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Manipulation of the Ubiquitin-Proteasome System by HIV-1: Role of the Accessory Protein Vpr

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SOMMAIRE

Le virus de l'immunodéficience humaine de type 1 (VIH-1), l'agent étiologique du SIDA, est un rétrovirus complexe arborant plusieurs protéines accessoires : Nef, Vif, Vpr, et Vpu. Celles-ci sont impliquées dans la modulation de la réplication virale, dans l'évasion immunitaire et dans la progression de la pathogenèse du SIDA. Dans ce contexte, il a été démontré que la protéine virale R (Vpr) induit un arrêt de cycle cellulaire en phase G2. Le mécanisme par lequel Vpr exerce cette fonction est l'activation, ATR (Ataxia telangiectasia and Rad3 related)-dépendante, du point de contrôle de dommage à l'ADN, mais les facteurs et mécanismes moléculaires directement impliqués dans cette activité demeurent inconnus.

Afin d'identifier de nouveaux facteurs cellulaires interagissant avec Vpr, nous avons utilisé une purification d'affinité en tandem (TAP) pour isoler des complexes protéiques natifs contenant Vpr. Nous avons découvert que Vpr s'associait avec CRL4A(VprBP), un complexe cellulaire d'E3 ubiquitine ligase, comprenant les protéines Cullin 4A, DDB1 (DNA damage-binding protein 1) et VprBP (Vpr-binding protein). Nos études ont mis en évidence que le recrutement de la E3 ligase par Vpr était nécessaire mais non suffisant pour l'induction de l'arrêt de cycle cellulaire en G2, suggérant ainsi que des événements additionnels seraient impliqués dans ce processus. À cet égard, nous apportons des preuves directes que Vpr détourne les fonctions de CRL4A(VprBP) pour induire la polyubiquitination de type K48 et la dégradation protéosomale de protéines cellulaires encore inconnues. Ces événements d'ubiquitination induits par Vpr ont été démontrés comme étant nécessaire à l'activation d'ATR. Finalement, nous montrons que Vpr forme des foyers ancrés à la chromatine co-localisant avec VprBP ainsi qu'avec des facteurs impliqués dans la réparation de l'ADN. La formation de ces foyers représente un événement essentiel et précoce dans l'induction de l'arrêt de cycle cellulaire en G2. Enfin, nous démontrons que Vpr est

capable de recruter CRL4A(VprBP) au niveau de la chromatine et nous apportons des preuves indiquant que le substrat inconnu ciblé par Vpr est une protéine associée à la chromatine.

Globalement, nos résultats révèlent certains des ménanismes par lesquels Vpr induit des perturbations du cycle cellulaire. En outre, cette étude contribue à notre compréhension de la modulation du système ubiquitine-protéasome par le VIH-1 et son implication fonctionnelle dans la manipulation de l'environnement cellulaire de l'hôte.

Mots clés: Virus, protéines accessoires, ATR, Point de contrôle de domage à l'ADN, cycle cellulaire, ubiquitination, ubiquitine ligase.

ABSTRACT

Human immunodeficiency virus 1 (HIV-1), the etiologic agent of AIDS, is a complex retrovirus with several accessory proteins. HIV-1 accessory proteins Nef, Vif, Vpr, and Vpu have been implicated in the modulation of viral replication, enhancement of viral fitness, immune evasion, and progression of AIDS pathogenesis. In that regard, viral protein R (Vpr) induces a cell cycle arrest in the G2 phase by activating the canonical ATR (Ataxia telangiectasia and Rad3 related)-mediated DNA damage checkpoint, but cellular factors and mechanisms directly engaged in this process remain unknown.

To identify novel Vpr-interacting cellular factors, we used tandem affinity purification (TAP) to isolate native Vpr-containing complexes. We found that Vpr hijacks a cellular E3 ubiquitin ligase complex, CRL4A(VprBP), composed of Cullin 4A, DDB1 (DNA damage-binding protein 1) and VprBP (Vpr-binding protein). Moreover, we observed that recruitment of the E3 ligase by Vpr was necessary but not sufficient for the induction of G2 cell cycle arrest, suggesting that additional events are involved. In this context, we provide direct evidence that Vpr usurps the function of CRL4A(VprBP) to induce the K48-linked polyubiquitination and proteasomal degradation of as-yet-unknown cellular proteins. These ubiquitination events mediated by Vpr were necessary for the activation of ATR. Moreover, we show that Vpr forms chromatin-associated foci that co-localize with VprBP and DNA repair factors. Our data indicate that formation of these foci represent a critical early event in the induction of G2 arrest. Finally, we show that Vpr is able to recruit CRL4A(VprBP) on chromatin and we provide evidence that the unknown substrate targeted by Vpr is a chromatin-associated protein.

Overall, our results reveal some of the mechanisms by which Vpr induces cell cycle perturbations. Furthermore, this study contributes to our understanding of the modulation of the ubiquitin-proteasome system by HIV-1 and its functional implication in the manipulation of the host cellular environment.

Keywords: Virus, accessory proteins, ATR, DNA damage checkpoint, cell cycle, ubiquitination, ubiquitin ligase.

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ABBREVIATIONS

53BP1	Tumor suppressor protein 53-binding protein 1
9-1-1	Rad9-Hus1-Rad1 complex
ABRA1	Abraxas
ANT	Adenine nucleotide translocator
AP-1	Adaptor protein 1
AP-2	Adaptor protein 2
APC/C	Anaphase-promoting complex/cyclosome
APOBEC3F	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3F
APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR-interacting protein
β-COP	Coatomer protein complex subunit beta
β-TrCP	Beta-Transducin repeat-containing protein
BARD1	BRCA1-associated RING domain 1
BER	Base excision repair
BRCA1	Breast cancer 1, early onset
BTB	Broad complex, tramtrack, bric-a-brac
Bub1	Budding uninhibited by benzimidazoles homolog 1
Bub3	Budding uninhibited by benzimidazoles homolog 3
BubR1	Bub1 homolog beta
CA	Capsid
CAND1	Cullin-associated and neddylation-dissociated
CCR5	C-C chemokine receptor type 5
Cdc25	Cell division cycle 25
CDK1	Cyclin-dependent kinase 1
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
Cdt1	Cdc10-dependent transcript 1
Cdt2	Cdc10-dependent transcript 2
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CKI	Cyclin-dependent kinase inhibitor
CRL	Cullin-RING E3 ubiquitin ligase
CRL1	Cullin 1-RING E3 ubiquitin ligase
CRL2	Cullin 2-RING E3 ubiquitin ligase
CRL3	Cullin 3-RING E3 ubiquitin ligase
CRL4A	Cullin 4A-RING E3 ubiquitin ligase
CRL4B	Cullin 4B-RING E3 ubiquitin ligase
CRL5	Cullin 5-RING E3 ubiquitin ligase

CRL7	Cullin 7-RING E3 ubiquitin ligase
CSN	COP9 signalosome
CSN6	COP9 signalosome subunit 6
CUL4A	Cullin 4A
CXCR4	Chemokine (CXC motif) receptor 4
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DC	Dendritic cells
DDA1	DET1 and DDB1 associated 1
DDB1	DNA Damage-Binding protein 1
DDB2	DNA Damage-Binding protein 2
DET1	De-etiolated homolog 1
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	DNA double-strand break
DUB	Deubiquitinase
EGFR	Epidermal growth factor receptor
Emi1	Early mitotic inhibitor 1
Env	Envelope
ERAD	Endoplasmic reticulum-associated degradation
ERK	Extracellular signal regulated kinase
ESCRT-I	Endosomal sorting complex I required for transport
ESCRT-III	Endosomal sorting complex III required for transport
FA	Fanconi anemia
Gag	Group-specific antigen
γ-H2AX	Phosphorylated H2AX
gp41	Glycoprotein 41
gp120	Glycoprotein 120
gp160	Glycoprotein 160
GR	Glucocorticoid receptor
H2AX	Histone 2A, variant X
HBV	Hepatitis B virus
HBx	Hepatitis B virus protein X
HCMV	Human cytomegalovirus
HECT	Homologous to the E6-AP carboxyl terminus
hHR23A	Human homolog A of RAD23
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	Human leukocyte antigen
IκBα	Inhibitor of NF-κB alpha
IKK	IκB kinase
KSHV	Kaposi's sarcoma-associated hespesvirus
LEDGF/p75	Lens epithelium-derived growth factor, p75
LTR	Long terminal repeat
LUBAC	Linear ubiquitin chain assembly complex

MA	Matrix
MAD1	Mitotic arrest deficient 1
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
MCM	Mini-chromosome maintenance complex
MDC1	Mediator of DNA damage checkpoint 1
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
Mps1	Monopolar spindle 1
MRN	Mre11-Rad50-NBS1
MVB	Multivesicular body
NBS1	Nijmegen breakage syndrome 1
NC	Nucleocapsid
NDC80	Ndc80 homolog, kinetochore complex component
Nef	Negative factor
NEDD8	Neuronal precursor expressed, developmentaly down-regulated 8
NEMO	NF-κB essential modulator
NER	Nucleotide excision repair
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
HR	Homologous recombination
NK	Natural killer cell
NKG2D	Natural killer group 2, member D
NLS	Nuclear localization signal
P-TEFb	Positive transcription elongation factor b
p300	E1A-binding protein 300
PARC	Parkin-like cytoplasmic protein
PBMCs	Peripheral blood mononuclear cells
PIC	Pre-integration complex
PIKK	Phosphoinositide-3-kinase-related protein kinase
PCNA	Proliferating cell nuclear antigen
PLK1	Polo-like kinase 1
Pol	Polymerase
PP	Protein phosphatase
Pre-RC	Pre-replicative complex
R	Repeated sequence
RAP80	Receptor-associated protein 80
Rb	Retinoblastoma tumor suppressor protein
Rev	Regulator of virion gene expression
RFC	Replication factor C
RING	Really interesting new gene
RNF8	RING finger protein 8
RNF168	RING finger protein 168

ROC1	Regulator 1 of Cullins
ROC2	Regulator 2 of Cullins
ROS	Reactive oxygen species
RPA	Replication protein A
RRE	Rev-responsive element
SCF	Skp1/Cullin 1/F-Box complex
SIV	Simian immunodeficiency virus
SIV_{agm}	SIV in African green monkeys
SIV_{cpz}	SIV in chimpanzees
SIV_{mac}	SIV in rhesus macaques
SIV_{sm}	SIV in sooty mangabeys
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
SOCS	Suppressor of cytokine signaling
SP1	Transcription factor SP1
STAT2	Signal transducer and activator of transcription 2
SV5	Simian virus 5
SV5 V	Simian virus 5 protein V
SUMO	Small ubiquitin-related modifier
TAP	Tandem affinity purification
Tat	Transcriptional transactivator
TLS	Translesion synthesis
TNF	Tumor necrosis factor
TopBP1	Topoisomerase 2 binding protein 1
TS	Template switching
U3	Unique to 3’end
U5	Unique to 5’end
Ub	Ubiquitin
UBD	Ubiquitin-binding domain
UNG2	Uracil-DNA glycosylase 2
UPS	Ubiquitin-proteasome system
VCP	Valosin-containing protein
Vif	Viral infectivity factor
Vpr	Viral protein R
VprBP	Vpr-binding protein
Vpu	Viral protein U
Vpx	Viral protein X
XPC	Xeroderma pigmentosum C

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INTRODUCTION

1. HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of the global epidemics of AIDS. In 2008, 33.4 million individuals were HIV-1-positive. An estimated 2.7 millions new HIV-1 infections occurred and 2 million people died of AIDS- related diseases. Due to the beneficial effects of the introduction of anti-retroviral therapy in low-income countries, the estimated number of new HIV-1 infection cases was 30% lower than at the peak of the epidemics in 1996 and the number of AIDS-related death was 10% lower than at the peak of mortality in 2004 [1].

HIV-1 is a highly heterogeneous virus and, based on genetic similarities, is subdivided into 4 groups: M, O, N, and P. Group M is responsible for the present epidemic and, due to founder effects, can be further subdivided into clades or subtypes (A to K). Clade B viruses are the most prevalent in Western countries whereas C is the most prominent globally. Because the epidemic originated in Africa, it has the most heterogeneous viral distribution and inter-clade or inter-group recombinants are commonly observed. Groups N, O, and P only represent a minority of cases and are typically restricted to some regions of Africa [2-4]. HIV is a zoonosis and each group is thought to have originated from a single independent cross-species transmission from a primate, the natural reservoir, to a human. Group M and N likely originated from chimpanzees (*Pan troglodytes troglodytes*) in South Cameroon, group O from either chimpanzees or gorillas (*Gorilla gorilla gorilla*) [5-8], and Group P from gorillas [2]. There exists a second human immunodeficiency virus called HIV-2. HIV-2 is characterized by a lower pathogenicity and is principally restricted to Western Africa. It probably arose from at least 8 independent cross-species transmissions from sooty mangabeys (*Cercocebus atys*) to humans [5,9]. Recent molecular clocking analyses situate the origins of the HIV-1 epidemic to the early 20th century in Belgian Congo (now Democratic Republic of Congo). Trans-species transmissions between chimpanzees and humans might have occurred before this period but social conditions

and population densities resulting from the establishment of colonial cities such as Leopoldville (now Kinshasa) probably generated the optimal conditions for the global spread of the sexually transmitted disease [10,11].

1.1. HIV-1 and the retrovirus family

HIV-1 belongs to the retrovirus family (Retroviridae), which comprises enveloped viruses with linear, non-segmented, positive, single-stranded RNA genomes. The hallmarks of this family of viruses are that they require reverse transcription of the viral genome into linear double-stranded DNA and subsequently stable integration of their genome into the host DNA. Retroviruses are ubiquitous and are present in all classes of vertebrates. All retroviruses possess three major genes: *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope). Particles vary in size from 80 to 120 nm in diameter and are covered by envelope glycoprotein spikes [12,13]. The genus lentivirus, of which HIV-1 is the prototypic example, comprises complex retroviruses that encode additional unique auxiliary proteins. Other defining characteristics of the lentiviral genus include a curved hexagonal viral core called a fullerene cone and a biphasic mode of gene expression [14,15]. All lentiviruses can infect macrophages and primate lentiviruses exhibit a strong tropism for CD4-expressing cells, including macrophages and T helper lymphocytes. In contrast to several other retroviruses, lentiviruses do not directly induce oncogenesis. Common manifestations of lentiviral diseases include long incubation time, persistent chronic viral replication, encephalopathy, and suppression of specific haematopoietic or immune cell types [16].

