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The HIV-1 Protein Vpr Targets the Endoribonuclease Dicer for Proteasomal Degradation to Boost Macrophage Infection

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The HIV-1 protein Vpr enhances macrophage infection, triggers G2 cell cycle arrest, and targets cells for NK-cell killing. Vpr acts through the CRL4 DCAF1 ubiquitin ligase complex to cause G2 arrest and trigger expression of NK ligands. Corresponding ubiquitination targets have not been identified. UNG2 and SMUG1 are the only known substrates for Vpr-directed depletion through CRL4 DCAF1. Here we identify the endoribonuclease Dicer as a target of HIV-1 Vpr-directed proteasomal degradation through CRL4 DCAF1. We show that HIV-1 Vpr inhibits short hairpin RNA function as expected upon reduction of Dicer levels. Dicer inhibits HIV-1 replication in T cells. We demonstrate that Dicer also restricts HIV-1 replication in human monocyte-derived macrophages (MDM) and that reducing Dicer expression in MDMs enhances HIV-1 infection in a Vpr-dependent manner. Our results support a model in which Vpr complexes with human Dicer to boost its interaction with the CRL4 DCAF1 ubiquitin ligase complex and its subsequent degradation.

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Introduction

Plants and insects were first found to employ RNA silencing, a nucleic acid-dependent immune response, to combat viral infections. Subsequent work demonstrated that mammals also exploit RNA silencing as a part of their anti-viral armamentarium (Andersson et al., 2005; de Vries and Berkhout, 2008; Lecellier et al., 2005; Lu and Cullen, 2004; Wang et al., 2006). To achieve RNA silencing, double-stranded RNAs are processed in mammalian cells by the nuclear endoribonuclease Drosha and exported into the cytosol. There they are further processed by the endoribonuclease Dicer into shorter 21–23 nucleotide duplexes. These are loaded into the RNA-induced silencing complex (RISC) to confer specificity to complementary sequences within messenger RNA. Assembly with RISC results in either destruction or translational inhibition of the targeted mRNA.

RNA silencing could restrict virus replication at least three ways: (1) by direct cleavage of viral RNA which could both destroy the RNA and process it to specifically target other viral or cellular RNA, (2) by cellular miRNA-mediated suppression of transcript expression or interference with viral processes that employ RNA, or (3) by use of cellular miRNAs to thwart expression of cellular proteins required for viral replication. Since most viral infections do not succumb to these restrictions, many viruses may have evolved strategies to evade or defeat restrictions imposed through RNA silencing. Indeed, plant and animal viruses that infect invertebrates encode suppressors of silencing (SRS). The mechanisms that the SRS factors use to inhibit RNA silencing are not well understood. SRS activity is encoded by a variety of mammalian viruses and include Ebola virus VP35 protein (de Vries and Berkhout, 2008), Adenovirus VA RNA I and RNA II (Andersson et al., 2005; Lu and Cullen, 2004), primate foamy virus type 1 Tas protein (Lecellier et al., 2005) and hepatitis C core protein (Wang et al., 2006). This indicates that battles between miRNA restriction and viral SRSs are taking place in organisms ranging from plants to mammals.

HIV-1 was among the first mammalian viruses shown to possess SRS activity. Yeung et al. (2005) demonstrated that HIV-1 hinders miRNA expression in HeLa cells. In the presence of HIV-1-specific siRNAs, HIV-1 adopted mutations to break the complementarity (Boden et al., 2003; Das et al., 2004; Jacque et al., 2002). Furthermore, HIV-1 encodes a double-stranded RNA transactivator response (TAR) loop that binds to, and may sequester TARBP, a Dicer co-factor that functions as part of the RISC complex (Bennasser et al., 2006). Other work attributes partial SRS activity...
to Tat (Benasser et al., 2005; Haasnoot et al., 2007; Qian et al., 2009; Schnettler et al., 2009), however, the results of another study conflict with these findings (Lin and Cullen, 2007). Finally, experimental evidence that miRNAs restrict HIV-1 in otherwise permissive cells suggests that this virus must encode SRS factors. This evidence demonstrates that cellular miRNAs target HIV-1 genes (Ahluwalia et al., 2008; Harirhan et al., 2005; Huang et al., 2007; Omoto et al., 2004; Yeung et al., 2009) or inhibit host factors that enable HIV replication (Sung and Rice, 2009; Triboulet et al., 2007). Furthermore, HIV encodes dsRNA regions that could be targeted by the RNA silencing machinery directly like those found in primate foamy retrovirus and vesicular stomatitis virus (Lecellier et al., 2005). Regardless of how miRNAs restrict HIV-1, it is clear that HIV-1 has evolved to overcome miRNA restriction to allow effective replication.

