HIV-1 Vpr Induces the K48-Linked Polyubiquitination and Proteasomal Degradation of Target Cellular Proteins to Activate ATR and Promote G2 Arrest

Jean-Philippe Belzile [1], Jonathan Richard [1], Nicole Rougeau, Yong Xiao, and Éric A. Cohen [1*]

Laboratory of Human Retrovirology, Institut de Recherches Cliniques de Montréal and [1]Department of Microbiology and Immunology, Université de Montréal, Montreal, Quebec, Canada.

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[*]Corresponding author: Mailing address: Laboratory of Human Retrovirology, Institut de Recherches Cliniques de Montréal, 110, Avenue des Pins Ouest, Montreal, Quebec, Canada H2W 1R7. Phone: (514) 987-5804; Fax: (514) 987-5691; E-mail: eric.cohen@ircm.qc.ca.

ABSTRACT

HIV-1 viral protein R (Vpr) induces a cell cycle arrest at the G2/M phase by a mechanism involving the activation of the DNA damage sensor ATR. We and others recently showed that Vpr performs this function by subverting the activity of the DDB1-CUL4A (VPRBP) E3 ubiquitin ligase. Vpr could thus act as a connector between the E3 ligase and an unknown cellular factor whose ubiquitination would induce G2 arrest. While attractive, this model is solely based on the indirect observation that some mutants of Vpr retain their interaction with the E3 ligase but fail to induce G2 arrest. Using a tandem affinity purification approach, we observed that Vpr interacts with ubiquitinated cellular proteins and that this association requires the recruitment of an active E3 ligase given that depletion of VPRBP by RNA interference or overexpression of a dominant-negative mutant of CUL4A decreased this association. Importantly, G2-arrest-defective mutants of Vpr in the C-terminal putative substrate-interacting domain displayed decreased association with ubiquitinated proteins. We also found that inhibition of proteasomal activity increased this association and that the ubiquitin chains were at least in part constituted of classical K48 linkages. Interestingly, inhibition of K48 polyubiquitination specifically impaired Vpr-induced phosphorylation of H2AX, an early target of ATR, but did not affect UV-induced H2AX phosphorylation. Overall, our results provide direct evidence that association of Vpr with the DDB1-CUL4A (VPRBP) E3 ubiquitin ligase induces the K48-linked polyubiquitination of yet-unknown cellular proteins resulting in their proteasomal degradation and ultimately leading to activation of ATR and G2 arrest.

INTRODUCTION

Viruses have evolved ways to modulate the host cellular environment in order to promote efficient viral replication and to disrupt elements of innate or acquired immunity. One strategy particularly favored by viruses to achieve these goals is to hijack components of the host ubiquitin-proteasome system in order to induce degradation, block the degradation, or modulate the expression and activity of specific cellular factors (4, 8, 16). Human immunodeficiency virus (HIV) is no exception to this precept. HIV harbors two extensively studied accessory proteins, viral protein U (Vpu) and viral infectivity factor (Vif), that are usurping the cellular ubiquitin-proteasome system in order to respectively degrade neo-synthesized CD4 and the cytidine deaminases APOBEC3F (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F) and 3G (37). Recently, we and several other investigators demonstrated that a third accessory protein of HIV, viral protein R (Vpr), would also exert its function by usurping the host ubiquitination machinery via recruitment of an E3 ubiquitin ligase complex composed of VPRBP (viral protein R binding protein, also known as DCAF1), damaged DNA binding protein 1 (DDB1) and Cullin 4A (CUL4A) (5, 14, 22, 31, 43, 49, 55).

Ubiquitination is a post-translational modification that involves the isopeptidic covalent linkage of the C-terminus of a small protein called ubiquitin most commonly to lysine acceptor residues. Conjugation of ubiquitin is performed by E3 ubiquitin ligase complexes, which also control specificity via direct interaction with substrates (24, 33). The Cullin-RING E3 ubiquitin ligases (CRL) structured around the scaffold protein CUL4A (CRL4A) interact with the 126-kDa DDB1 adaptor in order to recruit substrate receptors of the WDxR family (32). Mass spectrometric analyses of CUL4A-DDB1

complexes have revealed physical interactions to at least 30 different WDxR substrates receptors (3, 19, 21, 26), suggesting that CRL4A E3 ligases would likely regulate the function of hundreds of cellular proteins. Surprisingly however, relatively few cellular proteins have been shown to date to be regulated by these complexes. CRL4A (DDB2) promotes the ubiquitination of histone 2A, 3 and 4 (28, 53), of xeroderma pigmentosum group C protein (XPC) (48) and probably of xeroderma pigmentosum group A protein (XPA) (52) to facilitate UV damage repair. CRL4A (CSA) and CRL4A (DET1-COP1) induce the proteolysis of respectively Cockayne syndrome type B gene product (CSB) (18) and c-JUN (56). PCNA (proliferating cell nuclear antigen), via the recruitment of a CRL4A (CDT2) ligase, has been shown to regulate the degradation of the replication licensing factor CDT1 (20, 35) as well as of the CDK inhibitor p21 (1, 39). The 180-kDa VPRBP/DCAF1 WDxR substrate receptor was identified more than a decade ago as a Vpr-interacting protein and was found to be expressed at the mRNA level in most tissues (59, 61). However, its normal cellular functions remained elusive until recently. Huang and Chen demonstrated that CRL4A (VPRBP) induces the rapid degradation of the tumor suppressor Merlin (NF2, neurofibromin 2) following serum stimulation (23). Moreover, depletion of VPRBP reduced the rate of DNA replication, blocked cells in S-phase, and impeded cellular proliferation (22, 38). Consequently, genetic ablation of VPRBP in mouse (38) and in the evolutionary distant *Arabidopsis thaliana* (60) led to embryonic lethality, suggesting an essential role for VPRBP in cell cycle as well as in development.

The small HIV-1 accessory protein Vpr induces a cell cycle arrest in the G2/M phase in various cell types including transformed cell lines as well as primary lymphocytes (27, 41, 63). Notably, abnormal accumulation of infected cells in G2 can be observed in

tissues from patients infected by HIV-1 (63). Although the function of this cell cycle arrest has remained elusive, its molecular mechanism has recently begun to be elucidated. Several investigators reported that Vpr activates the canonical ATR (ataxia telangiectasia and Rad3 related) DNA damage/stress checkpoint (2, 29, 42, 62, 63). Vpr-mediated activation of ATR is accompanied by the formation of DNA repair foci that include RPA (replication protein A), HUS1, RAD17, BRCA1 (breast cancer 1, early onset), TP53BP1 (tumor protein p53 binding-protein 1), and γ-H2AX (phosphorylated histone 2A variant X) (2, 29, 62, 63) and by activation of the CHEK1 kinase (42). This series of events leads to inactivation of the CDC2/cyclin B complex, a master regulator of the G2 to M transition, and ultimately prevents entry into mitosis. In addition, Vpr was recently found to associate with DDB1 and CUL4A via a direct interaction with VPRBP (5, 14, 22, 31, 43, 49, 55). Depletion of VPRBP by RNA interference drastically impaired Vpr-mediated G2 arrest. Similarly, mutations of Vpr in the hydrophobic leucine-rich core region abrogated binding to CRL4A (VPRBP) and concomitantly impaired G2 arrest. In contrast, mutants of Vpr in the C-terminal arginine-rich domain were not compromised for the association with the E3 ligase but nevertheless failed to induce G2 arrest (5, 14, 22, 31, 43, 49, 55). These later mutants also displayed trans-dominant negative activity (14), thus indicating that the recruitment of this E3 ubiquitin ligase is essential but not sufficient to induce G2 arrest. The simplest explanation for these observations is that Vpr would recruit cellular substrates to be ubiquitinated by the complex. Ubiquitination of these yet-unknown proteins would lead to their degradation or modulation of their activity, ultimately resulting in ATR activation and G2 arrest (13). This is not the only instance in which Vpr would be implicated in the degradation of a cellular protein. Vpr directly interacts and induces the proteasomal degradation of the cellular enzyme UNG2 (uracil-DNA glycosylase 2; also

known as CCNO) independently of the induction of a cell cycle arrest (43, 44). Vpr-induced degradation of UNG2 was found to be mediated by DDB1 (43) but surprisingly did not require VPRBP (55). In addition, viral protein X (Vpx), a paralog of Vpr present in HIV-2 and some SIV isolates, is also able to recruit CRL4A (VPRBP) (6, 31, 45, 47). In contrast to Vpr, Vpx does not induce G2 arrest, but counteracts a putative cellular restriction factor expressed in macrophages and dendritic cells that targets a post-entry step critical for efficient reverse transcription (37). Importantly, the presence of an active proteasome system and the recruitment of CRL4A (VPRBP) by Vpx were shown to be required for the inhibition of the restriction factor, suggesting that, similarly to Vpr, Vpx would act via the CRL4A (VPRBP)-mediated ubiquitination and proteasomal degradation of cellular proteins (6, 17, 45, 47).

While very attractive, this model is however solely based on the indirect observation that some C-terminal mutants of Vpr retain their interaction with the E3 ligase but fail to induce G2 arrest. Other models for the activity of Vpr have also been proposed and include inhibition of DDB1 functions (43) or overall increased activity of CRL4A (VPRBP) (22). Moreover, it is unclear whether this association of Vpr with CRL4A (VPRBP) is the result of overexpression in transformed cell lines and whether this interaction would occur during infection of primary lymphocytes. Therefore, we sought to confirm the physiological significance of the Vpr-VPRBP interaction as well as obtain more direct evidence that Vpr is indeed recruiting cellular proteins and inducing their ubiquitination and proteasomal degradation. Herein, we show that Vpr interacts with VPRBP and DDB1 during infection of primary CD4+ T-lymphocytes. In addition, using a tandem affinity approach and overexpression of a dominant-negative tagged ubiquitin

mutant, we observed that wild type Vpr could specifically associate with unknown ubiquitinated cellular proteins and that this interaction required the recruitment of an active CRL4A (VPRBP) E3 ubiquitin ligase complex. Moreover, C-terminal G2-arrest-defective mutants of Vpr displayed reduced association to these ubiquitinated proteins. We also provide evidence that Vpr induces the K48-linked polyubiquitination of these cellular proteins leading to their rapid proteasomal degradation. Finally, these Vpr-induced ubiquitination events were specifically necessary for Vpr to induce phosphorylation of H2AX, an early marker of ATR-mediated DNA damage/stress checkpoint activation.