1.2. Genetic organization

As mentioned above, HIV-1 like all retroviruses harbours the *gag*, *pol*, and *env* genes (Figure 1, p.3). The HIV-1 *gag* gene encodes the viral structural proteins matrix (MA, p12), capsid (CA, p24), and nucleocapsid (NC, p7) expressed as part of a myristilated precursor polyprotein (p55). Individual components are released following the proteolytic processing of the polyprotein by the viral protease. The gag precursor protein also possesses p1, p2, and p6 domains which are not thought to play functions as individual proteins but rather contribute to the regulation or functions of the precursor polyprotein. The *pol* gene produces a precursor polyprotein fused to the Gag

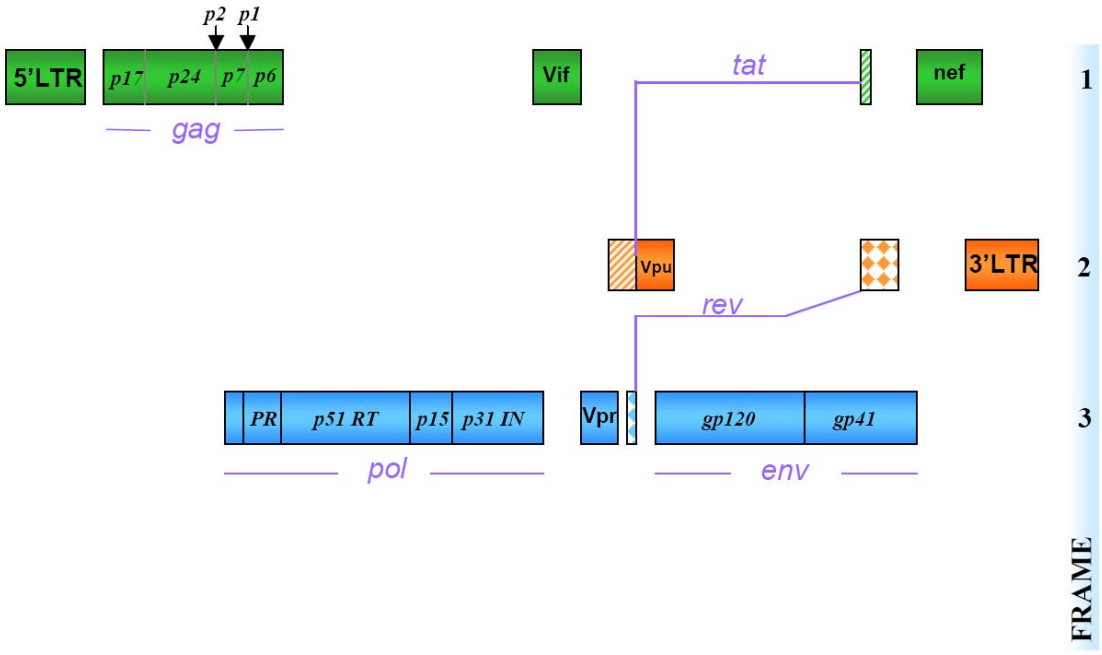

Figure 1. HIV-1 genomic organization.

The HIV-1 genome is flanked by two long-terminal repeats (LTR) and takes advantage of its three reading frames. HIV-1 possesses the conserved retroviral genes *gag*, *pol*, and *env*. The HIV-1 genome also contains six additional genes that encode two regulatory proteins (Tat and Rev) and four accessory proteins (Nef, Vif, Vpr, and Vpu).

polyprotein via a ribosomal frameshift. Proteolytic processing of Gag-pol yields the viral enzymatic proteins protease (Pr), reverse transcriptase (RT), and integrase (IN). The *env* gene encodes the two envelope subunits, gp120 and gp41, which are first expressed as a single precursor protein (gp160) and later cleaved by a cellular furin-like protease. The HIV-1 genome also contains six additional genes that encode the two regulatory proteins Tat (transcriptional transactivator) and Rev (regulator of virion gene expression) and the four accessory proteins: Nef (negative factor), Vif (viral infectivity factor), Vpr (viral protein R), and Vpu (viral protein U). HIV-2 (human immunodeficiency virus type 2) and some simian immunodeficiency virus (SIV) isolates of the HIV-2/SIV_{sm} (sooty mangabey) lineage possess a fifth accessory gene called Vpx (viral protein X), but do not encode for a Vpu protein. Tat and Rev are involved in HIV gene expression and proper splicing and export of the different mRNA species whereas the accessory proteins modulate host immune responses and facilitate viral replication in specific cell types. Except for Vpu and Env, which are expressed from the same mRNA, and Pol, which is expressed following a ribosomal frameshift, all the other HIV proteins are expressed from their own unique singly or multiply spliced mRNA. HIV genes are enclosed between two identical copies of the long terminal repeat (LTR). The LTR is subdivided in three regions: U3 (unique to 3'end), R (repeated sequence), and U5 (unique to 5'end). The transcription start site is located at the junction of U3 and R whereas the poly(A) signal is at the boundary of R and U5. Finally, the U3 region contains most of the transcriptional regulatory elements [13].

1.3. Overview of the HIV-1 replication cycle

The HIV-1 infection cycle (Figure 2, p.5) starts with the docking of the trimeric envelope glycoproteins (containing the gp41 and gp120 subunits) to its cognate cellular receptor CD4 [17,18]. The dendritic cell surface molecule DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) can also be used as an attachment factor for HIV-1. Although it can sometimes lead to productive infection of dentritic cells, it is generally exploited by HIV-1 as vehicle for dissemination of the virus within the host and as a mean to facilitate cell-to-cell transmission [19]. Binding of gp120 to CD4 induces a conformational change in gp120 that exposes its V3 loop

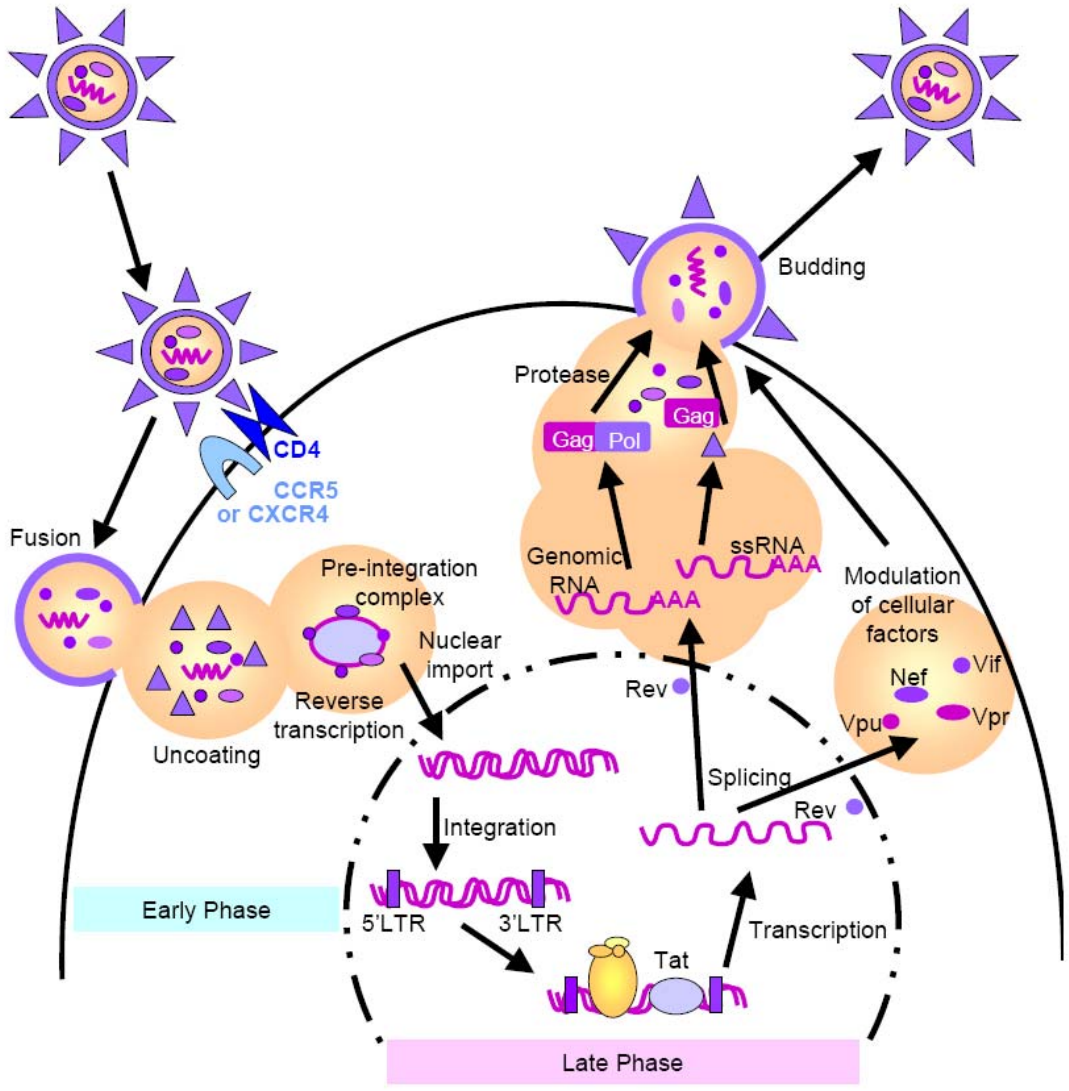

Figure 2. HIV-1 replication cycle.

The HIV-1 replication cycle can be subdivided in two phases: early and late. The early phase includes docking of the viral envelop to its primary receptor CD4 and a co-receptor, inducing fusion and released of the viral core into the cytoplasm. Entry is followed by reverse transcription of the viral genomic RNA into double-stranded DNA and ultimately by the stable integration of the viral genome into the host genomic DNA. The late phase of the infection consists of the expression of the various viral proteins and of the viral genomic RNA, resulting in the assembly and budding of progeny virions. Please refer to the main text for a detailed description of each step.

[20,21] enabling an interaction with its primary co-receptor CCR5 (C-C chemokine receptor type 5) [22]. A switch to CXCR4 (chemokine (CXC motif) receptor 4) in co-receptor usage happens late in infection in approximately 50% of infected individuals and is associated with rapid disease progression [23]. This second interaction event induces a series of conformation changes, this time in gp41, which induces the fusion of the viral membrane with the cellular plasma membrane following the insertion of the N-terminal fusion peptide [24-26]. It was long thought that HIV-1 was entering cells through fusion at the plasma membrane. However, recent evidence shows that although fusion might be initiated at the plasma membrane, it is completed after the virus has engaged the classical endocytic route [27,28]. Membrane fusion effectively releases the viral core into the cytoplasm of the cell [24,25]. There follows a series of poorly understood steps called uncoating whereby capsid proteins are shed from the viral core [29]. Once the RNA-capsid complex attains a certain level of maturation, reverse transcription is initiated [29,30]. It is a complex mechanism that involves multiple priming and initiation steps. The end product of reverse transcription is a double-stranded linear proviral DNA with a short overhanging structure called DNA flap on the positive strand [31]. As reverse transcription proceeds, the viral core continues its maturation and is transported along the microtubule networks toward the nuclear membrane. Once it reaches the nuclear membrane it is called the pre-integration complex and is now competent for nuclear import and integration into the host genome [29,30]. It is actively imported into the nucleoplasm via a nuclear pore and with the help of its karyophilic elements Vpr, MA, integrase, and the DNA flap [32]. As the provirus reaches the nucleoplasm it is tethered to chromatin via an interaction between the viral integrase and the stress-induced transcription factor LEDGF/p75 (Lens epithelium-derived growth factor, p75) [33-35]. Multimers of the viral integrase then trims two nucleotide at each end of the proviral DNA and catalyzes the nucleophilic attack of these ends onto the host genomic DNA [36]. The integration reaction leaves DNA overhangs that are subsequently repaired by the cellular DNA repair machinery. Which repair and sensor proteins are involved in this process remains highly controversial and is probably cell-type specific [37-40]. Once repair is complete, the late phase of the infection starts.

The dominant factor that promotes expression from the HIV-1 LTR is the viral regulatory protein Tat. It trans-activates the LTR by recruiting the positive transcription elongation factor b (P-TEFb) and histone acetyl transferase complexes and by activating NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). Overall, Tat increases LTR activity by several hundred folds [41-43]. Cis-acting elements in the viral RNA leads to the generation of unspliced, partially spliced or fully spliced RNA that are going to produce the different viral proteins [44]. Since nuclear export of RNA is tightly coupled with splicing, HIV has developed its own mechanism to promote efficient nuclear export of unspliced or partially spliced RNA. Multimers of the viral regulatory protein Rev binds to the rev-responsive element (RRE) and mediate nuclear export of RNA via an interaction with the nuclear export factor exportin 1. This mode of export is energy-dependent and requires Ran GTPase [45,46]. The primary sites of virion assembly are at the plasma membrane and appear to be concentrated in some membrane subdomains such as lipid rafts [47,48], tetraspanin-rich domains [49,50] and regions of cell-to-cell contact [51]. One of the defining features of viral assembly is the interaction of the MA portion of the myristoylated Gag precursor protein with membranes containing the lipid PI(4,5)P2. Gag-Gag and Gag-lipid interactions probably cooperate to stabilize Gag assembly at the plasma membrane [52-54]. Gag-Pol is similarly incorporated into assembling virions [55,56]. Encapsidation of the viral genomic RNA requires an interaction between its packaging signal and the NC domain of Gag [57-59]. The envelope precursor protein gp160 is translated in the endoplasmic reticulum and forms trimers via disulfide bonds. It is then transported through the Golgi secretory pathway where it is heavily glycosylated and cleaved into its individual subunits gp120 and gp41 [60]. The incorporation of envelope glycoproteins into virions is still not well understood but probably involves viral as well as cellular proteins [61]. Budding of virions is mediated by the direct recruitment of the cellular ESCRT-I (endosomal sorting complex 1 required for transport) and ESCRT-III (endosomal sorting complex 3 required for transport) complexes. This is achieved via interactions between late domains in the p6 region of Gag and the ESCRT protein TSG101 (Tumour susceptibility gene 101) and AIP1/Alix (ASK-interacting protein 1). The ESCRT

machinery then induces membrane curvature and finally release of virions [54,62,63]. The final step of the viral cycle is characterized by the maturation of the spherical viral core into a conical core. This process involves a major conformational rearrangement mediated by the viral protease. The protease sequentially cleaves the individual components of the Gag and Gag-Pol precursor proteins resulting in the realignment of capsid proteins around the RNA/protein complex [64,65].

