q65R). 48h post-transfection cells were harvested and the level of Mus81 was analyzed using anti-V5 antibody. B. Bar graph representation of the level of hMus81. The amount of Mus81 was normalized to the β-actin. Representative of three independent experiments.



S2.4. agmMus81 is not degraded by SIVagm Vpr.
A.293FT cells were transfected with human V5-Mus81 (hMUS81) or V5-agmMus81 in the presence of HIV-1 Vpr or SIVagm Vpr. 48h post-transfection cells were harvested and the level of Mus81 was analyzed using anti-V5 antibody. B) Bar graph representation of the level of hMus81 (dark grey) and agmMus81 (light grey) for the experiment above. The amount of Mus81 was normalized to the β-actin.

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CHAPTER 3

UNDERSTANDING THE MOLECULAR MANIPULATION OF DCAF1 BY THE LENTIVIRAL ACCESSORY PROTEIN VPR AND VPX

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Understanding the molecular manipulation of DCAF1 by the lentiviral accessory proteins Vpr and Vpx

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ABSTRACT

Vpr and Vpx are primate lentivirus proteins that manipulate the cellular CRL4 ubiquitin ligase complex. While Vpr is common to all primate lentiviruses, Vpx is only encoded by HIV-2 and a limited range of SIVs. Although Vpr and Vpx share a high degree of homology they are known to induce markedly different effects in host cell biology through the recruitment of different substrates to CRL4. Here we explore the interaction of HIV-1 Vpr and SIVmac Vpx with the CRL4 substrate receptor DCAF1. Through mutational analysis of DCAF1 we demonstrate that although Vpr and Vpx share a highly similar DCAF1-binding motif, they interact with a different set of residues in DCAF1. In addition, we show that Vpx recruits SAMHD1 through a protein–protein interface that includes interactions of SAMHD1 with both Vpx and DCAF1, as was first suggested in crystallography data by (Schwefel, D., Groom, H.C.T., Boucherit, V.C., Christodoulou, E., Walker, P.A., Stoye, J.P., Bishop, K.N., Taylor, I.A., 2014. Structural basis of lentiviral subversion of a cellular protein degradation pathway., Nature, 505, 234–238).

Introduction

Many viruses encode proteins that manipulate the cellular ubiquitin proteasome system (UPS) to degrade cellular restriction factors. Human Immunodeficiency Virus type-1 (HIV-1) encodes three such proteins (Vif, Vpu, and Vpr). In addition, some primate lentiviruses (including HIV-2 and SIVmac) encode the Vpr paralog, Vpx. While the mechanism of UPS manipulation and the cellular targets of Vif and Vpu have been well characterized, the functions of Vpr and Vpx have been more difficult to elucidate (reviewed in Guenzel et al. (2014), Malim and Emerman (2008) and Romani and Cohen (2012)). In 2011, two groups independently identified SAMHD1 as the cellular protein targeted by Vpx (Hrecka et al., 2011; Laguette et al., 2011). More recently, Laguette et al. (2014) proposed that Vpr activates the SLX4 complex in a ubiquitin dependent manner.

Vpr is a short, 96-amino acid protein that is highly conserved among primate lentiviruses, which is expressed late during viral replication and is present in virions (Cohen et al., 1990; Müller et al., 2000). Vpr and its function appear to be crucial for HIV infection as no primary isolates have been described which lack Vpr (reviewed in Andersen et al. (2008)). While Vpr induces cell-cycle arrest at the G_2/M transition through the activation of the DNA damage sensor Ataxia Telangiectasia and Rad3-related protein (ATR) (Roshal et al., 2003), the significance of this cell cycle arrest in the virus life cycle remains unclear (reviewed in Andersen et al. (2008)). Recently, it was proposed that G_2 arrest is initiated by the Vpr-mediated activation of the SLX4, presumably generating aberrant damage to the host genome and activation of the cellular DNA damage response (Laguette et al., 2014).

In 1994, Vpr was shown to interact with a novel cellular protein (Zhao et al., 1994), subsequently named DDB1-Cullin 4-Associated Factor 1 (DCAF1). The significance of this interaction remained uncertain until DCAF1 was identified as a substrate receptor for the Cullin 4-RING E3 ligase (CRL4) (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006). Subsequent studies demonstrated that induction of G_2 arrest by Vpr was dependent on the manipulation of $CRL4^{DCAF1}$ (Belzile et al., 2007; Dehart et al.,

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2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Tan 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 to viral reverse transcription (Yu et al., 1991; Goujon et al., 2008). Similar to Vpr, the Skowronski and Stevenson laboratories demonstrated that this Vpx-mediated effect requires the formation of a Vpx-CRL4^{DCAF1} complex (Sharova et al., 2008; Srivastava et al., 2008). In 2011, SAMHD1 was identified as the cellular protein targeted by Vpx, in the context of CRL4^{DCAF1} (Hrecka et al., 2011; Laguette et al., 2011).

Thus far, three mechanisms for viral directed ubiquitination 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 Simplex 1 (Boutell et al., 2002; Everett, 2000); 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 from primate lentiviruses (Mehle et al., 2004; Sheehy et al., 2003; Yu et al., 2003); and 3) mimicry of an endogenous substrate by the viral protein, which then ferries a cellular protein to be targeted for ubiquitination, as observed in the manipulation of CRL1$^{\beta TRCP}$ by HIV-1 Vpu to target CD4 (Bour et al., 2001; Margottin et al., 1998).

In this study we investigated the manner in which HIV-1 Vpr and SIVmac Vpx (hereafter referred to as "Vpr" and "Vpx", respectively) interact with DCAF1 resulting in the alteration of substrate specificity of CRL4^{DCAF1}. Using mutational analysis of the DCAF1 substrate-binding interface we found that although Vpr and Vpx share a highly homologous DCAF1-binding motif on their third alpha helix, Vpr and Vpx interact with CRL4^{DCAF1} using different residues on DCAF1. In addition, we identified the DCAF1 residues, D1092 which, when mutated, disrupted SAMHD1 degradation without impeding Vpx binding. Therefore, we surmise that the recruitment of SAMHD1 to DCAF1 by Vpx is mediated by a combination of residues in Vpx and DCAF1. Our results confirm and expand on a functional level the intermolecular interactions that were previously identified by Schwefel et al. (2014) via a co-crystal that included the DCAF1 WD40 domain, the C-terminal domain of SAMHD1 and Vpx.

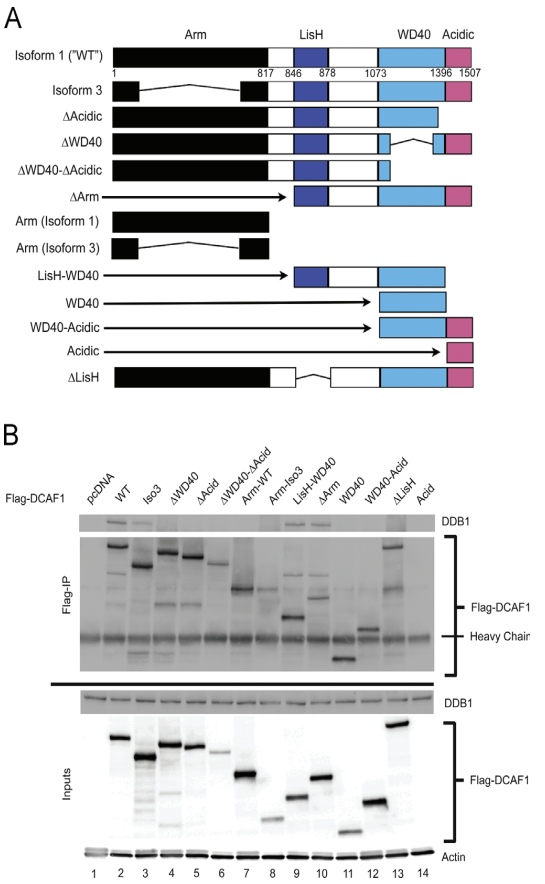

Fig. 1. The WD40 domain of DCAF1 is necessary for interaction with DDB1. (A) Domain architecture of WT DCAF1 (Isoform 1) and truncations. (B) 293FT cells were transfected with plasmids expressing FLAG-DCAF1 or the truncations described in (A). 36 h post-transfection cell lysates were subject to FLAG-immunoprecipitation (DCAF1) and analyzed for the presence of DDB1 by Western blot.

Materials and methods

Cell culture

Exponentially growing 293FT 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). Cells were harvested 36 h post-transfection, washed 2x with Phosphate Buffered Saline (PBS) and lysed as described below.

Plasmids

DCAF1 Iso1 cDNA (NCBI accession NM_014703) was amplified by PCR from a human cDNA library with the addition of an N-terminal 3x FLAG®-poly linker, then assembled into pCMV.Sport 6 (Invitrogen, Carlsbad, CA). DCAF1 truncations were made by generating unique restrictions sites which were indicated (Fig. 1A) using Quikchange Lightning site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). DCAF1 WD40 point mutants were also generated via Quikchange. Myc-huSAMHD1 was purchased from OriGene (Rockville, MD). pcDNA3.1 was purchased from Invitrogen. HA-Vpr of HIV-1 and HA-Vpx of SIVmac were subcloned from pHR-HA-Vpr-IRES-GFP and pHR-HA-Vpx-IRES-GFP, respectively (Dehart et al., 2007) into pFIN-EF1-GFP-2a-mCherH-WPRE (a kind gift of Dr. Semple-Rowland) (Verrier et al., 2011) in substitution of the mCherry gene.

