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The HIV-1 protein Vpr targets the endoribonuclease Dicer for proteasomal degradation to boost macrophage infection

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ABSTRACT

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.

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 trans-activator 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

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to Tat (Bennasser 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; Hariharan 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 $SCF^{\beta TrCP}$ 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 triphosphohydrolase, 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 $CRL4^{DCAF1}$ ubiquitin ligase complex to trigger G2 cell cycle arrest, to deplete cellular UNG2, and to boost expression of NKG2D 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, Vpu and Vpx commandeers 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 Langevin 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-elicited 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 $CRL4^{DCAF1}$ 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 $CRL4^{DCAF1}$ 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

A

protein ID	molecular wt (kDa)	no. of peptides	
		Vpr	FLAG–Vpr
DCAF1	169	0	357
DDB1	127	0	322
DDA1	12	0	12
UNG2	35	0	4
Dicer1	219	0	7

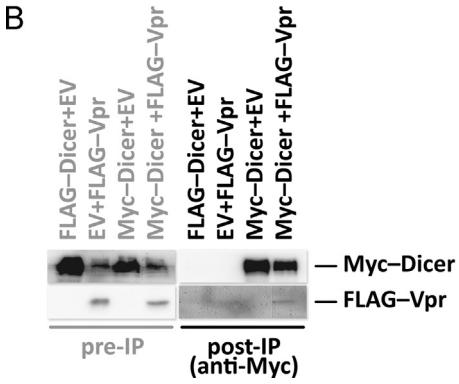


Fig. 1. HIV-1 Vpr assembles with human Dicer. HEK293T cells were transfected with expression vector for untagged Vpr or FLAG-epitope tagged Vpr. FLAG-specific antibody bound to beads was used to isolate proteins from the cell lysates. Proteins were eluted from the beads by competition with FLAG peptide 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).

absence and presence of Vpr. We saw a substantial decrease in the level of Myc-tagged Dicer in 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 $CRL4^{DCAF1}$ ubiquitin ligase complex, we hypothesized that Vpr recruits Dicer to $CRL4^{DCAF1}$ for ubiquitination and subsequent proteasomal destruction.