MATERIALS AND METHODS

Cell lines and antibodies

HEK293T and HeLa cells were cultured as previously described (58). The anti-HA tag and anti-Myc tag monoclonal antibodies were, respectively, clones 12CA5 and 9E10. Rabbit polyclonal antibodies against VPRBP and DDB1 were respectively obtained from Accurate Chemical and Scientific Corporation (Westbury, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Vpr was detected using a rabbit polyclonal antibody directed against a Vpr N-terminal peptide (12). The anti-GFP antibody was obtained from Molecular Probes (Invitrogen, San Diego, CA, USA) and the anti-phospho-H2AX (Ser139) antibody was clone JBW301 from Upstate (Millipore, Billerica, MA, USA). The rabbit anti-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA)

Plasmid construction

Plasmids SVCMV-TAP, SVCMV-TAP-Vpr (WT), and SVCMV-TAP-Vpr (Q65R) were described previously (5). Plasmids expressing TAP-Vpr (1-78), TAP-Vpr (1-86), and TAP-Vpr (R87A, R88A) were generated by subcloning the SalI-BamH1 fragment from respectively SVCMV-3HA-Vpr (1-78), SVCMV-3HA-Vpr (1-86), and SVCMV-3HA-Vpr (R87A, R88A) (57) into SVCMV-TAP-Vpr (WT). Plasmids expressing TAP-Vpr (1-84) and TAP-Vpr (1-90) were generated by PCR using a strategy described previously (5). Plasmids expressing Myc-His-tagged ubiquitin K48R (54) and HA-tagged ubiquitin (WT, K0, and K48R) (34) were kind gifts of Dr R. Kopito (Stanford University, CA, USA) and Dr T. Dawson (Johns Hopkins University, Baltimore, USA). The plasmid expressing HA-Ub (K63R) was constructed by site-directed mutagenesis using the Quickchange II mutagenesis kit (Stratagene, La Jolla, CA, USA). The GFP-expressing plasmid pQBI-25 was obtained from Qbiogene (Carlsbad, CA, USA). Vectors expressing scrambled shRNA and VPRBP shRNA were obtained from Open Biosystems (Huntsville, AL, USA). The CUL4A dominant-negative construct (55) was kindly donated by Dr C. de Noronha (University of Albany, NY, USA). The construction of the infectious molecular clone HxBru (Vpr-) was described previously (30). HxBru (HA-Vpr) was generated by PCR by adding an HA-tagged at the N-terminus of Vpr, which resulted in the addition of 9 extra amino acids at the C-terminus of Vif. The lentiviral vectors WPI and WPI-Vpr as well as the SVCMV-IN-VSV-G expression construct were described previously (5).

Transfection, tandem affinity purification (TAP) and immunoprecipitation

HEK293T cells were transfected using the calcium phosphate precipitation method. Forty-eight hours later, cells were harvested, washed and lysed in triton lysis buffer (50 mM Tris-

HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and complete protease inhibitors) or RIPA buffer (8 mM Na_2HPO_4 pH 7.2, 2 mM NaH_2PO_4, 140 mM NaCl, 1% NP-40, 0.05% SDS, 12 mM deoxycholate, and complete protease inhibitors). Tandem affinity purification was performed as described previously (5). In some experiments, where indicated, IgG pulldown assays were performed instead of the full tandem affinity purification, as described previously (5). Immunoprecipitations were performed using 50 ul of 50% anti-HA-coupled agarose beads (Sigma-Aldrich, St. Louis, MO, USA) followed by extensive washes in triton lysis buffer and elution with 100 μg/ml HA peptide. Eluted proteins were separated on a 12.5% SDS-PAGE gel and western blot analyses of eluted proteins were performed. Ubiquitinated proteins were revealed using monoclonal antibodies directed at Myc or HA tags depending on the ubiquitin construct used.

Production and titration of viruses and viral vectors

The production and titration of infectious viral particles as well as of the lentiviral vectors expressing GFP (WPI) or co-expressing GFP and Vpr (WPI-Vpr) were performed as described previously (5, 57). Murine leukemia virus-based retroviral vectors expressing VPRBP-targeting (ShVprBP) or scrambled control (shControl) shRNA were produced by transfection of 15 μg of vector, 12 μg of the packaging construct pCIG3-N (9), and 5 μg of the VSV-G-expressing plasmid pSVCMV-IN-VSV-G in 1.5 million HEK293T cells using the calcium phosphate precipitation method. Vector-containing supernatants were 0.45 micron-filtered and used immediately.

Infection of primary lymphocytes

Peripheral blood mononuclear cells (PBMCs) were extracted by the Ficoll method from whole blood obtained from consenting healthy adult donors who gave written informed consent under research protocols approved by the research ethics review board of the Institut de recherches cliniques de Montreal. CD4+ T-lymphocytes were purified from PBMCs by magnetic negative selection using the CD4+ T cell isolation Kit II and the AutoMACS Pro system according to the manufacturer instructions (Miltenyi Biotec, Auburn, CA, USA). CD4+ T-cells were cultured as previously described (57). Ten millions activated CD4+ cells were mock-infected or infected with HxBru (Vpr-) or HxBru (HA-Vpr) viruses at a MOI of 0.01. Five days after infection, cells were lysed in triton lysis buffer and subjected to anti-HA immunoprecipitation as described above.

Fluorescence immunohistochemistry

Fifty thousand HeLa cells were seeded on cover slips in 24-well plates and transfected with lipofectamine 2000 reagent according to the manufacturer's intruction. Twenty-four hours after transfection, cells were transduced with WPI or WPI-Vpr at a multiplicity of infection of 2.5 in presence of 8 μg/ml polybrene. Two days later, cells were processed for confocal fluorescence immunohistochemistry as previously described (15).

Generation of HEK293T cells with stable depletion of VPRBP

HEK293T cells were transduced with ShControl and ShVprBP retroviral vectors in presence of 8 μg/ml polybrene. Two days after transduction, cells were selected for 10 days with 1 μg/ml puromycin and single-cell clones were then isolated by the limited dilution method. Western blot were performed on ShControl- and ShVprBP-transduced

stable clones and the clone with the most significant decrease in VPRBP expression was selected.

Cell cycle, cell proliferation and apoptosis assays

Cell cycle profiles were determined by flow cytometry using propidium iodide staining as previously described (5). Apoptosis was assayed by flow cytometry using Annexin V and propidium iodide as a dead cell counter-stain as described previously (58). Cell proliferation kinetics was monitored by flow cytometry using a standard CFSE (carboxyfluorescein succinimidyl ester) assay.

RESULTS

Vpr associates with VPRBP and DDB1 in primary CD4+ lymphocytes.

The interaction between Vpr and the CRL4A (VPRBP) E3 ubiquitin ligase and its functional importance for Vpr-induced G2 arrest, although strongly established, were however solely demonstrated by overexpression studies in transformed cell lines. In order to determine whether Vpr could recruit the CRL4A (VPRBP) E3 ubiquitin ligase in more physiological conditions, primary CD4+ T-lymphocytes were infected with HxBru-derived virus expressing hemagglutinin (HA)-tagged Vpr (HA-Vpr) or deleted for Vpr at a multiplicity of infection of 0.01. Five days after infection, cells were harvested and subjected to immunoprecipitation using anti-HA-conjugated agarose beads and immunoprecipitated proteins were detected by western blot. Endogenous VPRBP and DDB1 could be detected in the lysate of activated primary CD4+ T-lymphocytes, thus confirming their expression at the protein level (Fig 1). Co-immunoprecipitation of

VPRBP and DDB1 could be observed in cells infected with HxBru (HA-Vpr) viruses but not in HxBru (Vpr-) or mock-infected cells (Fig 1). Therefore, recruitment of VPRBP and DDB1 by Vpr occurs in conditions mimicking an *in vivo* infection and is thus not the result of overexpression of Vpr in transformed cell lines.

Vpr interacts with ubiquitinated cellular proteins.

We next sought to obtain additional data on the mechanisms by which Vpr utilizes this complex to induce G2 arrest. If Vpr indeed acts as a connector between an E3 ubiquitin ligase complex and cellular proteins and that this association leads to ubiquitination of these substrates, we hypothesized that we should be able to observe Vpr interacting with some of these unknown ubiquitinated proteins. To assess this premise, we developed a tandem affinity purification (TAP) procedure (5) that takes advantage of a Myc-tagged ubiquitin K48R mutant with the unusual properties of acting as a weak chain terminator and stabilizing polyubiquitinated products (7, 54). Of note, the TAP-tagged Vpr (TAP-Vpr) construct used in the procedure is able to induce G2 arrest although to a lower extent than untagged native Vpr (Fig S1 and (5)). In these conditions, we were able to detect an interaction between TAP-Vpr and unknown cellular ubiquitinated proteins, as revealed by the anti-Myc-reacting protein smear in the TAP-Vpr pulldown, but not with the TAP tag alone (Fig 2A). This association was also detected in stringent extraction conditions such as in RIPA buffer and in high salt buffer (400 mM NaCl) (data not shown). The Myc-tagged ubiquitination signal detected in presence of pulled-down Vpr was the result of bound cellular ubiquitinated proteins and not of ubiquitination of Vpr itself since heat-denaturation of proteins in RIPA buffer prior to purification of TAP-Vpr complexes abolished this signal. (Fig 2B, compare lane 3 to lane 4) For this experiment, we could not

perform the full tandem affinity purification procedure because the TAP tag could not bind to calmodulin beads following denaturation. Consequently, these experiments involved solely an IgG pulldown step, hence explaining why the enrichment of ubiquitination observed with Vpr is less important in this particular condition (Fig 2B, compare lane 3 to lane 1). Nevertheless, retention of the ubiquitination signal under denaturating conditions would have indicated that this ubiquitination originated from ubiquitin covalently conjugated to Vpr itself and not to associated proteins. Therefore, in agreement with our model, Vpr is capable of associating with ubiquitinated cellular proteins.

