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DDB1 and Cul4A Are Required for Human Immunodeficiency Virus Type 1 Vpr-Induced G_2 Arrest[▽]

Lindi Tan, Elana Ehrlich, and Xiao-Fang Yu*

Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205

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Vpr-mediated induction of G_2 cell cycle arrest has been postulated to be important for human immunodeficiency virus type 1 (HIV-1) replication, but the precise role of Vpr in this cell cycle arrest is unclear. In the present study, we have shown that HIV-1 Vpr interacts with damaged DNA binding protein 1 (DDB1) but not its partner DDB2. The interaction of Vpr with DDB1 was inhibited when DCAF1 (VprBP) expression was reduced by short interfering RNA (siRNA) treatment. The Vpr mutant (Q65R) that was defective for DCAF1 interaction also had a defect in DDB1 binding. However, Vpr binding to DDB1 was not sufficient to induce G_2 arrest. A reduction in DDB1 or DDB2 expression in the absence of Vpr also did not induce G_2 arrest. On the other hand, Vpr-induced G_2 arrest was impaired when the intracellular level of DDB1 or Cullin 4A was reduced by siRNA treatment. Furthermore, Vpr-induced G_2 arrest was largely abolished by a proteasome inhibitor. These data suggest that Vpr assembles with DDB1 through interaction with DCAF1 to form an E3 ubiquitin ligase that targets cellular substrates for proteasome-mediated degradation and G_2 arrest.

Vpr is a virion-associated accessory protein of human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) (11, 60, 62). SIVmac with Vpr mutants replicate efficiently in peripheral blood mononuclear cells and macrophages in vitro (60) and are pathogenic in monkeys (17); however, in HIV type 1 (HIV-1), Vpr mutants have been shown to be less competent for replication in various systems (reviewed in references 3 and 27). A number of functions have been reported for Vpr, including mediating nuclear import of the viral preintegration complex (12, 15, 16, 20, 28, 41, 42, 56), inducing G_2 cell cycle arrest (5, 14, 19, 24, 45) and apoptosis (5, 10, 23, 39, 46, 50, 52, 53, 66), decreasing the viral mutation rate during reverse transcription by recruiting uracil DNA glycosylases into viral particles, and partially neutralizing the antiviral function of cytidine deaminase APOBEC3G by degrading uracil DNA glycosylases (8, 9, 36, 48).

Although the biological relevance of cell cycle G_2 arrest has yet to be elucidated, G_2 arrest has been suggested to allow for efficient HIV-1 transcription, a possibility that correlates with the observation that Vpr-induced G_2 arrest results in a high level of HIV-1 viral replication in T lymphocytes (18). The ability to elicit G_2 arrest appears to require the phosphorylation of Vpr (1, 65), and G_2 cell cycle arrest mutants tend to have mutations clustered in helix 3 of the Vpr C-terminal region. The Vpr-induced G_2 cell cycle arrest phenomenon appears to be mediated by inactivation of the cyclin-dependent kinase 1/cyclin B complex (19, 45). To date, Vpr has been reported to either affect the activity of or associate with Wee-1 kinase, cdc25 phosphatase, ataxia telangiectasia-mutated, ataxia telangiectasia-Rad3-related protein, and protein phosphatase 2, which are known to be upstream regulators of the cdk1/cyclin B complex. In addition, Vpr may exert its inhibitory effects on the cell cycle by affecting the functions of other known cell cycle regulators, such as 14-3-3, p53, and p21 (reviewed in references 2, 3, 27, and 63).

Interaction of HIV-1 Vpr with DDB1. HIV-1 Vpr has recently been shown to associate with Cullin 1 and Cullin 4A (Cul4A) as well as targeting UNG2 and SMUG1 for proteasomal degradation (48). Cul4A has been shown to associate with DNA binding protein 1 (DDB1) and Roc1, forming a functional E3 ubiquitin ligase complex to degrade the important cell cycle regulators cdt1 and p27Kip1 (4, 6, 21, 25, 30, 40, 43, 49). It is interesting to note that the association of paramyxovirus simian virus 5 V protein with the DDB1/Cul4A complex results in delayed cell cycle progression (32). Since Vpr is known to cause G_2 cell cycle arrest, we hypothesized that it may associate with DDB1 to recruit Cul4A, leading to the degradation of important cell cycle proteins and causing G_2 cell cycle arrest.

In order to determine whether Vpr can interact with DDB1, 293T cells were transfected with a Vpr-hemagglutinin (HA) or Vpr-myc expression vector, followed by coimmunoprecipitation analysis. Cell lysates were prepared 48 h after transfection and subjected to immunoprecipitation using an anti-HA antibody conjugated to agarose beads as previously described (59). The anti-HA affinity matrix (Roche) immunoprecipitated HA-tagged Vpr proteins from lysates of transfected 293T cells (Fig. 1A, lane 1). As expected, Vpr-myc was not immunoprecipitated by the anti-HA affinity matrix (Fig. 1A, lane 2). In repeated experiments, endogenous DDB1 coprecipitated with Vpr-HA (Fig. 1A, lane 1). DDB1 was not detected in the samples containing Vpr-myc (Fig. 1A, lane 2), attesting to the specificity of the interaction between Vpr and DDB1.

To confirm the interaction between Vpr and DDB1, 293T cells were cotransfected with the Vpr-myc expression vector plus DDB1-V5, DDB2-V5, or a green fluorescent protein

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205. Phone: (410) 955-3768. Fax: (410) 614-8263. E-mail: xfyu@jhsph.edu.