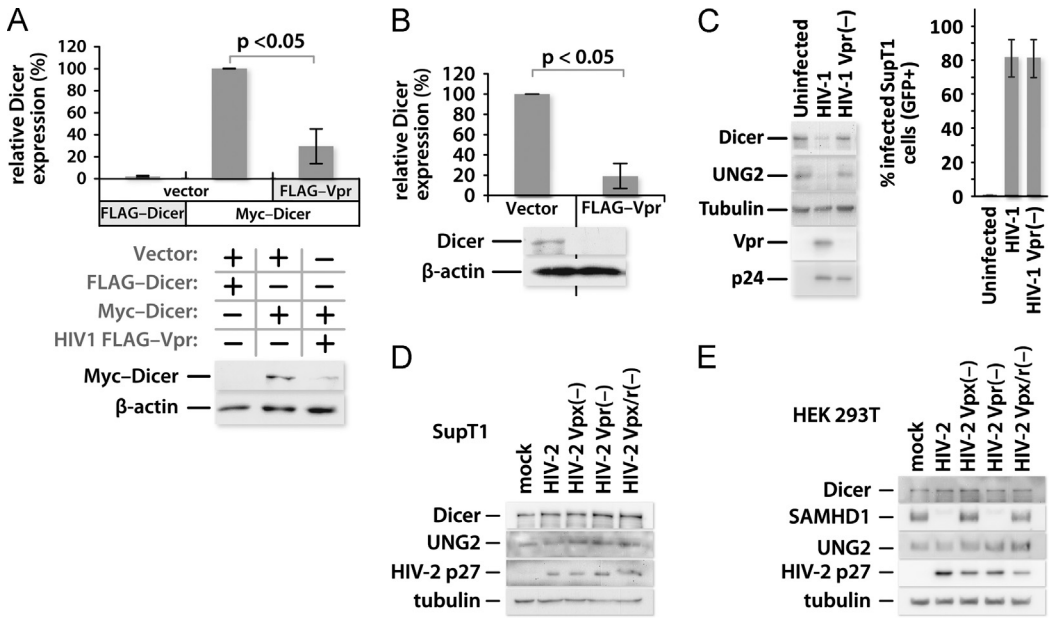


Fig. 2. Expression of HIV-1 Vpr triggers depletion of human Dicer both alone and in the context of an infection. Lysates of HEK293T cells co-transfected with the indicated expression vectors were immunoblotted for Dicer and β-actin. Dicer protein levels, relative to the β-actin protein signal, were calculated 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

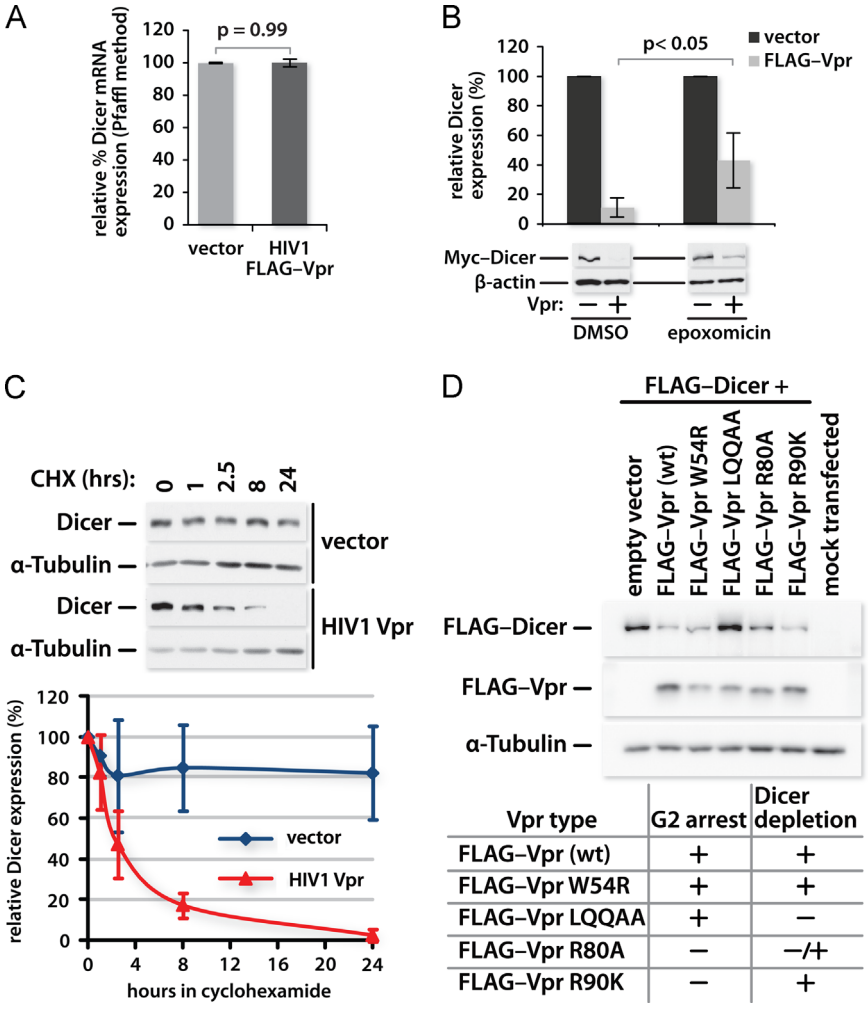
Vpr modulates transcription from numerous promoters (Agostini et al., 1996; Amini et al., 2004; Chowdhury et al., 2003; Cohen et al., 1990; Cullen, 1991; Fenard et al., 2009; Forget et al., 1998; Gummuluru and Emerman, 1999; Kino et al., 2005, 2002; Roux et al., 2000; Sawaya et al., 1998; Sherman et 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 ($T_{1/2}\approx 2.5$ h) compared to our vector control ($T_{1/2} > 24$ 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