1.4. Roles of HIV-1 accessory proteins in pathogenesis and immune evasion

The hallmarks of HIV-1 infection have long been considered as the progressive infection and destruction of the pool of CD4+ lymphocytes, thus inducing profound immune dysfunction and ultimately immunodeficiency [66,67]. However, recent observations in simian models have considerably changed our understanding of AIDS as well as of the immunobiological paradigms involved in progression towards this condition. Indeed, extensive studies of non-pathogenic infections in sooty mangabeys have revealed that despite high levels of viral replication and dramatic CD4+ T-cell depletion, these rarely develop AIDS [68]. One central phenotype that distinguishes pathogenic versus non-pathogenic infections is sustained immune activation. In this context, disease progression positively correlates with markers of T cells activation and is associated with widespread apoptosis in B and T cells as well as increased levels of pro-apoptotic and immunosuppressive tumour necrosis factor (TNF), TRAIL (TNF-related apoptosis-inducing ligand) and FAS ligand (FASL, CD154). Multiple factors and events likely contribute to the establishment of chronic immune activation early during HIV-1 infection. These include direct viral infection of immune cells, release of pro-inflammatory cytokines, perturbation of mucosal immunity, translocation of microbes across a damaged intestinal epithelium, and an aberrant balance between pro-inflammatory TH17 (T helper 17) and immunosuppressive TReg cells (regulatory T cells) [69-72]. The ability of the virus to establish a persistent infection and avoid immune eradication is therefore a prime contributor to the exhaustion of the immune system. The main culprits responsible for ongoing viral replication, immune evasion, and immunomodulatory adverse effects are likely to be the HIV-1 accessory proteins:

Vif, Vpu, Nef, and Vpr. They are referred to as 'accessory' because they generally only have a marginal role in viral replication *in vitro* but are essential for viral replication and pathogenesis in the host [73].

1.4.1. VIF

HIV-1 viral infectivity factor (Vif) is a 23-kDa acid cytoplasmic protein that is expressed at high levels late in the infection [74,75]. Vif is also incorporated at low levels in budding virions [76,77]. The most significant function of Vif was recently uncovered at the molecular level but had been evident for some years. Vif was known to be essential for the replication of HIV-1 in peripheral blood lymphocytes, macrophages, and some cell lines characterized as 'non-permissive' cells [78-80]. Vif was capable of increasing viral infectivity of virions produced from 'non-permissive' cells but had no effect on virions produced in 'permissive' cells [81-84]. Heterokaryon experiments between permissive and non-permissive cells suggested that Vif was able to counteract a cellular factor restricting viral infectivity [85,86].

This cellular 'restriction factor' was later identified as APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G) [87]. APOBEC3G is a ssDNA cytidine deaminase of the APOBEC family which also includes AID. In the absence of Vif, ABOBEC3G is incorporated into progeny virions and impairs the efficiency and accuracy of reverse transcription [88]. The mechanistic nature of this inhibition has been however controversial. Some investigators found that APOBEC3G could restrict HIV-1 replication independently of its deaminase activity [89-93], whereas others, using stable expression systems mimicking physiological conditions, observed that the enzymatic function was critical for the restriction [94,95]. APOBEC3F, another protein of the same family, was also found to restrict HIV-1 replication and to be inactivated by Vif [96-99]. Both enzymes preferentially target CC dinucleotides although not exclusively and induce G-to-A hypermutations in the plus-strand of the proviral DNA [96,97,100-102]. In absence of Vif, they are packaged

inside virions and associate with the viral core [103,104]. The mechanism of encapsidation appears to rely mainly on the packaging of viral genomic RNA [103,104], although other components including NC and the cellular 7SL RNA might also be required [105]. Vif from HIV-1 and SIV_{agm} (SIV African green monkey) directly interacts with APOBEC3G in a highly species-specific manner. On the other hand, SIV_{mac} (SIV macaque) Vif can inhibit APOBEC3G from humans, African green monkeys, and rhesus macaques [106] . Surprisingly, a single amino acid residue at position 128 in human APOBEC3G was responsible for the species specificity of the interaction with Vif. Conversion of this residue to match the agm sequence (D128K) was sufficient to exchange the sensitivity to HIV-1 and SIV_{agm} Vif [107-110]. Vif targets APOBEC3G and 3F to a Cullin 5 RING (really interesting new gene)–E3 ubiquitin ligase complex (CRL5) through a direct interaction with some of its components [111-114]. A SLQ(Y/F)LA motif similar to a conserved motif in the BC box of the suppressors of cytokine signalling (SOCS) proteins was found to mediate association of Vif to elongin C, an adaptor of CRL5 ligases [113]. Vif also directly associate with Cullin 5 via its HCCH zinc-binding domain [112,113]. Vif would target APOBEC3G and 3F to the CRL5 complex and induce their polyubiquitination and resulting in their proteasomal degradation [106,111,113,115]. Evidence for APOBEC3G polyubiquitination have however only been obtained *in vitro* [116,117]. Mutations of all the possible lysine acceptor sites yielded inconsistent results with some investigators finding that it prevented APOBEC3G degradation [118] whereas others observed no effect [119]. Some have proposed that Vif itself could be ubiquitinated and could act as the degradation signal [119]. Additionally, increasing evidence showed that Vif can also inhibit APOBEC3G in a degradation-independent manner. Expression of Vif led to reduced viral incorporation of a degradation-insensitive mutant of APOBEC3G [120]. Vif (S144A) induces degradation of APOBEC3G but is unable to prevent the restricting activity of APOBEC3G on progeny virions [113]. Therefore, Vif has the ability to inactivate APOBEC3G and 3F using degradation-dependent as well as degradation-independent mechanisms.

Besides the inactivation of APOBEC3G and 3F, Vif performs other functions in the viral replication cycle. Vif was shown to induce cell cycle perturbation in the G2 phase in infected cells [121,122]. In contrast to Vpr, which arrests cells in G2, Vif would only cause delays in cell cycle progression [123]. Recruitment of the CRL5 ubiquitin ligase by Vif was found to be essential for this activity but the presence of APOBEC3 family members was dispensable [123]. It is therefore thought that Vif would target a yet-unknown cellular factor for ubiquitination and degradation in order to induce this cell cycle delay [123]. The role of this activity of Vif and its potential interplay with Vpr-induced G2 arrest is not understood. Interestingly, a recent report shows that Vif might modulate Vpr-induced cell cycle arrest by inducing the degradation of Vpr [124]. Other poorly understood functions of Vif include contribution to viral assembly [125,126] and protection of viral cores during uncoating [127].

1.4.2. VPU

Vpu is a 16-kDa class I transmembrane protein present in HIV-1 and some SIV lineages (chimpanzee, greater spot-nosed monkey, mustached monkey, mona monkey, Dent's mona monkey, and gorilla). The protein is amphipathic and composed of an N-terminal hydrophobic domain and C-terminal hydrophilic cytoplasmic domain. Vpu is expressed late during infection from an Env-Vpu bicistronic RNA and is inserted in membranes at the level of the endoplasmic reticulum [73,128,129]. The C-terminal domain of Vpu contains two cysteine residues (S52 and S56) that are phosphorylated by casein kinase II [130]. Vpu performs two main biological functions during infection: down-regulation of neo-synthesized CD4 and enhancement of viral release by counter-acting the cellular restriction factor Tetherin.

Down-modulation by HIV-1 of its own primary receptor, CD4, is of capital importance for replication and pathogenesis, as evidenced by the fact that the virus devotes three proteins (Vpu, Nef, and the envelop precursor gp160) to this process [131]. Nef is expressed early during infection and rapidly removes cell-surface CD4

molecules by enhancing their endocytosis and lysosomal degradation (see section 1.4.3). On the other hand, gp160 and Vpu are expressed late in the infection and interfere with transport of neo-synthesized CD4 by respectively sequestering it in the endoplasmic reticulum and inducing its proteasomal degradation [132]. The exact beneficial role of CD4 down-modulation has remained debated but might include enhancement of virion release and infectivity [133-138], diminution of super-infection [139-141], and interference with T-cell activation [142]. Degradation of CD4 by Vpu involves the recruitment of the cellular Cullin 1-based E3 ubiquitin ligase complex SCF (Skp1/Cullin 1/F-box) via a direct interaction with the substrate receptor β-TrCP (β-transducin repeat-containing protein) [143]. Recruitment of this complex requires phosphorylation at S52 and S56 on Vpu [143]. Vpu was found to act as a bridge between this complex and neo-synthesized CD4, inducing its ubiquitination and proteasomal degradation [143-145]. Additional membrane dislocation events reminescent of ERAD (endoplasmic reticulum associated degradation) might also be required for CD4 degradation [146,147].

Vpu had been known for several years to enhance viral particle release in a cell type-dependent manner [148-151]. Heterokaryon experiments between Vpu-sensitive and insensitive cells have shown that Vpu was counteracting a putative cellular restriction factor [152]. This factor was later discovered to be the interferon-inducible cell-surface protein BST-2/CD317, renamed Tetherin because of its ability to trap budding viruses onto the plasma membrane and to subsequently induce their endocytosis [153-156]. Tetherin activity is not specific to HIV-1 but can also restrict release of other retroviruses or even of non-related enveloped viruses including KSHV (Karposi's sarcoma-associated herpesvirus) and Ebola [157-160]. Owing to its unusual topology, which includes both transmembrane and GPI (glycosylphosphatidylinositol) anchor domains [161], Tetherin is incorporated into nascent viral buds and effectively retain them by acting as a bridge to the plasma membrane [162]. The mechanism by which Vpu is able to counteract Tetherin has been the subject of an intense debate. Some investigators found that Vpu could enhance the endocytosis of cell-surface Tetherin, targeting it for lysosomal degradation [163,164] whereas others found that Vpu, via recruitment of $SCF^{\beta\text{-}TrCP}$, could induce the proteasomal degradation of neo-

synthesized Tetherin at the level of the endoplasmic reticulum [165,166] or target Tetherin for lysosomal degradation from the *trans*-Golgi network or early endosomes [167]. The results of this last study are supported by the observation that localization of Vpu to the *trans*-Golgi network correlates with its anti-Tetherin activity [168]. In contrast, other studies did not find that cell-surface down-modulation or intracellular degradation of Tetherin could fully account for the antagonizing effect of Vpu [169]. Moreover, the effect of Vpu was found to be specific to human Tetherin and could not relieve the restriction imposed by simian or rodent Tetherins [166,170-172]. Interestingly, Vpu from SIVcpz (SIV chimpanzee) does not antagonize Tetherin. Rather, Nef performs this activity [129]. In HIV-2 or in most other SIV isolates, Nef [170,173] or the envelope glycoproteins [174,175] are responsible for inhibiting Tetherin, suggesting that intense selective pressure during interspecies transmission would have driven different evolutional solutions [129]. Initially, another cellular protein called CAML (calcium-modulating cyclophilin ligand) had also been proposed to act as an additional Vpu-sensitive viral release restriction or perhaps as a cofactor for Tetherin [176], but it has now become apparent that it is probably not the case [177].

Besides its two main biological functions, Vpu has also been shown to down-modulate cell-surface expression of MHC-I (major histocompatibility complex class I) [178] and mature MHC-II (major histocompatibility complex class II) molecules [179]. However, the mechanisms and functional implications of these events still remain poorly understood. Moreover, expression of Vpu was shown to induce apoptosis by sequestering β-TrCP and thus inhibiting its normal function in cell cycle regulation [180]. It remains unknown whether Vpu-induced apoptosis in infected cells would contribute to HIV-1 pathogenesis and what would be the interplay with other pro-apoptotic viral factors (i.e. Vpr, Tat, and gp41).

1.4.3. NEF

The erroneously named negative factor (Nef) is a 27-kDa accessory proteins present in all lentiviral lineages and expressed very early in the viral replication cycle.

Myristoylation of its N-terminus is essential for its association to the cytoplasmic side of membranes and is critical for all of Nef functions [181]. A striking array of biological and immunomodulatory activities have been attributed to Nef including down-modulation of CD4, MHC class I and MHC class II as well as enhancement of viral infectivity and replication [142]. The importance of these Nef functions for viral immune evasion and pathogenesis has been illustrated by the observed prolonged survival of humans [182,183] or rhesus macaques [184] infected with viral strains lacking fully functional Nef proteins. Nef is however not absolutely required for progression to AIDS but instead appears to accelerate it given that individuals [185] or macaques [186] infected with Nef-defective viruses eventually develop immunodeficiency in absence of reversion to wild type Nef.

As mentioned above, down-modulation of cell-surface CD4 molecules by Nef complements the activity of Vpu and gp160 on neo-synthesized CD4 [131]. Nef is able to accelerate the endocytosis of CD4 by directly interacting with the cytoplasmic tail of the receptor [187,188] and by rerouting a number of intracellular trafficking factors [189]. CD4 cell-surface down-modulation requires the recruitment of AP-2 (adaptor protein 2) [190-193] by dileucine and diacidic motifs present on the Nef C-terminal flexible domain and targets CD4 for endocytosis via clathrin-coated vesicles [194,195]. Nef subsequently directs CD4 to the multivesicular body (MVB) pathway leading to its degradation in the lysosomes in a process that requires ESCRT complexes but was surprisingly independent of CD4 and Nef ubiquitination [196]. In contrast, another study showed that ubiquitination of Nef on lysine 144 was necessary for CD4 down-modulation [197]. Finally, other investigators reported that targeting to lysosomes involved the direct recruitment of β-COP (coatomer protein complex subunit beta) by Nef [198]. It is unclear at present whether the MVB and β-COP pathways might somehow be functionally linked.

Nef has also been implicated in the selective down-modulation of the MHC-I molecules HLA-A and HLA-B without affecting HLA-C and HLA-E [199]. This selective preservation of HLA-C and HLA-E would permit immune evasion from

cytotoxic T-lymphocytes but would protect infected cells against subsets of natural killer (NK) cells [199,200]. In contrast to CD4, Nef does not primarily target cell-surface MHC-I molecules but rather reroute them from the *trans*-Golgi network to lysosomes [189]. Nef accomplishes this by interacting directly with the cytoplasmic tail of MHC-I and by recruiting the clathrin adaptor protein 1 (AP-1) [201,202]. Recruitment of β-COP by Nef would then be necessary to target MHC-I to lysosomes [198]. Additionally, Nef marginally accelerates the endocytosis of MHC-I from the cell surface in an AP-1-dependent but clathrin-independent pathway and targets it to lysosomes [203]. This latter mechanism is thus distinct from cell-surface CD4 internalization and requires the GTPase ARF6 *(*ADP-ribosylation factor 6) [204].