Recruitment of a catalytically active CRL4A (VPRBP) complex is required to observe Vpr-associated ubiquitinated proteins.

This association of Vpr with cellular ubiquitinated proteins suggests that these proteins might be ubiquitinated by Vpr following recruitment of the CRL4A (VPRBP) E3 ubiquitin ligase. On the other hand, these cellular proteins might be already ubiquitinated as part of their normal metabolism before their interaction with Vpr and would thus not constitute substrates of the Vpr ubiquitin ligase complex. To distinguish between these two possibilities, we analyzed the effect of knocking down VPRBP on the association of Vpr with ubiquitinated proteins. Transient depletion of VPRBP with shRNA (small hairpin RNA) resulted in a significant decrease in the association of Vpr with cellular ubiquitinated proteins (Fig 3A, compare lanes 3 and 4), suggesting that the association of ubiquitinated proteins to Vpr involves the recruitment of the CRL4A (VPRBP) E3 ligase. Moreover, overexpression of a dominant negative construct of CUL4A (Cul4A DN) produced a similar decrease in the association of TAP-Vpr with ubiquitinated proteins, thus corroborating the results obtained with the depletion of VPRBP and indicating that the

CRL4A (VPRBP) E3 ligase must be catalytically active in order to detect these Vpr-associated ubiquitinated proteins (Fig 3B, compare lanes 3 and 4). However, these results do not exclude the possibility that this association of Vpr with ubiquitinated proteins might result from the sole association with ubiquitinated components of the E3 ligase complex without necessarily implicating ubiquitinated substrates.

To address this issue, we investigated this association in the context of Vpr mutants defective for their interaction with the E3 ligase or for the putative G2 arrest substrate protein. Surprisingly, the Q65R mutation in Vpr, which results in a strong reduction of the interaction with VPRBP and DDB1 (5, 14, 31, 49), displayed an increased association with ubiquitinated proteins (Fig 4A), independently of its higher level of expression. These unexpected results may be explained by non-specific interactions that might result from the accumulation of this non-functional mutant protein. To test whether the association of Vpr (Q65R) with ubiquitinated proteins was independent of the recruitment of CRL4A (VPRBP), we constructed 293T-based stable monoclonal cell lines expressing shRNA against VPRBP or non-targeting scrambled shRNA control (Fig S2A). Importantly, we did not detect any significant effect of VPRBP depletion on the cell cycle profile (Fig S2B). We did however observe a slight decrease in the growth kinetics of these cells (Fig S2C) but it did not result in a significant increase in apoptosis (Fig S2D). The level of stable VPRBP knockdown achieved in this cell line was sufficient to almost completely abolish Vpr-mediated G2 arrest compared to the scrambled control cell line (Fig S2E). We then used this pair of cell lines to assess the levels of cellular ubiquitinated proteins associated with the Q65R mutant of Vpr. As expected, the increased association of ubiquitinated proteins with Vpr (Q65R) was independent of the recruitment of the E3 ligase given that

depletion of VPRBP did not affect the levels of ubiquitinated proteins associated with this mutant of Vpr (Fig 4A, compare lanes 5 and 6). In comparison, in this system, we observed a significant reduction of the association of wild type Vpr with ubiquitinated proteins in the VPRBP-knocked-down cell line versus the control cell line (Fig 4A, compare lanes 3 and 4), corroborating results obtained with transient knockdown experiments. Therefore, we hypothesize that the Q65R mutation, in addition to its effect on the binding to VprBP, might also results in major conformational defects that would lead to this non-specific association to ubiquitinated proteins. Indeed, we observed that, in contrast to wild type Vpr which oligomerizes and displays a nuclear localization, Vpr (Q65R) accumulates in part in the cytoplasm as discrete puncta and also fails to efficiently oligomerize (manuscript in preparation). We also investigated the effect of a C-terminal deletion of Vpr, Vpr (1-78), a mutant that fails to induce G2 arrest (Fig S1) while maintaining the interaction with VPRBP (31). Importantly, Vpr (1-78), which should thus not interact with the putative substrate responsible for G2 arrest, failed to interact with ubiquitinated proteins (Fig B, compare lanes 2 and 3). Other deletions (1-84, 1-86, 1-90) or point mutations (R87A, R88A) in the C-terminal domain impaired G2 arrest (Fig S1) and led to a significant decrease of the association with ubiquitinated proteins (Fig. 4C) while maintaining their association to VPRBP (data not shown). Our data indicate that these proteins are not solely ubiquitinated UNG2 molecules given that C-terminal deletions of Vpr retain their interaction with UNG2 (10). Therefore, in agreement with our model, these data further indicate that the putative G2 arrest substrate(s) is ubiquitinated in presence of Vpr and that abrogation of Vpr-substrate(s) or Vpr-CRL4A (VPRBP) interactions impairs Vpr-mediated ubiquitination. Overall, these results provide direct

interaction-based evidence that Vpr acts as a connector between an E3 ubiquitin ligase complex and substrate proteins.

Vpr induces the K48-linked polyubiquitination of cellular proteins leading to their proteasomal degradation.

To determine the fate of proteins ubiquitinated by Vpr, we used the tandem affinity purification method in combination with an HA-tagged ubiquitin construct which does not significantly stabilize polyubiquitinated products and does not protect substrates from proteasome degradation (34). In this system, we detected ubiquitinated proteins associated with Vpr (Fig 5A), but the ubiquitination signal detected in these conditions did not rely on the recruitment of the E3 ligase complex since knockdown of VPRBP did not have any effect on the levels of ubiquitination (data not shown), suggesting that in absence of interference with polyubiquitination, the Vpr-targeted substrates might be rapidly degraded. In support of this scenario, a 16-hour treatment of cells co-expressing HA-Ub and TAP-Vpr with the proteasome inhibitor MG132 resulted in an increased association of Vpr with cellular ubiquitinated proteins (Fig 5A, compare lanes 3 and 4). We could also observe an increase of polyubiquitinated proteins for shorter MG132 treatment (5 hours) but to a lesser extent (data not shown). Most notably, this significant increase in levels of HA-polyubiquitinated proteins associated with Vpr following treatment with MG132 required the recruitment of the E3 ligase because depletion of VPRBP with shRNA drastically reduced the extent of polyubiquitination (Fig 5B). Therefore, these results suggest that following polyubiquitination by the Vpr-CRL4A (VPRBP) complex, substrates are rapidly degraded by the proteasome.

To determine whether the observed degradation of Vpr-associated ubiquitinated proteins resulted from a classical K48-linked polyubiquitination, we analyzed the effect of the ubiquitin K48R mutation on Vpr-associated ubiquitination. Cells were co-transfected with plasmids expressing TAP or TAP-Vpr with either of HA-Ub (wt) or HA-Ub (K48R). In contrast to Myc-tagged Ub (K48R) which acts as a weak chain terminator and stabilizes polyubiquitin products (7, 54), this HA-Ub (K48R) construct has been previously shown to potently block ubiquitin chain elongation through lysine 48 (34). We also used the polyubiquitination-null construct HA-Ub (K0), in which all lysines were mutated for arginines, as control. Following MG132 treatment, the K48R mutation in ubiquitin significantly reduced the levels of ubiquitinated proteins associated with Vpr, compared to wild type ubiquitin (Fig 6, compare lanes 6 and 8). However, the reduction of ubiquitination did not reach the levels achieved with the K0 mutation (Fig 6, compare lanes 7 and 8). These data suggest that Vpr induces at least in part a classical K48-linked polyubiquitination of cellular substrate proteins, leading to their proteasomal degradation. However, given that the levels of ubiquitination observed with the K48R mutant did not reach the ones observed with the polyubiquitination-null mutant K0, we cannot exclude the possibility that other lysine residues in ubiquitin might be involved in the formation of mixed ubiquitin linkages.

Vpr-induced K48-polyubiquitination is required for phosphorylation of H2AX.