Vpr type	G2 arrest	Dicer depletion
FLAG–Vpr (wt)	+	+
FLAG–Vpr W54R	+	+
FLAG–Vpr LQQAA	+	–
FLAG–Vpr R80A	–	–/+
FLAG–Vpr R90K	–	+

Fig. 3. Vpr reduces Dicer levels after protein translation in a manner that is not linked to the cell cycle. HEK293T cells were transfected with either empty expression vector or expression vector for HIV-1 FLAG–Vpr. Dicer mRNA levels were measured by real-time PCR and were normalized to β-actin mRNA. Quantification was calculated using the Pfaffl method and the relative mean was calculated from three experiments (A). HEK293T cells, transfected with either empty expression vector or expression vector for HIV-1 FLAG–Vpr, were treated with 1 μM of proteasome inhibitor, epoxomicin, or DMSO. Lysates were prepared as described in Materials and methods and immunoblotted for exogenous Myc–Dicer expression. A representative immunoblot is shown, $n=3$ (B). HEK293T cells were treated with 25 μg/ml cycloheximide and harvested at the time points indicated. Lysates were prepared and immunoblotted for endogenous Dicer and α-tubulin expression (C). HEK293T cells were transfected with 1 μg of expression vector for FLAG–Dicer and 3 μg of empty expression vector or expression vector for the Vpr mutant indicated. Cells were harvested 48 h after transfection and lysates were probed for Dicer, Vpr, or tubulin, as indicated. The table summarizes the phenotypes of Vpr mutants used (D).

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 $_{64}$LQQAA$_{68}$ caused no depletion. Wild-type, Vpr W54R and Vpr $_{64}$LQQAA$_{68}$ 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 time-frame 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 CRL4^{DCAF1} 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 CRL4^{DCAF1} 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 CRL4^{DCAF1} 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 CRL4^{DCAF1} 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).