HIV-1 Nef was also shown to down-regulate cell-surface MHC-II molecules, while increasing cell-surface levels of the invariant chain (Ii). Both mechanisms would lead to defective antigen presentation in macrophages, dentritic cells as well as activated helper T-lymphocytes [205]. Additionally, HIV Nef can down-regulate the co-stimulatory molecule CD28 and chemokine receptors including CXCR4 from the cell surface via its interaction with AP-2 (adaptor protein 2)[206,207]. Various other cell-surface molecules have also been shown to be down-modulated by Nef including NKG2D (natural-killer group 2, member D) ligands [208] and NKp44L (natural killer protein 44 ligand) [209], effectively promoting viral immune evasion from cytotoxic effector cells. Importantly, down-modulation of CD28, CD4 and MHC-I by Nef are genetically separable, suggesting that these molecules are selected independently rather than targeted by an overall increase in endocytosis [207]. Conversely and despite down-modulation of these cell-surface receptors, expression of HIV-1 Nef has been associated with an increased T-cell activation following its recruitment to the immunological synapse [210-212]. Another recently elucidated function of Nef is its effect on isotype-class switching in B-lymphocytes. Nef induces the formation of intercellular long-range actin-propelled conduits between infected macrophages and B-lymphocytes in systemic as well as gastrointestinal lymphoid follicles. Nef can penetrate B-cells by travelling along these structures in both clathrin-dependent and clathrin-independent vesicles [213]. Nef inhibits immunoglobulin class-switch DNA recombination by up-regulating

IκBα (inhibitor of NF-κB alpha) and SOCS proteins, thus blocking CD154 and cytokine signalling and inhibiting AID expression [214]. It remains to be seen if Nef-mediated formation of long-range intercellular conduits could have other implications for viral replication and pathogenesis.

Significant phenotypic and structural differences exist between the various alleles of Nef. Notably, most SIV Nef alleles encode longer Nef species of approximately 35 kDa that display only low levels of amino acid identity (30%) compared to those of the HIV-1 allele [142]. The down-regulation of CD4, CD28, and MHC-I is generally well conserved between HIV-1 Nef and several SIV alleles [215]. One striking difference however is the efficient down-modulation of CD3-TCR by most SIV and HIV-2 Nef alleles but not by those of pathogenic HIV-1 or SIV of the chimpanzee lineage [215]. Some investigators have recently proposed that down-modulation of CD3-TCR by SIV would lead to reduced CD4+ T-cell activation, proliferation and apoptosis, allowing the host to maintain functional immune responses [142].

In addition to its immunomodulatory role, Nef additionally increases viral infectivity at an early step of infection [216-218] by a poorly understood mechanism, which requires its expression in virus-producing cells [219] but does not necessitate its incorporation in viral particles [220,221]. Increased virion cholesterol content [222], increased viral entry [223], reduced susceptibility of viral cores to proteasomal degradation [224] as well as facilitated transport of the viral genome through the cortical actin network [225] have all been proposed to account for this effect of Nef. Recently, an interaction between Nef and the cellular protein dynamin-2 has also been implicated in this function [226]. Finally, Nef can increase viral production by at least two distinct mechanisms. HIV-1 Nef is transcribed very early from pre-integrated proviral DNA [227] in a selective process mediated by Vpr [228] and modulates the transcriptional activity of resting T-cells [227]. The second mechanism involves the enhancement of Tat-mediated transactivation of the LTR [229].

1.4.4. VPR

Viral protein R (Vpr) is a small protein of 96 amino acids (in HIV-1) that is well conserved among the different HIV and SIV lineages (Figure 3, p.18) [230,231]. The mRNA encoding for Vpr is expressed late during the course of infection [232]. Vpr is however present in the early phase of the infection as a PIC (pre-integration complex)-associated proteins [233-238] since it is encapsidated in virions [239,240] via a direct interaction with the p6 domain of the Gag precursor protein [241-244] and is a component of the viral core [245,246]. It is also released in the extracellular medium of *in vitro* infected cells [247] and can be found in the plasma and cerebrospinal fluid of infected patients [248,249]. Interestingly, Vpr, in its soluble form, has the ability to transduce non-infected cells [250-252]. Vpr performs several roles in viral replication and pathogenesis including the induction of G2 arrest and apoptosis, the promotion of viral replication, the up-regulation of LTR activity and immunomodulation.

Vpr has a simple secondary structure composed of disordered N- and C-termini and of three central alpha helices [253-256]. The tertiary structure of a full-length synthetic Vpr in 30% acetonitrile has recently been resolved by nuclear magnetic resonance (Figure 4, p.19). In these conditions, Vpr forms a compact hydrophobic core centred on the third helix (residues 56-77) with stabilizing hydrophobic intramolecular interactions with the first (residues 17-33) and second helices (residues 38-50) [253]. This closed tertiary structure of Vpr is thought to be important for its functions [253,257]. The determinants responsible for viral incorporation have been mapped to the first helix [242,258] but may required additional structural elements in the second helix [259]. Moreover, Vpr possesses two non-classical nuclear localization signals (NLS), in the first and third helices [258,260-264]. The third helix also contains a nuclear export signal (NES) and is involved in the nucleocytoplasmic shuttling of the protein [260]. A hydrophobic leucine/isoleucine-rich region in the third helix is also thought to act as a leucine-zipper, mediating interactions with several cellular proteins including Sp1 transcription factor, E1A-binding protein 300 (p300), GR

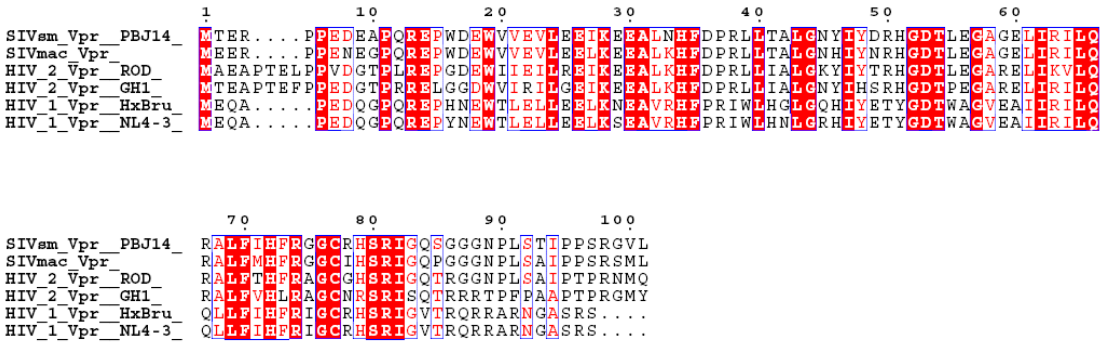

Figure 3. Conservation of Vpr throughout primate lentiviral evolution.

The amino acid sequences of HIV-1 Vpr (laboratory strains NL4-3 and HxBru) were aligned with Vpr sequences from HIV-2 (strains ROD and GH1), SIV_{sm} (isolate PBJ14), and SIV_{mac} (isolate 239) using the ClustalW software. Amino acids highlighted in red display perfect identity whereas amino acids written in red show conservation of charges.

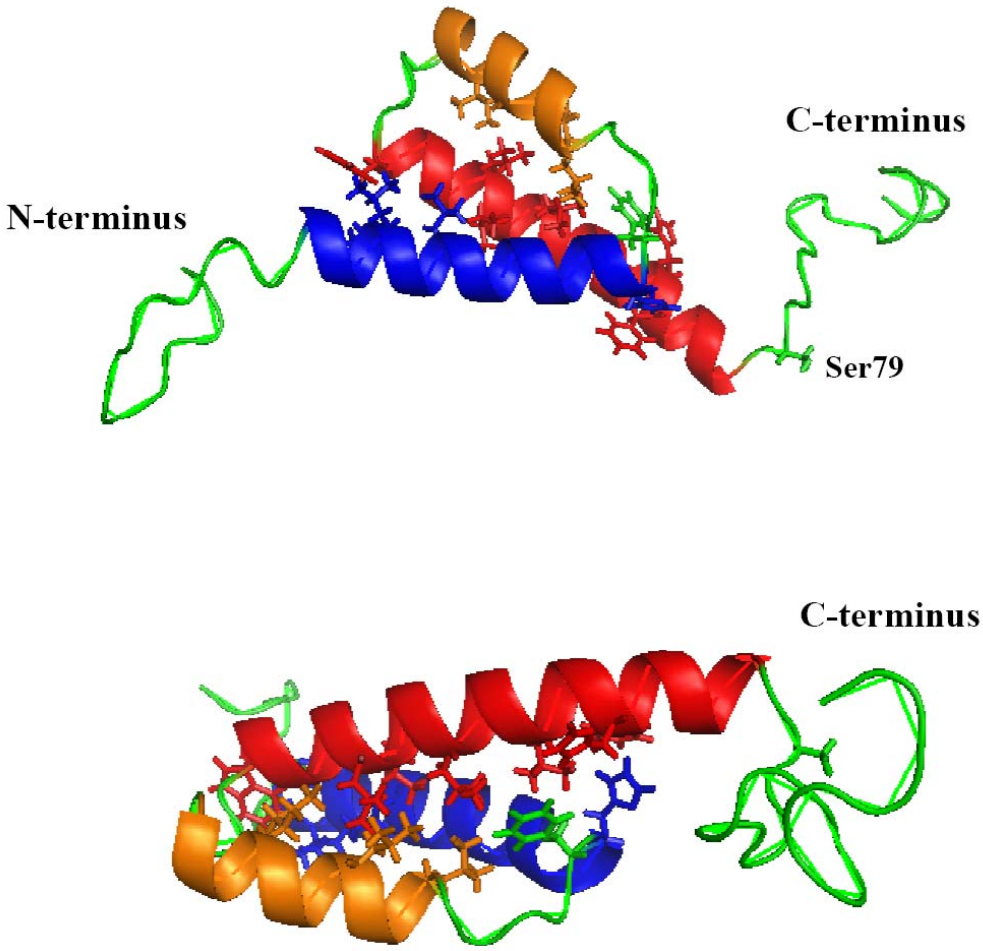

Figure 4. NMR structure of HIV-1 Vpr.

Two different views of the NMR structure of Vpr are shown. The first alpha-helix (residues 17-33 in blue), the second alpha-helix (residues 38-50 in orange) and the third alpha-helix (residues 56-77 in red) are displayed in ribbon form. Flexible disordered structures, including the N- and C-termini, are shown in green. The side chains of residues (T19, L20, L23, L26, H33, F34, L39, L42, I46, W54, I60, L64, L68, H71, and F72) stabilizing the hydrophobic core are depicted. The position of the phospho-residue S79 is also indicated.

(glucocorticoid receptor), and VprBP (Vpr-binding protein) [265-269]. The flexible C-terminus of Vpr is enriched in arginine residues and is critical for its biological functions [262,270-273]. Finally, the role of the flexible N-terminus is poorly understood [257]. Several groups have also reported that Vpr can form dimers as well as higher-order multimers *in vitro* as well as *in vivo* [274-278]. The exact regions of Vpr responsible are not known but appear to involve all three alpha helices, implying that formation of the hydrophobic core would be essential [257,274-278]. The physiological relevance of this oligomerization is however poorly understood but might be important for its interaction with Gag and incorporation into budding particles [276].

In 1995, several groups reported that Vpr, when expressed alone or in the context of infection, arrested cells in the G2/M phase of the cell cycle [279-282]. This activity of Vpr is conserved among all five primate lentiviral lineages [283,284] and can be recapitulated with soluble Vpr [251,252]. Abnormal accumulation of cells in G2 has also been observed in infected patients, suggesting that Vpr-induced G2 arrest would play an important role in replication and pathogenesis [285]. The most likely mechanism by which Vpr would perturb cell cycle is that it would induce the canonical DNA damage checkpoint by activating ATR (ataxia telangiectasia and Rad3 related**)** but not its homolog ATM (Ataxia telangiectasia mutated) [285-287]. Activation of ATR by Vpr leads to phosphorylation of several effector molecules including H2AX (histone 2A, variant X), RPA (replication protein a), and Chk1 (checkpoint kinase 1) and is accompanied by the formation of DNA repair foci containing γ-H2AX (phosphorylated H2AX), 53BP1 (tumour suppressor protein 53-binding protein 1), BRCA1 (breast cancer 1, early onset), RPA as well as the 9-1-1 (Rad9-Hus1-Rad1), and Rad17-RFC (Rad17-replication factor C) complexes [285,286,288-290]. The downstream result of this series of events is the up-regulation of Wee1 following its phosphorylation and the 14-3-3-dependent inhibition of inactive phosphorylated Cdc25 (cell division cycle 25), resulting in the stable phosphorylation of CDK1 (cyclin-dependent kinase 1, also known as cdc2 in yeast) to prevent entry into mitosis [279,280,291,292]. Several mechanisms have been proposed to explain how Vpr would induce checkpoint activation. These include direct inactivation of Cdc25 by Vpr [293] or direct modulation of the activity of

the cellular factors 14-3-3 [294], Wee1 [295], CSN6VIP/mov34 (COP9 signalosome subunit 6) [296], hHR23A (human homolog A of RAD23) [297,298], and SAP145 (spliceosome-associated protein 145) [299]. However, in most cases, interaction between Vpr and these cellular proteins did not correlate with the induction of G2 arrest [266,300,301]. These interactions might potentiate checkpoint function by affecting downstream effectors but are unlikely to be responsible for ATR activation [302]. Finally, Vpr was shown to induce the formation of transient nuclear membrane deformations or herniations by a yet-unknown mechanism [303]. Although it is conceivable that perturbation or the laminar network might result in DNA damages or DNA replication stress recognized by ATR, the link between these two possible causative events has not been formally established. Therefore, the proximal causes of the activation of the ATR checkpoint by Vpr remain poorly understood.

