Lentiviral Vpr usurps Cul4–DDB1[VprBP] E3 ubiquitin ligase to modulate cell cycle


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The replication of viruses depends on the cell cycle status of the infected cells. Viruses have evolved functions that alleviate restrictions imposed on their replication by the host. Vpr, an accessory factor of primate lentiviruses, arrests cells at the DNA damage checkpoint in G2 phase of the cell cycle, but the mechanism underlying this effect has remained elusive. Here we report that Vpr proteins of both the human (HIV-1) and the distantly related simian (SIVmac) immunodeficiency viruses specifically associate with a protein complex comprising subunits of E3 ubiquitin ligase assembled on Cul1. Vpr binding to Cul4–DDB1[VprBP] leads to increased neddylation and elevated intrinsic ubiquitin ligase activity of this E3. This effect is mediated through the VprBP subunit of the complex, which recently has been suggested to function as a substrate receptor for Cul4. We also demonstrate that VprBP regulates G2 phase and is essential for the completion of DNA replication in S phase. Furthermore, the ability of Vpr to arrest cells in G2 phase correlates with its ability to interact with Cul4–DDB1[VprBP] E3 complex. Our studies identify the Cul4–DDB1[VprBP] E3 ubiquitin ligase complex as the downstream effector of lentiviral Vpr for the induction of cell cycle arrest in G2 phase and suggest that Vpr may use this complex to perturb other aspects of the cell cycle and DNA metabolism in infected cells.

Cullin 4

The replication of HIV and other lentiviruses is restricted to some degree by the cell cycle status of the target cells. Although lentiviruses can infect nondividing cells, and thus establish stable reservoirs in CD4 T cells and terminally differentiated macrophages, they fail to replicate in quiescent cells (1). The observation that cells in G2 phase support lentivirus replication more efficiently than those in G1 phase further illustrates how the cell cycle status influences primate lentiviruses (2). HIV replication is also constrained by host cell mechanisms that detect and repair damaged DNA. For instance, the reverse-transcribed retroviral genomes are targeted for degradation by DNA repair proteins XBP and XPD (3), and the final steps of the integration of the reverse-transcribed retroviral genome into chromosomal DNA are thought to be catalyzed by DNA repair enzymes of the host cell (4). Viruses have evolved functions that partially alleviate various restrictions imposed on their replication by the host. In particular, accessory proteins of primate lentiviruses, such as Vpr, Vif, Nef, and Vpu, execute several such functions (5).

Vpr accessory proteins are multifunctional regulators located in the nuclei of the infected cells (6). Although Vpr is not required for lentivirus replication in cultured cells, its conservation in HIV-1, HIV-2, and simian immunodeficiency viruses (SIV) indicates that a strong selective pressure to preserve these proteins must operate in vivo. Indeed, Vpr was shown to accelerate progression to AIDS in rhesus macaques infected with SIV in the absence of a closely related SIV Vpx protein (7). How Vpr contributes to lentiviral pathogenesis is still under intense investigation. One conserved function of lentiviral Vpr is its ability to arrest the infected cells in the G2 phase of the cell cycle (8, 9). Vpr was also reported to suppress HIV-1 mutation rate, and this effect was correlated with its interaction with host-derived uracil DNA glycosylase, UNG2 (10). This enzyme is involved in the base excision repair pathway that specifically removes uracil from DNA (11). A recent study suggested that Vpr directs degradation of UNG2 to stabilize HIV reverse-transcription products (12). Finally, Vpr has been implicated as one of the factors that facilitates infection of terminally differentiated macrophages possibly by promoting entry of the HIV preintegration complex into the nucleus (13).