Immunoprecipitation and Western blots

For immunoprecipitation, cells were gently detached by incubation in PBS, pelleted and lysed with FLAG IP buffer (50 mM Tris HCl, pH 7.4, 15 0 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 subjected to immunoprecipitation using Anti-FLAG® M2 Magnetic Beads (SIGMA-ALDRICH, St. Louis, MO). Briefly, lysates were incubated with beads for 2 h (at RT) to overnight (at 4 °C). Beads were washed 5x with lysis buffer and proteins eluted with 3x-FLAG® Peptide, 100 μg/ml, for 1 h at RT. Cells used in degradation 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 immunoprecipitation samples were resolved by SDS-PAGE on 4–10% Criterion™ TGX™ gels (Bio Rad, Hercules, CA) as 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), and DDB1 and VprBP/DCAF1 (Abcam).

DCAF1 depletion

DCAF1 was knockdown using an shRNA expression plasmid previously described (Ward et al., 2009). Briefly, 293FT cells were transfected as described above with FG12 DCAF1_3590 and FG12 DCAF1_Scambled vectors graciously provided by Dr. Edward Barker (Rush University Medical Center, Chicago, Illinois). DCAF1_3590 target and scrambled sequences were previously described (Hrecka et al., 2007; Ward et al., 2009). Transfected cells were analyzed for DCAF1 depletions 48 h later by Western blot using rabbit anti-DCAF1 (Abcam).

Results and discussion

DCAF1 interacts with DDB1 through a region N-terminal of the WD40 domain

Most DCAFs 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, DCAF1 has a complex domain architecture, comprised of an N-terminal armadillo domain ("Arm"), a central LisH domain, a WD40 domain and a highly acidic C-terminal tail (Jin et al., 2006) (Fig. 1A, isoform 1). Several groups have previously shown that Vpr and Vpx interact with the DCAF1 WD40 domain (Gérard et al., 2014; Le Rouzic et al., 2007; Schwefel et al., 2014). Whether the other domains are required for viral proteins to alter substrate specificity remains unknown. In order to better understand how Vpx manipulates the $CRL4^{DCAF1}$ ubiquitin ligase, we generated a number of FLAG-tagged DCAF1 constructs in which each domain was removed individually or in conjunction with others. We also generated a construct reflecting the structure of the annotated DCAF1 isoform 3 (Uniprot identifier Q9Y4B6-3), which is characterized by a large truncation within the Arm domain (Fig. 1A).

DCAF1-mediated substrate ubiquitination is dependent on the interaction of DCAF1 with the ubiquitin ligase backbone comprised of DDB1-Cullin 4-Roc1 (Angers et al., 2006; Hu et al., 2004). To determine the minimal DCAF1 construct capable of interacting with DDB1, we performed immunoprecipitation studies. 293FT cells were transfected with expression vectors encoding the indicated DCAF1 truncations, subjected to FLAG immunoprecipitation and analyzed for the presence of DDB1. As expected, full length DCAF1, both isoform 1 ("WT") and isoform 3, were capable of interacting with DDB1 (Fig. 1B, lanes 2 and 3). Consistent with previous observations indicating a role of the WD40 domain in DDB1 interaction (Angers et al., 2006), constructs lacking the WD40 domain lost the ability to interact with DDB1 (Fig. 1B, lanes 4, 6–8). Surprisingly, the FLAG-DCAF1 WD40 construct did not interact with DDB1 (Fig. 1B, lane 11). This is in contrast to earlier reports in which the WD40 domain by itself was shown to be able to interact with DDB1 (Gérard et al., 2014; Le Rouzic et al., 2007). Most likely this discrepancy is due to differences in residues spanned by the constructs used in these different studies (residues 1041–1377 (Le Rouzic et al., 2007); 1041–1393 (Gérard et al., 2014); and 1073–1396 in this study). 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. (2014) recently determined that the putative DCAF1 Hbox spans residues 1049–1062, a stretch of residues which is absent from our WD40 construct. In agreement with this hypothesis the minimal truncation we generated of DCAF1 was capable of interacting with DDB1 containing the LisH and WD40 domains (Fig. 1B, lane 9). The Δ-acidic DCAF1 construct was unable to interact with DDB1 (Fig. 1B, lane 5). This was a surprising result in view of the observation that the construct containing LisH-WD40 domains was sufficient to bind to DDB1. The simplest explanation for these observations would be that Δ-acidic DCAF1 construct is incorrectly folded and lost the ability to bind to DDB1 even though all the necessary domains may be present.

Mutations in the predicted DCAF1 WD40 substrate interface disrupt Vpx-mediated SAMHD1 degradation

We then tested the DCAF1 LisH-WD40 and WD40 constructs for their ability to facilitate SAMHD1 degradation by Vpx. To determine the residues necessary for SIVmac Vpx to manipulate the substrate specificity of DCAF1, we also generated a number of point mutations in the WD40 domain of DCAF1 (Table 1). Substrate binding to WD40 domains is typically mediated by polar interactions on the "top" side (by convention) of the β-propeller structure (Patel et al., 2008; Pons et al., 2008). Previous reports have demonstrated that the top of WD40 domains form a shallow groove that mediates interactions with proteins to be targeted for ubiquitination (reviewed in Stirnimann et al. (2010) and Trievel and Shilatifard (2009)). We utilized two independent methods to

Table 1
Summary of DCAF1 mutants. DCAF1 mutations were screened for their ability to facilate Vpx-mediated SAMHD1 degradation or interact with DDB1, Vpx and Vpr as indicated. "+" indicates that SAMHD1 degradation was observed in the presence of the DCAF1 construct or the DCAF1 construct was able to interact with indicated protein. "−" denotes that SAMHD1 degradation was not observed in the presences of the DCAF1 construct or the DCAF1 construct was unable to co-IP the indicated protein. The two constructs for which SAMHD1 degradation was observed to be intermediated are denoted with "+/−". ND indicates that the DCAF1 construct was not tested for the indicated activity.

	Function	Interaction		
	SAMHD1 Deg	Vpx	Vpr	DDB1
Iso1	+	+	+	+
Iso3	ND	ND	ND	+
ΔWD40	ND	ND	ND	−
ΔAcid	ND	ND	ND	−
ΔWD40-ΔAcid	ND	ND	ND	−
Arm-Iso1	ND	ND	ND	−
Arm-Iso3	ND	ND	ND	−
Lish-WD40	−	+	+	+
ΔArm	ND	ND	ND	+
WD40	−	−	+	−
WD40-Acid	ND	ND	ND	−
Acid	ND	ND	ND	−
ΔLish	ND	ND	ND	−
D1092A	−	+	+	+
H1134A	+	+	+	+
N1135A	+/−	−	+	+
T1141A	+	ND	+	ND
T1155A	+	ND	+	ND
W1156H	−	−	+	+
E1178A-D1179A	+	ND	+	ND
H1180A	+	ND	+	ND
K1196A	+	ND	+	ND
N1221A-N1222A	+	ND	+	ND
R1225A	+	ND	+	ND
N1226A	+	ND	+	ND
D1256A	+	+	+	+
K1257A	+	ND	+	ND
S1263A	+	ND	ND	ND
D1295A	+	ND	ND	ND
Q1296A	+	ND	+	ND
Q1316A	+	ND	+	ND
D1320A	+	ND	ND	ND
F1334Y	+	ND	+	ND
V1350T	+	ND	+	ND
N1379A	+	ND	ND	ND