Other investigators demonstrated that blocking the activity of the proteasome using small molecule inhibitors or blocking polyubiquitination via expression of a dominant negative mutant of ubiquitin (Ub K48R) abrogated Vpr-mediated G2 arrest (14, 49). However, caution has to be used when interpreting these results given that inhibition of

polyubiquitination or proteasome function might have pleiotropic effects on checkpoint function without necessarily implicating the direct inhibition of Vpr's activity. Therefore, to evaluate the direct role of K48-linked polyubiquitination in Vpr's activity, we instead monitored phosphorylation of H2AX (γ-H2AX), an early marker of ATR–mediated checkpoint activation previously implicated in Vpr-mediated G2 arrest (29, 62). Following exogenously induced DNA damages, phosphorylation of H2AX occurs in absence of any ubiquitination events (36), and should thus not be directly affected by the K48R mutation in ubiquitin. Indeed, ectopic expression of HA-Ub (K48R) or HA-Ub (K63R) in HeLa cells did not have any effect on the number of cells displaying γ-H2AX foci following UV irradiation (Fig S3). To analyze the effect of Ub (K48R) on Vpr-induced γ-H2AX focus formation, we transiently transfected HeLa cells with a plasmid expressing HA-Ub (K48R) or expressing HA-Ub (K63R) (used as negative control). Twenty-four hours after transfection, cells were transduced with a lentiviral vector expressing GFP alone (WPI) or co-expressing Vpr and GFP (WPI-Vpr). Two days later, cells were processed for immunofluorescence detection using anti-HA antibody to detect HA-Ub-expressing cells, anti-GFP (to amplify the GFP signal, a marker of transduced cells) and with anti-phospho-H2AX (Fig 7A). Cells with greater than ten γ-H2AX foci were considered positive. In mock-transfected cells, transduction with the lentiviral vector expressing Vpr induced a significant increase in the percentage of cells positive for γ-H2AX compared to the control lentiviral vector (83.9% vs 12.3%, $P<0.0001$) (Fig 7A and 7B). In presence of HA-Ub (K48R), we observed a drastic decrease in the number of Vpr-expressing cells with γ-H2AX foci (25.9% vs 83.9%, $P<0.0005$) (Fig 7A and 7B). In contrast, overexpression of HA-Ub (K63R) only weakly altered the ability of Vpr to induce γ-H2AX foci,

demonstrating the specific requirement for K48-linkages over other types of linkages. Therefore, overexpression of the K48R mutant of ubiquitin specifically inhibited Vpr-induced phosphorylation of H2AX (Fig 7A and 7B) without affecting UV-induced phosphorylation of H2AX (Fig S3). These observations thus suggest that K48-linked ubiquitination would be specifically essential for the activity of Vpr toward early checkpoint activation but not for exogenously induced DNA damages. Therefore, taken together, our results provide direct evidence that Vpr recruits the CRL4A (VPRBP) E3 ubiquitin ligase complex to induce the K48-linked polyubiquitination of one or several yet-unknown cellular proteins resulting in their proteasomal degradation and ultimately leading to ATR-mediated phosphorylation of H2AX and G2 arrest.

DISCUSSION

Identification of the substrates targeted by the Vpr-CRL4A (VPRBP) complex represents an important aim not only to fully understand how Vpr activates ATR signaling and promote G2 arrest but also to comprehend the functional relevance of these biological activities. Given that Vpr induces a G2 cell cycle arrest, it is not conceivable to develop approaches that rely on the differential expression pattern of proteins in the presence or absence of Vpr because Vpr cytostatic activity may affect the expression profile of numerous proteins without necessarily implicating a direct recruitment to the Vpr-CRL4A (VPRBP) E3 ligase. On the other hand, identification of substrates of E3 ubiquitin ligases by interaction-based proteomic analyses remains a long-standing challenge due to several different inherent and technical problems. Notably, ubiquitinated proteins are present at low abundance, display a rapid turnover rate and are subjected to rapid deconjugation (25, 40). Therefore, special care must be taken to enrich and stabilize ubiquitin conjugates

using tagged ubiquitin constructs or proteasome inhibitors. To demonstrate a potential interaction of Vpr with its cognate ubiquitinated substrates, we used a combination of both approaches. First, we used a Myc-tagged ubiquitin K48R mutant with the unusual properties of acting as a weak chain terminator and stabilizing polyubiquitinated products (7, 54) coupled with a highly specific tandem affinity purification procedure (5) in order to enrich Vpr-interacting ubiquitinated proteins. With this method, we were able to show a specific interaction between Vpr and cellular ubiquitinated proteins (Fig 2A and 2B). Secondly, using a HA-tagged ubiquitin construct concomitantly with treatment with the proteasome inhibitor MG132, we were also able to reveal a specific association of Vpr with cellular ubiquitinated proteins (Fig 5A). In both cases, a significant part of the Vpr-associated ubiquitinated signal was dependent on the recruitment of an active CRL4A (VPRBP) ligase since depletion of VPRBP by shRNA (Fig 3A and Fig 5B) as well as overexpression of a dominant-negative form of CUL4A (Fig 3B) drastically reduced this association. Surprisingly, the Q65R mutation in Vpr, which virtually abrogates the interaction with VPRBP and should thus reduce binding to ubiquitinated proteins, had the opposite effect: it increased the interaction with ubiquitinated proteins (Fig 4A). However, we observed that the Q65R mutation led to accumulation of substantial amounts of Vpr in the cytoplasm and to inefficient oligomerization of the protein (manuscript in preparation), indicating that this mutation has pleiotropic effects on the functions of Vpr and probably induces conformational defects. Therefore, knockdown of VPRBP and the use of a dominant-negative mutant of CUL4A, both of which reduced binding to ubiquitinated proteins, represent a more reliable assessment of the role of the E3 ligase in the association of Vpr with ubiquitinated proteins. Importantly, deleting the entire putative substrate-interacting C-terminal domain of Vpr resulted in an abrogation of G2 arrest (Fig S1) and of

the association with ubiquitinated proteins (Fig 4B), indicating that these Vpr-associated ubiquitinated proteins are probably not components of the E3 ligase itself. Shorter deletions or point mutation in the C-terminal domain of Vpr also led to an inhibition of G2 arrest (Fig S1) and to a significant reduction in binding to ubiquitinated proteins (Fig 4C) suggesting that at least a significant fraction of these Vpr-associated ubiquitinated proteins would be substrates forcibly recruited by Vpr to the E3 ligase. Given that C-terminal deletions of Vpr retain their interaction with UNG2 (10), our data indicate that these ubiquitinated proteins do not contain detectable levels of ubiquitinated UNG2, therefore excluding the possibility that they are solely ubiquitinated UNG2. These are most likely substrates independently recruited by Vpr rather than an increased ubiquitination of VPRBP's own substrates because overexpression of the minimal Vpr-interacting domain of VPRBP was reported to increase the association of Vpr with DDB1 as well as the efficiency of Vpr-mediated G2 arrest (31). It is arguably unlikely that this minimal domain, which also contains the WDxR motif responsible for the association with DDB1 (26, 31), would also possess the determinants mediating substrate recognition. Finally, our results do not contradict the previous observations that Vpr would increase neddylation of CRL4A (VPRBP) (22) given that recruitment of substrates as well as substrate adaptors to CRL complexes, including CRL4A ligases, were shown to markedly promote neddylation (11).

As mentioned above, we found that MG132 stabilized Vpr-associated HA-tagged ubiquitinated protein (Fig 5A), suggesting that in absence of MG132, Vpr's substrates would be degraded by the proteasome. Indeed, in support of this interpretation, in absence of proteasome inhibition, the association of Vpr with cellular ubiquitinated proteins was

independent of the recruitment of CRL4A (VPRBP) (data not shown) whereas, when cells were treated with MG132, depletion of VPRBP significantly decreased the levels of Vpr-associated ubiquitinated proteins (Fig 5B). Moreover, Vpr was found to induce, at least in part, the classical K48-linked polyubiquitination of its substrates (Fig 6), thus further supporting our evidence that these substrates are degraded by the proteasome given that this type of homopolymeric ubiquitin chain generally leads to proteasomal proteolysis (50, 51).

Several investigators reported that Vpr activates ATR in a variety of cell types including primary CD4+ T-lymphocytes. Vpr-mediated activation of ATR was accompanied by the formation of DNA repair foci that included RPA, HUS1, RAD17, BRCA1, TP53BP1, and ϒ-H2AX. In contrast to other investigators that have used accumulation of cells in G2/M as a marker of Vpr activity (14, 49), we reasoned that using an early marker of checkpoint activation would likely constitute a more direct and less ambiguous strategy to assess the role of Vpr-mediated ubiquitination in its G2 arrest function. Phosphorylation of H2AX represents such a marker. Indeed, Mailand et al recently reported that MG132-mediated depletion of nuclear ubiquitin did not impair phosphorylation of H2AX in response to exogenous genotoxic stresses such as DNA double-strand breaks induced by ionizing radiation (36). Moreover, overexpression of HA-tagged Ub (K48R) or Ub (K63R) in HeLa cells did not inhibit H2AX phosphorylation following UV irradiation (Fig S3). Therefore, H2AX phosphorylation appears to be independent of ubiquitination. In contrast, in the case of Vpr-induced H2AX phosphorylation, overexpression of Ub (K48R) but not Ub (K63R) significantly reduced H2AX phosphorylation (Fig 7A and 7B), suggesting that this effect was most probably due

to a direct inhibition of Vpr-induced K48-polyubiquitination rather than a pleiotropic inhibition of checkpoint function. These functional data demonstrating the role of Vpr-induced ubiquitination in its G2 arrest activity are also supported by biochemical evidence whereby G2-arrest-defective mutants of Vpr in the putative C-terminal substrate-recruitment domain, still competent for association with CRL4A (VPRBP), failed to interact with ubiquitinated cellular proteins (Fig 4B). Therefore, taken together, our results strongly suggest that Vpr-mediated K48-linked ubiquitination and degradation of one or several putative substrates are responsible for Vpr-induced G2 arrest.