The most extensively studied Vpr function is its ability to arrest cycling cells in the G2 phase of the cell cycle (8, 9). One candidate mechanism for this function is suggested by the observation that Vpr activates the ataxia telangiectasia and Rad3-related (ATR) protein kinase (14). ATR triggers checkpoint signaling on genotoxic stress to stop the progression of the cell cycle until the damaged DNA is repaired (15). Recent evidence suggests that Vpr leads to ATR activation by interfering with the DNA replication machinery of the infected cell (16) and implicates Cullin 4 ubiquitin ligase containing VprBP/DCAF1 as potentially important for this effect (17, 18). It should be pointed out, however, that Vpr was also reported to interact with signaling molecules downstream of ATR, such as 14–3–3 proteins and Cdc25C, raising the possibility that additional mechanisms may be usurped by the virion protein to perturb the progression of the cell cycle (19–21).

The 3D structure of HIV-1 Vpr implies that it functions as an adaptor protein (22). Hence, we used a proteomic approach to identify the key cellular proteins that Vpr binds to subvert DNA replication and cell cycle regulation. Here we show that Vpr binds and deregulates an unstudied Cullin 4 ubiquitin ligase complex containing damaged DNA binding protein 1 (DDB1), WD40-repeat containing VprBP, and DDA1 subunits. The DDA1–DDB1–VprBP complex we purified is probably the most abundant Vpr-containing protein complex in the cell. The previously characterized Cull4 ligase complexes control DNA replication and DNA repair through ubiquitination of key substrates in these pathways (23–28). The Cull4 complex targeted by Vpr appears to regulate the G1 phase of the cell cycle and is essential for completion of DNA replication in S phase. We further demonstrate that, through the interaction with this Cull4


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complex. Vpr arrests cells in the G2 phase. Together our studies identify the immediate downstream effector of Vpr for G2 cell cycle arrest and suggest additional scenarios for how Vpr may use this complex to perturb other aspects of the cell cycle and DNA metabolism in infected cells.

**Results**

HIV-1 and SIV Vpr Proteins Bind a Common Set of Proteins. To address the molecular mechanisms used by the lentiviral Vpr proteins, we purified protein complexes containing HIV-1 Vpr or its orthologue encoded by the pathogenic SIVmac 239 strain. Vpr proteins were tagged at their N-termini with FLAG and HA epitopes in tandem and expressed stably in U937 monocytes or transiently in human embryonic kidney 293 T (HEK293T) cells. Next, Vpr and their associated proteins were purified from detergent extracts by sequential immunoprecipitations with α-HA- and then α-FLAG-epitope antibodies, followed each time by elution with the respective peptide epitope.

The immunoprecipitates were analyzed by Multidimensional Protein Identification Technology (MudPIT). MudPIT is a combination of chromatographic and mass spectrometric procedures that allow unbiased and sensitive identification of proteins in complex mixtures. Three relatively abundant polypeptides, DDB1, DDA1, and VprBP, that were specifically associated with Vpr proteins from both HIV-1 and SIVmac, which are two distantly related primate lentiviruses (29), in both U937 and HEK293T cells and absent in purifications from negative control cells were thus identified (SI Table 1). The conservation of these interactions suggested that they are important mediators of a common Vpr function. Therefore, we focused on these proteins.

**DDB1, DDA1, and VprBP Form a Ternary Complex That Vpr Binds.** The finding that Vpr binds DDB1, VprBP, and DDA1 linked Vpr to Cullin-4 RING E3 (Cul4 E3) ubiquitin ligase complexes. DDB1 is an obligatory subunit of Cul4 E3 ligases (30). These enzymes regulate DNA repair and replication and cell cycle progression through ubiquitination of key substrates in these processes. VprBP, a known HIV-1 Vpr binding protein (31), and DDA1 have been found recently to bind DDB1 (26); however, their normal functions remain unknown. Therefore, to gain further insights into Vpr interactions with these polypeptides, we characterized Vpr association with VprBP, DDB1, and DDA1.