Early on, the role of Vpr in facilitating viral replication was established by the observation that it weakly increased replication in transformed CD4+ T-lymphocytes [304,305]. This effect of Vpr was linked to its ability to transactivate the HIV LTR [304]. Infection of rhesus macaques with Vpr-defective mutant viruses reduced viral replication and delayed disease progression [306,307]. Moreover, Lang and colleagues reported that Vpr mutant viruses quickly reverted to wild type in a majority of cases [306]. Mutations affecting Vpr functions have also been identified in individuals naturally controlling the infection (long-term non-progressors) [308-313], thus emphasizing the relevance of this accessory protein for viral replication and pathogenesis *in vivo*. It is generally accepted that Vpr, particularly at low multiplicity of infection, has a weak (2 to 4 folds) stimulating effect on replication in transformed T-cell lines, primary CD4+ lymphocytes, PBMCs (peripheral blood mononuclear cells), and human lymphoid tissues (HLT) [304,305,314-316]. In contrast, other investigators did not observe a significant effect of Vpr on viral replication in quiescent or activated primary T-cells nor in cell lines [273,317,318]. Vpr, either as a virion-associated protein [236,270,314,317] or as a soluble factor [319], has also been implicated in the nuclear import of the pre-integration complex in non-dividing cells such as macrophages producing a significant increase in viral replication in these cell types. Finally,

extracellular Vpr can re-activate viral gene expression in latently infected cell lines and PBMCs [319]

As mention above, one of the first functions attributed to Vpr is its ability to transactivate the HIV-1 LTR and a variety of other viral and cellular promoters [304]. Although several mechanisms, including interaction with SP1, TFIIB (Transcription factor IIB), p300, and GR have been proposed [320], Vpr-induced modulation of transcription is likely a direct consequence of the induction of G2 arrest since the phenotype can be recapitulated by artificially arresting cells in G2 [321]. Vpr was also shown to induce apoptosis of infected cells [302]. The underlying molecular mechanism still remains controversial with some investigators finding that it is a direct consequence of prolong G2 arrest [288,322,323] whereas others observing that Vpr can induce apoptosis independently of G2 arrest [278,324,325]. The latter scenario was correlated with the ability of Vpr to bind ANT (adenine nucleotide translocator) and perturb the mitochondrial membrane potential [326,327]. Several investigators described an interaction between Vpr and the cellular DNA repair enzyme UNG2 (uracil-DNA glycosylase 2) [328]. While this interaction does not correlate with the induction of G2 arrest [329], its potential functional implications has remained controversial. Some investigators found that Vpr could mediate the incorporation of UNG2 in viral particles leading to an increased fidelity of reverse transcription [330,331] whereas others found that Vpr would instead induce down-modulation of UNG2 by proteasomal degradation [332,333] or by transcriptional repression [334]. Down-modulation of UNG2 was found to be associated with an up-regulation in LTR activity [333] or with an inhibition of APOBEC3G activity [332]. On the other hand, other investigators did not see any effect of Vpr on the expression and viral incorporation of UNG2 [335]. Finally, expression of Vpr was shown to perturb many immune functions in infected cells but also in bystander non-infected cells. These immune functions include T-cell activation, DC (dendritic cell) maturation, and NK cell function [336-339]. Recently, we and other investigators demonstrated that Vpr-induced G2 arrest induced the up-regulation of cell-surface NKG2D ligands, leading to increased NK cell cytolytic activity [340,341]. However, the functional consequences

of all these immunomodulatory functions of Vpr still need to be addressed in a physiologically relevant animal model.

2. THE UBIQUITIN-PROTEASOME SYSTEM

Vpu, Vif, and as will be exposed later in this thesis, Vpr, all perform their roles by hijacking Cullin-RING E3 ubiquitin ligases, which are components of the host ubiquitin-proteasome system, in order to inactivate cellular factors. In contrast, Nef appears to bypass the requirement for ubiquitination by directly linking its cellular target molecules to trafficking adaptors. A thorough understanding of the activities and regulatory mechanisms of ubiquitin-conjugating enzymes (ubiquitin ligases) is therefore warranted to fully appreciate the molecular processes involved in accessory protein functions.

2.1. Principles and mechanisms of ubiquitination

Ubiquitination (also known as ubiquitylation) is characterized by the covalent post-translational conjugation of a small protein called ubiquitin (Ub) to target proteins. Ubiquitin is 76-amino acid long and is highly conserved among all eukaryotes. Ubiquitin is expressed from four different genes: Ubc, Ubb, Uba52, and Uba80. These genes encode polyubiquitin precursors with C-terminal extensions or single ubiquitin molecules fused to ribosomal proteins. These must be proteolytically processed by several classes of ubiquitin-specific proteases known as deubiquitinases (DUBs) in order to release functional ubiquitin monomers [342-344]. Ubiquitin adopts a common structure known as a ubiquitin beta-grasp or fold, exposing its carboxy-terminal tail to form covalent linkages with target proteins [345].

In order to be conjugated to target proteins (Fig 5, p.24), ubiquitin must first be ‘activated’ by the enzymes Uba1 (ubiquitin-activating enzyme 1) or Uba6 (ubiquitin-

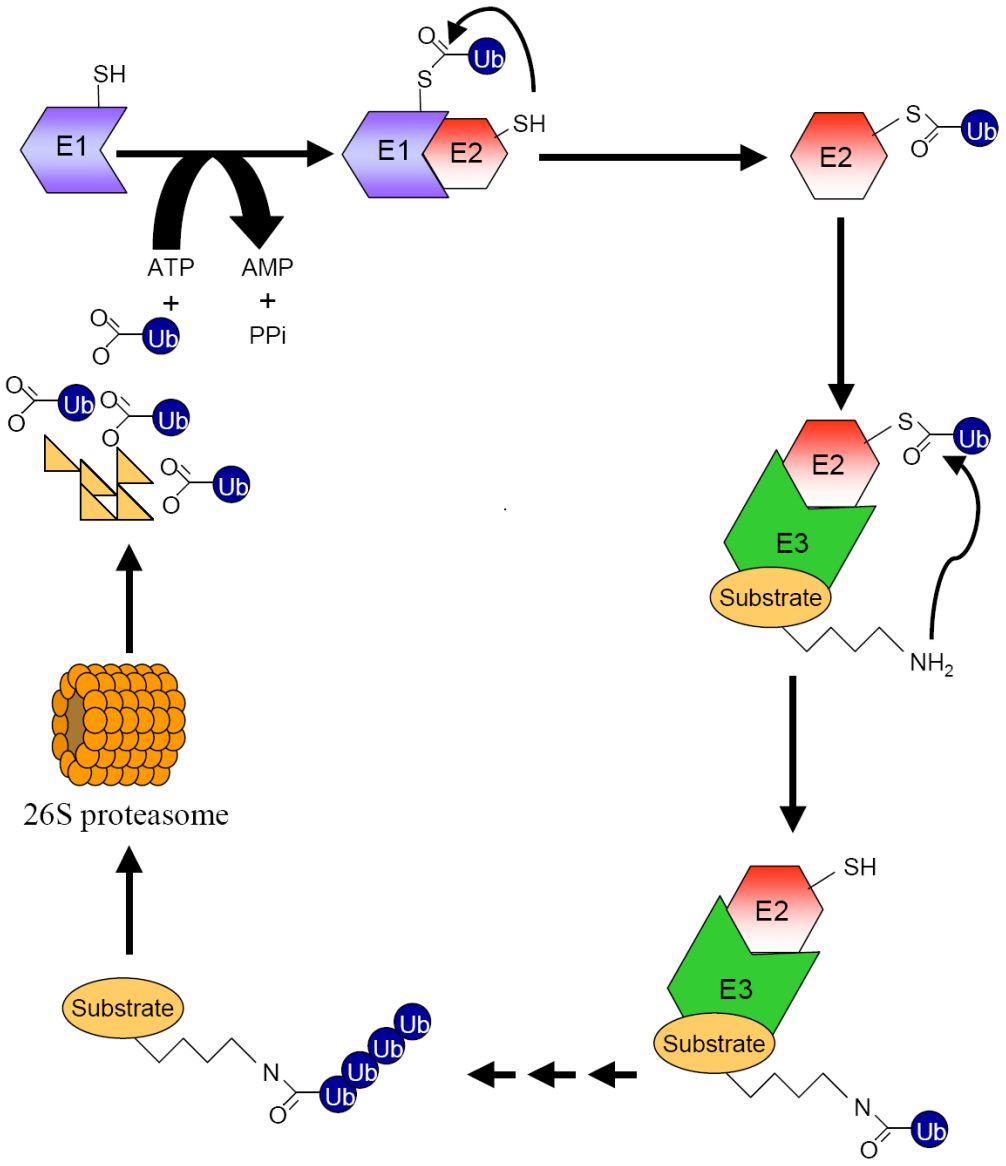

Figure 5. The ubiquitin conjugation cycle.

An E1 ubiquitin-activating enzyme 'activates' ubiquitin by forming a high-energy thioester bond on its catalytic cysteine. Ubiquitin is then transferred to the active site of an E2 conjugating enzyme. The E2 interacts with an E3 RING ligase and transfer the ubiquitin to an acceptor amino group on the E3-bound substrate protein. Multiple rounds of ubiquitination (polyubiquitination) target the substrate for proteasomal degradation. Finally, ubiquitin is recycled by the proteasome and can be used again for additional cycles of conjugation.

activating enzyme 6) commonly referred to as activating enzymes or E1. This is accomplished by the ATP-dependent attachment of the carboxyl group at the extreme C-terminus of ubiquitin to the sulfhydryl group of the active-site cysteine residue, forming a high-energy thioester conjugate [346,347]. The E1-conjugated ubiquitin is then transferred to the active-site cysteine residue in the ubiquitin-conjugating domain of an E2 conjugating enzyme. The human genome encodes at least 38 E2s. Once ubiquitin is transferred, the E1 is ejected from the E1-E2 complex, allowing the E2 to specifically interact with one of the estimated 600-1000 E3 ubiquitin ligases [346,348,349]. E3 ubiquitin ligases direct the specificity of ubiquitination by directly interacting with target substrate proteins or via multiple cofactors. They are subdivided in three main classes: HECT (Homologous to the E6-AP Carboxyl Terminus), RING, and U-Box. HECT ligases possess a conserved cysteine residue that first forms a thioester intermediate with ubiquitin and then catalyses the isopeptidic bond formation between an acceptor group and ubiquitin. HECT ligases represent a minor fraction with only 28 identified members to date. Notable examples include NEDD4, NEDD4L and HUWE1 [350]. RING ligases are characterized by their zinc-binding RING finger globular domain and are structurally and functionally related to the zinc-free U-Box ligases. RING-containing proteins are the most prevalent and constitute more than 95% of the E3 ubiquitin ligases. In contrast to HECT E3s, which directly catalyses transfer of ubiquitin to the target protein, RING and U-Box E3s merely serve as adaptors, connecting a catalytically active E2 with a target cellular protein. Although RING E3 ligases sometimes function as independent units, they are usually part of multiprotein complexes in which the E2- and substrate-interacting domain are on different subunits, as exemplified by APC/C (anaphase-promoting complex/cyclosome) and Cullin-RING ligases (CRLs), [351-353]. Mechanistically, ubiquitin transfer involves the E2- or E3-catalyzed deprotonation of the acceptor group, usually an amino group, and its nucleophilic attack on the ubiquitin-cysteine thioester conjugate, resulting in an isopeptidic (i.e. amide) bond between the C-terminal carboxy group of ubiquitin and the amino acceptor group [354]. The acceptor group is usually the ε-amino group of the lysine side-chain. However, more and more examples of ubiquitin transfer at the free N-terminal amino group of proteins are uncovered [355,356]. Moreover, a few reports

have documented ubiquitin transfer to cysteine, threonine, and serine residues. Because of the unstable nature of the thioester or ester bonds formed between ubiquitin and non-lysine residues, it is unclear at present whether such modifications are widespread or if they are only rare occurrences [357-361].

Not only can E3 ubiquitin ligases promote the transfer of a single ubiquitin (monoubiquitination) to a lysine residue on the target protein, but they can also induce the formation of an isopeptidic linkage on any of the seven lysine residues of a ubiquitin already conjugated to a target protein, forming different types of ubiquitin chains (K6, K11, K27, K29, K33, K48, and K63) [362,363]. The functional consequences of this diversity in ubiquitin modifications will be discussed in the next section. The molecular processes underlying the processivity and specificity of polyubiquitin chain synthesis (polyubiquitination) have been the subjects of considerable work and several models have already been proposed to explain the differential behaviours of E3 ligase complexes. 1) Rapid E2-RING E3 assembly and disassembly through high affinity electrostatic interactions can lead to efficient elongation of the ubiquitin chain. Specialized domains in the E2 might interact with the elongating ubiquitin chain and help to orient the attacking lysine residue for optimal transfer. 2) The E2 dissociates from the RING domain allowing re-loading of ubiquitin but remains in association with other domains of the E3 ligase complex. This topology would permit rapid association and dissociation of the E2 at the RING interface. 3) A first E2 initiates chain elongation by adding one or a few ubiquitin moieties. The substrate or the E3 is then bound by a chain-elongating E2 (sometimes called E4) to complete the formation of the polyubiquitin chain. 4) The ubiquitin chain is pre-formed on the E2 active site and transferred en bloc to the substrate. These models are not mutually exclusive and probably explain the different modes of action of E2-E3 pairs [348,364-373]. As for the selection of correct linkages, the intrinsic properties of some E2s might be responsible for the specificity. For instance, whether or not cdc34 and UBE2S (ubiquitin-conjugating enzyme E2S) are in complex with an E3, they specifically catalyze K48- and K11 polyubiquitination, respectively [366,367,374]. It is thought that the preference for a specific linkage is probably the result of the interaction between the E2s

and the acceptor ubiquitin, orienting it to expose solely a specific lysine to its active site. Other E2s including UBC13 (ubiquitin-conjugating enzyme 13) form a complex with the pseudo-E2 MMS2. In these cases, MMS2 binds to the acceptor ubiquitin to provide linkage specificity [375,376]. On the other hand, E2s like UBCH5 do not show any linkage specificity when bound to their cognate E3s and can induce the assembly of homotypic chains on all seven lysine residues as well as mixed-linkage chains [377,378]. Various combinations of E2s with the same E3 can therefore induce the formation of a variety of linkages. The E3 ligase BRCA1, when in complex with the E2s UBCH6 and UBE2E2, induces monoubiquitination of its substrate, whereas when it is in complex with MMS2-UBC13 and UBE2K, catalyses the formation of K63- and K48-linkages respectively [379]. In the case of HECT ligases, the E3 but not the E2 appears to be fully responsible for chain elongation processivity and specificity [380,381].

2.2. Functional implications of ubiquitin chain topologies

In contrast to phosphorylation, which primarily acts as a binary switch, ubiquitination constitutes a versatile multimodal class of post-translational modifications. The different types of ubiquitination can be subdivided in five main categories: monoubiquitin, homotypic polyubiquitin chains, mixed chains, head-to-tail linear chains, and heterologous chains.