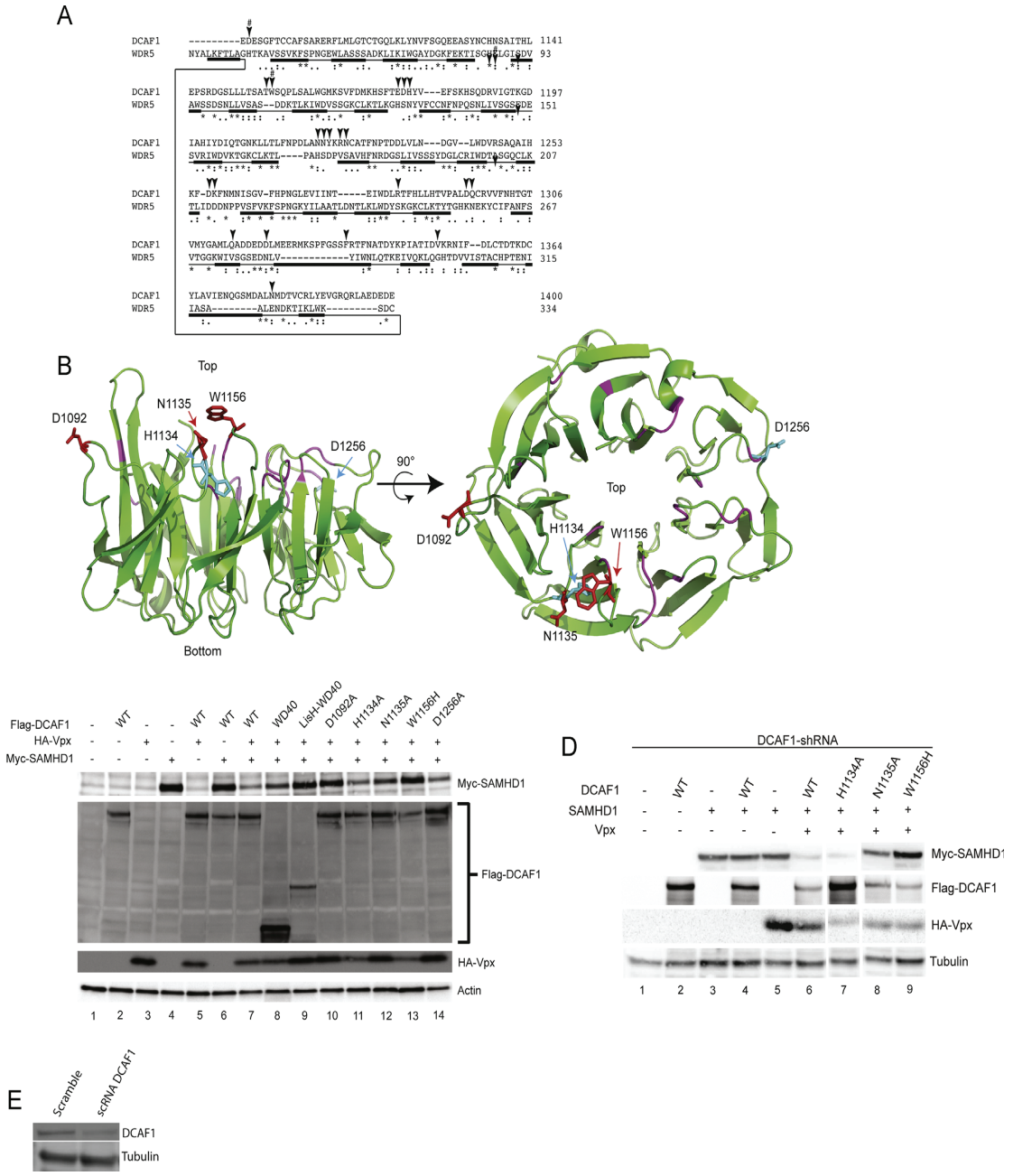

Fig. 2. Analysis of DCAF1 mutations for their ability to facilitate Vpx-mediated SAMHD1 degradation. (A) DCAF1 WD40 domain and WDR5 protein sequences were aligned and used to predict DCAF1 WD40 secondary structure. WDR5 tertiary structure is indicated below (bold lines – β-sheets, narrow lines connect β-sheets which form respective "blades" of the β-propeller. Homology is indicated below: asterisks (*) indicate identity, colons (:) and periods (.), similarity. Arrowheads indicate residues mutated in DCAF1. Residues observed to be important for Vpx mediated SAMHD1 degradation are indicted with a pound symbol (#) above the arrowhead. B) DCAF1 WD40 domain as determined by Schwefel et al. (2014) (PDB accession 4CC9). The side chains for the three loss-of function mutants (D1092, N1135 and W1156) are shown in red, and those for two control mutants (H1134 and D1256) are shown in cyan, additional residues screened are shown without side chains in purple. (C) 293FT cells were transfected with human Myc-SAMHD1, HA-Vpx and/or FLAG-DCAF1 mutants. 36 h post-transfection, cells were lysed and analyzed for the presence of SAMHD1 by Western blot. (D) FLAG-DCAF1 constructs were tested for the ability to facilitate Vpx-mediated degradation Myc-SAMHD1 in DCAF1 depleted 293FT cells. (E) Transfection of DCAF1 shRNA expression construct efficiently depleted DCAF1 in 293FT cells.

select a number of polar/charged residues, which we predicted may be involved in binding to Vpx based on their predicted location within the top face of DCAF1 WD40 domain: 1) homology modeling between DCAF1 and the most closely related DCAF with a known crystal structure (WDR5; Fig. 2A) (Patel et al., 2008; Schuetz et al., 2006; Song and Kingston, 2008) by alignment of primary sequences using ClustalW2 (Larkin et al., 2007) and 2) an *in silico* (ModBase) structural prediction of the DCAF1 WD40 domain (Pieper et al., 2014). Selected residues were then mutated to alanine. In addition, three hydrophobic residues (W1156, F1334 and V1350) were mutated, to polar amino acids of similar size, based on their predicted proximity to and orientation towards the

predicted substrate-binding groove. Finally, based on the recent observations by Schwefel et al., we mutated residue D1092 in order to functionally test its role in SAMHD1 recruitment by Vpx (Schwefel et al., 2014). Mutated residues are represented as arrowheads in Fig. 2A and colored (other than green) residues in Fig. 2C.

293FT cells were transfected with DCAF1 expression constructs, SAMHD1 and Vpx as indicated. Because endogenous DCAF1 was not depleted in this experiment, this method evaluated the abilities of indicated constructs to dominantly inhibit degradation of SAMHD1. 36 h post-transfection cells were lysed and SAMHD1 degradation was analyzed by Western blot. As LisH-WD40 or WD40 (Fig. 2C, lanes 8 and 9) constructs were unable to mediate SAMHD1 degradation we propose that DCAF1 does not merely serve as a bridge by which Vpx brings SAMHD1 to the $CRL4^{DCAF1}$ ubiquitin ligase. Rather, additional domains of DCAF1 appear to be necessary to facilitate this activity.

Two DCAF1 point mutants (D1092A, and W1156H) were identified which failed to mediate the degradation of SAMHD1 in the presence of Vpx while a third point mutant (N1135A) showed reduced ability to facilitate SAMHD1 degradation by Vpx (Fig. 2C, lanes 10, 12–13). Other point mutants (H1134A and D1256A) in the DCAF1 WD40 domain (Fig. 2C, lanes 11 and 14), as well as all additional point mutations in the DCAF1 WD40 domain (Table 1) retained the ability to support Vpx-induced degradation of SAMHD1. For simplicity, data corresponding to the three loss-of-function mutants (side chains shown in red in Fig. 2B), as well as two control mutants (side chains shown in cyan in Fig. 2B), are shown and the overall results for all mutants are summarized in Table 1.

In order to confirm these results in the absence of potential contribution of endogenous DCAF1 to the degradation of SAMHD1, 293FT cells were co-transfected with a DCAF1 shRNA expressing plasmid, Myc-SAMHD1, FLAG-DCAF1 constructs and HA-Vpx as indicated. FLAG-DCAF1 constructs were protected from shRNA depletion by the generation of silent mutations at the shRNA-targeting site. 48 h post-transfection, cells were lysed and analyzed for SAMHD1 degradation by Western blot (Fig. 2D–E). As expected, Vpx was unable to facilitate the degradation of SAMHD1 in the absence of endogenous DCAF1 while the expression of WT DCAF1 was able to restore Vpx mediated SAMHD1 degradation (Fig. 2D lane 5 vs. 6). Confirming the observations depicted in Fig. 2C, DCAF1 W1156H was unable to elicit the degradation of SAMHD1 and DCAF1 N1135A exhibited an intermediate phenotype (Fig. 2D, lanes 9 and 8, respectively). The mutant DCAF1 H1134A was able to recover Vpx mediated SAMHD1 degradation in DCAF1-depleted cells (Fig. 2D lane 7). The efficiency of DCAF1 depletion by shRNA is shown in Fig. 2E.

Select DCAF1 WD40 domain mutations disrupt Vpx binding

To better understand how the above DCAF1 truncations/substitutions (LisH-WD40, WD40, D1092A, H1134A, N1135A, W1156H and D1256A) disrupt Vpx function, we analyzed the ability of each to interact with Vpx by co-IP. As expected, immunoprecipitation of full-length FLAG-DCAF1 was able to co-precipitate Vpx (Fig. 3, lane 4). Interestingly, in contrast to previous reports regarding the highly related lentiviral protein HIV-1 Vpr (Le Rouzic et al. (2007) and this report), the WD40 domain of DCAF1, alone, was not sufficient to co-IP with Vpx (Fig. 3, lane 5), while the longer construct containing both LisH and WD40 domains retained this function (Fig. 3, lane 6). These observations suggest that the Vpx–DCAF1 interaction may involve residues outside the WD40 region in addition to those within. Alternatively, it is also possible that the presence of the LisH domain is required for the WD40 domain to retain its native conformation.

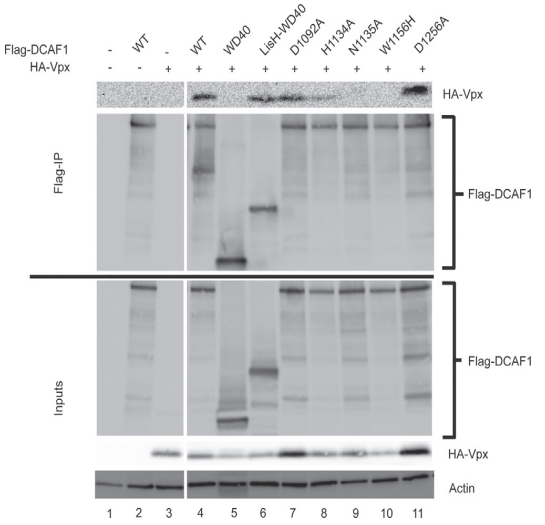

Fig. 3. DCAF1 mutations differentially effect interaction with Vpx. 293FT cells were transfected with plasmids expressing FLAG-DCAF1 or mutants and HA-Vpx as indicated. Cell lysates were subject to FLAG-immunoprecipitation and analyzed for the presence of HA-Vpx by Western blot.

While DCAF1 mutations D1092A, N1135A and W1156H all disrupted Vpx-mediated SAMHD1 degradation, only N1135A and W1156H (Fig. 3, lanes 9 and 10) resulted in a loss of Vpx interaction. Residue D1092 in DCAF1 was recently shown to form a direct ionic interaction with residue K622 of SAMHD1 (Schwefel et al., 2014). This is consistent with our observation that DCAF1 D1092A was still able to bind to Vpx (Fig. 3, lane 7) but not to facilitate the degradation SAMHD1. Therefore, our results functionally confirm and extend the findings by Schwefel et al. (2014) and suggest that the interaction of Vpx with DCAF1 leads to the formation of a new protein–protein interface that recruits SAMHD1 via interactions with both DCAF1 and Vpx residues (Fig. 4). These observations taken together functional confirm the crystallographic data of Schwefel et al. demonstrating the importance of residues N1135 and W1156 in binding the Vpx Q76 residue.

Vpr interacts with different residues in DCAF1 than those utilized by Vpx

While a recently published crystallographic study identified the Vpx–DCAF1 binding interface (Schwefel et al., 2014), no structural data exists regarding the Vpr–DCAF1 interaction. The existence of a high degree of homology between HIV-1 Vpr and SIVmac Vpx led us to hypothesize that both viral proteins would interact with DCAF1 in a similar manner. We speculated that the same set of residues in DCAF1 might mediate interactions with both viral proteins. In support of this hypothesis, analogous mutations in the third helix of both Vpr (Q65R) and Vpx (Q76R) have been shown to abrogate interaction with DCAF1 (Belzile et al., 2007; Dehart et al., 2007; Le Rouzic et al., 2007; Schröfelbauer et al., 2007; Srivastava et al., 2008).