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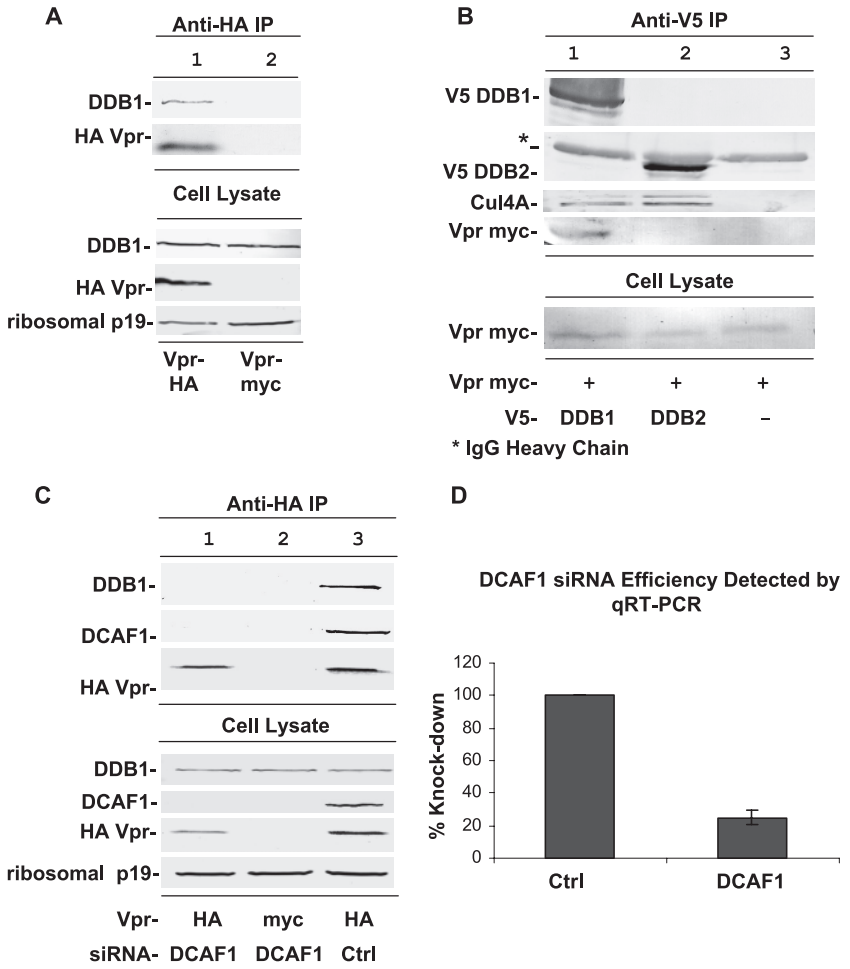


FIG. 1. Vpr does not interact with DDB2, but interacts with DDB1 through DCAF1. (A) Interaction between endogenous DDB1 and Vpr-HA. 293T cells were transfected with the Vpr-HA or Vpr-myc expression vector. Cell lysates from transfected cells were immunoprecipitated with anti-HA affinity matrix, and eluted samples were analyzed by immunoblotting using anti-HA antibody to detect Vpr-HA. Coprecipitated samples were also separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and reacted with DDB1 antibody. Intracellular expression levels of Vpr-HA, DDB1, and ribosomal proteins were detected with anti-HA, anti-DDB1, and anti-ribosomal protein antibodies, respectively. (B) Vpr interacts with DDB1 and not DDB2. 293T cells were cotransfected with the Vpr-myc expression vector plus the DDB1-V5, DDB2-V5, or GFP expression vector. Cell lysates from transfected cells were immunoprecipitated with anti-V5 antibody. Coprecipitated samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with an antibody against the V5 tag to detect DDB1-V5 or DDB2-V5. Coprecipitated samples were also analyzed by immunoblotting by using anti-myc antibody to detect Vpr-myc and antibody against Cul4A. Intracellular expression of Vpr-myc was also detected by immunoblotting using anti-myc antibody. IgG, immunoglobulin G. (C) Interaction of Vpr with DDB1 occurs through DCAF. 293T cells were transfected with either DCAF1 siRNA or control siRNA for 24 h, followed by a second round of transfection with the same siRNA, along with either Vpr-HA or Vpr-myc expression vector. Cell lysates from transfected cells were immunoprecipitated with anti-HA affinity matrix 72 h after the second transfection. The coprecipitated samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with antibodies against DDB1, DCAF1, and HA (to detect Vpr-HA). Intracellular expression levels of DDB1, DCAF1, ribosomal proteins, and HA-Vpr were also detected by immunoblotting using the appropriate antibodies. (D) Efficiency of DCAF1 mRNA knockdown detected by quantitative real-time PCR. Approximately 10^6 transfected cells were harvested and subjected to RNA extraction according to the protocol for TRIzol reagent (Invitrogen). One-fifth of the RNA was reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems), and the cDNA was amplified using TaqMan Universal PCR master mix (Applied Biosystems) and an ABI Prism 7000 sequence detection system (Applied Biosystems). The primers/probe sets were prevalidated TaqMan gene expression assays specific for DCAF1. The amplification of DCAF1 was normalized to β-actin as an endogenous control (human ACTB β-actin endogenous control 6-carboxyfluorescein/MGB probe; Applied Biosystems). Error bars indicate standard deviations. qRT-PCR, quantitative reverse transcription-PCR.

(GFP) expression vector. Cell lysates were prepared 48 h after transfection and immunoprecipitated with anti-V5 antibody (Invitrogen). Coprecipitated samples were analyzed by immunoblotting with anti-myc (to detect Vpr-myc) (Upstate), anti-V5 (to detect DDB1-V5 or DDB2-V5), or anti-Cul4A antibody. Reciprocal immunoprecipitation of DDB1-V5 (Fig. 1B, lane 1) but not the negative control GFP (Fig. 1B, lane 3) coprecipitated Vpr-myc.