Host cells maintain mechanisms aside from miRNA to inhibit HIV replication and HIV encodes several specialized proteins to counter them. Some viral defenses act through host cell ubiquitin ligases. Vif, for example, associates with an elonginBC-Cul5 ubiquitin ligase complex to trigger destruction of the host antiviral factor APOBEC3G (Conticello et al., 2003; Kobayashi et al., 2005; Marin et al., 2003; Mehle et al., 2004; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). In the absence of APOBEC3G degradation, this cytidine deaminase is incorporated into viral particles and deaminates viral cytosines to form uracils during the reverse transcription. APOBEC3G hinders reverse transcription, but the exact mechanism has not been determined (reviewed in Wissing et al., 2010). Vpu, another specialized HIV-1 protein, partners with the SCF<sup>WPI</sup> ubiquitin ligase complex to facilitate virus release by keeping the cellular protein tetherin/BST-2 from the cell surface (Goffinet et al., 2009; Gupta et al., 2009; Mangeat et al., 2009). HIV-2/SIV protein Vpx supports virus replication by depleting cellular SAMHD1 through the CRL4 ubiquitin ligase complex (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1, a deoxynucleoside triphosphate phosphohydrolase, acts to hinder retroviral infection by depleting dNTP stores in non-dividing cells (Goldstone et al., 2011; Hofmann et al., 2012). While HIV-1 Vpr does not deplete SAMHD1, it aids macrophage infection albeit less efficiently than Vpx (Balliet et al., 1994; Connor et al., 1995; Heinzinger et al., 1994). HIV-1 Vpr also acts through the CRL4<sup>DCAF1</sup> ubiquitin ligase complex to trigger G2 cell cycle arrest, to deplete cellular UNG2, and to boost expression of NGK2D cell surface ligand on infected cells. Vpr-mediated enhancement of macrophage infectivity was originally attributed to Vpr-mediated transport of the viral pre-integration complex (PIC) into the nucleus of non-dividing cells (Nie et al., 1998; Popov et al., 1998; Subbramanian et al., 1998). Other findings, however, show that Vpr is not required for nuclear transport of the PIC (Riviere et al., 2010; Yamashita and Emerman, 2004). Based on the association of Vpr with the CRL4 ubiquitin ligase complex we hypothesized that Vpr, like Vif, Vpx and Vpco commandeer a host ubiquitin ligase complex to relieve a host cell restriction to infection.

UNG2 and SMUG1 are the only substrates identified for HIV-1 Vpr-mediated protein degradation through the CRL4 ubiquitin ligase complex. Neither of these enzymes has been shown to impact Vpr-mediated G2 cell cycle arrest (Selig et al., 1997). The role of UNG2 in HIV infection remains unclear. Early work showed that Vpr brings UNG2 into HIV-1 virions (Chen et al., 2004; Mansky et al., 2000). More recent work showed that Vpr triggers degradation of UNG2 through the CRL4 complex to protect the viral genome against UNG2-mediated processing of APOBEC3G-mediated damage (Schrofelbauer et al., 2005). Fenard et al. (2009), however, demonstrated that UNG2 suppresses transcription from the HIV-1 LTR while Landeven et al. (2009) showed that Vpr suppresses UNG2 gene transcription. Furthermore, UNG2, in association with integrase, was shown to be vital for HIV propagation (Priet et al., 2005). Interestingly (Jones et al. (2010) linked the requirement for UNG2 to co-receptor usage while other work showed that UNG2 has little or no effect on HIV replication (Kaiser and Emerman, 2006; Mbisa et al., 2007). In contrast, Weil et al. (2013) showed that UNG2 triggers degradation of uracil-containing HIV-1 cDNA and prevents its integration. Finally, Vpr-elicted expression of NK-cell ligands (Pham et al., 2011; Ward et al., 2009) requires both UNG2 and Vpr (Norman et al., 2011).

While working to discover targets for CRL4 action in the presence of HIV-1 Vpr, we identified human Dicer as a cellular Vpr partner. We discovered that it is a substrate for Vpr-mediated degradation through the CRL4<sup>DCAF1</sup> ubiquitin ligase complex. Probing the impact of the interaction between Dicer and Vpr on HIV-1 replication in MDMs, we found that HIV infectivity increased in these cells upon depletion of Dicer. The increase in infectivity was greater for virus lacking Vpr than for wild-type virus, indicating that Vpr acts to suppress RNA silencing in MDMs.

**Results**

**HIV-1 Vpr interacts with human Dicer**

UNG2 and SMUG1 are the only known targets for Vpr-mediated proteasomal degradation (Schrofelbauer et al., 2005). Targeting of each requires the CRL4 ubiquitin ligase complex and both share a tryptophan-X-X-phenylalanine (WXXF) motif that is required for UNG2 assembly with Vpr (BouHamdan et al., 1998). We initiated work to determine whether other cellular proteins are also targets of Vpr-enhanced proteasomal degradation and whether the WXXF motif is required for Vpr-mediated targeting.

To discover candidates for Vpr-mediated ubiquitination, we first identified proteins that co-isolate with Vpr. FLAG-epitope-tagged HIV-1 Vpr was over-expressed in HEK293T cells from a transfected expression vector. FLAG–Vpr was isolated from cleared lysates with FLAG-specific antibodies conjugated to agarose beads. Bound proteins were eluted by competition with FLAG peptide. Analysis of the complete eluate by mass spectrometry identified known cellular partners of Vpr, including components of the CRL4<sup>DCAF1</sup> ubiquitin ligase complex and UNG2. Our results also, for the first time, revealed Dicer as a cellular Vpr partner (Fig. 1A). None of these proteins were detected in our negative control, an eluate prepared in parallel, originating from cells transfected with untagged Vpr.

To confirm that human Dicer assembles in a complex with HIV-1 Vpr, we performed co-immunoprecipitation assays to determine whether Vpr could be co-isolated with Dicer from lysates of cells transfected with expression vectors for both. Immunoprecipitation of Myc-tagged Dicer resulted in the co-purification of HIV-1 Vpr (Fig. 1B). Thus, we confirmed that Dicer and HIV-1 Vpr can assemble in the same protein complex.