In order to examine the molecular interaction between DCAF1 and Vpr, we tested our panel of DCAF1 truncations and point mutants for their ability to interact with Vpr. As a positive control, wild-type Vpr was able to co-IP with FLAG-DCAF1 (Fig. 5A, lane 4). In agreement with previous observations (Le Rouzic et al., 2007) the DCAF1 WD40 domain alone was sufficient to mediate Vpr binding (Fig. 5A, lane 5). The longer LisH-WD40-containing construct also retained Vpr binding (Fig. 5A, lane 6). In contrast to what we observed with Vpx, both N1135A and W1156H DCAF1 substitutions retained Vpr binding (Fig. 5A, lanes 9 and 10).

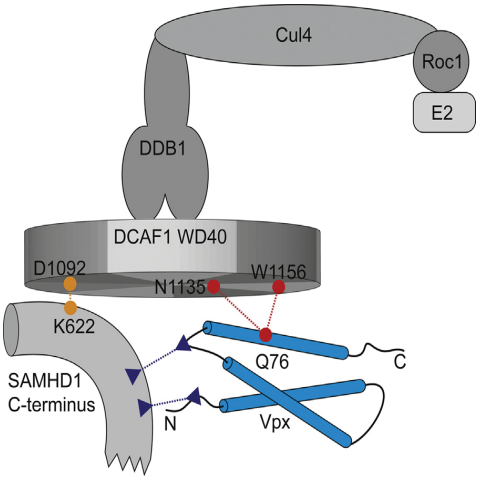

Fig. 4. Model of Vpx manipulation of the $CRL4^{DCAF1}$ ubiquitin ligase. Interaction of DCAF1 WD40 domain with Vpx is mediated primarily by interactions between DCAF1 N1135 and W1156 and Vpx (red lines). Interaction between Q76 of Vpx with residues N1135 and W1156 in DCAF1 was documented by Schwefel et al. (2014). In addition, mutation of DCAF1 D1092 was observed to disrupt the ability of Vpx to mediated SAMHD1 degradation without affecting binding to Vpx. D1092 was recently shown to form an ionic bond with SAMHD1 K622 by Schwefel et al. (2014) (orange line). Finally, based on previous reports interactions between Vpx and SAMHD1 are known to involve multiple residues within the unstructured N-terminus and linker between second and third α-helix of Vpx and SAMHD1 (blue lines) (Schwefel et al., 2014).

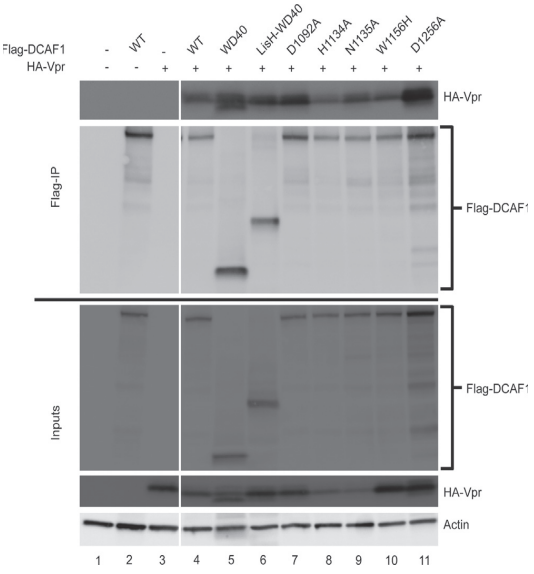

Fig. 5. DCAF1 residues required for Vpr interaction differ from those required for Vpx interaction. Plasmids expressing FLAG-DCAF1 or mutants and HA-Vpr were transfected into 293FT cells. FLAG-DCAF1 was then immunoprecipitated and analyzed for the presence of HA-Vpr by Western blot.

Therefore residues N1135 and W1156 are required for interaction of DCAF1 with Vpx but not with Vpr. We conclude that Vpr and Vpx interact with a non-identical set of residues in DCAF1 WD40 domain.

A previous study by Gérard et al. (2014) explored the interaction between DCAF1 and HIV-1 Vpr by investigating the potential role of F/YxxF/Y motifs within the DCAF1 WD40 domain, reminiscent of the motif identified in UNG2 as being necessary for Vpr

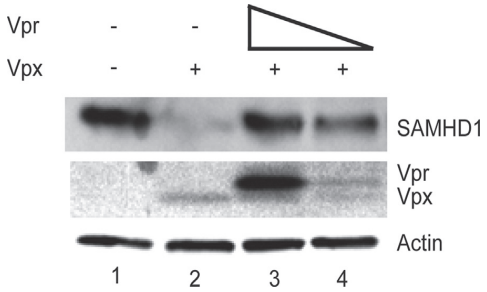

Fig. 6. Vpr is dominant over Vpx mediated SAMHD1 degradation. 293FT cells were transfected with a HA-Vpx plasmid in the absence or presence of increasing concentrations of HA-Vpr. All cells were transfected with equal amounts of total plasmid DNA by transfection of pcDNA3.1. Cells were lysed 36 h post-transfection and endogenous SAMHD1 levels were analyzed by Western blot.

interaction (BouHamdan et al., 1998). However, mutation of these motifs individually failed to identify residues of DCAF1 which interact with Vpr (Gérard et al., 2014). The generation of a DCAF1–Vpr co-crystal will be of great interest in order to more clearly resolve the molecular nature of this interaction and its differences in comparison to that of the DCAF1–Vpx complex.

Vpr and Vpx interact with DCAF1 in a competitive manner with each other

The above results indicate that the binding surfaces for Vpx and Vpr on DCAF1 are at least somewhat different. However, because none of the WD40 domain point mutants tested disrupted Vpr binding (data summarized in Table 1), we are unable to discern whether Vpx and Vpr bind to overlapping or non-overlapping regions on the WD40 domain. To test whether the binding regions may overlap, we designed the following competition experiment. We hypothesized that if the Vpr and Vpx binding regions on DCAF1 overlap, then over-expression of Vpr would to some degree interfere with the ability of Vpx to recruit SAMHD1 to the ubiquitin ligase complex and would therefore hinder SAMHD1 degradation. 293FT cells were transfected with HA-Vpx alone or in combination with HA-Vpr at 1:2 or 1:1 Vpx: Vpr expression plasmid ratios. As expected, Vpx expression resulted in the depletion of SAMHD1 (Fig. 6, lane 1 vs. lane 2). Co-expression of Vpr robustly inhibited Vpx induced SAMHD1 degradation at both ratios (Fig. 6, lanes 3 and 4 vs. lane 2). Therefore, although we were unable to define specific residues on DCAF1 that mediate interaction with Vpr, the ability of Vpr to interfere with Vpx function suggest that both viral proteins bind to overlapping areas within the top face of the DCAF1 WD40. However, these results, cannot distinguish whether this competition is for a shared/overlapping binding site of Vpr and Vpx or if this competition is due to conformational changes in DCAF1 induced by Vpr binding that may in turn disrupt Vpx binding at a distance. Based on the high homology between their third α-helices, we favor a model in which HIV-1 Vpr and SIVmac Vpx utilize a similar, but not identical, binding interface on DCAF1 (Le Rouzic et al., 2007; Schwefel et al., 2014; Srivastava et al., 2008) (reviewed in Morellet et al. (2009)).

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CHAPTER 4

HIV-1 VPU UTILIZES BOTH CULLIN RING LIGASE (CRL) DEPENDENT AND INDEPENDENT MECHANISMS TO ANTAGONIZE HOST PROTEINS

Abstract

Hijacking of the Cullin-RING E3 ubiquitin ldownmodulateigase (CRL) machinery is a common mechanism employed by diverse groups of viruses for the efficient counteraction and eventual degradation of host proteins. In particular, HIV-1 Vpu usurps the Skp1-Cullin1-F-box (SCF) E3 ubiquitin (Ub) ligase complex to ubiquitinate CD4 for eventual degradation by the 26S proteasome. Vpu also interacts with and downmodulates a number of other host proteins, including the restriction factor BST-2. Recent data have suggested a model through which Vpu mistrafficks BST-2 via adaptor-protein 1 (AP-1) for eventual lysosomal degradation, independently of cullin activity. However, whether Vpu primarily relies on a cullin-dependent or -independent mechanism to antagonize host proteins in primary $CD4^+$ T cells has not been fully elucidated.

We utilized a sulphamate AMP analog, MLN4924, to effectively block the activation and thus activity of CRLs within infected primary $CD4^+$ T cells. MLN4924 treatment, in a dose-dependent manner, efficiently relieved surface downmodulation and degradation of CD4 by Vpu. As expected, the drug had no effect on Nef's ability to counteract CD4, which does not rely on CRL activity. Interestingly, Vpu's capacity to downregulate BST-2, NTB-A, and CCR7 was not inhibited by MLN4924, suggesting that in primary $CD4^+$ T cells, AP-1-mediated mistrafficking, but not b-TrCP-dependent ubiquitination/degradation, is the main mechanism of Vpu activity.

The CRL complexes are an essential component of HIV-1 infection. While most accessory genes hijack this machinery to degrade host proteins and evade

immune detection, Vpu primarily antagonizes its target proteins via a cullin-independent mechanism. Therefore, drugs that target a combination of CRLs, such as MLN4924, as well as AP-1, may show promise in help promoting anti-viral defense.