During the preparation of this paper, the interaction between Vpr and DDB1 was also reported by other groups (26, 46). In addition to its role as a component of the Cul4A E3 ubiquitin ligase complex, DDB1 was originally identified as

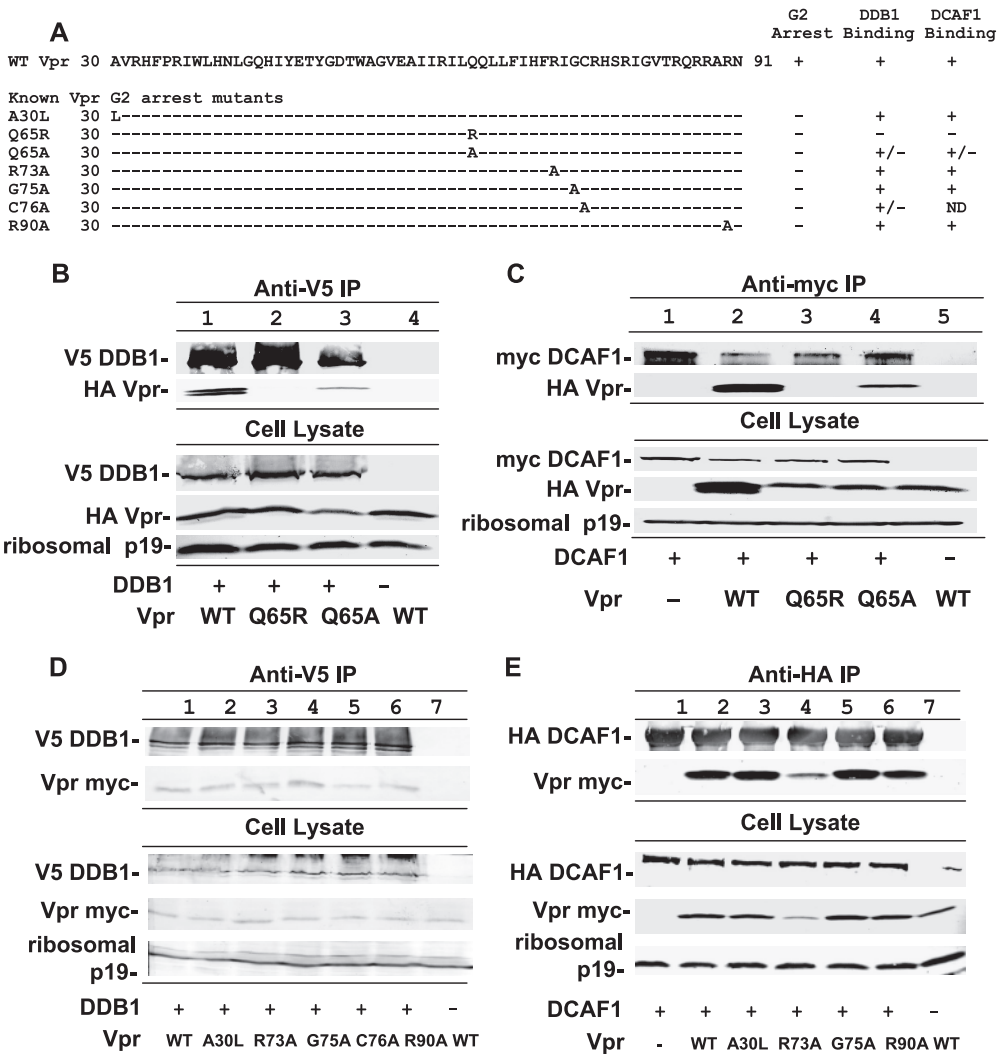
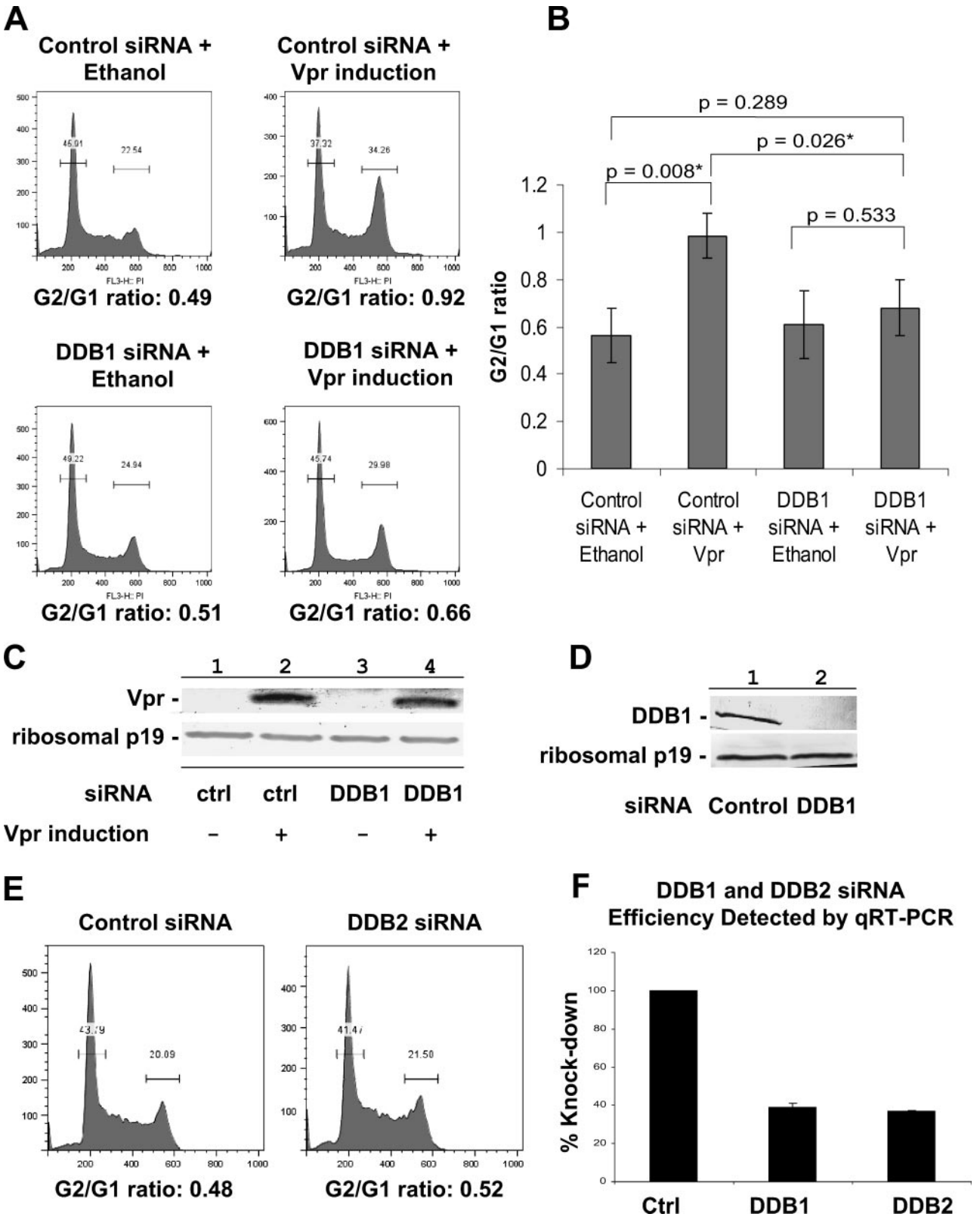