The observations that VprBP and DDA1, as well as DDB1, all copurified with Vpr, and that they were recovered at similar relative abundance based on relative spectral counts (see SI Table 1), suggested that they form a ternary complex, which is then targeted by Vpr. To address these possibilities, FLAG-tagged VprBP, DDB1, DDA1, and Vpr were individually expressed in HEK293T cells. Detergent extracts from the transfected and control cells were immunoprecipitated with α-FLAG beads and immune complexes analyzed by immunoblotting. As shown in Fig. 1A, each protein was capable of associating with each other (lanes 3–5). Next, the complexes purified via VprBP and HIV-1 Vpr (lanes 2 and 3) were separated on 10–40% glycerol gradients. As shown in Fig. 1B, DDB1 (≈127 kDa molecular mass), DDA1 (11.8 kDa), and VprBP (169 kDa) all peaked in fractions 6 and 7, indicating that they form a ternary complex of ≈300–400 kDa (Fig. 1B Upper). Notably, the bulk of the Vpr-bound VprBP, DDB1, and DDA1 co sedimented with similar velocities (Fig. 1B Lower). Their distributions, however, were shifted toward the bottom of the gradient, compared with those in the absence of Vpr, suggesting that the viral protein may recruit additional components to these complexes. We conclude that VprBP, DDB1, and DDA1 form a ternary complex, which Vpr binds, and that the viral protein may modulate interactions between the DDA1–DDB1–VprBP complex and other factors.

Vpr Elevates Neddylation of Cul4 in Cul4–DDB1[VprBP] E3 Complex. Surprisingly, although DDB1 functions through participation in the Cul4 E3 ligase platform (30), our MudPIT analyses detected only a few Cul4-derived peptides in α-Vpr immune complexes. This observation suggested that Cul4 is not a stoichiometric subunit of the Vpr complex we purified. To address a link to Cul4 ubiquitin ligase, we further characterized the interaction between Vpr and Cul4. Myc-tagged Cul4A was coexpressed together with VprBP and/or HIV-1 Vpr by transient transfection in HEK293T cells. In some experiments, Vpr, or VprBP, were FLAG-tagged to facilitate their immunoprecipitation, α-VFLAG immune complexes prepared from transfected cells were analyzed by immunoblotting for Cul4. As shown in Fig. 2A, Cul4 was found in FLAG–VprBP immune complexes, thus confirming that VprBP participates in Cul4 E3 ligase complex (lanes 6 and 7) (26). Significantly, Cul4 was also found in FLAG–Vpr immune complexes, but only when VprBP was also ectopically expressed, thereby confirming that Vpr association with Cul4 is bridged by VprBP (compare lanes 3 and 2).

The ubiquitin ligase activity of cullin complexes is regulated by cycles of Nedd8 moiety attachment to the cullin scaffold and its removal (32). The neddylated forms of cullins migrate slower than their unmodified forms during SDS/PAGE electrophoresis. Strikingly, ectopic expression of Vpr and VprBP together elicited a slower migrating Cul4A species readily detectable in detergent extracts prepared from the transfected cells (Fig. 2A top gels, lanes 3, 4, 7, and 12, and 13). This Cul4 species was less pronounced in control experiments where Vpr, or VprBP, were expressed separately (lanes 2, 6, 8, 10, and 11). Immunoprecipitation experiments revealed that both Cul4A forms were associated with VprBP (Fig. 2A, middle gels, lane 7) and with Vpr, but only when VprBP was also ectopically expressed (compare lanes 2 and 3). Immunoblotting of the immune complexes for Nedd8 confirmed that the slower migrating Cul4A species was indeed the neddylated form (Fig. 2A, bottom gel, lanes 3 and 7).

The data described above demonstrated that Vpr can increase Cul4 neddylation through its interaction with VprBP, but they did not rule out that Vpr causes generalized neddylation of Cul4 present in other Cul4–DDB1 complexes. To address this latter possibility, we tested the effect of Vpr on the Cul4A component of the previously well characterized Cul4 E3 complex that contains DDB2 subunit instead of VprBP and mediates repair of...
Vpr Elevates Intrinsic Ubiquitin Ligase Activity of Cul4–DDB1[VprBP] E3 Complex. We observed that Cul4 E3 ubiquitin ligases are thought to control DNA replication and progression of the cell cycle (23–28). We hypothesized that Vpr usurps VprBP and the Cul4–DDB1[VprBP] E3 complex to perturb these processes. Therefore, experiments were performed to characterize the normal function of VprBP.