Introduction

Viruses are intracellular obligate parasites that hijack host cellular machinery to promote their efficient replication and dissemination. In particular, a recurring theme between diverse species of viruses is their common ability to usurp the host cullin-ring finger ubiquitin ligase (CRL) complexes with the aim of evading host control mechanisms. Notably, the HIV-1 accessory protein Vif hijacks a cullin-5 containing ubiquitin ligase complex (CRL5) to target the cytidine deaminase APOBEC3G (A3G) for proteasomal degradation (Marin et al., 2003; Mehle et al., 2004a; Mehle et al., 2004b; Sheehy et al., 2002; Yu et al., 2003). In the absence of Vif, A3G is incorporated into virions and induces lethal dG to dA hypermutation within the viral genome (Harris et al., 2003; Stopak et al., 2003). Similarly, the HIV-2 accessory protein Vpx relies on a cullin-4 containing (CRL4) complex to degrade the restriction factor SAMHD1, thus preventing a decrease in intracellular dNTP levels via SAMHD1's deoxynucleoside triphosphohydrolase activity and allowing for efficient viral DNA synthesis by reverse transcriptase (RT) (Baldauf et al., 2012; Hrecka et al., 2011; Laguette et al., 2011; Lahouassa et al., 2012).

Indeed, cullin activity has also been shown to be an important factor for the HIV-1 Vpu protein. Vpu is an 81 amino acid type 1 integral membrane phosphoprotein expressed in most isolates of HIV-1, some SIVs such as SIVgsn, SIVmon, SIVmus, SIVden, (Strebel, 2014), including the closely related primate lentivirus SIVcpz, but not HIV-2 (Cohen et al., 1988; Maldarelli et al., 1993; Strebel et al., 1989).

Translation of Vpu and envelope (Env) occurs from a single bi-cistronic mRNA late in the viral life cycle (Schwartz et al., 1990). Biologically, Vpu induces the rapid degradation of newly synthesized CD4 within the endoplasmic reticulum (ER), relieving the formation of gp160-CD4 complexes in the ER and allowing envelope glycoprotein (gp) maturation and full viral assembly (Bour et al., 1991; Lenburg and Landau, 1993; Willey et al., 1992a, b). Vpu degradation pf CD4 requires two highly conserved, constitutively phosphorylated serine residues within its cytoplasmic domain that bind the F-box protein substrate receptor β-TrCP, which together with the adaptor protein Skp1, recruit a cullin-1 containing (CRL1) complex to target CD4 for proteasomal degradation (Margottin et al., 1998; Schubert et al., 1994; Schubert and Strebel, 1994).

Vpu is known to antagonize multiple host proteins. In addition to CD4, Vpu also counteracts the restriction factor BST-2 (tetherin) via cell surface downregulation, thereby increasing viral budding and release (Klimkait et al., 1990; Neil et al., 2008; Van Damme et al., 2008). Mechanistically, previous reports have shown that Vpu can antagonize BST-2 via proteasomal and / or β-TrCP and ESCRT-dependent lysosomal degradation (Caillet et al., 2011;

Douglas et al., 2009; Goffinet et al., 2009; Gupta et al., 2009; Iwabu et al., 2009; Janvier et al., 2011; Mangeat et al., 2009; Mitchell et al., 2009). Additionally, others found that sequestration of BST-2 within the *trans-golgi network* (TGN), was important for Vpu antagonism (Dubé et al., 2010; Dube et al., 2009; Vigan and Neil, 2010). More recent studies, however, have shown that a putative clathrin sorting motif, ExxxLV (ELV), present within the cytoplasmic domain of subtype B Vpu, is also critical to counteract BST-2 (Kueck and Neil, 2012). Indeed, Jia et al. recently found that the clathrin adaptor-protein-1 (AP-1), a molecule involved in the trafficking of cargo between the TGN and sorting endosomes, simultaneously binds Vpu via its ELV motif as well as BST-2, thereby causing the mistrafficking of BST-2 away from viral assembly sites for eventual lysosomal degradation (Jia et al., 2014). Yet, whether the major mechanism of BST-2 counteraction by Vpu primarily involves AP-1-mediated mistrafficking or β-TrCP-dependent degradation within primary $CD4^+$ T cells is still not fully understood.

Moreover, other than CD4 and BST-2, Vpu can negatively affect other important immune factors, such as NTB-A, CD1d, and CCR7. NTB-A is a co-activating signal molecule necessary for Natural Killer (NK) degranulation. Thus, through downregulation of cell surface NTB-A by Vpu, HIV-1 infected cells efficiently evade lysis by NK cells (Shah et al., 2010). Furthermore, downregulation of CD1d on HIV-1 infected dendritic cells (DCs), which is a coordinated effect of both Nef and Vpu, hinders lipid antigen presentation to invariant natural killer T (iNKT) cells (Moll et al., 2010). Finally, our recent studies

have shown that the chemokine receptor CCR7 is downmodulated on infected primary $CD4^+$ T cells by Vpu, thus impairing their ability to efficiently migrate towards a gradient of CCL19 and CCL21 (Ramirez et al., 2014).

Physiologically, CRLs constitute an important group of ubiquitin ligases and play a prominent role in the efficient regulation of protein turnover and homeostasis (Petroski and Deshaies, 2005). Their activation is dependent on a process known as neddylation. This posttranslational modification involves the addition of the NEDD8 protein, a relative of ubiquitin, onto cullins via a lysine residue linkage. This posttranslational modification induces a conformational change that renders the enzyme catalytically active (Bosu and Kipreos, 2008; Hori et al., 1999; Kamitani et al., 1997).

Indeed, the neddylation pathway, like the NEDD8 protein, occurs in a very similar fashion to protein ubiquitination. First, NEDD8 precursor processing occurs through a C-terminal hydrolase, followed by activation of Nedd-8 by the Nedd8-Activating Enzyme (NAE; E1), a heterodimer of APPBP1 and UBA3, in an ATP-dependent manner (Huang et al., 2004). This creates a NEDD8-AMP adenylate intermediate, which can then go on to form an NAE-NEDD8 thioester (Bohnsack and Haas, 2003; Brownell et al., 2010). Next, NEDD8 is transferred to one of two E2 conjugating enzymes, UBE2M (Ubc12) or UBE2F, via a transthiolation reaction (Huang et al., 2007; Liakopoulos et al., 1998). Finally, NEDD8 is shuttled to an E3 ligase, which confers substrate specificity.

In this work, we utilized MLN4924, a potent inhibitor of NAE (Nedd8-Activating Enzyme, NAE; E1), to selectively block CRL activity and thus

determine the necessity for β-TrCP in Vpu antagonism of host proteins endogenously expressed within primary $CD4^+$ T cells (Soucy et al., 2009). Our results indicate that CRL activity is absolutely required for Vpu-mediated CD4 cell surface downregulation and degradation. However, the ability of Vpu to downmodulate BST-2, CCR7, and NTB-A was completely independent of cullin activity, further suggesting that mistrafficking of these host proteins, presumably through AP-1, is the dominant mechanism employed by Vpu in primary $CD4^+$ T cells.

Results

Neddylation and $Scf^{\beta\text{-}Trcp}$ Activity Is Required for Vpu-mediated Cell Surface Downregulation of CD4 but Not for BST-2, CCR7 or NTB-A downmodulation

Previous studies involving HIV-1 and the neddylation inhibitor MLN4924 have shown that the drug can potently block Vif-mediated proteasomal degradation of A3G (A3G)(Stanley et al., 2012). In addition, Stanley et al. found that knockdown of the neddylation E2 conjugating enzyme UBE2F, but not UBE2M (Ubc12), can restore restriction by A3G and circumvent the effects of Vif (Stanley et al., 2012). Furthermore, in the context of HIV-2, MLN4924 inhibited the degradation of SAMHDI facilitated by Vpx, phenocopying an HIV-2 virus lacking the accessory protein and restoring viral restrictive potential in myeloid cells (Hofmann et al., 2013; Nekorchuk et al., 2013; Wei et al., 2014).

Additionally, MLN4924 inhibited the induction of G2 arrest by HIV-1 Vpr (data not shown).

We therefore wanted to address, in primary $CD4^+$ T cells, whether pharmacological inhibition of the $SCF^{\beta\text{-}TrCP}$ complex by MLN4924 would restore surface levels of proteins antagonized by Vpu. We hypothesized that Vpu counteraction of CD4, being dependent on β-TrCP and CRL activity, would be negatively affected by MLN4924. We also predicted that downregulation of BST-2, CCR7, and NTB-A, where targeting of the $SCF^{\beta\text{-}TrCP}$ complex may not be the dominant mode of counteraction, would either show an intermediate or no effect after drug treatment. To that end, primary $CD4^+$ T cells (generated as described in Materials and Methods) were infected with either an HIV-$1_{NL4\text{-}3}$ replication-defective molecular clone carrying GFP in place of Nef (DHIV GFPΔNef), or as a negative control, a virus lacking both Nef and Vpu (DHIV GFPΔNefΔVpu)(Ramirez et al., 2014). All viruses were pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) envelope. Particularly, we utilized these viruses because unlike BST-2, CCR7, and NTB-A, where antagonism is exclusive to Vpu, CD4 is counteracted by HIV-1 envelope, Nef, and Vpu, thus infection of cells with DHIV GFPΔNef pseudotyped with VSV-G would allow us to probe the effect of Vpu function only (Malim and Emerman, 2008). Two days post infection, cells were incubated in either DMSO or increasing concentrations of MLN4924 in DMSO, and then stained for surface proteins and analyzed by flow cytometry 24h later. As expected, a virus devoid of Nef and Vpu (DHIV GFPΔNefΔVpu) showed similar surface levels of CD4, BST-2, CCR7, and NTB-A

between GFP negative and positive cells (Figure 4.1A, panels ii,viii,xiv,xx). Furthermore, downregulation of CD4, BST-2, CCR7, and NTB-A was apparent in cells that were infected with DHIV GFPΔNef and treated with DMSO only (Figure 4.1A, panels iii,viv,xv,xxi). However, in a dose-dependent manner, MLN4924 completely relieved the downmodulation of CD4 mediated by Vpu, indicating that CRL activity is essential for Vpu to antagonize CD4 (Figure 4.1A, compare panels iii-vi; Figure 4.1B). In contrast, surface levels of BST-2, CCR7, and NTB-A were unaffected by MLN4924 treatment, suggesting that recruitment of the $SCF^{\beta\text{-}TrCP}$ complex is not the dominant mechanism employed by Vpu to antagonize these proteins (Figure 4.1A, panels vii-xxiv; Figure 4.1B). We were unable to assess the effect of MLN4924 on the downmodulation of the natural killer cell activating ligand CD155 (PVR), which has been shown to be a target of both Nef and Vpu, because we were unable to reproduce downregulation of cCD155 after infection with DHIV GFPΔNef (data not shown). Thus taken together, these results suggest that Vpu utilizes both a cullin-dependent and -independent mechanisms for antagonizing host proteins.