Zimmerman and colleagues previously observed that Vpr was unable to induce checkpoint activation in macrophages due to the absence of ATR in these cells, while gamma irradiation led to ATM (ataxia telangiectasia mutated) activation (63). Importantly, Vpr did not appear to cause DNA double-strand breaks in cycling cells in conditions where ATR was activated (29). The authors concluded that Vpr likely causes DNA replication stresses rather than direct DNA damages such as DNA double-strand breaks that would otherwise activate ATM (63). Thus, it would appear that the cellular substrate(s) targeted by Vpr might have important roles in DNA replication and that its degradation by Vpr would cause DNA replication stress as demonstrated by the formation of RPA foci (29), ultimately leading to ATR activation (42) and accumulation of cells in G2/M. Interestingly, Vpr was previously shown to form nuclear foci that co-localized with DNA repair foci containing γ-H2AX and RPA (29). It would be tempting to speculate that Vpr would recruit CRL4A (VPRBP) onto chromatin to degrade directly at this site a chromatin-bound component of the DNA replication machinery. Alternatively, the degradation of the

substrate(s) might not directly cause DNA replication stresses but might somehow mimic signals induced by those. Recently, forced tethering of DNA repair factors including ATM, MDC1 (mediator of DNA damage checkpoint 1) and NBS1 (Nijmegen breakage syndrome 1, nibrin) to chromatin was shown to induce the formation of fully competent DNA repair foci in absence of any DNA damages (46). It is thus conceivable that ubiquitination and degradation of a DNA repair regulator(s) by Vpr might somehow induce the incorrect recruitment of DNA repair proteins to chromatin in absence of any DNA replication stress.

In conclusion, using a tandem affinity purification approach, we provide additional and more direct evidence that Vpr recruits the CRL4A (VPRBP) E3 ubiquitin ligase complex to induce the K48-linked polyubiquitination of one or several putative substrates, resulting in their proteasomal degradation. Proteolysis of these putative substrates would lead to phosphorylation of H2AX, an early target of ATR activation, and ultimately G2 arrest. Identification of the cellular proteins degraded by Vpr will be central in the understanding of how Vpr triggers ATR activation and why Vpr induces a cell cycle arrest. The tandem affinity purification procedure presented in this study represents a powerful approach to isolate and identify cellular ubiquitinated substrates interacting with Vpr.

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FIGURE 1. Vpr interacts with VPRBP and DDB1 during infection of primary CD4+ T-lymphocytes.

Ten millions activated primary CD4+ T-lymphocytes were mock-infected or infected with viruses defective for Vpr (HxBru Vpr-) or encoding HA-tagged Vpr (HxBru Ha-Vpr) at a multiplicity of infection of 0.01. Five days after infection cells were harvested in triton lysis buffer and immunoprecipitation against HA was performed as described in Materials and Methods. Co-immunoprecipitated endogenous VPRBP and DDB1 were detected by western blot using specific rabbit polyclonal antibodies. HA-tagged Vpr was detected using a monoclonal anti-HA antibody.

FIGURE 2. Vpr interacts with unknown cellular ubiquitinated proteins.

A) HEK293T cells were transfected with plasmids encoding TAP (lanes 1 and 3) or TAP-Vpr (lanes 2 and 4). Cells were co-transfected with either an empty plasmid (lanes 1 and 2) or a Myc-Ub (K48R)-encoding plasmid (lanes 3 and 4). Two days later, tandem affinity purification was performed on cell lysates as described in Materials and Methods. The levels of ubiquitinated proteins were determined by western blot in crude lysates and pulled-down fractions using a monoclonal anti-Myc antibody. **B)** Cells were co-transfected with plasmids expressing TAP-Vpr and Myc-Ub (K48R) (lanes 3 and 4) or with plasmids expressing TAP and Myc-Ub (K48R) as control (lanes 1 and 2). Forty-eight hours post-transfection, cells were lysed in triton lysis buffer (lanes 1 and 3) or heat-denatured following lysis in RIPA buffer (lanes 2 and 4). Cell extracts were subjected to IgG pulldowns using pre-coupled beads and complexes were eluted following cleavage with tobacco etch virus (TEV) protease. The levels of ubiquitinated proteins were determined using a monoclonal anti-Myc antibody and Vpr was detected using a polyclonal antibody.

FIGURE 3. Association of Vpr with ubiquitinated proteins involves the recruitment of an active E3 ubiquitin ligase complex.

HEK293T cells were co-transfected with a plasmid encoding Myc-Ub (K48R) and with either TAP- (lanes 1 and 2) or TAP-Vpr-encoding plasmids (lanes 3 and 4). Cells were transcomplemented with **A)** plasmids expressing scrambled shRNA or shRNA targeting VPRBP and **B)** with an empty plasmid or a plasmid encoding a dominant-negative mutant form of CUL4A (Cul4A DN) as indicated. Two days after transfection, cell extracts were subjected to tandem affinity purification as described in Materials and Methods. The levels of ubiquitinated proteins were determined by western blot in crude lysates and pulled-down fractions using a monoclonal anti-Myc antibody. Vpr and VPRBP were detected using polyclonal antibodies. * Denotes a non-specific band detected by the anti-VPRBP antibody used as a loading control.

FIGURE 4. Analysis of the association of Vpr mutants with ubiquitinated proteins. **A)** HEK293T monoclonal cell lines stably expressing a control shRNA (ShControl) or a shRNA against VPRBP (ShVprBP) were co-transfected with a plasmid encoding Myc-Ub (K48R) and plasmids expressing TAP, TAP-Vpr (WT), or TAP-Vpr (Q65R) as indicated. Two days after transfection, cell extracts were subjected to tandem affinity purification as described in Materials and Methods. **B)** HEK293T cells were co-transfected with a plasmid encoding Myc-Ub (K48R) and plasmids expressing TAP, TAP-Vpr (WT), and TAP-Vpr (1-78) as indicated. Cell extracts were processed as in panel A. **C)** HEK293T cells were co-transfected with a plasmid encoding Myc-Ub (K48R) and plasmids expressing TAP, TAP-Vpr (WT), TAP-Vpr (R87A, R88A), TAP-Vpr (1-84), TAP-Vpr (1-86), and TAP-Vpr (1-90), as indicated. Two days after transfection, cell extracts were subjected to tandem affinity purification as described in Materials and Methods. Pulldown eluates (lanes 7, 8, 10, 12, 14, 16) and 2-fold dilutions of pulldown eluates (lanes 9, 11, 13, 15, 17) were resolved by SDS-PAGE for analysis. For all panels, the levels of ubiquitinated proteins were determined by western blot in crude lysates and pulled-down fractions using a monoclonal anti-Myc antibody. Vpr and VPRBP were detected using polyclonal antibodies. * Denotes non-specific bands used as loading controls.

FIGURE 5. Vpr-associated ubiquitinated proteins are degraded by the proteasome.

A) HEK293T cells were co-transfected with a plasmid encoding HA-Ub (WT) and with either TAP- (lanes 1 and 2) or TAP-Vpr-encoding plasmids (lanes 3 and 4). Twenty-four hours after transfection, cells were treated (lanes 2 and 4) or not (lanes 1 and 3) with 5 µM MG132 for 16 hours. **B)** Cells were co-transfected with a plasmid encoding HA-Ub (WT) and with either TAP- (lanes 1 and 2) or TAP-Vpr-encoding plasmids (lanes 3 and 4). Cells were transcomplemented with plasmids expressing scrambled shRNA or shRNA targeting VPRBP as indicated. Twenty-four hours after transfection, cells were treated with 5 µM MG132 for 16 hours. For both panels, cell extracts were subjected to tandem affinity purification. Ubiquitinated proteins were detected using a monoclonal anti-HA antibody. Vpr and VPRBP were detected using polyclonal antibodies. * Denotes a non-specific band detected by the anti-VPRBP antibody used as a loading control.

FIGURE 6. Vpr induces the K48-linked polyubiquitination of unknown cellular substrates.

HEK293T cells were transfected with a plasmid encoding either TAP (lanes 1 to 4) or TAP-Vpr (lanes 5 to 8). Cells were transcomplemented with plasmids expressing HA-Ub (WT) (lanes 2 and 6), HA-Ub (K0) (lanes 3 and 7), HA-Ub (K48R) (lanes 4 and 8) or with an empty plasmid as negative control (lanes 1 and 5). Twenty-four hours after transfection, cells were treated with 5 μM MG132 for 16 hours and cell extracts were subjected to tandem affinity purification. The levels of ubiquitinated proteins were determined by western blot in crude lysates and pulled-down fractions using a monoclonal anti-HA antibody. Vpr was detected using a polyclonal antibody.

FIGURE 7. K48-linked polyubiquitination is required for Vpr-induced H2AX phosphorylation.

A) HeLa cells were transiently transfected with an empty plasmid or plasmids expressing HA-Ub (K48R) or HA-Ub (K63R). Twenty-four hours after transfection, cells were transduced with a lentiviral vector expressing GFP alone (WPI) or co-expressing Vpr and GFP (Vpr). Two days later, cells were fixed, permeabilized and stained with antibodies against GFP (green), γ-H2AX (red), and HA (blue). Cells with more than ten γ-H2AX foci were considered positive for H2AX phosphorylation. **B)** Results depicted in the graphs are the means of three independent experiments. Error bars represent standard deviations. Statistical significance was determined with a Student t-test with 95% confidence ($p < 0.05$).