Fig. 2. Vpr elevates neddylation and intrinsic ubiquitin-ligase activity of the Cul4–DDB1[VprBP] E3 complex. (A) Vpr binds to and specifically elevates neddylation of Cul4A associated with VprBP. Myc-tagged Cul4A (m-Cul4) was coexpressed with Vpr, VprBP, and DDB2. In various combinations, in HEK293T cells. FLAG-tagged (f) versions of these proteins were used in some experiments to facilitate their immunoprecipitation. m-Cul4A (arrow) and its neddylated form (+) were detected in detergent extracts (Extr) and α-FLAG immunocomplexes (IP) by immunoblotting with α-myc and α-Nedd8 antibodies, respectively. (B) Vpr stimulates intrinsic ubiquitin ligase activity of the Cul4–DDB1[VprBP] E3 complex. m-Cul4–DDB1[VprBP] complexes were assembled with or without Vpr in HEK293T cells, purified via their FLAG-VprBP subunits, and incubated with E1, ubiquitin, and/or E2 as indicated. Cul4A and its ubiquitinated forms were detected by immunoblotting for Cul4A.

VprBP Depletion Leads to the Activation of DNA Damage Response. To assess whether VprBP depletion led to the activation of DNA damage checkpoints, we characterized the expression of serine 139-phosphorylated histone H2A.X variant (γ-H2A.X). H2A.X is phosphorylated early on in response to DNA damage and is involved in the recruitment of repair proteins to the vicinity of DNA lesions (34). As shown in Fig. 3C, levels of γ-H2A.X expression were elevated in the G2-phase VprBP-depleted cells compared with control cells. This observation supports the notion that a subpopulation of VprBP-depleted U2OS cells is arrested in the G2 phase at the DNA damage checkpoint. Next, we analyzed expression of key checkpoint proteins and cell cycle regulators by immunoblotting (Fig. 3D). Notably, the steady-state levels of p53 and p21 cyclin-dependent kinase inhibitor, which mediate p53-dependent DNA damage checkpoint (35), were both increased in VprBP-depleted cells. The levels of S-phase cyclin A and of cyclin B1 were relatively low, in agreement with a low frequency of S-phase cells (see Fig. 3B–D). The expression levels of cdc6 and geminin, which control replication complex assembly and replication licensing (36), were also relatively low, consistent with low expression levels of these proteins in G1 cells. Together our observations provide evidence that depletion of VprBP leads to the activation of cellular response to DNA damage and arrest of the cells in the G1 and G2 phases at DNA damage checkpoints.
VprBP depletion leads to G1 and G2 phase arrests. (A) The effect of RNAi to VprBP on the ability of Vpr to arrest cells in G2. U2OS cells expressing shRNAs to VprBP (Lower) or DOCK2 (Upper) were transduced with a lentiviral TEIG vector expressing HIV-1 Vpr (Vpr) or a control empty vector (Vector). Three days later, the cells were stained with PI, and the DNA content was analyzed by flow cytometry. (B) VprBP-depleted cells arrest in G1 and G2. VprBP-depleted (Lower) and control cell populations (Upper) were labeled with BrdU for 1 h. BrdU incorporation and DNA content were analyzed by flow cytometry either immediately after BrdU labeling or after 6 and 12 h chase. (C) Histone γ-H2A.X and cyclin A expression in VprBP-depleted (VprBP) and control cells (vector) were revealed by indirect fluorescence. Cells were counterstained with DAPI, and fluorescent signals were imaged with an iCyS laser scanning cytometer. Histograms of DNA content and bivariate distributions of γ-H2A.X or cyclin A fluorescence versus DNA content are shown. (D) Levels of cyclins, checkpoint, and replication licensing proteins in lysates of VprBP-depleted and control cells were analyzed by immunoblotting with antibodies to the indicated proteins. Splicing factor 2 (SF2) was used as a loading control. The asterisk indicates a band reacting nonspecifically with the α-VprBP IgG. Lanes 1 and 3 contain 3-fold dilutions of the amounts loaded in lanes 2 and 4, respectively.