MLN4924 Inhibits Vpu-Mediated CD4 Degradation by Blocking Neddylation of the $Scf^{\beta\text{-}Trcp}$ Complex

To determine whether MLN4924 prevented the actual degradation of CD4, as opposed to just the surface downregulation, primary $CD4^+$ T cells were infected as described in Figure 4.1 but were instead permeabilized, fixed, and stained for total levels of CD4. Figure 4.2 collectively shows that inhibition of

neddylation rescued CD4 from Vpu-mediated degradation in a dose-dependent manner that mirrored what we observed at the cell surface (Figure 4.2A; 4.2B, left panel). Furthermore, MLN4924 treatment did not have any adverse effects in modulating CD4 expression in uninfected (Mock) or DHIV GFPΔNefΔVpu infected cells. We did, however, observe that CD4 levels were slightly upregulated in GFP positive cells infected with DHIV GFPΔNefΔVpu, a phenomenon we and others have observed with other host proteins (Figure 4.2B right panel) (Kueck and Neil, 2012).

Nef-Mediated CD4 Antagonism Is Unaffected by MLN4924 Treatment

Nef is a 27kDa multifunctional myristoylated protein localized within the cytoplasmic side of cellular membranes and expressed early within the HIV-1 viral life cycle. Nef interferes with a number of cellular trafficking and signaling pathways that ultimately lead to increased viral spread and pathogenesis (Kirchhoff, 2010). One of Nef's key functions is the counteraction of CD4. However, unlike Vpu, which acts on newly synthesized CD4 molecules within the ER, Nef accelerates the endocytosis of target CD4 molecules present on the plasma membrane via a clathrin and Adaptor Protein 2 (AP2) pathway (Aiken et al., 1994; Chaudhuri et al., 2007; Rhee and Marsh, 1994). Moreover, Nef has been shown to shuttle CD4 through a multivesicular body (MVB) pathway that involves members of the endosomal sorting complex required for transport (ESCRT) machinery, eventually leading to CD4 lysosomal degradation (daSilva

et al., 2009). Yet, while accelerated endocytosis and targeting of CD4 through the MVB pathway are separate functions of Nef, neither rely on the recruitment of CRL complexes. We therefore hypothesized that a virus only encoding Nef would be unaffected by MLN4924 treatment in its ability to downregulate CD4. Primary $CD4^+$ T cells were infected with either HIV-1 (DHIV WT), a virus lacking Vpu (DHIV ΔVpu), or a virus lacking Nef (DHIV ΔNef). As expected, a ΔNef virus was unable to cause CD4 downregulation in the presence of MLN4924 (Figure 4.3, panel viii). Thus, our data clearly show that inhibiting CRL activity only prevents Vpu-mediated CD4 antagonism, but not counteraction by Nef, which is cullin independent.

Discussion

It is now well appreciated that hijacking of CRLs is a common theme for many viruses, regardless of their nucleic acid makeup or sense (Mahon et al., 2014). In particular, the Ad5 Adenovirus strain encodes two viral proteins, E4ORF6 and E1B55K, which together activate the CRL5 complex to degrade the master cell cycle and apoptosis regulator p53, thereby preventing cell cycle arrest and premature apoptosis (Querido et al., 2001a; Querido et al., 2001b). Similarly, Kaposi's sarcoma associated herpesvirus (KSHV) utilizes its virally encoded latency-associated nuclear antigen (LANA) protein to target p53 and VHL (Von Hippel-Lindau Tumor Supressor) for ubiquitination and subsequent proteasomal degradation through a CRL5 complex (Cai et al., 2006). Moreover, the Large T antigen (LTAg) of the polyoma virus SV40 has been associated with

the ability to bind CRL7, thereby leading to deregulation of its substrate, the insulin receptor substrate 1 (IRS-1), possibly to dampen downstream IRS-1 pathways related to oncogenesis (Kasper et al., 2005). Finally, the Epstein Barr Virus (EBV) protein BPLF1 mimics the deneddylase activity of the host COP9 signalosome (CSN), resulting in the accumulation of CRL substrates and the deregulation of the cell cycle, providing a favorable environment for viral DNA replication (Gastaldello et al., 2010).

Despite the requirement for cullin activity in HIV-1 Vif, HIV-2 Vpx, and HIV-1 Vpr function, the data presented here argue that HIV-1 Vpu targets host proteins, with the exception of CD4, through a cullin-independent mechanism. Indeed, inhibiting neddylation with MLN4924 clearly did not have any effect on restoring Vpu-dependent BST-2 surface levels, even at high concentrations. Interestingly, Schindler and colleagues found that endogenous BST-2 levels were comparatively lower within $CD4^+$ T cells as opposed to macrophages. Consequently, a Vpu mutant unable to bind β-TrCP hindered viral replication and counteraction of BST-2 within macrophages, but not within tissue $CD4^+$ T cells (Schindler et al., 2010). Our data support a model whereby within $CD4^+$ T cells, CRL1 is largely dispensable for Vpu mediated antagonism of BST-2.

Materials and Methods

Cells and Plasmids

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) containing 10% Fetal Bovine Serum (Atlas Biologicals) supplemented with 100 U/ml Penicillin, 100 mg/ml Streptomycin, and 2mM L-glutamine (Life Technologies). HeLa-CD4 (obtained through the NIH AIDS Reagent Division of AIDS, NIAID, NIH (Cat. #459)) were cultured in RPMI complete media in the presence of 1 mg/ml G418 (Life Technologies) while CCRF-CEM and primary $CD4^+$ T cells were cultured in RPMI complete media only. All cells were maintained at 5% CO_2 at 37°C. For all experiments involving primary $CD4^+$ T cells, coverage was maintained under protocol #IRB_00067637 approved by the University of Utah Institutional Review Board. The generation of cultured T_{CM} has been described previously (Ramirez et al; Cell Reports 2014).

All DHIV plasmids used in this manuscript have been described previously (Ramirez et al. 2014). Briefly, the "defective" HIV (DHIV) plasmid, derived from the HIV_{NL4-3} isolate, contains a frame-shift mutation within envelope/gp120 but maintains in-frame Tat, Rev and RRE ORFs.

Pharmacological Inhibitor MLN4924

MLN4924 was purchased from Cayman Biologicals. The dry solvent was then resuspended in DMSO at a stock concentration of 20 mM, further aliquotted and diluted at 200 mM, and used as indicated.

Flow Cytometry Antibodies

To measure relative surface levels of CD4, BST-2, CCR7, and NTB-A, primary $CD4^+$ T cells were stained with the human monoclonal antibodies anti-APC-CCR7 (Caltag, Burlingame CA), anti-APC-CD4 (Life Technologies), and anti-NTB-A (Life Technologies). Surface analysis of BST-2 was determined using anti-BST-2 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; Drs. Klaus Strebel and Amy Andrew (Cat. # 11722)) followed by staining with a goat-anti-rabbit secondary antibody coupled to Alexa Fluor 647 (Molecular Probes, Invitrogen). A viability dye, eFlour 450 (eBioscience), was then used to distinguish live from dead cells. Fixation was achieved using .5% Paraformaldehyde (PFA). All staining and fixation steps were performed at 4°C for 30 minutes in buffer (1x PBS + 3% FBS). Detection of CD4 and BST-2 on the surface of HeLa-CD4 was determined as described above.

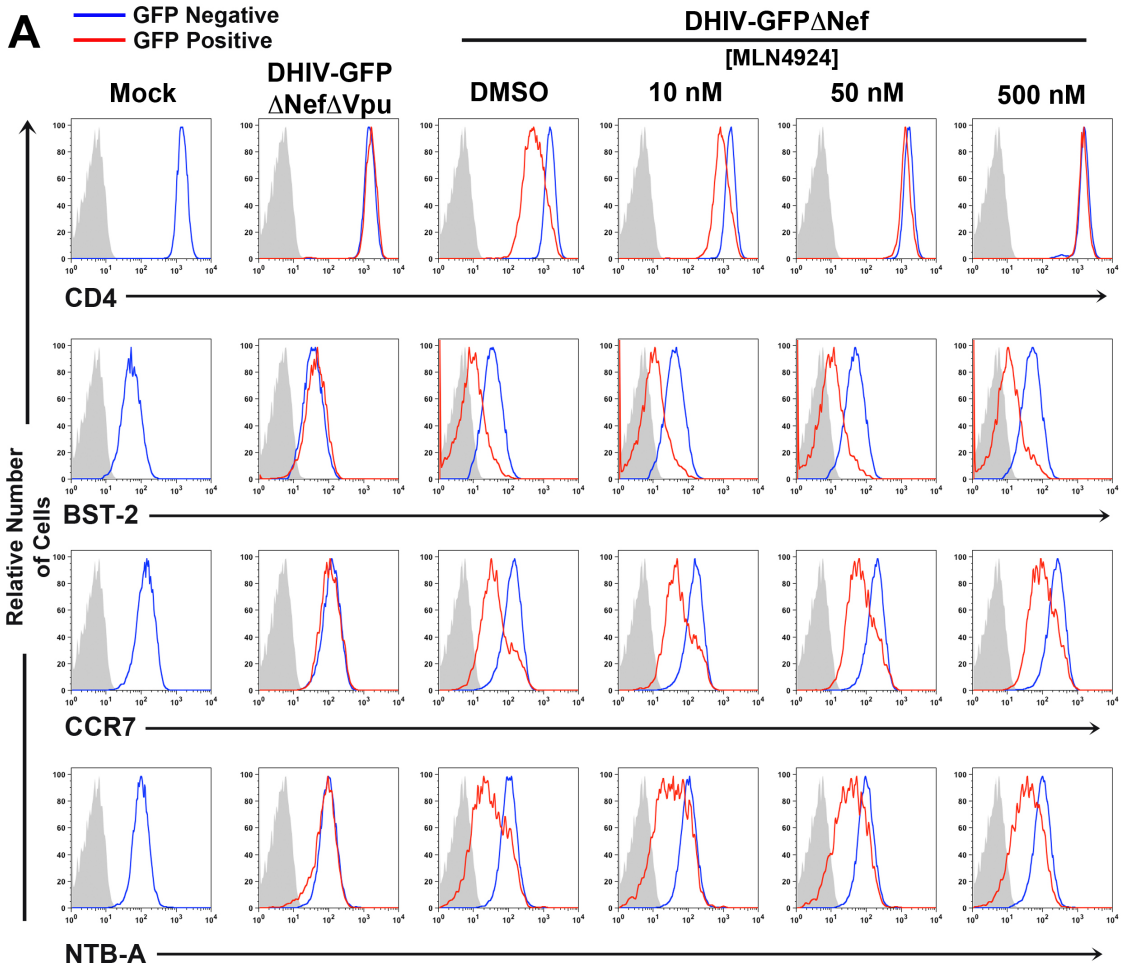
In experiments involving surface analysis of CD4 and detection of intracellular p24, cells were first probed with anti-APC-CD4, stained with eFluor 450, permeabilized (Cytofix/Cytoperm;), and then stained with mouse-anti-FITC-p24 (clone KC57, Beckman Coulter). Total levels of CD4 in primary $CD4^+$ T cells were measured by staining cells with eFlour 450, permeabilization, and then probing with anti-APC-CD4. All data were collected on a BD FACS Canto and analyzed with FlowJo software.