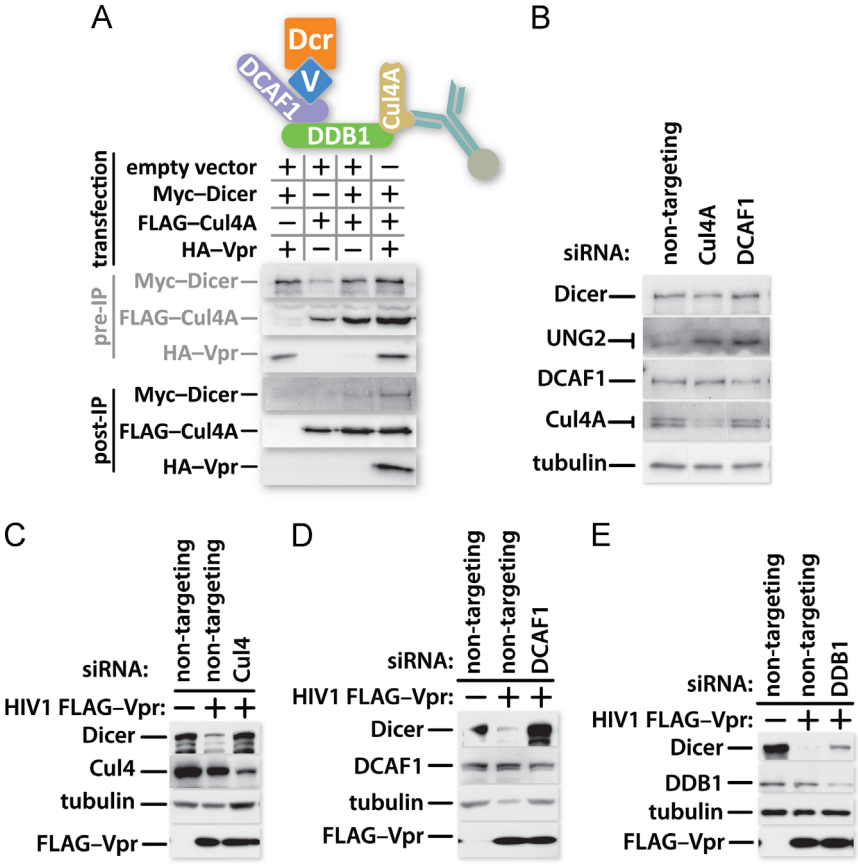


Fig. 4. HIV-1 Vpr-mediated depletion of Dicer relies on the CRL4^{DCAF1} ubiquitin ligase complex. HEK293T cells were co-transfected with Myc-Dicer, FLAG-Cul4A and HA-Vpr. FLAG-Cul4A was immunoprecipitated with anti-FLAG beads. Bound proteins were eluted and immunoblotted for Myc-Dicer and HA-Vpr. Myc-Dicer 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 Cul4A or for DCAF1. The levels of Dicer, UNG2, DCAF1, Cul4A, and α-tubulin were assessed by immunoblotting with protein-specific antibodies (B). 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 $CRL4^{DCAF1}$ 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 $CRL4^{DCAF1}$ 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 depletion. Overall, our results are consistent with a Vpr-specific abrogation of shRNA-mediated silencing. These data show that Vpr can act as a HIV-1-encoded suppressor of RNA silencing (SRS).

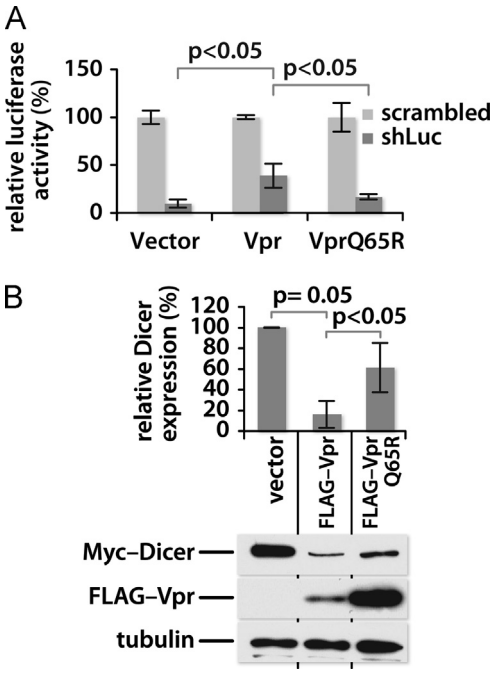


Fig. 5. HIV-1 Vpr suppresses shRNA function. HeLa cells were co-transfected with firefly and renilla luciferase reporter plasmids as well as with either scrambled or firefly luciferase-specific shRNA expression vectors and the indicated HIV-1 Vpr expression plasmids. Firefly luciferase activity was measured and normalized against the internal control, renilla luciferase activity. These data were normalized to the level of luciferase activity in cells transfected with scrambled shRNA, which was set at 100%. Means were calculated from at least three experiments performed in duplicate (A). HEK293T cells co-transfected with Myc–Dicer expression vector along with either empty vector, or expression vector for wild-type HIV-1 FLAG–Vpr or for HIV-1 FLAG–Vpr Q65R. Lysates were prepared and immunoblotted for expression of Myc–Dicer, FLAG–Vpr or α-tubulin as indicated. A representative immunoblot is shown, $n=4$ (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*).

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. 6C). Here, depletion of Dicer increased the levels of p24 for an infection with HIV-1 *vpr*(−) to levels comparable to those of an infection with Vpr-expressing HIV-1. In this analysis siRNA-mediated depletion of Dicer also increased the p24 output of infections with Vpr-expressing HIV-1. However, comparison of the ratios of p24 produced by cultures transfected with siRNA specific for Dicer with those transfected with the non-targeting siRNA control demonstrates that depletion of Dicer by siRNA had a much greater impact on HIV-1 *vpr*(−) virus production than on Vpr-expressing HIV-1 virus production (Fig. 6D). This suggests that the enhancement mediated by reduction of Dicer abundance is Vpr-dependent. Our observations underscore that the effects of Vpr or siRNA-mediated Dicer depletion on HIV-1 infectivity appear to determine whether a viable infection can be established.