In our co-immunoprecipitation experiments, we consistently observed lower Dicer levels in lysates from cells co-expressing Vpr (Fig. 1B). Consequently, less Dicer was immunoprecipitated in the presence of Vpr than in its absence. Vpr is, of course, known to engage the CRL4 ubiquitin ligase complex but the substrates of this complex relevant to HIV infection remain unknown. These observations led us to hypothesize that the expression of Vpr reduced Dicer levels by targeting this protein for degradation through the ubiquitin pathway.

**Expression of HIV-1 Vpr triggers depletion of human Dicer both alone and in the context of an infection**

To test our hypothesis that expression of HIV-1 Vpr triggers degradation of Dicer, we measured Dicer protein levels in the
Cells were harvested 48 h after infection for immunoblotting. The levels of Dicer, UNG2, SAMHD1, p27, and the data is representative of the presence of Vpr (Fig. 2A), consistent with our previous observations in the blots of the pre-IP lysates (Fig. 1B, left). In both sets of experiments we over-expressed Dicer. In order to rule out the possibility that Vpr-mediated depletion targets only over- or exogenously expressed proteins, we measured the effect of Vpr expression on endogenous Dicer levels. Immunoblotting lysates of mock- or Vpr-transfected cells with Dicer-specific antibody revealed a substantial decrease in endogenous Dicer levels in the presence of Vpr (Fig. 2B). Finally, in order to determine whether Vpr expressed in the context of an infection is sufficient to cause depletion of endogenous Dicer, we infected cultures of the SupT1 T cell line and probed cell lysates for Dicer. Here too we saw depletion of endogenous Dicer (Fig. 2C). As a control we also probed for UNG2, an established target of HIV-1 Vpr. Like Dicer, UNG2 was depleted only in cultures infected with HIV-1 encoding Vpr. The capacity of HIV-1 Vpr to reduce cellular Dicer levels was not shared by the Vpr and Vpx proteins of HIV-2. Infection of SupT1 or HEK293T cells with HIV-2 or HIV-2 lacking Vpr and/or Vpx did not change Dicer levels, whereas SAMHD1 was markedly depleted in HEK293T cells infected with HIV-2 encoding Vpx (Fig. 2 D and E). From this data we conclude that Dicer depletion is specific for HIV-1 Vpr like SAMHD1 depletion is for HIV-2 Vpx.

In summary, our observations show that levels of Dicer, exogenous or endogenous in origin, are significantly decreased in the presence of Vpr. Furthermore, we observed that expression of Vpr in the absence of other viral proteins is sufficient to trigger Dicer depletion, and that the quantities of Vpr present in an infection are sufficient to mediate this process as well. Based on the established interaction between Vpr and the CRL4<sup>DCAF1</sup> ubiquitin ligase complex, we hypothesized that Vpr recruits Dicer to CRL4<sup>DCAF1</sup> for ubiquitination and subsequent proteasomal destruction.

**Fig. 1.** HIV-1 Vpr assembles with human Dicer. HEK293T cells were transfected with expression vector for untagged Vpr or FLAG-epitope tagged Vpr. FLAG-specific antibody bound to beads was used to isolate proteins from the cell lysates. Proteins were eluted from the beads by competition with FLAG peptide and identified by mass spectroscopy. Proteins isolated from lysates expressing untagged Vpr served to identify non-specifically isolated proteins. The number of peptides identified in the negative control (untagged Vpr) and the experimental (FLAG-Vpr) samples are shown (A). Lysates from HEK293T cells co-transfected with the indicated expression vectors (EV designates empty expression vector) were immunoblotted directly or after immunoprecipitation of Myc-Dicer with Myc-specific antibody. Samples were resolved by SDS-PAGE and blotted for Dicer or HIV-1 FLAG-Vpr with FLAG-specific antibody (B).

**Fig. 2.** Expression of HIV-1 Vpr triggers depletion of human Dicer both alone and in the context of an infection. Lysates of HEK293T cells co-transfected with the indicated expression vectors were immunoblotted for Dicer and β-actin. Dicer protein levels, relative to the β-actin protein signal, were calculated based on the densitometric analysis of four separate experiments. A representative immunoblot is shown (A). Please note that in this and all other figures, error bars represent ± SD of the mean. Lysates of HEK293T cells transfected with either empty vector or HIV-1 FLAG-Vpr expression vector were immunoblotted for endogenous Dicer using antibody specific for Dicer. A representative immunoblot is shown, n=4 (B). Cultures of SupT1 cells were mock infected or infected with HIV-1 (pNL4-3 env(−) nef(−) gfp(+)) or HIV-1 with deleted Vpr sequences (pNL4-3 env(−) vpr(−) nef(−) gfp(+)) at a MOI=2. Cells were harvested 48 h after infection for immunoblotting and analysis by flow cytometry. The levels of Dicer, UNG2, p24, Vpr, and α-tubulin were assessed by western blot (left). The percentage of infected cells was determined by GFP expression as assessed by flow cytometry (right). The data is representative of n=2 (C). Cultures of SupT1 cells or HEK293T cells were mock infected or infected with HIV-2 or HIV-2 lacking Vpr or Vpx at equivalent MOIs. Cells were harvested 48 h after infection for immunoblotting. The levels of Dicer, UNG2, SAMHD1, p27, and α-tubulin were assessed by western blot (D and E).
Vpr reduces cellular Dicer levels after translation