FIG. 2. Interaction of Vpr mutants with DDB1. (A) Characterization of Vpr G_2 arrest mutant constructs with their DDB1 and DCAF1 binding abilities. "+" indicates substantial interaction, "−" indicates lack of interaction, "+/−" indicates reduced interaction, and "ND" indicates no data. (B and C) Interactions of VprQ65-HA mutants with DDB1 and DCAF1. 293T cells were transfected with WT Vpr-HA or VprQ65-HA mutant expression vectors plus DDB1-V5, myc-DCAF1, or a control pcDNA3.1 vector as indicated. Cell lysates from transfected cells were immunoprecipitated (IP) with either anti-V5 or anti-myc antibody. Coprecipitated samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with antibody against V5 tag to detect DDB1-V5, anti-myc antibody to detect myc-DCAF1, or anti-HA antibody to detect Vpr-HA. (D and E) Interactions of Vpr-myc mutants with DDB1 and DCAF1. 293T cells were transfected with WT Vpr-myc or Vpr-myc mutant expression vector plus DDB1-V5, DCAF1-HA, or a control pcDNA3.1 vector as indicated. Cell lysates from transfected cells were immunoprecipitated with either anti-V5 antibody or anti-HA affinity matrix. Coprecipitated samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with antibody against V5 tag to detect DDB1-V5, anti-HA antibody to detect HA-DCAF1, or anti-myc antibody to detect Vpr-myc.

FIG. 3. DDB1 is required for Vpr-induced G_2 arrest. (A) Effect of DDB1 siRNA shutdown in the presence or absence of Vpr induction. A Vpr-inducible 293VE-632 cell line was transfected for 72 h with either DDB1 or control siRNA for 3 days, followed by the addition of either ethanol or muristerone A to induce Vpr expression. The cells were harvested 72 h after induction of Vpr expression, fixed with ice-cold ethanol, and then subjected to RNase A treatment and propidium iodide staining before flow cytometry analysis. The G_2/G_1 ratios were calculated by dividing the proportion of cells in G_2/M by the proportion of cells in G_1. The analysis for flow cytometry data was carried out using FlowJo software. (B) Statistical analysis was carried out using the *t* test (two-sample assuming unequal variances, for the effect of DDB1 siRNA on Vpr-induced G_2 arrest). Error bars indicate standard deviations. (C) Vpr induction in 293VE-632 cell line following DDB1 or control siRNA knockdown. 293VE-632 cell lysates were immunoblotted with anti-Vpr antibody to determine the Vpr induction. Ribosomal p19 protein was used as the sample loading control (ctrl). (D) Efficiency of DDB1 siRNA shutdown. 293VE-632 cell lysates were immunoblotted with anti-DDB1 antibody to determine the efficiency of DDB1 siRNA shutdown. Ribosomal p19 protein was used as the sample loading control. (E) Effect of DDB2 siRNA shutdown in the absence of Vpr induction. The 293VE-632 cell line was transfected for 72 h with either DDB2 or control siRNA for 3 days, followed by the addition of ethanol for 3 days as a mock control for Vpr expression. The cells were harvested 72 h after mock induction of Vpr expression, fixed with ice-cold ethanol, and then subjected to RNase A treatment and propidium iodide staining before flow cytometry analysis. The G_2/G_1 ratios were calculated by dividing the proportion of cells in G_2/M by the proportion of cells in G_1. (F) Efficiency of DDB1 and DDB2 mRNA knockdown detected by quantitative real-time PCR. Approximately 10^6 transfected cells were harvested and subjected to RNA extraction



using TRIzol reagent (Invitrogen). One-fifth of the RNA was reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems), and cDNA was amplified using TaqMan Universal PCR master mix (Applied Biosystems) and an ABI Prism 7000 sequence detection system (Applied Biosystems). The primers/probe sets were prevalidated TaqMan gene expression assays specific for DDB1 and DDB2. The amplifications of DDB1 and DDB2 were normalized to β-actin as an endogenous control (human ACTB β-actin endogenous control 6-carboxyfluorescein/MGB probe; Applied Biosystems). qRT-PCR, quantitative reverse-transcription PCR. Error bars indicate standard deviations.