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

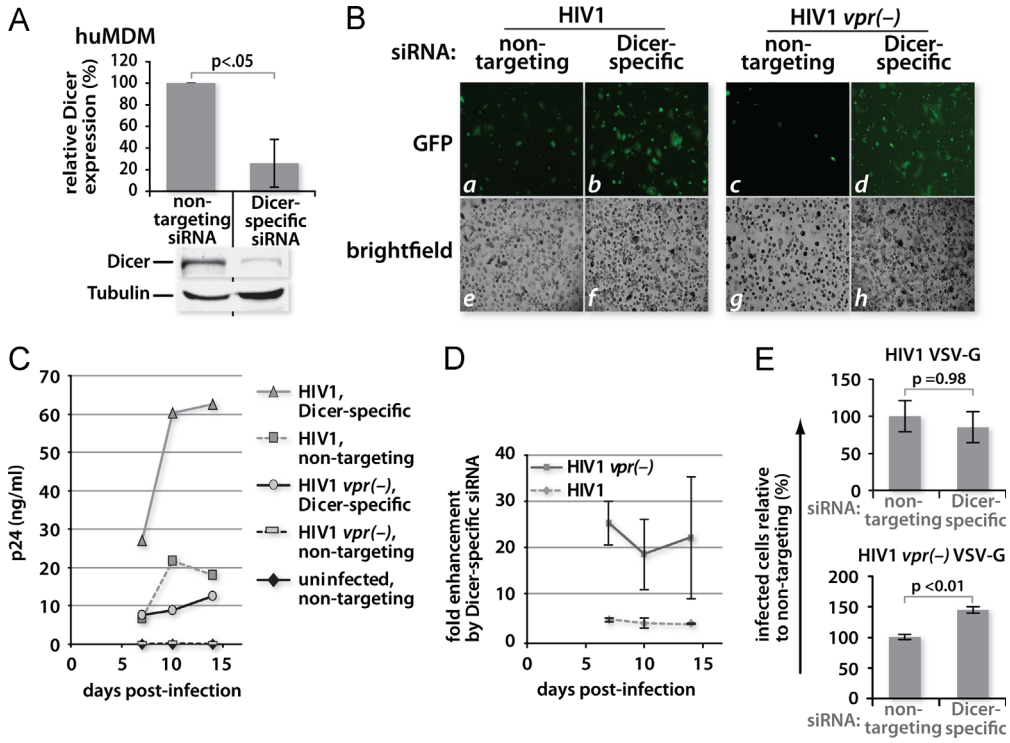


Fig. 6. Reduction of Dicer expression in human MDMs enhances HIV-1 infectivity and production in a Vpr-dependent manner. Primary human MDM cultures were transfected with either non-targeting siRNA or siRNA specific for Dicer. Forty-eight hours after transfection, lysates were prepared and immunoblotted for endogenous Dicer and tubulin. A representative immunoblot is shown, $n=2$ (A). Primary human MDM cultures were transfected with either non-targeting siRNA or siRNA specific for Dicer and infected 24 h after transfection at equivalent MOI with either macrophage-tropic HIV-1 GFP or HIV-1 GFP *vpr*(–). Infectivity was visualized 19 days after infection by phase and fluorescence microscopy. Images are shown from a representative experiment. The experiment was performed with primary cells from at least three different donors and equivalent results were obtained (B). Supernatants from MDM cultures infected in (B) were harvested at days 7, 10 and 14 post-infection and were analyzed for p24 Gag production by p24 antigen-capture ELISA (C). p24 Gag production data were normalized to the levels of p24 in MDM cultures transfected with non-targeting siRNA to obtain the fold enhancement of viral production by Dicer siRNA. Means were calculated from p24 analysis of two separate experiments (D). Primary human MDM cultures were transfected with either non-targeting siRNA or siRNA specific for Dicer. These were infected 24 h after transfection at an equivalent MOI with either VSV-G pseudotyped single cycle HIV-1 GFP or HIV-1 GFP *vpr*(–). Cells were harvested five days after infection and analyzed by flow cytometry. These data were normalized to the value of cells transfected with non-targeting siRNA, which was set to 100%. Means were calculated from five separate experiments using primary cells from five different donors (E).