Monoubiquitination or the conjugation of a single ubiquitin moiety to an acceptor lysine residue is one the most abundant types of ubiquitination. It is involved in multiple processes including protein trafficking, signalling, transcription, and DNA repair [382]. Although monoubiquitination has been implicated in receptor endocytosis, its exact role and its intricate interplays with other types of ubiquitin modifications has remained controversial [383,384]. On one hand, directly fusing a polyubiquitination-defective ubiquitin moiety to the N-terminal of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), demonstrated that monoubiquitination was

sufficient to induce internalization and degradation of most receptor-tyrosine kinases in response to ligand stimulation, albeit less efficiently than K63-polyubiquitination. Mass spectrometry analysis revealed that native EGFR was modified by monoubiquitin on multiple lysine residues (multiple monoubiquitination) as well as by K63-polyubiquitin chains on the receptor cytoplasmic tail [383,385,386]. Multiple monoubiquitination and K63-polyubiquitination are thought to increase the avidity of the ubiquitin binding domains (UBD) of endocytic adaptors such as Eps15 and epsin [387,388]. On the other hand, mutants of EGFR defective for ubiquitination were nevertheless internalized but did not undergo degradation. In this case, ubiquitination was found to induce EGFR internalization via a clathrin-independent pathway, leading to lysosomal targeting whereas, in absence of ubiquitination, EGFR was internalized in clathrin-coated pits, retained intracellularly in early endosomes and eventually recycled to the cell surface [389,390]. Finally, K63-polyubiquitination of other receptors such as TrkA (tyrosine kinase receptor A) induced its internalization but not its degradation whereas multiple monoubiquitination could induce its degradation [391,392]. It is therefore plausible that different types of ubiquitination might be needed along the endocytic pathways to direct the receptors to specific compartments. The outcomes of these ubiquitination events are also probably specific for a given ligand-receptor-adaptor system [383]. Consistent with this model, monoubiquitination and multiple monoubiquitination but not K63-polyubiquitination were found to be necessary for the sorting of membrane proteins into multivesicular bodies (MVB) and for the targeting for lysosomal degradation via interactions with Vps27/Hrs and other components of the ESCRT machinery [393-395]. Monoubiquitination has also a central role in DNA replication and repair. Monoubiquitination in the DNA clamp PCNA (proliferating cell nuclear antigen) was shown to induce the recruitment of error-prone translesion polymerases whereas its K63-polyubiquitination at the same residue induced error-free DNA repair [396]. Moreover, in response to DNA damage, subunits of the 9-1-1, RFC, and Fanconi anemia (FA) complexes are also monoubiquitinated to coordinate DNA damage signalling and repair [397-399]. Finally, as much as 10-15% of H2A and H2B are monoubiquitinated. While H2A monoubiquitination is associated with transcriptional repression, H2B monoubiquitination promotes transcription initiation and elongation [400].

Homotypic polyubiquitination is characterized by the formation of a polyubiquitin chain on a unique lysine on ubiquitin, forming a homogeneous type of linkage (K6, K11, K27, K29, K33, K48, and K63) [401]. All of these linkages can be observed *in vivo* but K48, K11, and K63 are the most abundant [402,403]. K48-polyubiquitination of proteins generally leads to their degradation by the proteasome. The compact topology adopted by four ubiquitin moieties conjugated through K48-linkages is recognized by multiple specific ubiquitin receptors on the regulatory 'lid' of the ring-shaped mega-complex termed proteasome. The bound substrate is then unfolded and translocated into the core proteolytic domain by six distinct ATPases. The substrate is finally degraded by proteolytic subunits belonging to the N-terminal nucleophile hydrolase family. Removal of the ubiquitin moieties by deubiquitinases (DUBs) is tightly coupled with degradation and permits recycling of ubiquitin [404]. K48 linkages can also be involved in non-proteolytic functions. Most of these functions involve the recognition and segregation of K48-linked proteins by the AAA (ATPase associated with various cellular activities) p97/VCP (valosin-containing protein, also known as Cdc48 in yeast). VCP interacts with several cofactors that differentially control the fates of its substrates [405]. This segregase function of VCP is implicated in the extraction of misfolded ubiquitinated membrane proteins during ERAD [406]. Most of the released substrates are then degraded by the proteasome but some, like Spt23, are deubiquitinated and remain stable [407]. Additionally, a recent report shows that VCP in complex with the cofactors Ufd1-Np14 (ubiquitin fusion degradation 1-nuclear protein localization 4) can extract K48-polyubiquitinated Aurora B kinase from chromatin at the end of mitosis, promoting formation of a new nuclear membrane [408].

In addition to K48-linkages, K63-linkages can also direct proteins for degradation. While a few reports have shown that K63-linkages can induce proteasome degradation, most instances of proteolysis following K63-ubiquitination involved autophagy [362]. NBR1 and p62 acts as autophagy receptors, binding simultaneously to K63-polyubiquitin chains and to the ubiquitin-like autophagy-specific modifiers LC3 (microtubule-associated protein light chain 3) and GABARAP (Gamma-aminobutyric

acid A receptors-associated protein). These mediate the maturation of autophagosomes, fusion to lysosomes, and degradation of the ubiquitinated substrates [409]. K63-linkages are also involved in a variety of other processes including receptor internalization (described above), signalling, and DNA repair. Two notable examples include the NF-κB signalling pathway and the ubiquitin-dependent recruitment of BRCA1 to sites of DNA damage. NEMO (NF-κB essential modulator), a subunit of IKK (IκB kinase), is K63-polyubiquitinated by TRAF6, in response to TNF-alpha stimulation. Polyubiquitination of NEMO activates IKK, inducing the phosphorylation of the NF-κB inhibitor IκB, targeting it for K48-polyubiquitination and proteasomal degradation [410,411]. Upon induction of DNA damage, the E3 ligases RNF8 (RING finger protein 8) and RNF168 (RING finger protein 168) are both recruited to DNA repair foci in an MDC1(Mediator of DNA damage checkpoint 1)-dependent manner and collaborate to induce the K63-polyubiquitination of H2A and H2AX. These K63-linked chains are then recognized by the ubiquitin-binding domain of RAP80 (receptor-associated protein 80), mediating the recruitment of the BRCA1-RAP80-ABRA1(Abraxas) complex to DNA repair foci [398,412].

Other types of linkages have also been documented but their functions remain poorly understood. K11-polyubiquitination of several substrates by APC/C has been shown to be central for progression of cells through mitosis [365,413]. K29-polyubiquitination of the Notch signalling modulator Deltex was found to target it for lysosomal degradation [414]. During DNA damage repair, BRCA1 induces the formation of K6-polyubiquitin homotypic chains, but the functional relevance of these has not been determined [415-417]. Mixed K29/K33-polyubiquitination (a ubiquitin chain containing branched K29- and K33-linkages) of the AMPK (AMP-activated protein kinase) family members ARK5 (AMP-related kinase 5) and MARK4 (MAP/microtubule affinity-regulating kinase 4) kinases blocks their kinase activation by interfering with phosphorylation of the activation-loop [418]. Autoubiquitination of the polycomb protein Ring1B by K6/K27/K48-mixed linkages does not lead to degradation of the E3 ligase but is instead essential for the efficient monoubiquitination of H2A [419]. The LUBAC (linear ubiquitin chain assembly complex) E3 ligase complex

catalyses the assembly of linear chains (conjugation of the C-terminus of ubiquitin on the free N-terminal amino group of another ubiquitin) [420]. LUBAC-mediated modification of NEMO with these linear ubiquitin chains was found to play an important role in NF-κB activation [421,422]. Finally, the formation of heterologous chains between ubiquitin and other ubiquitin-like proteins such as SUMO (small ubiquitin-related modifier) have also been observed. RNF4 (RING finger protein 4) can induce the ubiquitination of a conjugated SUMO moiety on PML (promyelocytic leukemia protein), generating Ub/SUMO chains. Such modifications of PML led to its degradation by the proteasome [423].

2.3. Cullin-RING ubiquitin ligases (CRLs)

2.3.1. Structure and composition

Cullin-RING ubiquitin ligases (CRLs) are characterized by their extended and rigid architecture (Figure 6, p.32). They are based on a large scaffold protein called Cullin. The human genomes encode seven Cullin proteins (Cullin 1, 2, 3, 4A, 4B, 5, and 7) and one Cullin-like protein (Parkin-like cytoplasmic protein, PARC). In addition to the recruitment of the ubiquitination machinery, these serve as platform to engage multiple and distinct sets of adaptors, receptors, and substrates [424]. Cullins have curved N-terminal stacks of alpha-helices known as Cullin repeats linked to a globular domain at its C-terminus. The RING E3 ligases ROC1/RBX1/HRT1 (regulator of Cullins 1) and ROC2/RBX2 (regulator of Cullins 2) (for Cullin 5 only) bind to the Cullin globular head. The E2 is recruited by the Cullin-bound E3 via its RING domain [425-427]. Both cdc34 and UBCH5 are utilized as E2s by CRLs, suggesting that these complexes have the capacity to generate a variety of ubiquitin linkages [378]. The N-terminus of Cullins are involved in the interaction with adaptors such as Skp1 (S-phase kinase-associated protein 1) for Cullin 1 or DDB1 for Cullin 4A. These adaptors will in turn mediate interactions with a multitude of substrate receptors that are recruiting substrate proteins. It is believed that the rigid architecture of Cullin molecules might juxtapose the E2 and the substrate to favour ubiquitin transfer [425-427].

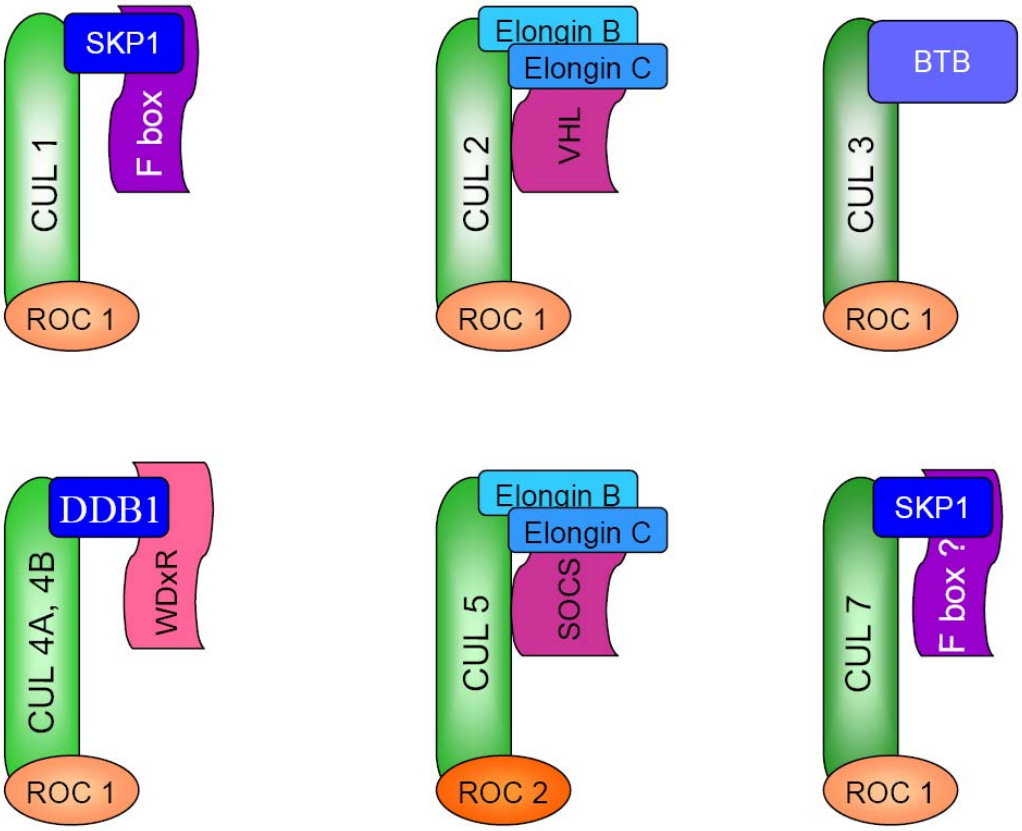

Figure 6. Composition and architecture of Cullin-RING E3 ubiquitin ligases.

The C-terminus of Cullins (1, 2, 3, 4A, 4B, 5, and 7) recruits various E2s via an interaction with the RING E3 ligases ROC1 or ROC2. The N-terminus of Cullins engages adaptors and substrates receptors to specifically recruit substrates. Cullin 1 recruits substrates through the adaptor protein SKP1 and an F-box-protein substrate receptor. Cullin 2 recruit substrates through an elongin-BC adaptor and a VHL/BC/Cullin 2-Box substrate receptor. Cullin 3 recruits substrates through BTB-domain-containing substrate receptor proteins without the need of an adaptor. Cullin 4A and 4B recruits substrates through the DDB1 adaptor and a WDxR substrate receptor. Cullin 5 recruits substrates through an elongin-BC adaptor and a SOCS/BC/Cullin 5-Box substrate receptor. Cullin 7 recruits substrates through the adaptor protein Skp1 and presumably an F-box-protein substrate receptor.

The prototypic Cullin 1-RING E3 ligase complexes (CRL1) are generally called SCF (Skp1-Cullin 1-F Box). They use the adaptor Skp1 to bind substrate receptors containing an F-Box, a motif that includes WD40 repeats. F-Box proteins usually bind to phosphorylated residues and substrate recognition is therefore regulated by phosphorylation [428,429]. Cullin 2 and Cullin 5 complexes are similar in their compositions. They both bind the adaptor elongin C in complex with elongin B and recruit substrate receptors containing a BC-box subdomain [430,431]. Despite sharing the same adaptor proteins, they bind to two different classes of BC-box substrate receptors. VHL (von Hippel-Lindau protein)-box receptors are recruited to CRL2 complexes via a C-terminal Cul2-box domain and SOCS-box receptors interact with CRL5 complexes via a Cul5-box. These specific subdomains are thought to mediate a direct interaction with their respective Cullin scaffold proteins [432,433]. Another notable difference between CRL2 and CRL5 complexes is the apparent preference of CRL5 for the E3 ROC2 instead of ROC1 [430]. CRL3 complexes have a different architecture than the rest of the CRL family. They do not appear to use an adaptor protein. Instead, they bind directly to BTB (broad complex, tramtrack, bric-a-brac) proteins, which act simultaneously as adaptor and substrate receptor [434-437]. Cullin 4A contains a limited number of Cullin repeats and use DDB1 as its adaptor. The proper substrate-E2 distance appears to be maintained through the larger size of its adaptor [438]. The substrate receptors recruited by DDB1 were recently characterized and share a conserved WDxR motif, a subclass of WD40 motif [439]. In addition to its role as an adaptor, DDB1 can also bind directly to substrates such as Chk1 [440]. DDB1 has also been implicated in the formation of E3 ligase complexes independently of Cullin. The kinase DYRK2 (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2) serves as a scaffold to recruit DDB1 and the HECT E3 ligase EED (embryonic ectoderm development) to induce the phosphorylation and subsequent degradation of its downstream substrate, katanin p60 [441]. Cullin 4B is a longer paralog of Cullin 4A. CRL4A and 4B share DDB1 and numerous substrate receptors and can regulate identical substrates. It is unclear at present whether Cullin 4B can target unique substrates or whether both ligases have complementary roles. Genetic evidence implicating Cullin 4B in mental retardation and Cullin 4A in the development of breast

and liver cancers support the likelihood of both scenarios [442,443]. CRL7 complexes are poorly characterized. Cullin 7 uses Skp1 as adaptor and can presumably bind to F-box substrate receptors, although only Fbw8 (F-box and WD repeat domain containing 8) has been observed to date [444,445]. There is also an eight putative CRL structured around the Cullin 7 homolog PARC, but the adaptors and substrate receptors associated with this complex are not known [446].