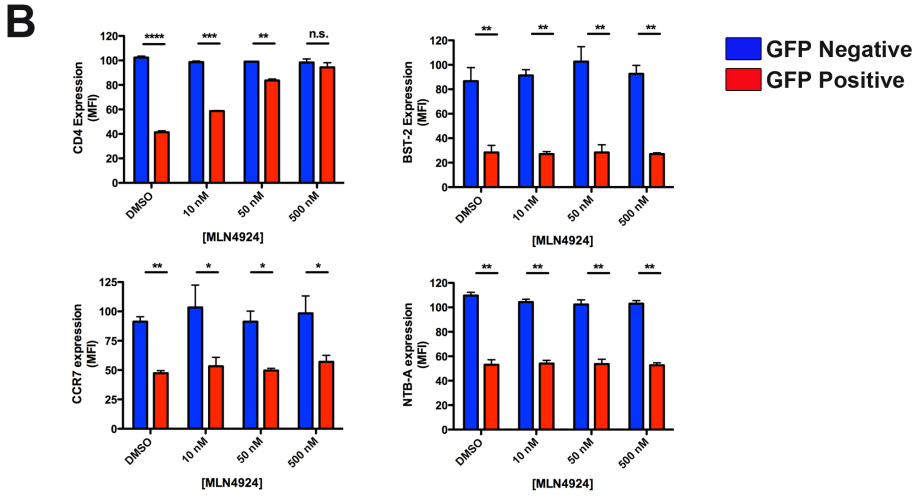
Viruses and Infections

Viral stocks were generated by co-transfection of 20 mg DHIV along and 5mg of a construct expressing vesicular stomatitis virus G-protein (VSV-G) by calcium phosphate-mediated transfection into HEK293T cells. Media (DMEM) was replaced after 16 h and the cellular supernatant collected, aliquotted, and stored at -80°C 48h post-transfection. Viral titers and MOI were determined via infection of CCRF-CEM cells. Primary $CD4^+$ T cells generated as described above were infected five days post-activation at an MOI of either 1 (Flow Cytometry) or 3 (Immunoblotting) via spinoculation: 10^6 cells (1 ml final volume) for 2h at 37°C in the presence of 8mg/ml Polybrene (Sigma). After infection, cells were then resuspended in RPMI complete medium supplemented with IL-2 (30 U/ml).

For infection of HeLa-CD4, 2 x 10^5 cells were plated in a 6 well plate. 24h later, the cells were infected at an MOI = 2 for 5h at 37°C in the presence of 8mg/ml Polybrene (Sigma). Following infection, the cells were resuspended in RPMI complete medium supplemented with 1 mg/ml G418.

Figure 4.1. HIV-1 Vpu utilizes both a cullin-dependent and -independent mechanism to antagonize host proteins.
A. Primary $CD4^+$ T cells were either mock infected or infected at an MOI of 1 with DHIVGFPDNef or DHIVGFPDNefDVpu. 2 days post infection, either DMSO or increasing concentrations of MLN4924 were added to cell cultures. 24h later, surface expression of CD4, BST-2, CCR7, or NTB-A was analyzed by flow cytometry. Histograms depict a comparison of GFP negative (blue line) and GFP positive (red line) cells along with an IgG matched isotype control (gray shaded histogram). Unless otherwise noted, all experiments involving primary $CD4^+$ T cells are representative of three distinct experiments performed in three separate healthy donors. B. Mean Fluorescence Intensity (MFI) values of surface expression of CD4, BST-2, CCR7, or NTB-A from DHIVGFPDNef infected cells (A). Data were normalized by setting the MFI values from uninfected (mock) cells to 100% and is depicted graphically as +/- SEM.


A
GFP Negative
GFP Positive
DHIV-GFPΔNef
[MLN4924]
Mock
DHIV-GFP ΔNefΔVpu
DMSO
10 nM
50 nM
500 nM
CD4
BST-2
CCR7
NTB-A
Relative Number of Cells


B
GFP Negative
GFP Positive
CD4 Expression (MFI)
BST-2 Expression (MFI)
CCR7 expression (MFI)
NTB-A expression (MFI)
DMSO
10 nM
50 nM
500 nM
[MLN4924]
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n.s.

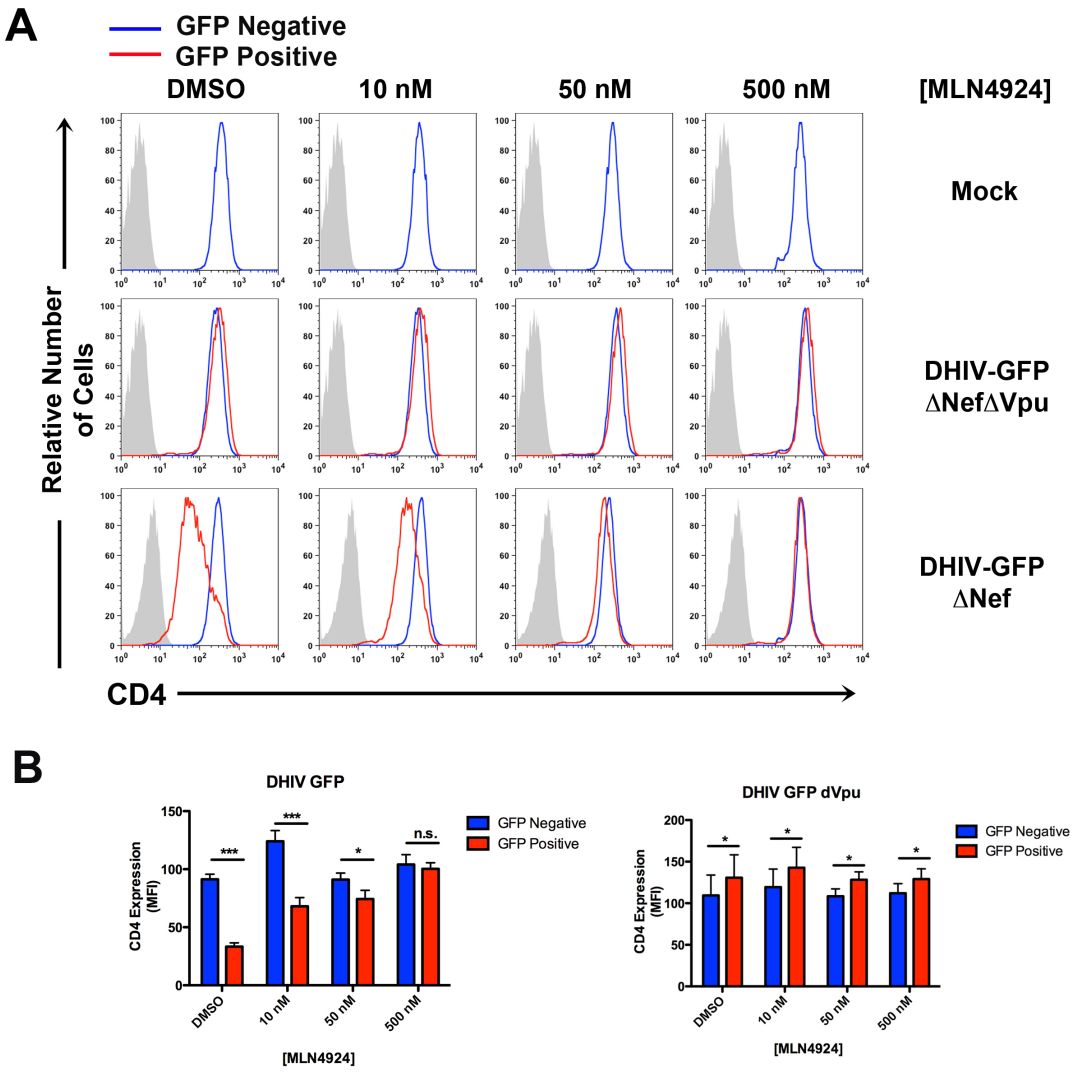

Figure 4.2. MLN4924 inhibits Vpu-mediated degradation of CD4.
A. Cultured T_{CM} were either mock infected or infected at an MOI of 1 with DHIVGFPDNef or DHIVGFPDNefDVpu. 48h after infection, cell cultures were treated with either DMSO or increasing concentrations of MLN4924. To assess total levels of CD4, cells were permeabilized and stained 24 h after addition of MLN4924 and analyzed by flow cytometry. Histograms represent GFP negative cells (blue line), GFP positive cells (red line), or an IgG matched isotype (gray-shaded histogram). B. MFI values of total (intracellular) CD4 expression levels from either DHIVGFPDNef (left) or DHIVGFPDNefDVpu (right). Data were normalized by setting the MFI values from uninfected (mock) cells to 100% and is depicted graphically as +/- SEM.