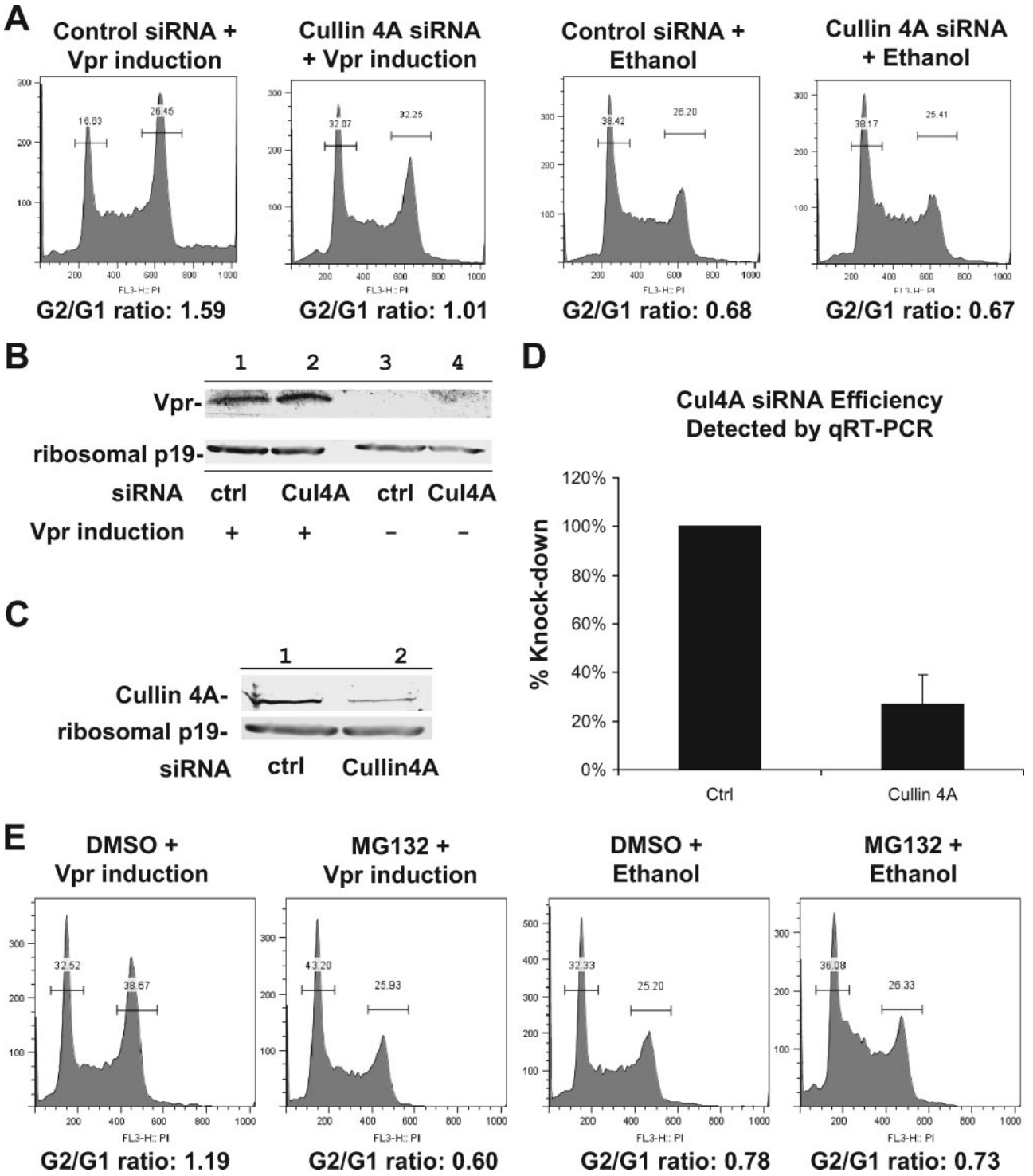


FIG. 4. Vpr-induced G_2 arrest is dependent on Cul4A-mediated proteasome degradation. (A) Effect of Cul4A siRNA shutdown in the presence or absence of Vpr induction. The Vpr-inducible 293VE-632 cell line was transfected for 72 h with either Cul4A or control siRNA for 3 days, followed by the addition of either ethanol or muristerone A to induce Vpr expression. The cells were harvested 72 h after the induction of Vpr expression, fixed with ice-cold ethanol, and then subjected to RNase A treatment and propidium iodide staining before flow cytometry analysis. The G_2/G_1 ratios were calculated by dividing the proportion of cells in G_2/M by the proportion of cells in G_1. (B) Vpr induction in the 293VE-632 cell line following Cul4A or control siRNA knockdown. 293VE-632 cell lysates were immunoblotted with anti-Vpr antibody to determine the Vpr induction. Ribosomal P19 protein was used as the sample loading control. (C) Efficiency of Cul4A siRNA shutdown. 293VE-632 cell lysates were immunoblotted with anti-Cul4A antibody to determine the efficiency of Cul4A siRNA shutdown. Ribosomal P19 protein was used as the sample loading control. (D) Efficiency of Cul4A mRNA knockdown detected by quantitative real-time PCR (qRT-PCR). Approximately 10^6 transfected cells were harvested and subjected to RNA extraction using TRIzol reagent (Invitrogen). One-fifth of the RNA was reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems), and the cDNA was amplified using TaqMan Universal PCR master mix (Applied

playing an important role in nucleotide excision repair (22, 31, 44, 57). In response to DNA damage caused by UV light, DDB1 is translocated into the nucleus through an association with nuclear import protein DDB2, forming the UV-DDB repair complex, which exhibits a high affinity for UV-damaged DNA (33, 54, 57, 58). The simian virus 5 V protein and hepatitis B virus X protein have both been shown to compete directly with DDB2 for binding to DDB1 (7, 29). Consistent with the idea that Vpr and DDB2 competitively bind DDB1, we observed that DDB1, but not DDB2, interacted with Vpr (Fig. 1B, lanes 1 and 2). As expected, both DDB1 and DDB2 interacted with Cul4A (Fig. 1B, lanes 1 and 2).