2.3.2. Regulation

Since each CRL can associate with over one hundred different substrate receptors, they likely regulate the stability and activity of a vast number of proteins. Accordingly, these complexes have been implicated in several cellular processes including DNA replication, cell cycle regulation, signal transduction, development, and transcription. The activity of these complexes is therefore tightly regulated by several independent mechanisms: sequestration, dimerization, proteolysis, and post-translational modifications of CRL components as well as of substrates [427].

One of the main regulatory systems of CRL activity is the CAND1-Nedd8-CSN cycle. In their inactive form, CRLs are bound to the inhibitor molecule TIP120A/CAND1 (Cullin-associated and neddylation-dissociated 1). CAND1 wraps around Cullins, masking the adaptor binding site and completely blocking the Nedd8-conjugation site [447]. Recruitment of the adaptor-receptor-substrate complex displaces the inhibitor CAND1 and allows Nedd8 (neural precursor cell expressed, developmentally down-regulated 8) conjugation [448-450]. Nedd8 is a small ubiquitin-like protein that is conjugated to Cullin at a conserved lysine residue. This process is termed neddylation and is mediated by the sequential action of E1 (NAE1–UBA3), E2 (UBE2F or UBC12/UBE2M), and E3 (ROC1 or ROC2) enzymes [346,355,451]. Conjugation of Nedd8 to Cullin subunits plays multiple roles in favouring ubiquitination. First, it induces a conformation shift in Cullin that masks the CAND1 binding site and that bridges the gap between the initiator ubiquitin at the E2 active site and the acceptor amino group on the substrate. Nedd8-induced Cullin flexibility also

enhances recruitment of E2s and formation of the amide bond at the E2 active site, promoting ubiquitin chain elongation. The overall effect of neddylation is that it increases the probability that a substrate will acquire sufficient number of ubiquitins (four or more) in a single encounter with a CRL [452-454]. The COP9 signalosome (CSN) is an eight-subunit complex homologous to the proteasome regulatory subunit. It can also form smaller complexes of two to four subunits. Individual subunit can bind to multiple cellular proteins and probably have functions other than as component of CSN. The regulation of CSN interactions with CRLs is poorly understood but its functional implications have been extensively studied. Once the substrate is ubiquitinated, one of the CSN subunits, CSN5, catalyses the removal of Nedd8 (deneddylation) from Cullin molecules [455]. Deneddylation of Cullin allows CAND1 to somehow displace the adaptor/receptor complex and to sequester Cullin in an inactive form [456].

CAND1 and the CSN complex could thus intuitively be considered as negative regulators of CRLs. However, surprisingly, down-modulation of CAND1 or CSN inhibits CRL activity. This apparent paradox can be explained by several observations on the stability of substrate receptors. Indeed, in absence of CSN, substrate receptors are auto-ubiquitinated by CRLs and eventually degraded by the proteasome. In contrast, in presence of substrates, substrate receptors are protected from auto-ubiquitination, presumably because substrates would out-compete substrate receptors as acceptor molecules [427]. It appears that the deneddylating activity of CSN is sufficient to prevent uncontrolled ubiquitination and catalytic turnover of substrate-bound receptors [457]. It is possible that, in some cases, USP15 (Ubp12 in yeast), a deubiquitinase associated with the CSN complex, might also participate in this regulation by inducing ubiquitin removal from CRL components [458]. In the case of CAND1, the mechanism of activation/inhibition is not as evident. While some substrate receptors are degraded in absence of CAND1, most retain their normal levels of expression but are nevertheless inactive. Recent data by Schmidt and colleagues sheds light on the mechanisms of CAND1-mediated activation of CRLs [457]. In absence of CAND1, highly abundant substrate receptors are preferentially recruited to CRLs compared to less abundant receptors. CAND1 functions by increasing the access of less

abundant receptors to CRLs by presumably changing Cullin conformation to increase its affinity for rare substrate receptors. Activation/inhibition cycles of CRLs would therefore promote formation of CRLs composed of diverse substrate receptors and would allow the substrates to regulate their own ubiquitination and degradation by controlling the levels of substrate receptors [427,459].

CRLs are additionally regulated by several mechanisms controlling receptor availability and substrate recognition. In addition to autocatalytic regulations by CRLs, the levels of substrate receptors can be further down-modulated by the activity of other E3 ligases. For instance, APC/C^{Cdh1} induces the degradation of the CRL1 receptor Skp2 in G1 [427]. Moreover, the activity of several CRL1 complexes is regulated by post-transcriptional modification of substrates. Most F-Box receptors can only engage their cognate substrates when the binding interface on the substrate is phosphorylated. This specialized motif is called a phosphodegron and has the evolutionary advantage of coupling phosphorylation-mediated signalling cascade with regulatory ubiquitination [428,429]. Substrate recognition can also be limited by spatial constrains [426]. The DNA replication-licensing factor Cdt1 (cdc10-dependent transcript 1) is degraded during S-phase by CRL4 (Cdt2) to prevent repeated firing of the same origin of replication. Loading of CRL4 (Cdt2) on the origins of replication is controlled by a conformational switch in PCNA to ensure that Cdt1 is degraded only during S-phase and only on origins where DNA replication has been initiated [460].

Other regulatory mechanisms involve the modulation of CRL catalytic activity by cofactors or by changes in complex architecture. The CSN complex is directly involved in the stable inhibitory sequestration of a subset of CRLs, including CRL4A (DDB2), CRL4A (VprBP), and CRL3 (Btbd1/2), that are implicated in DNA replication and repair [461]. Indeed, in the case of CRL4A (DDB2), UV irradiation induces the disengagement of CSN [462], promoting the association of the ligase with damaged DNA and ubiquitination of several substrates including the DNA repair protein XPC (Xeroderma pigmentosum C) [463]. DDB2 (DNA damage-binding protein 2) is then rapidly degraded following autoubiquitination, probably as a consequence of CSN

uncoupling [461,463]. The activity of several CRL4A complexes is also modulated by the small chromatin-associated protein DDA1. DDA1 (DET1 and DDB1 associated 1) forms a core complex with DDB1 and Cullin 4A and is essential for the activity of CRL4 ligases [461]. Conversely, when in association with DET1 (de-etiolated homolog 1), DDA1 has the opposite effect on CRL4A-mediated ubiquitination [464]. The mechanisms underlying the divergent effects of DDA1 are however unclear. Dimerization of Cullins (mediated by neddylation or by homo- and hetero-dimerization of substrate receptors) and dimerization of substrate receptors on monomeric Cullin are other mechanisms that modulate the kinetics of ubiquitin transfer. These alternate complex architectures can enhance ubiquitination of substrates by spatially accommodating substrates with divergent sizes or with multiple acceptor residues. The structural flexibility induced by CRL dimerization can also favour transfer of ubiquitin to elongating ubiquitin chains [465]. Finally, ubiquitin transfer by CRLs can be reversed by deubiquitinases [342]. One notable example is IκBα, which is subjected to continuous ubiquitination by CRL1 (β-TRCP). In presence of exogenous stimuli, the USP15 (ubiquitin carboxyl-terminal hydrolase 15) subunit of the CSN complex deubiquitinates IκBα and prevents its proteasomal degradation [466]. Another example is the inhibition of CRL3-mediated caspase-8 polyubiquitination by the deubiquitinase A20 [467].

3. CELL CYCLE REGULATION

HIV-1 encodes two accessory proteins, Vpr and Vif, which perturb the host cell cycle. HIV-1 is not an exception in this regard. Many viruses, including adenoviruses, HCMV (human cytomegalovirus), EBV (Epstein-Barr virus), and HBV (hepatitis B virus), have found means to interfere with the host cell cycle, either by blocking it or by stimulating it. The main advantage of controlling host cell cycle is probably to optimize the cellular environment for efficient viral replication [468-470].

3.1. Regulation of cell cycle progression

The different phases of the cell cycle are organized by a series of highly regulated proteins called cyclins and their associated cyclin-dependent kinases (CDK). At each phase, CDKs are responsible for the activation of the different mediators and effectors, precisely regulating the expression of essential genes and promoting passage through the different cell cycle transitions (G1/S, S, G2/M, M). Complex combinations of CDKs with phase-specific cyclins (Figure 7, p.39) and their sequential inactivation by CRL and APC/C ubiquitin ligases are responsible for this temporal regulation [471-474].

In the G1 phase, CDK4 (cyclin-dependent kinase 4) and CDK6 (cyclin-dependent kinase 6) associates with cyclin D and CDK2 associates with cyclin E, activating their kinase activity. These CDKs then phosphorylate Rb (retinoblastoma tumor suppressor protein) and the other members (p107 and p130) of the pocket protein family in a sequential and concerted manner (see Section 3.2 for further details). Pocket proteins are transcriptional repressor of a number of transcription factors including E2Fs (E2F-1, -2, and –3). Binding of pocket proteins to the transactivation domain of E2Fs and recruitment of chromatin repression factors such histone deacetylases on E2F-responsive genes block the E2Fs transcriptional activity [475,476]. Hyperphosphorylation of pocket proteins dissociates the complex and therefore induces the transcription of numerous genes, including cyclin A, DNA polymerase subunits, cdc6, thymidine kinase, and MCM (mini-chromosome maintenance complex) subunits, which are essential for the initiation and proper completion of DNA replication [477,478]. At the end of G1, E2F-mediated transcription is terminated by CDK2/cyclin A. E2F6 and possibly E2F7 and E2F8 then represses transcription of E2F target genes [479-481].

Loading of the ORC complex, Cdt1, cdc6, and the MCM2-7 helicase onto the origins of replication forms the Pre-Replicative Complex (Pre-RC) in G1. At the G1/S

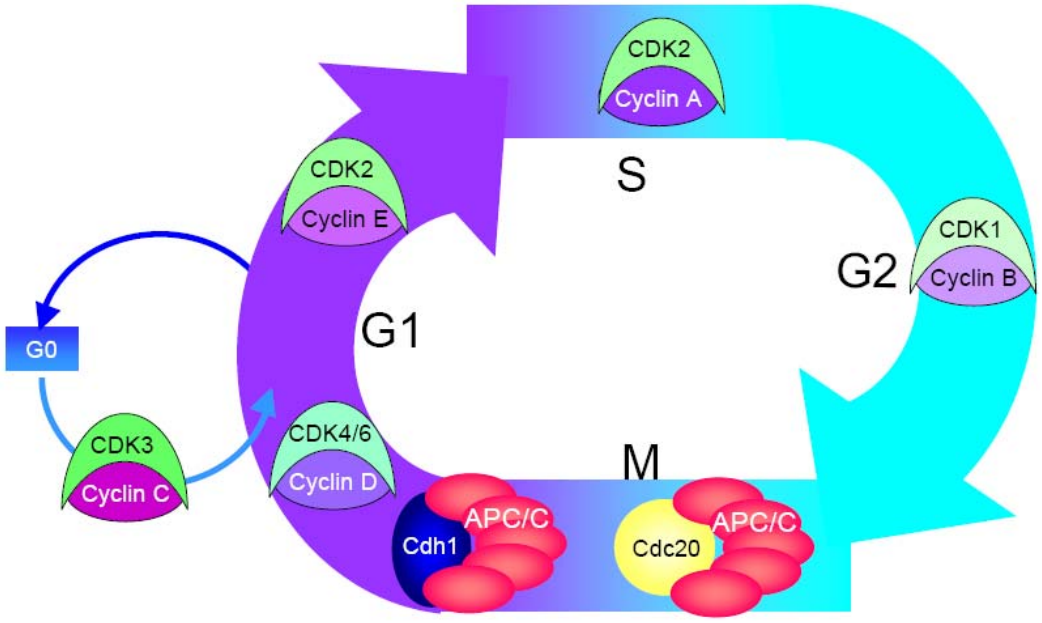

Figure 7. Regulation of cell cycle progression by CDKs and APC/C

In G1, the mitogen-dependent sequential activation of CDK4/6-Cyclin D and CDK2-Cyclin E initiates the replication of DNA. Efficient DNA replication requires CDK2-Cyclin A. When DNA replication is complete, CDK1-Cyclin B is activated and promotes entry into mitosis by inducing chromatin condensation and breakdown of the nuclear envelope. After chromosome alignment and microtubule attachment, APC/C^{cdc20} induces sister chromatid separation and cytokinesis. At the end of mitosis, APC/C^{cdh1} is activated to promote completion of mitosis and initiation of a new G1 phase. See main text for a more detailed description.

boundary, CDK2-cyclin E and cdc7-Dbf4 cooperate to recruit MCM10, cdc45, and other factors to the pre-RC, inducing unwinding of the origin and stabilization of ssDNA by RPA and by the RPA-interacting protein TopBP1 (DNA topoisomerase 2-binding protein 1). Elongation factors, an array of DNA polymerases, and the RFC clamp loader complexes are then recruited to these chromatin structures. Processive DNA initiation and replication involves the recruitment of the clamp protein PCNA [482,483]. Once replication as started, Cdt1 is ubiquitinated by CRL4A (Cdt2) in a PCNA-dependent manner and degraded by the proteasome. In S/G2/M phases, free Cdt1 and cdc6 are phosphorylated by CDK1-cyclin A. This results in the translocation of cdc6 to the cytoplasm and to the degradation of Cdt1 following ubiquitination by CRL1 (Skp2). Moreover, Geminin levels increases during S-phase and further inhibit Pre-RC formation by sequestering free Cdt1. Cyclin E is also degraded following ubiquitination by CRL1 (Skp2) and CRL1 (Fbw7). The goal of these feedback mechanisms, globally referred to as origin licensing, is to prevent DNA re-replication [484,485].

As DNA replications progresses, several transcription factors, including Lin-9 and B-myb, up-regulate expression of cyclin B [486,487]. Cyclin B forms a complex with CDK1 (also known as cdc2 in yeast) and is phosphorylated by the CDK-activating kinase (CAK) and PLK1 (Polo-like kinase 1). This allows the nuclear import of CDK1-cyclin B. As nuclear levels of CDK1 increases, Wee1 and Cdc25 compete to respectively inhibit and activate CDK1. At the onset of mitosis, when enough nuclear CDK1 and PLK1 are active, Cdc25 is activated and Wee1 is inhibited by PLK1- and CDK1-mediated phosphorylation events, tipping the balance towards a full activation of CDK1. Phosphorylation of Wee1 ultimately results in its ubiquitination by CRL1 (β-TRCP) and degradation by the proteasome [488,489]. Once activated, CDK1-cyclin B mediates breakdown of the nuclear envelope and associates with mature centromeres in an Aurora A- and PLK1-dependent manner where it assists the formation of the kinetochores. Until all kinetochores are attached to their respective spindle, the mitotic checkpoint remains active (see section 3.4) and blocks the separation of sister chromatids [472,490,491]. The last stages of mitosis are governed by a large ubiquitin ligase complex called the anaphase-promoting complex/cyclosome (APC/C).