HIV-1 Vpr Arrests Cell Cycle by Interacting with the Cul4–DDB1[VprBP]

E3 Complex. Observations from our genetic and biochemical studies indicated that VprBP influences the transition from S to G2 phase probably through modulation of the DNA replication and/or damage repair processes. Significantly, RNA interference mediated depletion of the DDA1 and DDB1 subunits of the E3 complex targeted by Vpr also leads to cell cycle perturbations and activation of DNA damage response (SI Figs. 6 and 7) (28). This evidence strongly suggested that Vpr arrests cells in the G2 phase through its interaction with the DDA1–DDB1–VprBP complex and its associated Cul4 E3 ubiquitin ligase. To address this possibility, a panel of HIV-1 vpr alleles were tested for their abilities to arrest cells in G2, associate with VprBP and DDB1, and elevate Cul4A neddylation via VprBP. U2OS cells were transduced with retroviral MIG vectors expressing wild-type or mutant HIV-1 Vpr proteins, and their cell cycle profiles were analyzed 3 days later. As shown in Fig. 4A, ~75% of cells transduced with wild-type vpr were arrested in G2. Notably, Vpr also caused accumulation of cells with >4n DNA content, suggesting that the viral protein can interfere with replication licensing (36). Next, we tested two mutations, one substituting arginine for histidine H71 (H71R) and the other attaching a tandem FLAG-HA epitope tag to the C terminus of the Vpr molecule (C-tag). Both mutations disrupted the ability of Vpr to arrest cells in the G2 phase, in agreement with a previous report (37). Notably, neither of the two proteins was able to associate with VprBP or DDB1, or to increase the levels of neddylated Cul4A (Fig. 4 B and C). Substitution of proline for alanine A30 (A30P) slightly diminished the ability of Vpr to arrest cells in G2 and also slightly decreased Vpr binding to VprBP and DDB1 and Cul4 neddylation. In contrast, no such effects were seen with two other mutations (W54R and I63G) that did not affect the ability of Vpr to arrest cells in G2. Of note, the W54R substitution was found to elevate the association of the viral protein with VprBP and DDB1, but did not elevate Cul4 neddylation above the level seen with wild-type Vpr. Thus, the binding of Vpr to VprBP probably is not sufficient to increase Cul4 neddylation, and some additional Vpr function is likely required for this effect. These data provide genetic and biochemical evidence in support of a model in which Vpr arrests cells in G2 by targeting the Cul4–DDB1[VprBP] E3 ubiquitin ligase.

Discussion

In a search for downstream effectors of lentiviral Vpr, we purified an abundant Vpr-associated protein complex and identified its components as subunits of a specific ubiquitin ligase complex assembled on Cul4 scaffold (Cul4–DDB1[VprBP]). The VprBP subunit of this complex contains canonical WD40/WDXR motifs, similar to known Cul4 substrate receptors, such as DDB2, CSA, and Cdt2, which use these motifs to dock to DDB1 (23, 38). DDB1, in turn, connects them to Cul4, thus, by analogy to these polyepitides, VprBP probably functions as a substrate receptor for Cul4 (17, 26, 38). Importantly, our data show that Vpr positively regulates the ubiquitin ligase activity of this specific E3 complex probably by elevating neddylation of Cul4. Covalent conjugation of a Ned8 moiety to cullin is thought to mediate the recruitment of the E2-conjugating enzyme for the ubiquitin transfer reaction, thereby up-regulating ubiquitin ligase activity of the E3 complex (23, 39, 40). Regarding the mechanism that underlies the increased neddylation of Vpr-associated Cul4, Vpr could promote Ned8 ligation to Cul4 or stabilize the neddylated Cul4 by inhibiting Ned8 deconjugation by COP9 signalsome or another isopeptidase (40). Of note, we observed that COP9 subunits were undetectable in the E3 complexes associated with Vpr, but present in those assembled in the absence of Vpr expression (see Fig. 1, lanes 2 and 3; and data not shown). This evidence suggests that Vpr up-regulates the catalytic activity of Cul4–DDB1[VprBP] by interfering with Ned8 deconjugation by COP9.