Vpr modulates transcription from numerous promoters (Agostini et al., 1996; Amini et al., 2004; Chowdhury et al., 2003; Cohen et al., 1990; Cullen, 1991; Fenard et al., 2009; Forget et al., 1998; Gummulu and Emerman, 1999; Kino et al., 2005, 2002; Roux et al., 2000; Sawaya et al., 1998; Sherman et al., 2000; Wang et al., 1995; Zhu et al., 2003). Langevin et al. (2009) even demonstrated that Vpr expression specifically down-modulates transcription from the UNG2 promoter. We therefore tested whether Vpr acts to reduce Dicer levels by decreasing transcription. Real-time PCR analyses demonstrated that Dicer mRNA levels do not differ significantly in cells expressing Vpr as compared to those expressing vector alone (Fig. 3A). This finding indicates that Vpr does not act as a modulator of Dicer mRNA production.

In order to determine whether Vpr-mediated Dicer depletion proceeds through proteasomal degradation, we treated cells with the irreversible proteasome inhibitor epoxomicin prior to lysis. Inhibition of proteasomal function restored Dicer levels in the presence of Vpr, suggesting that Vpr decreases Dicer via a proteasome-dependent pathway (Fig. 3B).

We established that expression of HIV-1 Vpr reduces cellular Dicer levels in a proteasome-dependent manner, but did not significantly impact levels of Dicer mRNA. In order to confirm that HIV-1 Vpr impacts Dicer after translation, we measured the stability of Dicer protein in the presence or absence of HIV-1 Vpr. Cells were transfected with either an expression vector for FLAG-tagged Vpr or an empty expression vector. Twenty-four hours later, the cells were seeded into separate plates. Forty-eight hours after transfection, the cultures were treated with the translation inhibitor cycloheximide and then harvested 1, 2.5, 8 and 24 h later. An untreated control was also harvested for each transfection (T=0). In Fig. 3C, we show that the rate of Dicer depletion is greatly enhanced in the presence of Vpr (T1/2≈2.5 h) compared to our vector control (T1/2>24 h). Together with the previous experiments these data show that Vpr targets Dicer for depletion after protein translation by dramatically shortening its half-life.

Vpr-mediated Dicer depletion is not dependent on G2 cell cycle arrest

Since the levels of some proteins are regulated in a cell cycle-dependent manner, we tested whether the decrease in
Dicer levels is directly linked to Vpr-mediated G2 arrest. We transfected HEK293T cells with an expression vector for FLAG-epitope-tagged Dicer together with empty expression vector or expression vector encoding wild-type Vpr or well-characterized Vpr mutants (Fig. 3D). Among the Vpr species, wild-type Vpr, Vpr W54R and Vpr R90K caused depletion of Dicer while Vpr R80A produced partial depletion and Vpr R64LQQAA68 caused no depletion. Wild-type, Vpr W54R and Vpr R64LQQAA68 cause G2 arrest while Vpr R80A and Vpr R90K do not (Schrofelbauer et al., 2005; Selig et al., 1997; Sherman et al., 2000). Thus, both arresting and non-arresting mutants can trigger depletion or fail to do so, demonstrating that Dicer-depletion is not dependent on the cell cycle. Additionally, the Vpr W54R mutant, which does not bind the WXXF-motif of UNG2 and therefore fails to deplete UNG2 (BouHamdan et al., 1998; Schrofelbauer et al., 2005), causes Dicer depletion. This, together with the observation that Dicer has no WXXF motifs indicates that Vpr is not strictly dependent on this motif for the recruitment of target proteins.

In summary, Vpr expression causes depletion of cellular Dicer levels after translation by decreasing Dicer half-life. This phenotype was not amplified by changes in Dicer transcription. The levels of Dicer could be at least partially restored, in the timeframe tested, by adding proteasome inhibitor to the culture media. Finally, the mechanism responsible for accelerated Dicer turnover does not depend on the ability of Vpr to cause G2 cell cycle arrest.

Vpr-mediated depletion of Dicer depends on the CRL4DCAF1 ubiquitin ligase complex

Vpr-directed G2 cell cycle arrest, depletion of UNG2, and expression of NK-cell ligands have all been linked to the assembly of Vpr with the CRL4DCAF1 ubiquitin ligase complex. We therefore tested whether Vpr is required for the recruitment of Dicer to the CRL4 complex. We expressed, in cultures of HEK293T cells, FLAG epitope tagged Cul4A alone, together with Myc-Dicer or with both Myc-Dicer and HA epitope tagged Vpr. We then isolated FLAG–Cul4A and probed for the presence of Dicer and Vpr among the co-isolated proteins. In order to minimize HIV-1 Vpr-mediated depletion of Dicer in these experiments, we harvested cells at 18 h post-transfection, to capture the proteins as they assembled but before extensive degradation was observed. In the absence of FLAG–Cul4A neither Dicer nor Vpr was isolated. Small quantities of Dicer were co-isolated with FLAG–Cul4A alone, but these were increased in the presence of HIV-1 Vpr (Fig. 4A). We have previously shown that UNG2 assembles with and is turned over through the CRL4DCAF1 complex in the absence of Vpr but that Vpr enhances this interaction. This observation prompted us to investigate whether Dicer is also a substrate for the CRL4DCAF1 complex in the absence of Vpr. To test this, we depleted HEK293T cells of Cul4A or DCAF1 by siRNA transfection. A non-targeting siRNA was used as control. Upon depletion of either Cul4A or DCAF1, the steady state levels of endogenous UNG2 increased, whereas those of Dicer remained constant (Fig. 4B).