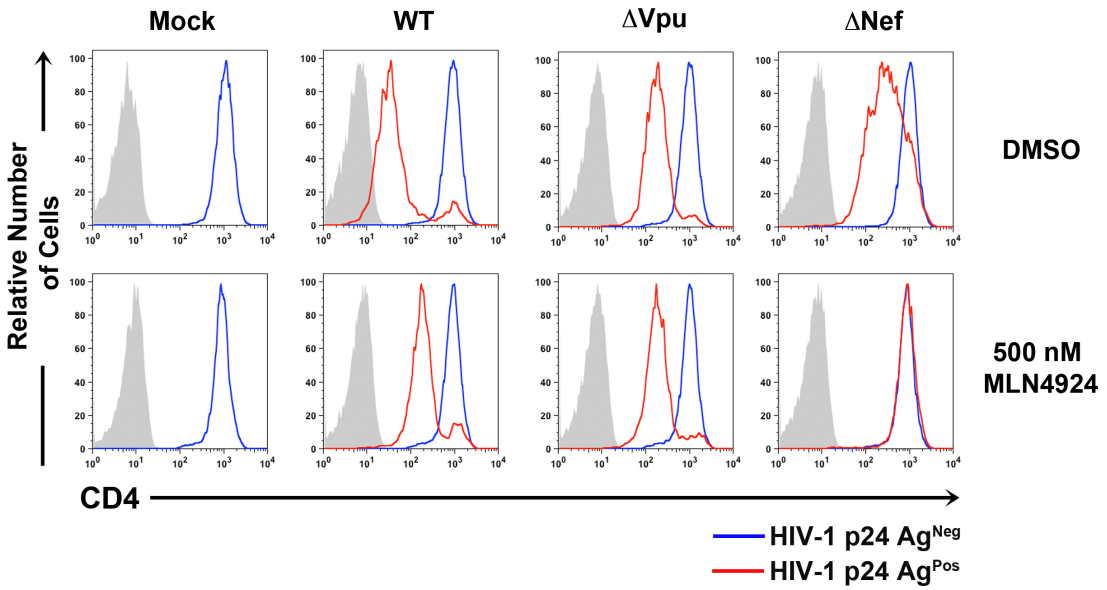

Figure 4.3: Inhibition of CRL activity negatively affects Vpu, but not Nef-mediated antagonism of CD4.
A. Primary $CD4^+$ T cells were either mock infected or infected at an MOI of 1 with DHIV WT, DHIVDVpu, or DHIVDNef. 2 days post infection, cell cultures were treated with either DMSO or 500 nM MLN4924. To determine the impact of MLN4924 treatment on viruses lacking Vpu or Nef, surface levels of CD4 were assessed by flow cytometry. Histograms represent GFP negative cells (blue line), GFP positive cells (red line), or an IgG matched isotype (gray-shaded histogram). Shown is one representative experiment out of three.

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CHAPTER 5

DISCUSSION

HIV/SIV lentiviruses encode accessory proteins that were shown to overcome host restriction factors to favor viral replication. Understanding how accessory proteins avoid immune recognition could be used for new drug development. Although the mechanisms used by accessory proteins to target APOBEC3G, CD4, and SAMHD1 (degraded by Vif, Vpu, and Vpx, respectively) are well established, the exact host proteins used by Vpr to induce G_2 arrest remain unknown.

Vif degrades APOBE3G by hijacking Cul5 (Sheehy et al., 2003; Yu et al., 2003); Vpu degrades CD4 through Cul1 (Margottin et al., 1996; Margottin et al., 1998); Vpx degrades SAMHD1 by associating wth Cul4 via DCAF1 via a direct interaction (Hrecka et al., 2011; Laguette et al., 2011). Considering that all these accessory proteins usurp the ubiquitin proteasome system (UPS) to degrade a specific host factor, and since Vpr interaction with $Cul4^{DDB1/DCAF1}$ is necessary to induce cell cycle arrest (DeHart et al., 2007; Hrecka et al., 2007; Wen et al., 2007), one hypothesis was that Vpr recruits a host protein to the Cul4 ubiquitin E3 ligase for ubiquitination/degradation.

Recently, it was reported that Vpr targets the SLX4 complex to arrest the cell cycle (Laguette et al., 2014). SLX4com contains the endocuclease Mus81, which was shown to be ubiquitinated and degraded in the presence of Vpr (Laguette et al., 2014). Surprisingly, our results showed that degradation of Mus81 is independent of Vpr-induction of G_2 arrest. The well-known Vpr point mutants (R80A and Q65R), which are unable to arrest cells in G2/M, were found in our studies to degrade Mus81. It was demonstrated that Vpr interaction with

SLX4 was required for induction of cell cycle arrest (Berger et al., 2014). However, ubiquitination of the SLX4com induces its activation but not degradation. This result is in contradiction with the current model of activity. Indeed, according to the prevailing model, knockdown of the putative target would mimic the cell cycle arrest induced by Vpr. However, siRNA depletion of each of the SLX4com components inhibited the G_2 arrest-induced by Vpr (Laguette et al., 2014).

In our studies, we confirmed the necessity of the C-terminus of Vpr to cause cell cycle arrest. Earlier reports have suggested the requirement of the C-terminal unstructured region of Vpr to recruit the putative G_2 arrest target (DeHart et al., 2007; Di Marzio et al., 1995; Le Rouzic et al., 2007). We took advantage of the SIVagm Vpr, a subset of primate lentivirus that encode a bifunctional Vpr (SIVagm Vpr degrades SAMHD1 and induces G_2 arrest) that is species-specific, arresting cell cycle in AGM cell but not in human cells (Planelles et al., 1996). Using chimeric proteins between HIV-1 Vpr and SIVagm Vpr, transfer of the C-terminus of HIV-1 Vpr onto SIVagm Vpr conferred upon SIVagm Vpr a *de novo* ability to induce G_2 arrest in human cells. Furthermore and in agreement with SIVmac Vpx (Ahn et al., 2012; DeLucia et al., 2013), we found that the N-terminal domain of SIVagm Vpr is required for degradation of agmSAMHD1.

A more refined study is necessary to answer some unresolved questions: Does SLX4 interact with the C-terminal domain of Vpr? Does the interaction between SLX4 and Vpr(R80A) lead to SLX4com activation? Does SLX4

activation by Vpr result in ATR activation, a hallmark of the G_2 arrest-induced by Vpr?

To understand the manipulation of the Cul4-DCAF1 ubiquitin ligase by Vpr and its paralog Vpx, we generated several DCAF1 point mutants and truncations (Cassiday et al., 2014). Vpr and Vpx are known to interact with DCAF1 through a high conserved region found in their 3rd alpha helix (Le Rouzic et al., 2007; Zhao et al., 1994). Surprisingly, our results revealed a novel aspect of the interaction of Vpr and Vpx with DCAF1. The point mutations N1135A and W1156H in DCAF1 inhibited the binding of DCAF1 to Vpx but not to Vpr. When we looked at SAMHD1 degradation by Vpx in the presence of Vpr, we observed that Vpr was able to decrease Vpx function. However, Vpx was not able to compete Vpr's induction of G2 arrest, suggesting that Vpr binds to DCAF1 with higher affinity than Vpx (Cassiday et al., 2014). Furthermore, in agreement with a recently solved DCAF1-Vpx-SAMHD1 crystal structure (Schwefel et al., 2014), we confirmed on a functional level that Vpx-binding to DCAF1 creates a new interface that accommodates SAMHD1, which interacts directly with both Vpx and DCAF1.

The activity of cullins is controlled by the conjugation of a ubiquitin-like molecule called NEDD8 (Hori et al., 1999; Huang et al., 2009). A small molecule inhibitor of the NEDD8 pathway, MLN4924 (Soucy et al., 2009), was shown to suppress the capacity of Vif and Vpx to degrade APOBEC3G (Stanley et al., 2012) and SAMHD1 (Wei et al., 2014), respectively. In agreement with the findings by Laguette et al., (Laguette et al., 2014), we have shown that MLN4924

blocks Mus81 degradation, suggesting the action of a Cullin-based ubiquitin E3 ligase.

In the context of MLN4924, we also asked whether this drug would have any effect on previously described targets of Vpu. Vpu was shown to downregulate a number of cellular host protein such as CD4 (Margottin et al., 1996; Margottin et al., 1998), NTB-A (Shah et al., 2010), BST-2/Tetherin (Neil et al., 2008), and CCR7 (Ramirez et al., 2014). MLN4924 was able to inhibit downregulation of CD4 by Vpu but not that of CCR7, BST-2/Tetherin, or NTB-A. These results suggest that while Vpu hijacks Cul1 to ubiquitinate and degrade CD4, a mechanism independent of cullin is used to antagonize CCR7 and BST-2/Tetherin and NTB-A.

An alternative mechanism used by Vpu to downregulate protein expression is trans-golgi entrapment. In the absence of Vpu, newly synthesized proteins migrate from the ER to the Golgi and then, under normal conditions, anterograde trafficking moves them to the cellular membrane (Ramirez et al., 2014). However, when Vpu is present, it sequesters these proteins in the trans-golgi network, preventing its expression on the plasma membrane.

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