Two lines of evidence suggest that Vpr usurps the Cul4–DDB1[VprBP] E3 to arrest cells in the G2 phase. First, our data link VprBP and its associated Cul4 E3 ubiquitin ligase to the regulation of DNA replication and the cell cycle. Second, we found that mutant Vpr proteins deficient for binding the Cul4–DDB1[VprBP] complex and elevating neddylation of its associated Cul4 are unable to arrest cells in G2. These findings support a model in which Vpr perturbs the normal function of Cul4–DDB1[VprBP] E3, and thereby interferes with the completion
of DNA replication, which leads to the activation of DNA damage checkpoint and cell cycle arrest in G2 phase.

Available evidence suggests three not exclusive scenarios for how Vpr may interfere with the normal function of the Cul4–DDB1[VprBP] E3 substrate that is involved in DNA replication. First, our finding that Vpr specifically stimulates catalytic activity of the Cul4–DDB1[VprBP] E3 implies that the viral protein may cause premature ubiquitination of proteins that are natural substrates of this ubiquitin ligase complex and mediate DNA replication/repair. However, the ability of Vpr to activate Cul4 is probably not sufficient to explain how it arrests cells in G2 because we observed that the Vpx protein, a Vpr orthologue encoded by HIV-2 and SIVsm viruses, also binds and stimulates neddylation of Cul4 in the Cul4–DDB1[VprBP] complex, but does not arrest cells in G2 (data not shown). Second, Vpr may recruit novel protein substrates that normally are not recruited by VprBP for ubiquitination by Cul4. This possibility is supported by recent evidence that Vpr directs ubiquitination of UNG2 and SMUG protein substrates that normally are not recruited by VprBP for ubiquitination of a physiological Cul4–DDB1[VprBP] E3 substrate that is involved in DNA replication. This possibility is consistent with our observation that RNAi-mediated loss-of-VprBP function leads to G2 arrest. It is conceivable that a combination of two or more of the above effects leads to the activation of DNA damage checkpoint and triggers G2 arrest.

How Vpr, by subverting the VprBP-linked Cul4 E3 ubiquitin ligase, benefits replication of primate lentiviruses is not known, but can be speculated on given the existing data. Our evidence firmly links VprBP to the control of G1-phase progression. Significantly, previous studies established that a certain threshold of cellular activation is needed to support productive lentiviral infection. Although quiescent cells in the G0 state are not permissive, just a partial stimulation that drives them into the G1 phase without triggering proliferation is frequently sufficient to establish competence for lentiviral replication (41, 42). Because cells in late G1 are permissive, whereas those in early G1 are not, the position of cells in the G1 phase probably determines how efficiently they support viral replication (41). Thus, Vpr could benefit HIV replication by manipulating progression through G1, early G0, and late G0 phases in noncycling cells and in minimally activated cells. Such an effect would benefit the virus early in natural infection, when the levels of immune activation are low and the majority of the infected cells are thought to be quiescent.