Fig. 4. HIV-1 Vpr-mediated depletion of Dicer depends on the CRL4DCAF1 ubiquitin ligase complex. HEK293T cells were co-transfected with Myc-Dicer, FLAG–Cul4A and HA-Vpr. FLAG–Cul4A was immunoprecipitated with anti-FLAG beads. Bound proteins were eluted and immunoblotted for Myc-Dicer and HA-Vpr. Myc-Dicer was detected using Dicer specific antibody. FLAG–Cul4A and HA-Vpr were detected using antibodies specific for their respective tags (A). HEK293T cells were transfected with either non-targeting siRNA or siRNA specific for Cul4 (C), DCAF1 (D), or DDB1 (E). Lysates were prepared and corresponding blots were immunoblotted for endogenous Dicer, Flag–Vpr and α-tubulin. Representative immunoblots are shown.
Having demonstrated that Vpr enhances the assembly of Dicer with the CRL4 complex and that the depletion of CRL4 complex components in the absence of Vpr does not alter steady-state Dicer levels, we determined whether we could protect Dicer from Vpr-mediated degradation by interfering with CRL4 function. To ascertain whether the CRL4 complex is important for Vpr-directed depletion of Dicer, we individually depleted three of its components and probed the impact on Dicer degradation. We transfected HEK293T cells with siRNA specific for Cul4, DCAF1, or DDB1 and then used western blotting to determine whether the lack of specific CRL4 components interfered with Vpr-directed Dicer degradation (Fig. 4C–E, respectively). Treatment with siRNA specific for the ubiquitin ligase components, but not with non-targeting siRNA, restricted the capacity of HIV-1 Vpr to cause Dicer depletion.

Taken together, these data support a model in which Vpr assembles with Dicer and the CRL4 complex, and that this complex is required for Vpr-mediated Dicer degradation. Importantly, Dicer is only the third protein target identified for Vpr-directed proteasomal degradation through this ubiquitin ligase complex and Dicer is the first target identified that lacks a WXXF motif.

**Vpr inhibits shRNA function**

Dicer processes double stranded RNAs to products that ultimately lend specificity to RNA interference. Vpr-directed depletion of Dicer should thus impede processing of double stranded RNAs. We therefore investigated the functional consequence of Vpr expression on Dicer activity. Using a luciferase reporter assay, we tested whether Vpr suppresses the silencing function of firefly luciferase-specific shRNA. We transfected HeLa cells with a firefly luciferase expression plasmid together with either a shRNA designed to target firefly luciferase or a scrambled, non-targeting shRNA as well as a plasmid expressing renilla luciferase to control for transfection efficiency and cell viability. In the absence of Vpr, the luciferase-directed shRNA reduced luciferase activity by approximately 80% relative to the scrambled control shRNA (Fig. 5A). When we co-expressed Vpr, the efficacy of the luciferase-specific shRNA was significantly decreased, resulting in increased luciferase expression relative to the vector control (shLuc/Vector versus shLuc/Vpr in Fig. 5A).

The Vpr Q65R mutant was significantly impaired in its capacity to cause Dicer depletion (Fig. 5B), likely due to its impaired interaction with DCAF1 (Le Rouzic et al., 2007). Vpr Q65R showed significantly less repression of Dicer activity than wild-type HIV-1 Vpr did, paralleling the observation that it is less effective at promoting Dicer levels comparable to those of an infection with Vpr-expressing HIV-1 (Fig. 6B panels a and b). Depletion of Dicer, however, also enhanced infectivity of Vpr-encoding HIV-1 suggesting that Vpr may not completely deplete Dicer (Fig. 6B panels a and b).

We further tested the impact of Vpr-mediated Dicer depletion on infection by measuring the quantity of p24 produced by cells during spreading infections (Fig. 6). Here, depletion of Dicer increased the levels of p24 for an infection with HIV-1 vpr(−) to a much greater extent (Fig. 6B panels c and d) than the infectivity of Vpr-encoding HIV (Fig. 6B panels a and b). Depletion of Dicer, however, also enhanced infectivity of Vpr-encoding HIV-1 suggesting that Vpr may not completely deplete Dicer (Fig. 6B panels a and b).

**Reduction of Dicer levels enhances macrophage infection in a Vpr-dependent manner**

Vpr enhances HIV-1 infectivity in macrophages and Dicer impairs HIV infectivity in CD4+ T cells. We therefore investigated how Dicer depletion impacts HIV-1 infection of macrophages and whether this effect is Vpr-dependent.

We transfected primary human monocyte-derived macrophages (MDM) with either an siRNA that specifically targets human Dicer or a non-targeting control siRNA. Dicer-specific siRNA reduced Dicer protein levels to approximately 20% of those found in cultures treated with the non-targeting siRNA (Fig. 6A). We used these cultures to test whether Dicer hinders HIV-1 in MDMs in the context of a spreading infection. For these experiments we used vpr(+) or vpr(−) forms of NL81A, a HIV-1 which encodes envelope sequences from the macrophage-tropic BaL strain in place of the V1–V3 loops of the NL4-3 envelope. The viruses also contained a GFP gene in place of 5’ nef sequences. Images of the infected cells were recorded 19 days after infection. Depletion of Dicer increased infectivity of HIV-1 vpr(−) to a much greater extent (Fig. 6B panels c and d) than the infectivity of Vpr-encoding HIV (Fig. 6B panels a and b). Depletion of Dicer, however, also enhanced infectivity of Vpr-encoding HIV-1 suggesting that Vpr may not completely deplete Dicer (Fig. 6B panels a and b).