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 $CRL4^{DCAF1}$ 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 a viral protein while our data provides evidence that reducing Dicer expression offers a greater advantage to the virus in the absence of Vpr than in its presence. This indicates that Vpr counteracts the Dicer-dependent restriction. Viral protein-directed depletion of RNA silencing pathway components has not been described. Other mechanisms for SRS function rely on 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 flock

house virus B2 protein more generally targets double stranded RNA to prevent its processing by Dicer (Li et al., 2002). The cucumber mosaic virus 2b protein interacts with and inhibits Argonaute in the RISC complex (Zhang et al., 2006). Thus, a variety of mechanisms are emerging for SRS function and curiously none appear to be specific for a particular RNA sequence.

Dicer may not be the only factor that Vpr blocks to aid HIV-1 infection in macrophages. Infection with Vpr-expressing HIV was more efficient than with *vpr*(−) HIV-1, even when Dicer was depleted with siRNA. It is likely that depletion of Dicer resulted in a smaller improvement of wild-type virus infectivity because virus-encoded Vpr could deplete at least a portion of cellular Dicer. Furthermore, while Dicer depletion aided single-round infection with *vpr*(−) HIV, we cannot rule out that Vpr-mediated Dicer depletion can also boost virus production. Since viral RNA is a key component for both incoming and outgoing virus, it is conceivable that Dicer function could impact both. Furthermore, it is also possible that if Dicer was not sufficiently depleted in the cultures to which the corresponding siRNA was applied, that the wild-type virus had the advantage of carrying Vpr to neutralize remaining Dicer.

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 (BouHamdan 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 integrase, 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 (Schrofelbauer 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 (Hrecka 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-mediated 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 $CRL4^{DCAF1}$ 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-XhoI 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 XhoI. 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 Tuschl, 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, pNL4-3GFP *env*(−) and pNL4-3GFP *env*(−)*vpr*(−), were kindly provided by Dr. Vicente Planelles (University of Utah, Salt Lake City, UT). pGL-AN *nef*(−)*GFP*, pGL-St *nef*(−)*GFP* pGL-Ec *nef*(−)*GFP* and pGL-St/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 ug/ml streptomycin.

Human monocytes were obtained from healthy donors at the University of Nebraska Medical Center, Omaha, NE. Elutriated 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 × 10^6 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% IGEPAL 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 antibody-conjugated beads and incubated overnight at 4 °C followed by competitive elution with peptide corresponding to the epitope tag. Immunoprecipitates or total cell lysates were resolved by SDS/PAGE and transferred to PVDF membranes (Millipore). The following primary antibodies were used for immunoblotting: c-myc-specific monoclonal antibody 9E10 (Stratagene), FLAG-specific monoclonal antibody M2 (Sigma-Aldrich), HA-specific monoclonal antibody 12CA5 (Roche Applied Science), Dicer-specific polyclonal anti-serum (Cell Signaling), Tubulin-specific monoclonal antibody N356 (Amersham) and β-actin-specific monoclonal antibody (Sigma-Aldrich). The primary antibodies were detected with corresponding horseradish peroxidase-conjugated secondary antibodies. These were visualized using Immobilon Western horseradish peroxidase substrate (Millipore).

Protein levels are reported as relative densitometric intensity measured by NIH Image J software. Each protein level was corrected against the corresponding loading control. Means and standard deviation of means were calculated from at least three separate experiments.

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%.

Dicer forward: 5′-CATGGATAGTGGGATGTCAC-3′ (Chendrimada et al., 2005)
Dicer reverse: 5′-CTACTTCCACAGTGACTCTG-3′ (Chendrimada et al., 2005)
β-actin forward: 5′-AAAGACCTGTACGCCAACAC-3′ (Chendrimada et al., 2005)
β-actin reverse: 5′-GTCATACTCCTGCTTGCTGAT-3′ (Chendrimada et al., 2005)

The Pfaffl method was used to quantify mRNA levels, and Dicer expression was normalized to β-actin expression.