Interaction of Vpr with DDB1 requires VprBP (DCAF1). Although Vpr has recently been shown to associate with both DDB1 and DCAF1 (26, 46), the molecular assembly of the Vpr/DDB1/DCAF1 complex has yet to be resolved. In order to address this issue, we transfected 293T cells with DCAF1 (Dharmacon) or control short interfering RNA (siRNA) for 24 h, followed by transfection with the same siRNA, along with either Vpr-HA or Vpr-myc expression vector. Cell lysates were prepared 72 h after the second transfection and immunoprecipitated with anti-HA affinity matrix. Coprecipitated samples were analyzed by immunoblotting using anti-HA (to detect Vpr-HA) (Covance), anti-DDB1 (Zymed), and anti-DCAF1 (Shanghai Genomics) antibodies. We showed that Vpr-HA was able to coprecipitate with both DDB1 and DCAF1 in the presence of control siRNA (Fig. 1C, lane 3). In contrast, in the presence of DCAF1 siRNA knockdown, Vpr-HA was unable to immunoprecipitate DDB1 and DCAF1 (Fig. 1C, lane 1). DCAF1 siRNA efficiently reduced endogenous DCAF1 expression, as indicated by immunoblotting and quantitative real-time PCR (Fig. 1C and D). This result suggests the model in which Vpr interaction with DDB1 occurs through DCAF1 (Fig. 5A). During the revision of the manuscript, DeHart et al. also reported similar findings suggesting that Vpr recruitment of DDB1 requires DCAF1 (13).

Interaction with DDB1 is not sufficient for Vpr-induced G_2 arrest. It has been previously reported that VprQ65 residue is important for binding to DCAF1 (26). Since it has been postulated that Vpr recruitment of DDB1 requires DCAF1, we predict that VprQ65 mutants that are defective for DCAF1 binding will also show defects in DDB1 binding. In comparison to wild-type (WT) Vpr interaction with DDB1 (Fig. 2B, lane 1), the VprQ65R mutant, which did not interact with DCAF1 (Fig. 2C, lane 3), also lost interaction with DDB1 (Fig. 2B, lane 2). In addition, the VprQ65A mutant, which has reduced interaction with DCAF1 (26), also showed reduced interaction with DDB1 (Fig. 2B, lane 3). The Vpr mutation analysis (summarized in Fig. 2A) further supports the model in which Vpr recruits DDB1 through DCAF1, as Vpr mutants which retained interaction with DCAF1 also retained interaction with DDB1 (Fig. 2D and E), while VprQ65 mutants with defective DCAF1 interactions displayed similar defects in DDB1 interactions (Fig. 2B and C).

As proposed by Schröfelbauer et al., it is plausible that Vpr binds to DDB1 and interferes with DDB1's normal cellular function in triggering G_2 arrest (46). In such a case, all G_2 arrest-defective Vpr mutants would be expected to have lost the ability to bind DDB1. Alternatively, Vpr could recruit DDB1 as a copartner to form an E3 ubiquitin ligase complex, thereby destroying a cell cycle regulator that induces G_2. If this second scenario is correct, then Vpr would require at least two functional domains: one to recruit DDB1-containing E3 ligase components and another to recognize the substrate. We therefore tested several Vpr mutants known to be defective in inducing G_2 arrest (summarized in Fig. 2A), comparing their interaction with DDB1 to that of WT Vpr by coimmunoprecipitation analysis. The comparison to WT Vpr showed that VprA30L, VprR73A, VprG75A, and VprR90A all maintained the ability to bind to DDB1 (Fig. 2D). VprC76A showed a slight reduction in DDB1 binding compared to WT Vpr (Fig. 2D). The Vpr mutants VprA30L, VprR73A, VprG75A, and VprR90A, which were able to bind DDB1 (Fig. 2D), were also able to retain efficient interaction with DCAF1 (Fig. 2E). Therefore, although Vpr may disrupt the formation of normal cellular DDB1 complexes by binding to DDB1 (46), its interaction with DDB1 is not sufficient to induce G_2 arrest.

DDB1 is important for Vpr-induced G_2 arrest. To further examine the role of DDB1 in Vpr-induced G_2 arrest, we determined the effect on G_2 arrest of silencing DDB1 with siRNA (Dharmacon) in the absence and presence of Vpr. Using a Vpr-inducible expression system comprised of a 293VE-632 cell line stably transfected with an inducible Vpr expression vector, we were able to study the effects of Vpr induction on G_2 cell cycle arrest (64). The 293VE-632-inducible Vpr cells were transfected with either DDB1 or control siRNA for 3 days, followed by the induction of Vpr expression with 1 μM muristerone A (Invitrogen) for 3 days. In the absence of Vpr induction (ethanol control), both the control siRNA- and the DDB1 siRNA-transfected cells exhibited similar G_2/G_1 ratios of 0.49 and 0.51, respectively (Fig. 3A). DDB1 siRNA efficiently reduced endogenous DDB1 expression, as indicated by immunoblotting and quantitative real-time PCR (Fig. 3D and F). These results indicated that DDB1 knockdown in 293VE-632 cells did not cause G_2/M arrest. Thus, although DDB1 knockdown has been shown to induce G_2 arrest in HeLa cells (34, 46), 293 cells provide a unique system to study the role of DDB1 in Vpr-induced G_2 arrest.