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In order to ascertain whether HIV is inhibited by Dicer before GFP expression from the provirus, we performed single-round infections in the presence or absence of Dicer and determined the number of infected cells using flow cytometry. Two days after
transfection with either Dicer-specific or non-targeting siRNA, we infected the primary human MDMs with VSV-G pseudotyped, env(−) HIV-1 that either encodes or fails to encode Vpr. Both of these viruses have a green fluorescent protein (GFP) reporter gene in place of nef. Five days after infection, we harvested the cells and used flow cytometry to determine the fraction of GFP-expressing cells as a measure of infection. We found that transfection with Dicer-specific siRNA significantly enhanced infectivity of vpr(−) HIV-1 but not that of HIV-1 capable of expressing wild-type Vpr (Fig. 6E).

Consistent with our data from the spreading infections, depletion of Dicer very reproducibly enhanced single-round infection with vpr(−) HIV-1 in primary human MDMs, albeit modestly. This suggests that the anti-viral function of Dicer can act on the virus on its way to establishing a functional provirus in the absence of Vpr, but leaves open the possibility that Dicer impacts HIV-1 at other phases of the infection cycle. The observation that depletion of Dicer does not enhance single-round infection with wild-type virus, however, suggests that Vpr action is already maximal during this phase of infection. Our ability to enhance the infectivity of wild-type virus in spreading infections by depleting Dicer suggests that Vpr may be less efficient at protecting the virus in another phase of replication such as during virus production.

**Discussion**

In this work we showed for the first time that HIV-1 Vpr assembles with Dicer. The primary functional consequence of this interaction is depletion of Dicer through the CRL4DCAF1 ubiquitin ligase complex. Indeed we showed that Dicer, Vpr and Cul4 all assembled into the same protein complex. We further showed that Vpr-mediated depletion of Dicer interferes with shRNA function. Importantly, we found that siRNA-mediated depletion of Dicer boosts HIV-1 infectivity in primary human macrophage cultures and that this enhancement is significantly more pronounced in infections with vpr(−) virus than with wild-type virus. This of course suggests that the Vpr associated with the wild-type virus alleviates the need for Dicer inhibition that is vital for infection with vpr(−) virus. Of note, Coley et al. (2010) previously showed that Vpr prevents expression of Dicer upon differentiation of monocytes. Our work offers an explanation for this finding. Rather than inhibiting Dicer production, Vpr triggers its elimination.

Our observations are also consistent with previous reports showing enhancement of HIV-1 infection after Dicer depletion in peripheral blood mononuclear cells and the cell lines, Jurkat and HEK293T (Nathans et al., 2009; Triboulet et al., 2007). Importantly, these earlier studies did not link the Dicer restriction directly with viral protein binding to either RNA or protein targets. The tomato bushy stunt virus protein p19, targets double-stranded RNA with 2-nucleotide 3′ overhangs to prevent their assembly into the RISC complex (Scholthof et al., 1995; Silhavy et al., 2002). The floc
miRNA pathway can restrict HIV replication in mammalian cells. Another Vpr function impacted by its interaction with Dicer. Regulation of ULBP-1 and ULBP-2 are linked could reveal yet examining whether Dicer degradation and Vpr-mediated up-
ATR in these cells (Zimmerman et al., 2006). Future experiments unclear. Of note, macrophages may not trigger the Dicer-
ligands are up-regulated in response to DNA damage remain
ULBP-2 (Wu et al., 2011). The factors that determine which NKGD2
response to up-regulate NKGD2 ligands albeit not ULBP-1 or
decreasing Dicer expression can also trigger a DNA damage
lysis (Richard et al., 2010; Ward et al., 2009). Interestingly,
ligands in infected cells to enhance natural killer cell-mediated
ing the DNA damage response, up-regulates ULBP-1 and ULBP-2
example, recent work demonstrated that Vpr expression, trigger-
Vpx in monocyte-derived dendritic cells (Laguette et al., 2011).
Vpr directs depletion of both UNG2 and SMUG1 proteins, both
of which encode WXXF motifs. The WXXF motif on UNG2 is
critical for its assembly with Vpr (BouHamlan et al., 1998). The
WXXF motif on SMUG1 has not been tested but may perform a
similar function in assembly with Vpr. Addition of this motif to
integrate, allowed its assembly with Vpr (Kulkosky et al., 1999).
Dicer, the third target for Vpr-mediated destruction by
CRL4-DCAF1, does not have a WXXF motif. Furthermore, Dicer
was depleted efficiently by the Vpr W54R mutant which fails to bind
UNG2 or to deplete UNG2 (Schoelbauer et al., 2007). Dicer
therefore is the first protein to be identified that is susceptible to
Vpr-mediated depletion by CRL4-DCAF1 in the absence of the
WXXF motif. This observation suggests that Vpr can flag a range of
proteins for destruction rather than one specific target.

Previous work showed that Tat can partially suppress Dicer activity
although the significance of this finding has not been fully resolved. Future experiments comparing the SRS activity of Tat to that of Vpr
will be necessary to determine their respective contributions to
alleviating miRNA restriction, especially in macrophages.