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′-TCTCAAGTGTTGTTCCATTCCATAAGTTCTCTATGGAATGGAACAA-CACTTCT-3′
Scrambled luciferase shRNA:
5′-TCTCGATTTTAGCCGTACTTCGTAAGTTCTCTACGAAGTACGGC-TAAAATCCT-3′
The following siRNAs were purchased from Dharmacon:
Non-targeting control siRNA (CAT# D-001210-02-20)
Dicer: 5′-UGCUUGAAGCAGCUCUGGA(dTdT)-3′
DCAF1: 5′-AUAUGGCCGUUUCCGUAAA(dTdT)-3′
DDB1: 5′-CCCAGUUUCUGCAGAAUGAATA(dTdT)-3′
Cul4 (mix): 5′-CGGCUUCAGCUUUGAGGAGAUA(dTdT)-3′
and 5′-AGGGACUACAUGGAAAGAGAUA(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.

Virus preparation and infection

HEK293T cells were transfected with pNL81A HIV-1GFP, pNL81A HIV-1*vpr*(−)GFP, pGL-AN*nef*(−)GFP, pGL-St*nef*(−)GFP pGL-Ec*nef*(−)GFP or pGL-St/Ec*nef*(−), using calcium phosphate or FuGENE HD. Viruses for single round infections were made by co-transfecting HEK293T cells with viral expression vector and an expression plasmid for vesicular stomatitis virus G-protein. Viruses for both spreading and single-round infections were harvested 48 h after transfection. Supernatants were centrifuged at 800 × g for 5 min to sediment cellular debris. Clarified supernatants were concentrated with centrifugal filter columns (Amicon Ultra 100 K from Millipore) using manufacturer's protocol. Concentrated virus was stored at –80 °C. Viruses were titered on the GHOST X4/R5 indicator cell line (kindly provided by the NIH AIDS Research and Reference Reagent Program).

Prior to infections, MDMs that had been differentiated for 7 days were transfected with either non-targeting or Dicer-specific siRNA using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were infected at an equivalent multiplicity with *vpr*(+) and *vpr*(−) viruses. After 5–7 h, cells were washed twice with PBS and incubated in fresh DMEM supplemented with 10% human AB serum.

SupT1 cells were incubated with VSV-G pseudotyped *vpr*(+) and *vpr*(−) viruses at a multiplicity of infection of two, as measured by titration on GHOST indicator cells. After 2 h of incubation, cells were washed with PBS and resuspended in RPMI media. Forty-eight hours post-infection, flow analysis was performed on a small fraction of the cells to analyze infectivity. The rest of the cells were harvested and lysed. The lysates were separated by SDS/PAGE, and immunoblotted as previously described.

Single round MDM infections were analyzed, using flow cytometry, five days after infection. Infected cells were released from their substrate by incubating cultures for 30 min in a 1:1 solution of 10 mM EDTA in PBS (pH 7.4) and RPMI containing 20% fetal bovine serum. Cells were fixed for 30 min at room temperature in 2% formaldehyde and washed twice in PBS. 30,000 events were recorded on a LSRII flow cytometer (BD Biosciences) and data was analyzed using FlowJo v7.5 software (Tree Star, Inc., Ashland, OR).

Spreading MDM infections were monitored by fluorescence microscopy using a Zeiss Axio Observer.Z1 microscope. GFP-expressing cells were visualized using 488 nm excitation with a FITC emission filter set (Zeiss). Images shown were recorded at 19 days after infection.

Virus production was monitored by measuring viral p24gag protein in cell-free supernatants harvested from cultures at the specified time-points. HIV-1 p24 ELISA Assay Kits were used as per manufacturer's instructions (AIDS Vaccine Program, National Cancer Institute (NCI) at Frederick, MD, USA).

Statistical analysis

All analysis was performed using the unpaired Student *t* test with a two-tailed distribution.

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