Figure 1

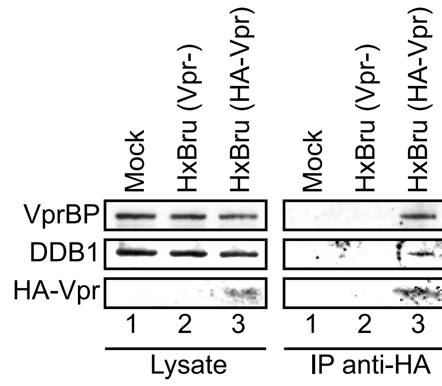
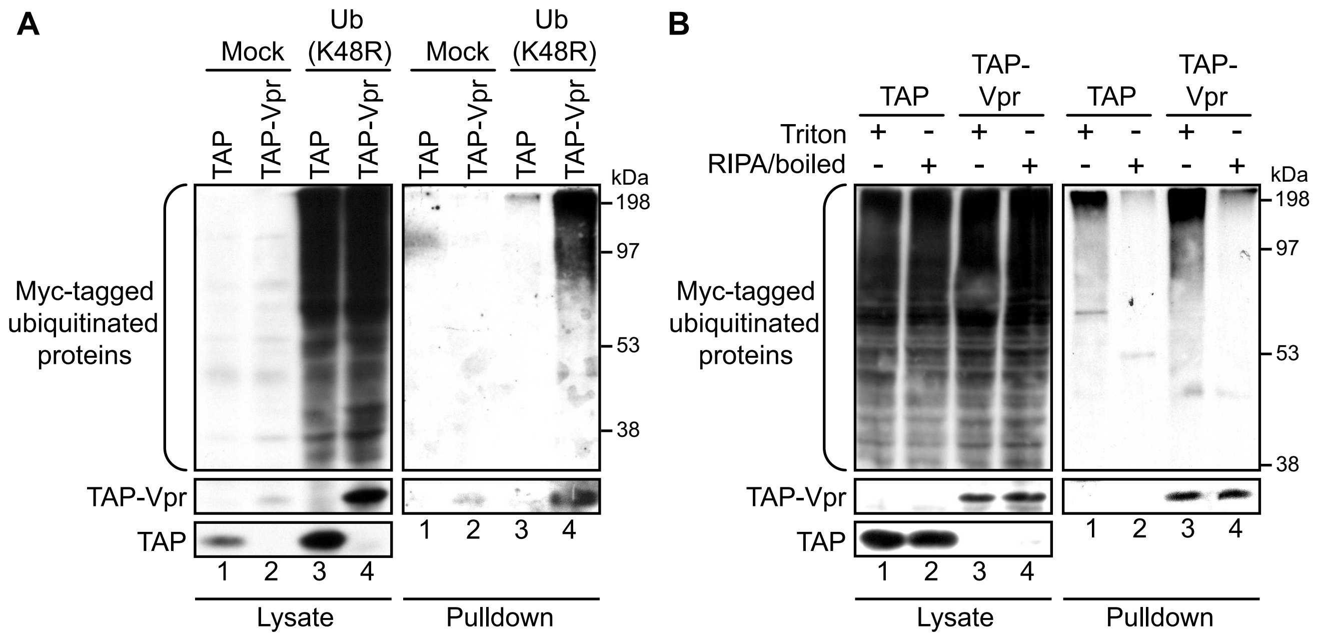


Figure 2



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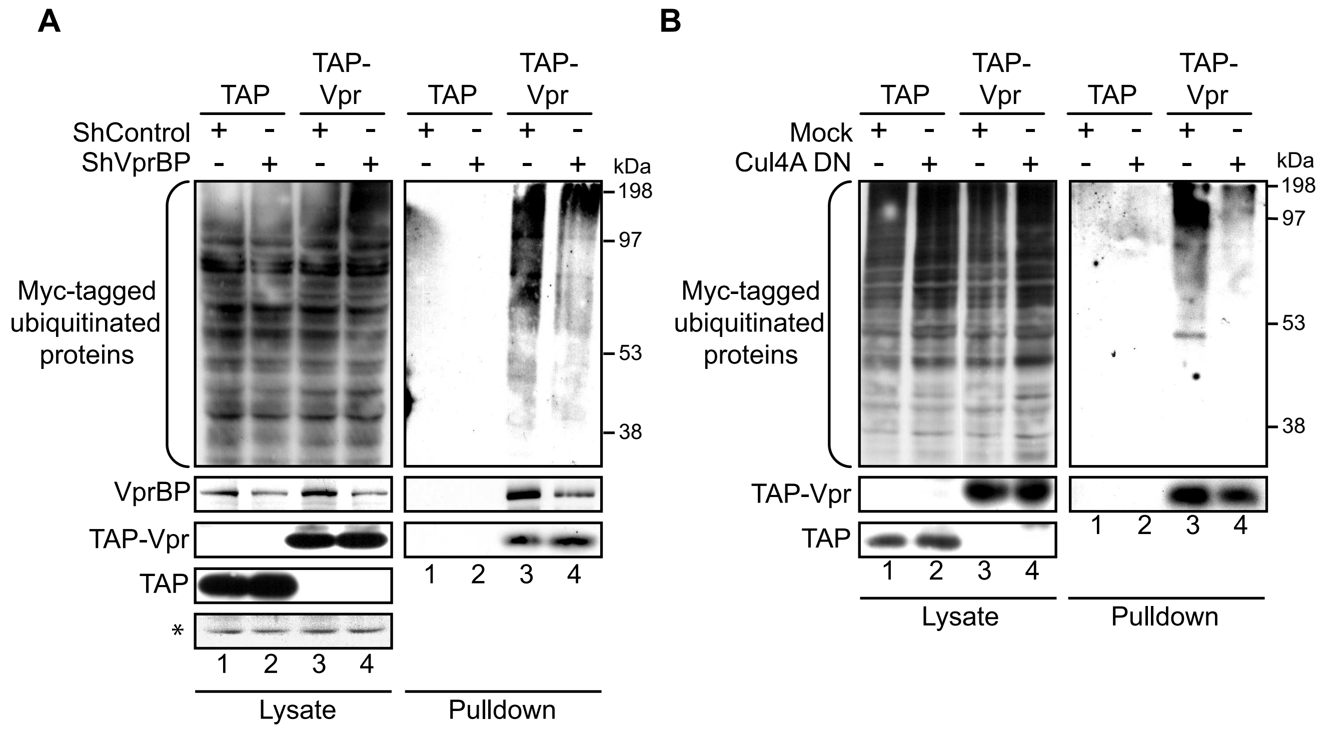
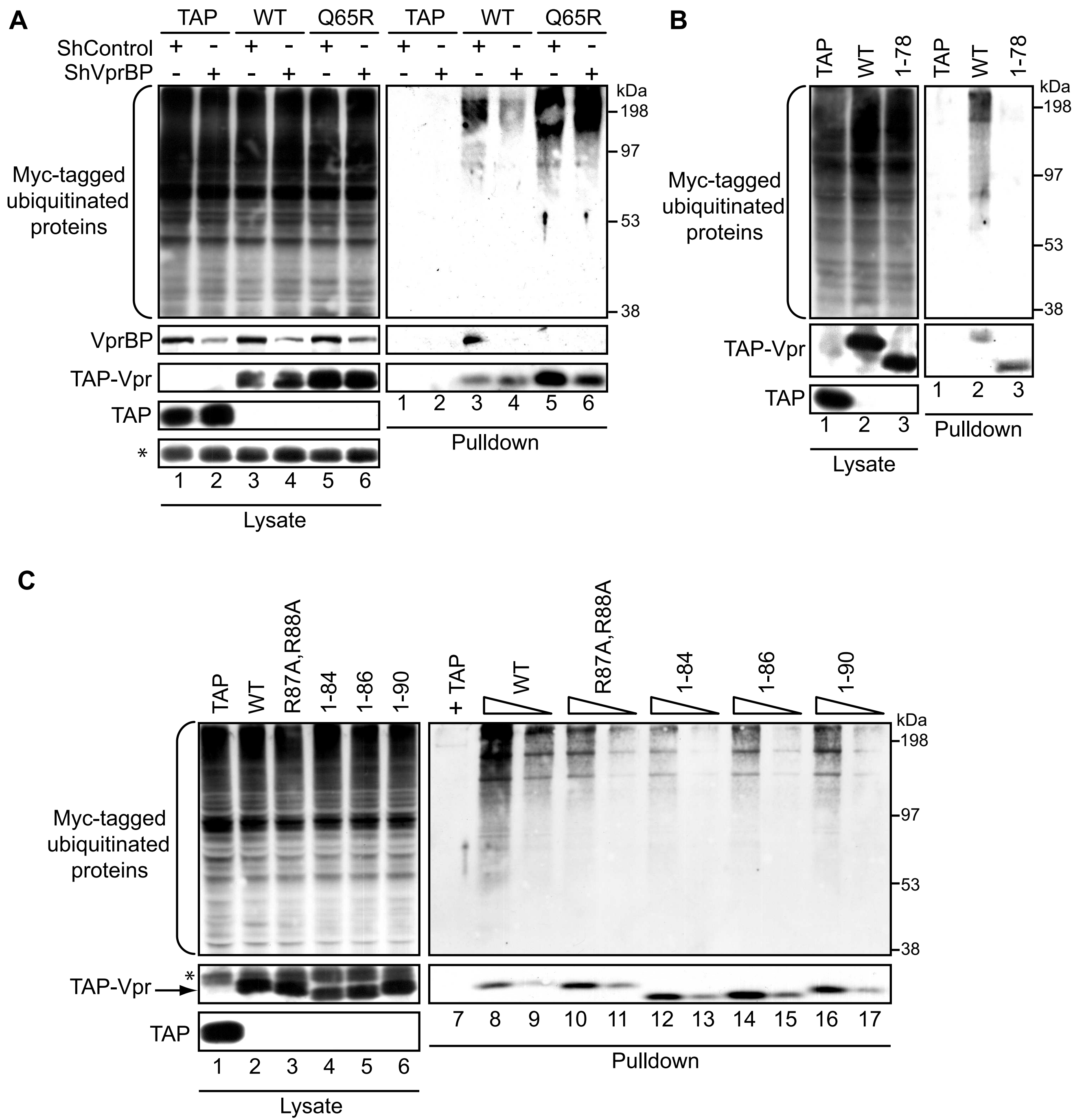