HIV-1 Vpr paralog HIV-2 Vpx does not deplete Dicer. Vpx
enhances macrophage infection albeit more dramatically than
HIV-1 Vpr (Sharova et al., 2008; Srivastava et al., 2008) by
combating antiviral protein SAMHD1 through the CRL4-
DCAF1 complex. SAMHD1 (Hrecza et al., 2011) also acts as a restriction that can be overcome by Vpx in monocyte-derived dendritic cells
(Laguette et al., 2011).

While work presented here is focused on the role of the Dicer interaction with HIV-1 Vpr and its effect on macrophage infection efficiency, this interaction may also impact other Vpr functions. For example, recent work demonstrated that Vpr expression, triggering the DNA damage response, up-regulates ULBP-1 and ULBP-2 ligands in infected cells to enhance natural killer cell-mediated lysis (Richard et al., 2010; Ward et al., 2009). Interestingly, decreasing Dicer expression can also trigger a DNA damage response to up-regulate NKGD2 ligands albeit not ULBP-1 or ULBP-2 (Wu et al., 2011). The factors that determine which NKGD2
ligands are up-regulated in response to DNA damage remain
unclear. Of note, macrophages may not trigger the Dicer-
derived DNA damage response because Vpr does not activate ATR in these cells (Zimmerman et al., 2006). Future experiments
examining whether Dicer degradation and Vpr-mediated up-
regulation of ULBP-1 and ULBP-2 are linked could reveal yet
another Vpr function impacted by its interaction with Dicer.

In summary, accumulating evidence has established that the
miRNA pathway can restrict HIV replication in mammalian cells.

Here we identified Vpr as an HIV-encoded protein that possesses SRS activity and determined its mechanism for defeating the
miRNA restriction, specifically triggering destruction of Dicer. Furthermore, this work identifies a novel substrate of Vpr-
mediated degradation via the CRL4DCAF1 complex. Future studies to identify miRNAs that are impacted by Vpr expression will offer
new insights into the mechanism of this evolutionarily conserved antiviral response.

Materials and methods

Ethics statement

All primary human monocyte samples were from de-identified
donors at the University of Nebraska Medical Center, Omaha, NE. Our protocol for the use of primary human monocytes was
approved by the Albany Medical College Committee on Research
Involving Human Subjects and granted a category 4 exemption from consent procedures based on the anonymous nature of the samples.

Proviral clones and expression plasmids

Macrophage-tropic proviral clones, pNL81A GFP and pNL81A
vpr(-) GFP, were constructed by inserting a BamHI-Xhol fragment containing eGFP from pNL4-3 GFP env(-) (kindly provided by Dr. Dana Gabuzda, Dana-Farber Cancer Institute, Boston, MA) into
pNL81A or pNL81A vpr(-) that had been digested with BamHI and
Xhol. The Nef open reading frame was thus replaced with the eGFP
gene. pNL81A and its vpr-deficient counterpart were previously
described (Eckstein et al., 2001; Toohey et al., 1995). FLAG-HA–
Dicer was purchased from Addgene (Addgene plasmid #19881,
provided by Dr. Thomas Tuschi, Rockefeller University, New York
NY). 5′ Myc–Dicer pcDNA3.1 was kindly provided by Dr. Patrick
Provost (Université Laval, Quebec, Canada). The expression vector for codon-optimized FLAG-epitope tagged Vpr, pcDNA3.1(-)
huFLAG–Vpr was previously described (Wen et al., 2007). The Vpr mutants tested in Fig. 3 were similarly generated or derived by
PCR-based mutagenesis of the original codon-optimized clones. The proviruses used in single-round infections, pNL4-3 GFP env(-)
and pNL4-3 GFP env(-)vpr(-), were kindly provided by Dr. Vicente
Planelles (University of Utah, Salt Lake City, UT). pGL-AN nef(-)/GFP,
GPl-ST nef(-)/GFP plg-L-Ec nef(-)/GFP and plg-LSt/Ec nef(-)/GFP
originated as pGL-AN, pGL-St, pGL-Ec and pGL-St/Ec, a kind gift of Dr.
Mikako Fujita, but have GFP inserted in place of nef sequences
upstream of the 3′ LTR.

Cell culture

HEK293T and HeLa cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

SupT1 cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Human monocytes were obtained from healthy donors at the University of Nebraska Medical Center, Omaha, NE. Eutiated
monocytes were differentiated for 7 days in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% human serum, and recombinant human macrophage colony-stimulating factor (rhM-CSF, Cell Sciences, Canton, MA). Cultures were maintained by replacing one half of the cell culture medium with fresh medium
every 2–3 days. After 7 days in differentiation media, MDMs were
maintained in DMEM supplemented with 10% human serum without rhM-CSF.

**Immunoprecipitation and Tandem mass spectroscopy analysis**

4 × 10⁶ HEK293T cells were seeded into each of 10, 10 cm plates. Each culture was transfected, using calcium phosphate, with 20 μg of pCDNA3.1(−)HIV-1huVpr or pCDNA3.1(−)HIV-1FLAG–huVpr. Forty-eight hours after transfection the cells were lysed with 1 ml cold RIPA buffer (25 mM Tris–HCl pH 8.0, 250 mM NaCl, 10 v/v% glycerol, 1 v/v% IGEPA CA-630, 0.25 w/v% deoxycholic acid, 2 mM EDTA, 1 mM NaF, 50 mM β-glycerophosphate and Complete™ protease inhibitor cocktail (Roche)). The lysates were cleared twice by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatants were incubated with 50 μl of anti-FLAG M2 agarose resin (Sigma–Aldrich) for 2 h at 4 °C with constant rotation. The resin with the bound proteins was washed three times with lysis buffer. The remaining bound proteins were eluted by competition with 50 μl of 200 mg/ml FLAG peptide (Sigma–Aldrich) at room temperature for 10 min. The eluted proteins were identified using tandem mass spectroscopy (NextGen Sciences, Ann Arbor, Michigan).