As expected, the induction of Vpr expression (Fig. 3C) in control siRNA-transfected 293VE-632 cells produced an in-

Biosystems) and an ABI Prism 7000 sequence detection system (Applied Biosystems). The primers/probe sets were prevalidated TaqMan gene expression assays specific for Cul4A. Amplifications of Cul4A were normalized to β-actin as an endogenous control (human ACTB β-actin endogenous control 6-carboxyfluorescein/MGB probe; Applied Biosystems). Error bars indicate standard deviations. (E) Effect of MG132 proteasome inhibitor in the presence or absence of Vpr induction. The 293VE-632 cell line was treated for 56 h with either ethanol or muristerone A to induce Vpr expression before the addition of either DMSO or MG132. The cells were harvested 16 h later, fixed with ice-cold ethanol, treated with RNase A, and stained with propidium iodide before flow cytometry analysis. The G_2/G_1 ratios were calculated by dividing the proportion of cells in G_2/M by the proportion of cells in G_1.

creased G_2/G_1 ratio of 0.92 (compared to a G_2/G_1 ratio of 0.49 in the absence of Vpr [$P = 0.008$]) (Fig. 3A and B), consistent with the previously reported Vpr-induced G_2 arrest in 293VE-632 cells (64). However, Vpr-induced G_2 arrest was significantly inhibited in 293VE-632 cells transfected with DDB1 siRNA, with a G_2/G_1 ratio of 0.66; the G_2/G_1 ratio was 0.51 in the control cells (Fig. 3A). In repeated experiments, Vpr-induced G_2 arrest was inhibited by 60 to 75% in the presence of DDB1 siRNA, compared to the control siRNA (P value = 0.026) (Fig. 3B). Quantitative real-time PCR analysis indicated that DDB1 siRNA reduced the level of DDB1 mRNA by approximately 61% (Fig. 3F), consistent with the incomplete inhibition of Vpr-induced G_2 arrest by DDB1 siRNA. Thus, DDB1 is apparently required for Vpr-induced G_2 arrest.

Schröfelbauer et al. proposed that Vpr-induced G_2 arrest occurs through the disruption of the normal function of the DDB1/DDB2 complex via Vpr recruitment of DDB1 (46). Our data are more consistent with the model proposed by Le Rouzic et al. (26) in which Vpr-induced G_2 arrest occurs through recruiting the DDB1/DCAF1 complex and not through the disruption of DDB1/DDB2 function. Our results lend support to the model of Vpr recruiting DDB1 for G_2 arrest, as DDB2 siRNA knockdown (Fig. 3F) in the absence of Vpr did not result in G_2 arrest (Fig. 3E). These data are also in agreement with a recent publication demonstrating that cells from xeroderma pigmentosum complementation group E (XPE), which lacks DDB2/XPE function, can still undergo Vpr-induced G_2 arrest (13).

Cul4A is required for Vpr-induced G_2 cell cycle arrest. Since Vpr was shown to associate with both DDB1 and DCAF1, which are known to interact with Cul4A, we sought to investigate whether Cul4A is required for Vpr-induced G_2 arrest. The 293VE-632-inducible Vpr cells were transfected with either Cul4A (Dharmacon) or control siRNA, followed by the induction of Vpr expression with 1 μM muristerone A. In the absence of Vpr induction, the control siRNA- and Cul4A siRNA-transfected cells exhibited G_2/G_1 ratios of 0.68 and 0.67, respectively (Fig. 4A). Cul4A siRNA knockdown showed a moderate reduction in endogenous Cul4A expression, as indicated by immunoblotting and quantitative real-time PCR (Fig. 4C and D). The induction of Vpr expression by muristerone A (Fig. 4B) in control siRNA-transfected 293VE-632 cells produced an increased G_2/G_1 ratio of 1.59; the G_2/G_1 ratio was 0.68 in the absence of Vpr (Fig. 4A). We show for the first time that Vpr-induced G_2 arrest was inhibited in 293VE-632 cells transfected with Cul4A siRNA, with a G_2/G_1 ratio of 1.01; the G_2/G_1 ratio was 1.59 for the control siRNA-transfected cells (Fig. 4A). Both the Cul4A immunoblotting and the quantitative real-time PCR analysis indicated that Cul4A siRNA did not completely shut down endogenous Cul4A levels (Fig. 4C and D), consistent with the ~64% inhibition of Vpr-induced G_2 arrest by Cul4A siRNA (Fig. 4A). We hereby suggest that an E3 ubiquitin ligase, Cul4A, is involved in Vpr-induced G_2 arrest.

To further support the hypothesis that Vpr recruitment of DDB1-containing E3 ubiquitin ligase complexes causes G_2 arrest through the degradation of cell cycle regulatory proteins, we examined the effect of the proteasome inhibitor MG132 on Vpr-induced G_2 arrest. In the absence of Vpr induction, the control (dimethyl sulfoxide [DMSO]) and MG132-treated cells exhibited similar G_2/G_1 ratios (Fig. 4E). In the presence of Vpr induction and an intact functioning proteasome, control (DMSO-treated) cells had a G_2/G_1 ratio of 1.19, clearly demonstrating a significant increase in the percentage of cells arrested in G_2, compared to a G_2/G_1 ratio of 0.78 in control cells without Vpr induction (Fig. 4E). In contrast, the cells had a G_2/G_1 ratio of 0.60 in the presence of MG132 and Vpr induction; the ratio was 0.73 in cells without Vpr induction (Fig. 4E).

We are cautious regarding the interpretation of the data from MG132 treatment, as inhibition of the proteasome is known to interfere with the normal degradation of cell cycle control proteins, which will in turn affect cell cycle progression. Although there are caveats in the interpretation of the results from treating Vpr-expressing cells with MG132, we attempt to minimize the nonspecific effects of a proteasome inhibitor on cell cycle progression. We first induced Vpr expression for 56 h to promote G_2 arrest before transient treatment with the proteasome inhibitor MG132 for 16 h to see whether MG132 could relieve Vpr-induced G_2 arrest.