Figure 4



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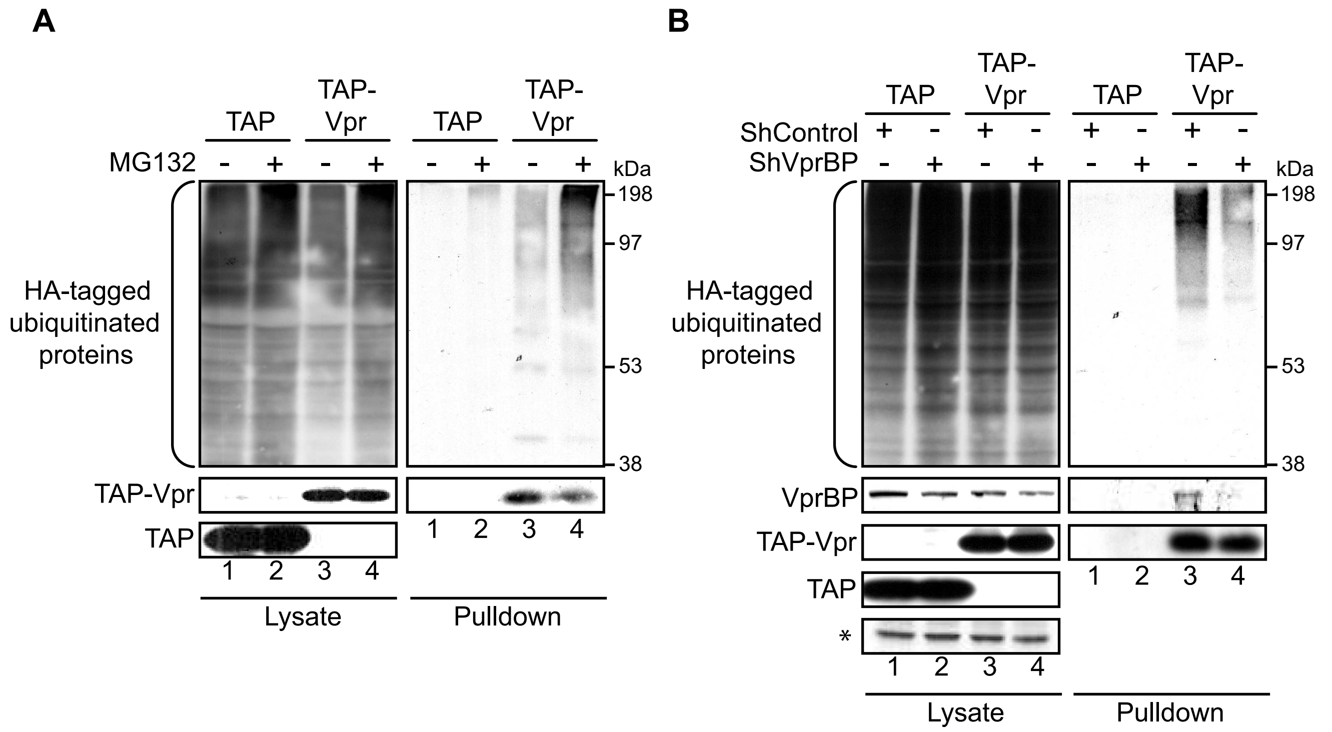

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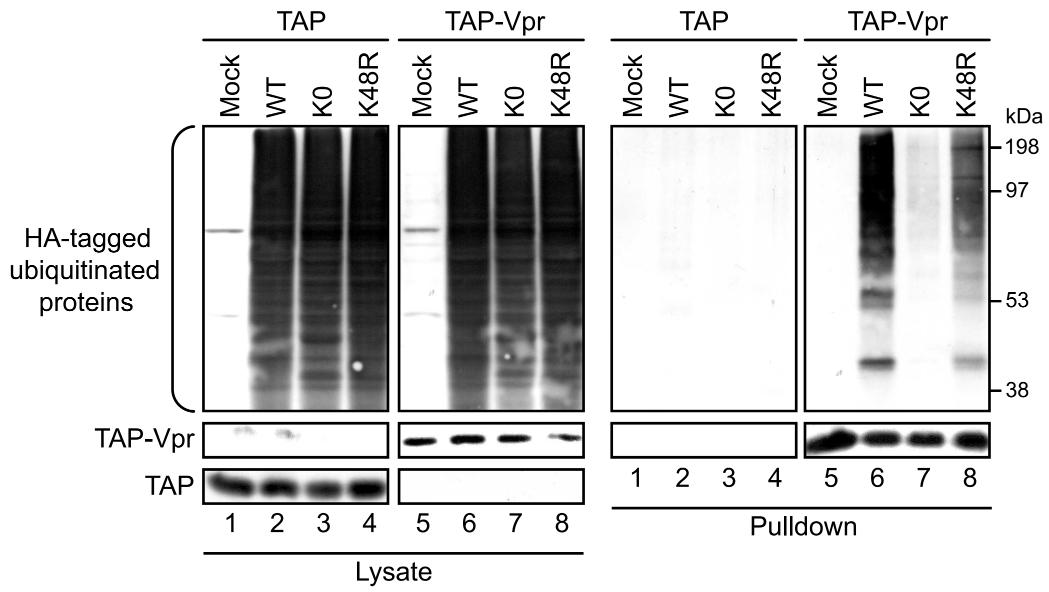
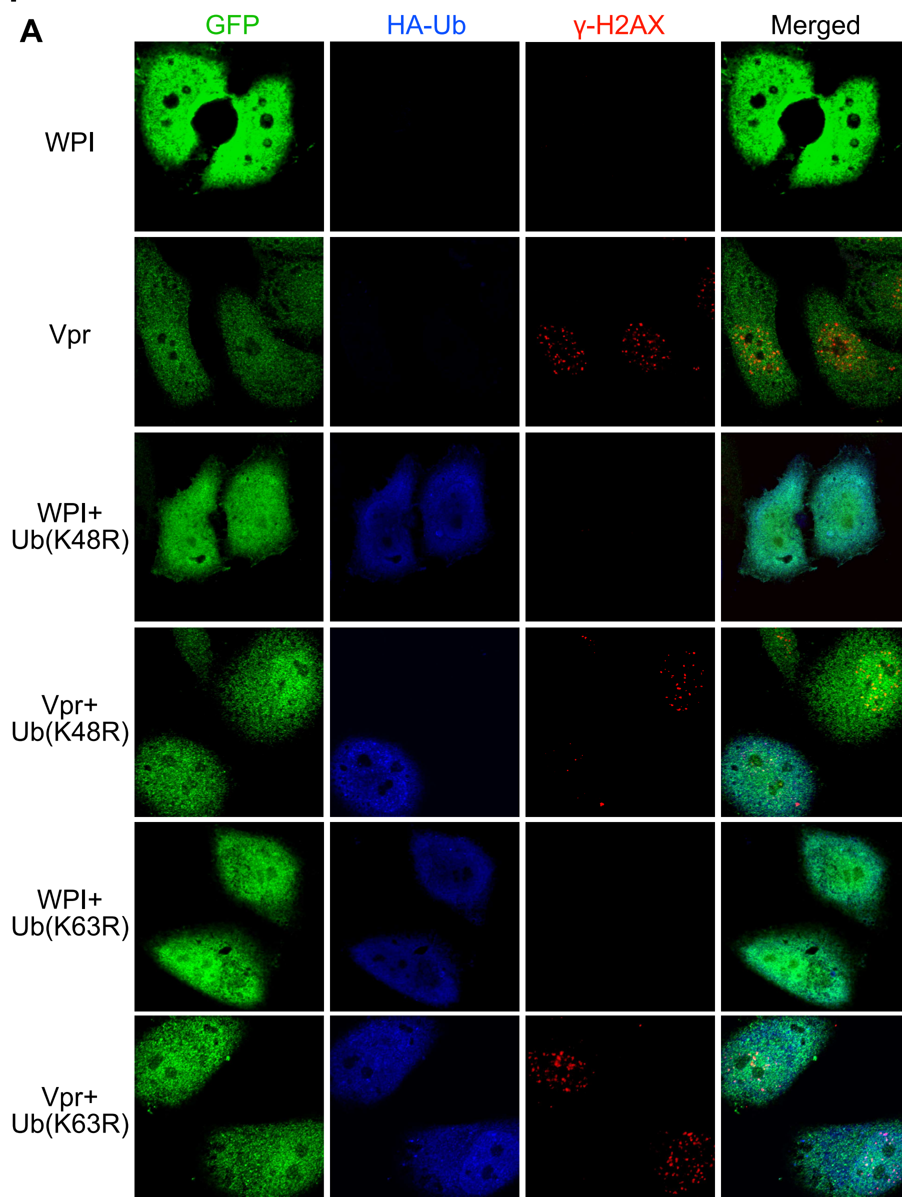
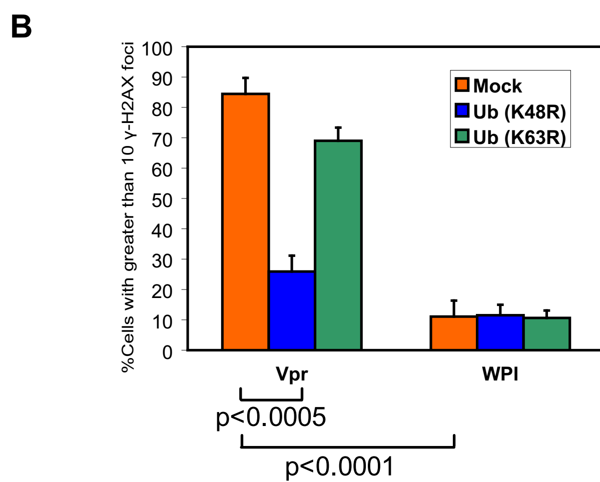