**Immunoprecipitation and immunoblotting**

HEK293T cells were transfected using calcium phosphate. Cells were lysed in ELB buffer (50 mM HEPES pH 7.3, 400 mM NaCl, 0.2% NP-40, 5 mM EDTA, 0.5 mM DTT) for 15 min and then centrifuged for 10 min to pellet debris. Clarified supernatants were either stored as a total cellular lysates or mixed with 50 μl of anti-FLAG M2 agarose resin (Sigma–Aldrich) for 2 h at 4 °C with constant rotation. The resin with the bound proteins was washed three times with lysis buffer. The remaining bound proteins were eluted by competition with 50 μl of 200 mg/ml FLAG peptide (Sigma–Aldrich) at room temperature for 10 min. The eluted proteins were identified using tandem mass spectroscopy (NextGen Sciences, Ann Arbor, Michigan).

**Proteasome inhibition**

HEK293T cells were treated with 1 μM epoxomicin (Sigma–Aldrich) or an equivalent volume of DMSO for 5−7 h at 37 °C.

**Cycloheximide time course**

10 cm plates of HEK293T cells were transfected with either empty vector or with an expression vector for FLAG-epitope-tagged HIV-1 Vpr. Twenty-four hours later, the cells were replated into 60 mm dishes. Forty-eight hours later, pre-warmed media containing 25 μg/ml cycloheximide was added to all cells at time = 0. Cells were harvested at the indicated time-points and immediately frozen at −20°C. Once all samples were harvested, cells were lysed in ELB buffer (50 mM HEPES pH 7.3, 400 mM NaCl, 0.2% NP-40, 5 mM EDTA, 0.5 mM DTT) for 10 min on ice. Cell debris was pelleted by centrifugation at 14 K rpm for 10 min. Supernatants were mixed with 2 × Laemmli buffer and boiled to ensure complete denaturation and lysis. Samples were resolved by SDS/PAGE and transferred to PVDF membranes (Millipore) for immunoblotting.

**Real-time PCR**

Cytoplasmic RNA was isolated from transfected HEK293T cells using the RNeasy Mini Kit according to manufacturer’s instructions (Qiagen, Inc., Valencia, CA). DNase-treated RNA was quantified by Nanodrop™ spectrophotometer, and 1 μg of RNA was reverse transcribed using the iScript cDNA synthesis kit, following manufacturer’s instructions (BioRad, Hercules, CA). Dicer and β-actin mRNA was amplified from 20 ng of cDNA with an Applied Biosystems 7500 real-time PCR cycler, using SYBR green reagent (Bio-Rad). The sequence of Dicer- and β-actin-specific primers is shown below. These were used at 250 nM or 400 nM concentrations, respectively. Standard curves were established to determine the amplification efficiency of each primer set. The efficiency for Dicer was 93.8% and that for β-actin was 93%.

**Short hairpin RNA and siRNA**

The firefly luciferase specific and scrambled luciferase shRNA vector was constructed using the GeneClip™ U1 Hairpin Cloning System. The oligonucleotides corresponding to the sequences shown below were synthesized and annealed with complementary oligonucleotides, as specified in manufacturer’s instructions, and ligated into the expression vector provided. The integrity and identity of the inserts was confirmed by DNA sequencing.

Luciferase-specific shRNA:

5′-TTCACAGTGTGTTCTCATTCCAATGATTCTCTACTGAATGGAACAA-CACTTCT-3′

Scrambled luciferase shRNA:

5′-TTCGGATTTAGCGTACTTCTCTAGATTGCTGCAAGCTGCCGTTACGCAAC-CATCATTCT-3′

The following siRNAs were purchased from Dharmacon: Non-targeting control siRNA (CAT# D-001210-02-20) Dicer: 5′-UGCUUGAAGCUGUGA(dTdT)-3′ DCF1: 5′-AUAUGGCCGUUUCGCUAAA(dTdT)-3′ DDB1: 5′-CCAGUUGUACUGAAGAATGATG(dTdT)-3′ Cul4 (mix): 5′-CCGUUCAGCUGGAGAUAU(dTdT)-3′ and 5′-AGGGACUACUGGAAAGAUA(dTdT)-3′

**Dicer activity reporter assay**

HeLa cells were seeded in 12-well plates and transfected using FuGene HD (Roche), in triplicate, with 2.5 ng EF-1 renilla luciferase plasmid, 25 ng of pGL3 control plasmid (Promega), 375 ng of scrambled or luciferase-specific shRNA and either 100 ng of vector or expression plasmid encoding HIV-1 Vpr or HIV-1 VprQ65R. Two
days after transfection, the cells were harvested in 1 × Renilla lysis buffer (Promega). Lysates were mixed with either Firefly luciferase assay buffer or Renilla luciferase assay buffer (Promega) and luciferase activity was measured using a plate reader luminometer. All samples were normalized to Renilla luciferase activity. Data is expressed as luciferase activity in luciferase-specific shRNA transfected cells relative to scrambled shRNA transfected cells.

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