Another possibility is suggested by the observation that the depletion of VprBP expression levels by RNAi leads to the activation of the DNA damage checkpoint in G2. This evidence links VprBP to the control of DNA synthesis and/or repair processes that are essential for the completion of S phase. Notably, recent studies have begun to reveal the unexpected complexity of the interactions between the replicating retroviral genomes and cellular machineries that repair damaged DNA. For example, integration of cDNA copies of retrovirus genomes is thought to be aided by cellular machineries that mediate repair of damaged DNA (4). However, it appears that enzymes that normally mediate repair of damaged DNA target the incoming retroviral nucleic acids to inhibit, rather than promote, the infection (3, 43). Moreover, retroviral genomes are substrates for DNA editing enzymes and additional repair reactions (10, 12, 44). It will be important to determine whether the Cul4–DDB1[VprBP] complex controls any of these processes. Ultimately, the identification of substrate proteins recruited for ubiquitination by VprBP, both in the absence and presence of Vpr, should lead to a better understanding of the roles of Cul4–DDB1[VprBP] E3 in DNA metabolism, cell cycle control, and lentivirus infection.

Materials and Methods

Expression Vectors and Viruses. HIV-1 NL4–3 and SIVmac 239 vpr tagged with N-terminal FLAG-HA-AU1 (hfa) epitopes in tandem (45) and other epitope-tagged cDNAs were cloned into pBABE-puro, pCG, MIG, and TEIG bicistronic vectors expressing GFP (46). shRNAs targeting sequences, listed in supporting information (SI) Text, were subcloned into TRIP lentiviral vectors (47). MIG and TEIG are modified MSCV and TRIP vectors containing a polylinker, followed by internal ribosome entry site element and GFP cassette derived from pCG. VSV-G pseudotyped viral particles were produced in transiently transfected HEK293T cells, and viral titers to U2OS cells were determined by flow-cytometry analysis of GFP/CFP expression.

Immunofluor Purification of Epitope-Tagged Proteins and MudPIT Analysis. Protein complexes were purified by two sequential immunoprecipitations via the FLAG and HA epitope tags from 7 g to 12 g of U937 cells stably expressing hfa-tagged Vpr proteins (or control cells), or transiently transfected HEK293T cells as described.
Flow-Cytometry Analysis. To visualize cells in S phase, cells were labeled with BrdU for 30 to 60 min. BrdU was detected with APC-conjugated α-BrdU antibody (Becton Dickinson, San Jose, CA) and DNA counterstained with 0.1 mg/ml propidium iodide (PI). For DNA content analysis only, cells were fixed with ethanol, and DNA was then stained with PI. The cells were analyzed on a Becton Dickinson LSRII Flow Cytometer, and data were processed with FlowJo software.

Transient Transfections, Immunoprecipitations, and Immunoblotting. Detergent extracts from transiently transfected HEK293T cells were immunoprecipitated with α-FLAG M2 affinity gel (Sigma–Aldrich, St. Louis, MO) (45). For preparation of lysates, U2OS cells were harvested by trypsinization, washed with PBS, and boiled in five volumes of Laemmli sample loading buffer. Antibodies used for immunoblot analyses are listed in SI Text.

Fluorescent Microscopy. U2OS cells grown on coverslips were fixed in 2% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X in PBS for 5 min on ice. Incubations with primary and secondary antibodies were performed as described (46). Cells were counterstained with 0.5 μg/ml DAPI (Sigma–Aldrich). Slides were imaged with a Zeiss Axioplan2 or iCy5 laser scanning cytometer (Carl Zeiss, Thornwood, NY).

In Vitro Ubiquitin Ligase Activity Assay. To measure ubiquitin ligase activity, Cul4–DDB1/[VprBP] complexes assembled in the absence or presence of HIV-1 NL43 Vpr expression in HEK293T cells were purified by immunoprecipitation via their FLAG-VprBP subunits and incubated at 30°C for 60 min with 0.2 μg of UbclE1, 0.03 μg of Ubch5b, 5 μg of ubiquitin (BIOMOL Research Laboratories, Plymouth Meeting, PA) in the medium of 50 mM TrisHCl (pH 8.0), 5 mM MgCl2, 0.2 mM CaCl2, 1 mM DTT.

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