Figure 7





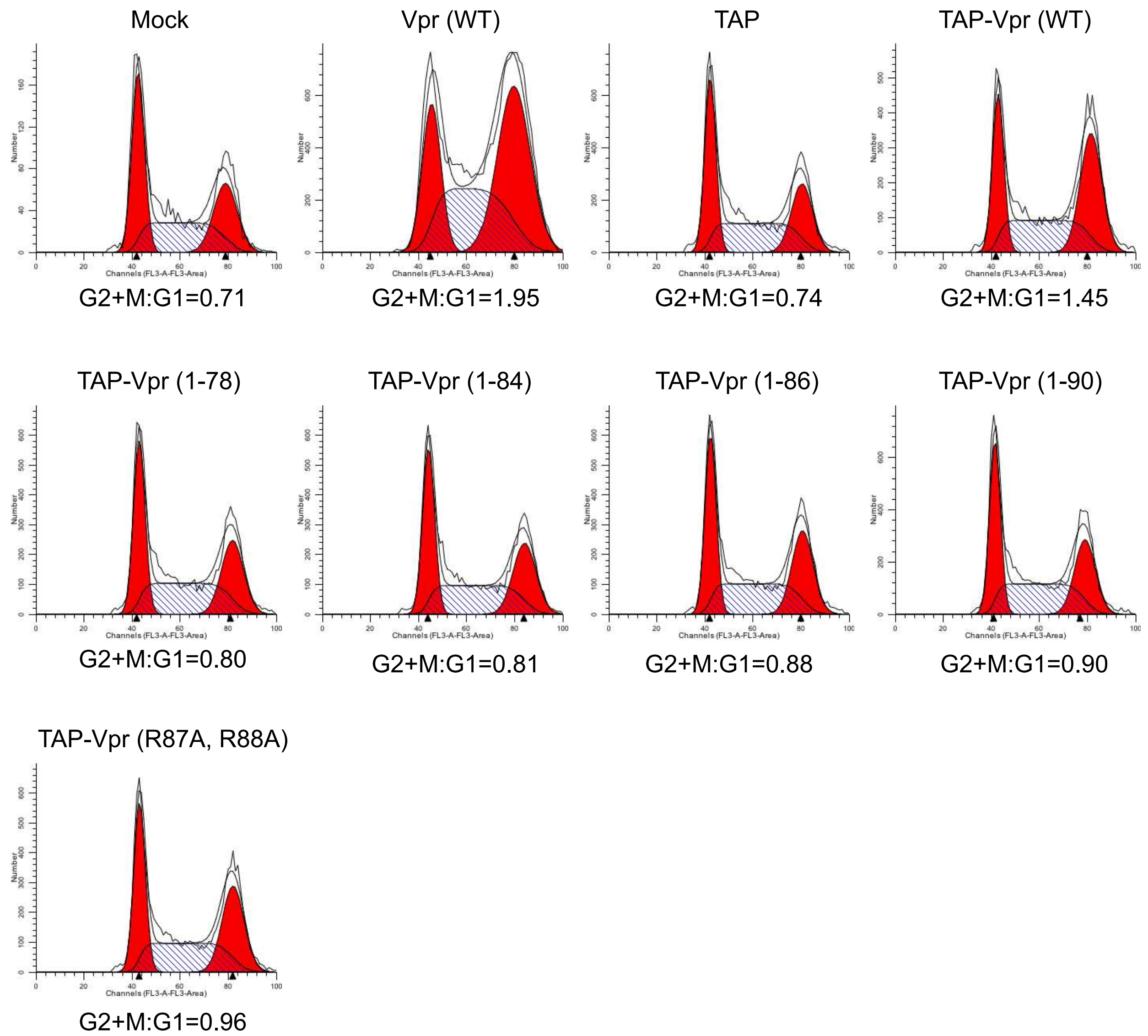
SUPPLEMENTAL FIGURE 1. Analysis of the induction of G2 arrest by TAP-tagged wild type Vpr and mutants. HEK293T cells were co-transfected with 1.5 ug of the GFP-expressing plasmid pQBI-25 and with 15 ug of control plasmid or plasmids expressing native Vpr (WT), TAP tag, or TAP-tagged Vpr (WT) and mutants, as indicated. Two days after transfection, cell cycle profiles were determined on the GFP-positive population by flow cytometry and PI staining.

SUPPLEMENTAL FIGURE 2. Characterization of stable cell lines expressing control and VPRBP-targeting shRNA. A) Western blot analysis of VPRBP levels in control non-targeting shRNA-expressing cell line (shControl) and VPRBP-knocked down cell line (shVprBP). Actin and VPRBP were detected using rabbit polyclonal antibodies. **B)** The cell cycle profiles of shControl and shVprBP cell lines were determined using flow cytometry and propidium iodide (PI) staining. Percentages of cells in G1, S, and G2 phases, as determined by the Modfit software, are shown in each graph. **C)** The growth kinetics of ShControl and ShVprBP cell lines was monitored by flow cytometry using CFSE (carboxyfluorescein succinimidyl ester) staining. Percentages of cells in generations 3, 4, and 5 (G_3, G_4, and G_5) are shown in each graph. **D)** Levels of apoptotic cells were determined by flow cytometry using a standard PI and Annexin V staining. PI^- Annexin V^+ cells represent the apoptotic population. **E)** shControl and shVprBP cell lines were transfected with an HA-Vpr-expressing plasmid or a control plasmid. Two days after transfection, cell cycle profiles were determined by flow cytometry and PI staining. Western blot analysis was performed on the transfected cells to monitor levels of HA-Vpr.

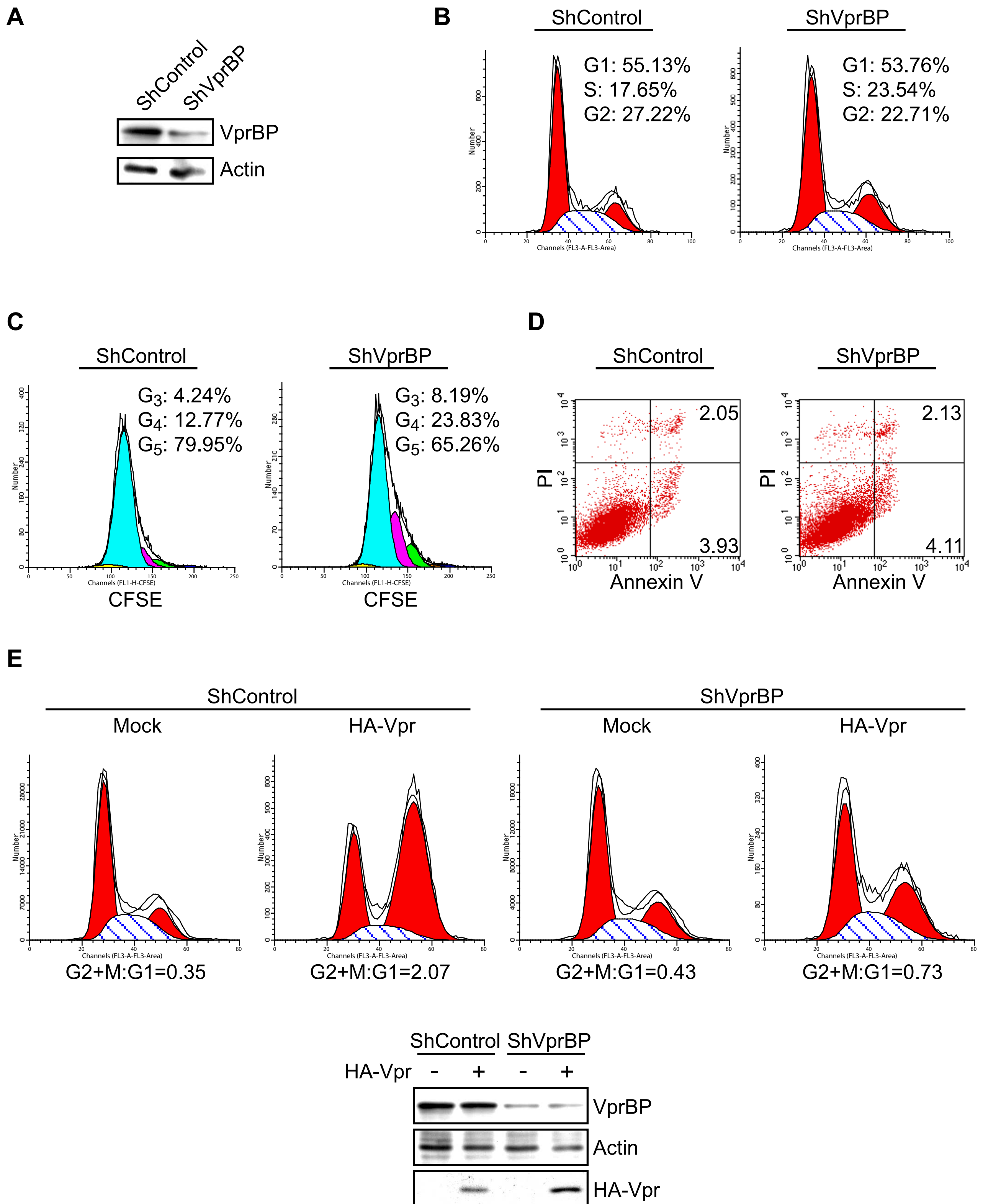
SUPPLEMENTAL FIGURE 3. K48- or K63-linked polyubiquitination is not required for UV-induced H2AX phosphorylation.

HeLa cells were transiently transfected with a plasmid expressing HA-Ub (K48R) **(A)** or HA-Ub (K63R) **(B)**. Seventy-two hours after transfection, cells were irradiated or not with 25 J/m^2 ultraviolet light at 254 nm. Four hours after irradiation cells were fixed, permeabilized and stained with antibodies against γ-H2AX (red) and HA (blue).

Supplemental figure 1



Supplemental figure 2



Supplemental figure 3