The collective results from proteasome inhibitor and Cul4A siRNA knockdown experiments suggest that Vpr-induced G_2 cell cycle arrest is dependent on an intact Cul4A-mediated ubiquitin proteasome pathway. The data substantiate the hypothesis that Vpr recruits the DDB1/DCAF1/Cul4A-containing complex to facilitate the ubiquitination and subsequent proteasomal degradation of cell cycle regulatory proteins, resulting in G_2 arrest.

The data presented here, and those published recently by others (13, 26, 46), indicate that an interaction occurs between HIV-1 Vpr and DDB1. Two models have been proposed to explain the role of this Vpr/DDB1/DCAF1 interaction in G_2 cell cycle arrest. The first suggests that Vpr binding to DDB1 induces G_2 arrest by interfering with the normal cellular functions of DDB1 (46); however, this scenario is unlikely to occur in 293 cells, since several Vpr mutants were as efficient as WT Vpr in binding DDB1 but were unable to induce G_2 arrest (Fig. 2D). Thus, binding to DDB1 is not sufficient for Vpr-induced G_2 arrest. Furthermore, reducing DDB1 levels with siRNA did not induce G_2 arrest in the absence of Vpr (Fig. 3A). A similar siRNA treatment of DDB2, another component of the UV-induced DNA damage repair complexes, also did not induce G_2 arrest in the absence of Vpr (Fig. 3E).

The second model proposes that Vpr recruits DDB1/DCAF1 to form an E3 ubiquitin ligase complex that can target cell cycle regulatory protein(s) for destruction and induce G_2 arrest (13, 26). The data presented here support this model. Although we and others established that Vpr interaction with DDB1 requires DCAF1 (Fig. 5A) (13), it is plausible that Vpr might interact with both DDB1 and DCAF1 (Fig. 5B). In this case, like other WD40 repeat proteins, Vpr would act as a substrate receptor within the Cullin-DDB1 complex to dictate the substrate specificity of the Cullin-DDB1-DCAF1-Vpr E3 ligase. One class of Vpr targets that has already been identified is the uracil DNA glycosylases, such as UNG2 and SMUG1 (46). Our results suggest that Vpr-induced G_2 arrest is mediated by Cullin-DDB1-DCAF1-Vpr E3 ligase activity; knocking down the DDB1 expression level impaired Vpr-induced G_2 arrest (Fig. 3A). More importantly, we demonstrated for the first time that proteasomal activity, which is a downstream effector of E3 ubiquitin ligases, and Cul4A, a putative E3

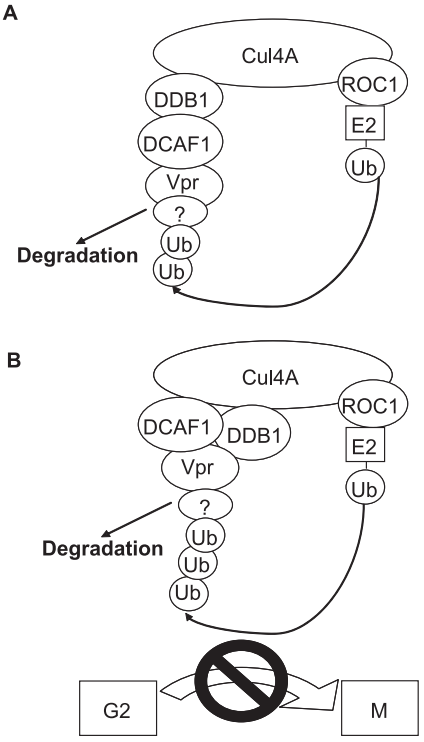


FIG. 5. HIV-1 Vpr acts as a substrate receptor in a DDB1/DCAF1/Cullin containing E3 ubiquitin ligase. (A) Vpr interacts with DDB1 through DCAF1, forming an E3 ubiquitin ligase complex to target the unknown cellular factors for polyubiquitination and proteasomal degradation to induce G_2 arrest. (B) An alternative model in which Vpr is required to interact directly with both DDB1 and DCAF1 to form an E3 ubiquitin ligase complex leading to polyubiquitination and proteasomal degradation of unknown cellular factors resulting in G_2 arrest.

ubiquitin ligase in the Vpr/DDB1/DCAF1 complex, were found to be required for Vpr-induced G_2 arrest (Fig. 4A). Although Cul4A is shown to play a role in Vpr-induced G_2 arrest, Cul4A knockdown did not completely inhibit Vpr-induced G_2 arrest. It is plausible that other Cullins could also be recruited by the DDB1/DCAF1/Vpr complex to degrade target substrates, resulting in a G_2 arrest phenotype.

HIV-1 Vif also recruits cellular Cul5, elongin B, and elongin C to form an E3 ubiquitin ligase that mediates APOBEC3G polyubiquitination and degradation (59). The C-terminal region of Vif contains a BC-box and an HCCH motif, which bind elongin B/elongin C and Cul5, respectively (35, 38, 61). The N-terminal region of Vif mediates APOBEC3G/APOBEC3F recognition (37, 47, 51, 55). Although a Vpr mutant (L64P) that has been identified to lose the ability to interact with DDB1 is defective for G_2 arrest (46), our results indicate that other Vpr mutants that are defective for G_2 arrest can still interact with DDB1 (Fig. 2D). This suggests that the DDB1 association is important but not sufficient for Vpr-induced G_2 arrest. By analogy to HIV-1 Vif, some of these Vpr mutants may have lost the ability to interact with the putative cellular target protein(s) whose destruction by proteasomes is critical for the induction of G_2 arrest. The identification of these Vpr-targeted cellular proteins will not only further our understanding of Vpr-mediated G_2 arrest but may also shed light on the normal regulation of the cell cycle.

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