At the end of DNA replication, PLK1 phosphorylates the inhibitory heterodimeric complex Evi5 (ecotropic viral integration site 5)-Emi1 (early mitotic inhibitor 1), targeting Emi1 for ubiquitination and degradation via CRL1 (β-TRCP) [484,492]. This partially activates APC/C^{cdc20} which can trigger cyclin A degradation [484]. APC/C^{cdc20} localized on kinetochores is phosphorylated at multiple sites by PLK1 and CDK1. As the spindle checkpoint remains active cdc20 is inhibited, but after proper chromosomal attachment, the checkpoint is silenced and cdc20 is released [493-495]. This induces the APC/C^{cdc20}-mediated ubiquitination of several substrates including cyclin B and securin, promoting sister chromatids separation and cytokinesis. Following degradation of cyclin B, APC/C^{cdh1} is activated by a poorly understood mechanism and will sequentially degrade mitotic factors such as cdc20, PLK1, Aurora A and B. These degradation events are necessary for spindle disassembly, chromosomal decondensation and reformation of the nuclear envelope [496]. APC/C^{cdh1} remains active throughout G1 and induces the degradation of geminin to promote re-assembly of Pre-RC on origins of replication [484,496].

3.2. Mitogenic restriction point

Unicellular organisms primarily respond to concentrations of nutrients to determine whether to undergo cell division or to remain in a state of latency (quiescence). In multicellular metazoans however, nutrients are generally plentiful, and other regulatory mechanisms have evolved to ensure that cells do not undergo division in an uncontrolled manner but only when required for the development of the whole organism or maintenance of its homeostasis. The decision to initiate cell division encompasses two opposing forces: pro-mitotic signals (mitogens) generally in the form of soluble molecules (growth factors, pro-inflammatory cytokines, etc.) and cellular regulatory network that inhibits initiation of S-phase (checkpoint). Not only must cell division be regulated temporally but also spatially to restrict growth to tissue microenvironment and to conserve tissue organization and architecture. This checkpoint

therefore additionally integrates multiple signalling pathways responding to extracellular anti-proliferative signals, cellular confluency, and cell adhesion. Failure to respond to any of these signals can lead to the development of proliferative syndromes, aberrant or invasive cellular masses, and cancer [497-499]. Initiation of DNA replication is ultimately regulated by the E2F family of transcription factors, which induces the expression of S-phase essential genes. Control of E2Fs activity is therefore the prime objective of an intricate regulatory loop involving pocket proteins, CDKs, CDK inhibitors, CRL1 (Skp2), and APC/C^{Cdh1} [500-502].

During quiescence (G0), expression and translation are mainly repressed except for a subset of genes involved in housekeeping. Consequently, cells in G0 are generally smaller in size and have a lower metabolism. One protein that is highly expressed in G0 is the pocket protein p130. Binding of p130 to E2Fs suppresses transcription of cell cycle genes. It was generally accepted that mitogenic induction of CDK4/6-Cyclin D would permit the re-activation of cell cycle [500]. However, a recent study showed that, in physiological conditions, cyclin D and CDK4/6 are absent in most quiescent cell types and could therefore not be involved in the phosphorylation-mediated inhibition of p130. Instead, another complex, CDK3-Cyclin C, would hypophosphorylate p130 at sites distinct from those of CDK4/6 and CDK2, partially relieving the inhibition imposed by p130. The regulation or even the ubiquity of the CDK3-mediated G0 exit remains unclear at present [501,502]. In early G1, mitogen-dependent expression of cyclin D permits the formation of CDK4/6-Cyclin D complexes. CDK4/6-cyclin D induces a second round of phosphorylation of pocket proteins, notably Rb, permitting the E2F-mediated expression of cyclin E. Finally, CDK2-cyclin E then completely inhibits pocket proteins (hyperphosphorylation) and fully transactivates E2F-mediated transcription of S-phase essential genes. The pocket proteins p107 and p130 also negatively regulate G1/S progression by directly binding and inhibiting cyclin E/CDK2 and cyclin A/CDK2. Phosphorylation of both pocket proteins by cyclin D-CDK4/6 relieves this repression [475,476]. Finally, E2Fs are regulated at the level of translation

by cellular miRNAs. Mitogen-mediated up-regulation of miR-17 and miR-20a down-modulates expression of E2F-1 to prevent its premature accumulation [503,504].

Besides transcriptional regulation of their activating cyclins or direct inhibition by pocket proteins, CDKs are also negatively regulated by the cyclin-dependent kinase inhibitors (CKIs). There are two classes of these inhibitors: the INK4 family (p15Ink4b, p16Ink4a, p18Ink4c and p19Ink4d) and the Cip/Kip family (p21Cip1, p27Kip1 and p57Kip2). INK4 members are specific for CDK4/6 whereas Cip/Kip proteins have a broader CDK inhibitory spectrum [505,506]. In G0 and early G1, Cip/Kip proteins bind to CDK4/6-cyclin D and block the CDK active site. Phosphorylation of Cip/Kip members by several mitogenic pathways, including MAPK (mitogen-activated protein kinase) and ERK (extracellular signal-regulated kinase), induces a change in conformation that reveals the CDK active site, therefore activating the CDK4/6-cyclin D complex [507]. In addition to their activation following an absence of mitogenic signalling (serum starvation), Cip/Kip proteins are also induced by cell-to-cell contact (confluency) and by lost of cell adhesion [508]. INK4 proteins bind directly to CDK4/6 and induce an allosteric change that prevents association to cyclin D [506,509]. In contrast to Cip/Kip members, INK4 proteins are not expressed at high levels in most conditions. However, antiproliferative signals such as those induced by TGF-β (tumor growth factor beta) or over-activation of a proto-oncogene up-regulates INK4 genes. Binding of INK4 to CDK4/6 displaces Cip/Kip proteins and allows the latter to inhibit CDK2-cyclin E and –cyclin A complexes [505,510]. Cip/Kip proteins are also targeted for proteasomal degradation by the CRL1 (Skp2) ubiquitin ligase to further increase CDK activity [508]. But before this can happen, Skp2 needs to be induced by increased transcription and decreased proteolysis. In early G1, Spk2 transcription is actively repressed by pocket proteins, notably Rb. At the same time, APC^{Cdh1} is also active, ubiquitinating Skp2 and targeting it for proteasomal degradation. Mitogen-dependent CDK4/6-cyclin D-mediated phosphorylation of pocket proteins promotes E2F-mediated expression of Skp2 as well as of the APC/C inhibitor Emi1, resulting in a transcriptional up-regulation of Skp2 and an increased stability of the protein [473,496,501]. Moreover, CDK2-mediated phosphorylation of Skp2 protects it from degradation by

APC^{Cdh1} [511]. Finally, in late G1, phosphorylation of Cdh1 by CDK2-Cyclin A dissociates the activating subunit from the complex to further inhibit the E3 ligase [512]. Besides its role in mid-late G1, inactivation of APC^{Cdh1} is also necessary for the stabilization of cyclin A, an event essential for proper replication of DNA and progression into mitosis [473,484,513].

The inhibition of CDK4/6-cyclin D and CK2-cyclin E complexes by CKIs and the regulation of these CKIs by the Skp2-APC^{Cdh1} axis entail constant mitogenic stimulation to maintain the activity of these CDK complexes. If stimulation is deprived, E2F-mediated transcription is shut down, re-establishing a quiescent state (G0) until sufficient mitogenic signalling can re-induce cell cycling. Mitogen-dependent cell cycle progression is required until the cell cycle reaches a certain point in mid-late G1, the restriction point. Past the restriction point, mitogenic signalling becomes dispensable until the end of mitosis, probably because pocket proteins are already hyperphosphorylated and because of high E2F transcriptional activity.

3.3. DNA damage checkpoint

The genome is under constant threat from exogenous agents such as genotoxic chemicals or radiation. Endogenous processes such as base deamination, DNA replication problems or the generation of reactive oxygen species (ROS) can also generate DNA damages. These genotoxic lesions must be repaired, preferentially before mitosis, to avoid the transmission of deleterious mutations to progeny cells. Cells have developed a series of regulatory mechanisms to halt cell cycle progression and promote repair of these different genotoxic insults. These mechanisms are collectively known as the DNA damage checkpoint and are coordinated by two damage-sensing kinases of the PIKK (phosphoinositide-3-kinase-related protein kinase) family: ATM and ATR (Figure 8, p.45). The activities of ATM and ATR can be artificially divided in three stages: damage recognition, signal amplification and promotion of DNA repair, and checkpoint

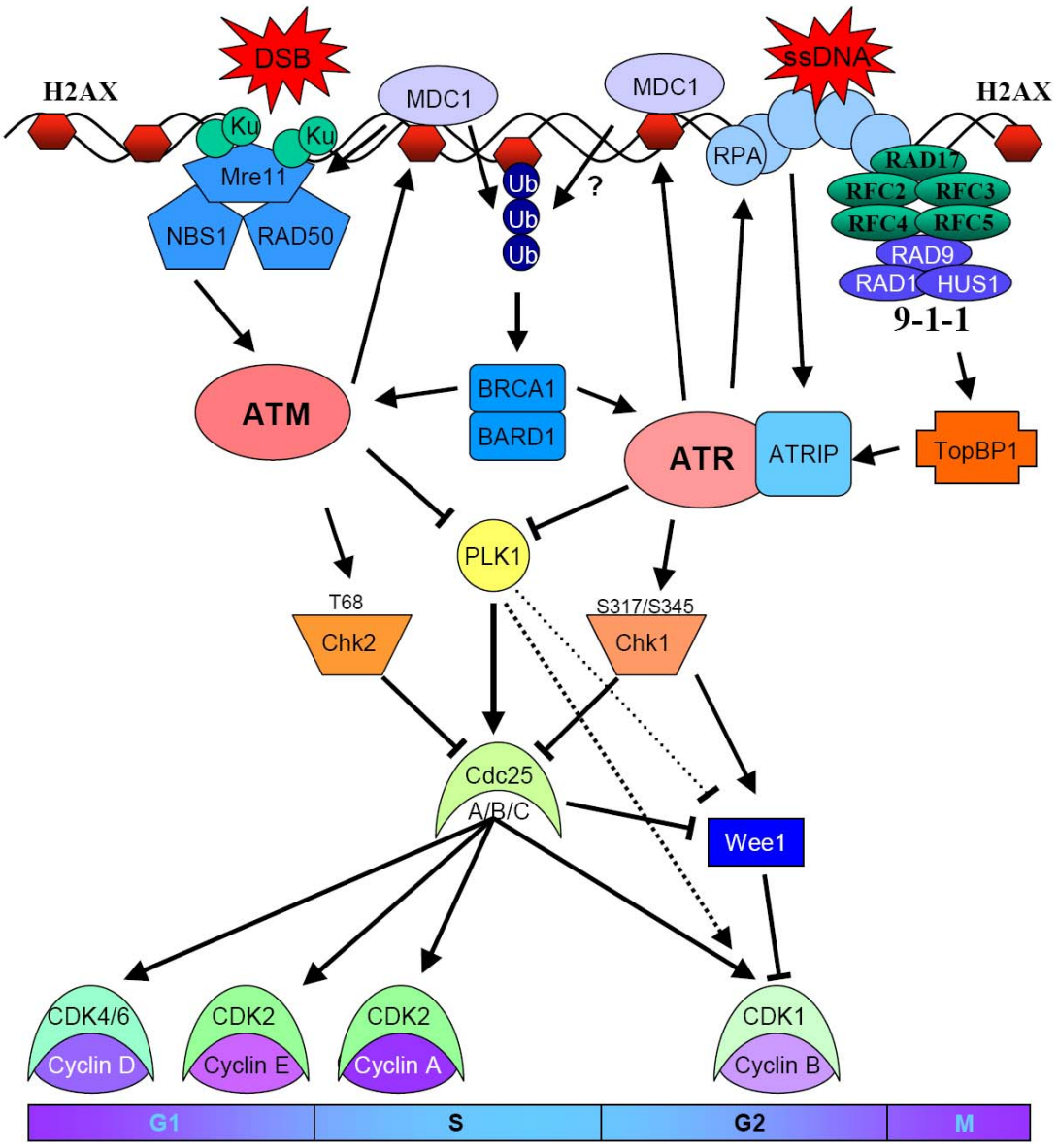

Figure 8. The DNA damage checkpoint.

The DNA damage sensor ATM is recruited to DNA double-strand breaks by the MRN complex and is activated by an amplification cascade involving H2AX, MDC1, ubiquitin ligases, and BRCA1. ATR and its cofactor ATRIP are loaded on RPA-coated ssDNA by TopBP1, the Rad17-RFC complex, and the 9-1-1 complex. ATR is then activated by a poorly understood mechanism involving H2AX, MDC1 and BRCA1. ATM- and ATR-mediated phosphorylation activates respectively Chk2 and Chk1. ATM, ATR, Chk1 and Chk2 inactivate cdc25 and PLK1 and activate Wee1, resulting in inhibition of CDK activity at various stages of the cell cycle.

activation. Extensive levels of cross-talk exist between the two sensors but each appears to respond to specific damages in a cell-cycle phase-dependent manner. ATM primarily recognizes DNA double-strand breaks (DSBs) in G1 whereas ATR reacts to a variety of genotoxic stresses including base damages, bulky adducts, stalled replication forks, and DSBs in S and G2 phases [514-516].

In order for ATR and ATM to be activated, they first need to recognize or sense DNA damages. They do not however directly bind to damages but are instead recruited to processed intermediates. In absence of lesions, ATM forms an inactive homodimer. Upon the occurrence of DSBs, binding of the Ku70/Ku80 heterodimers to both ends of the DSB protects them from resection by exonucleases. The MRN complex (Mre11-Rad50-NBS1) then associates to the ends of DSBs, presumably independently of the Ku heterodimers. The NBS1 (Nijmegen breakage syndrome 1) subunit is responsible for the direct recruitment of ATM to DSBs [517]. Both NBS1 and the presence of DNA induce ATM autophosphorylation (at S367, S1893 and S1981), leading to dissociation of the homodimer into active monomers [518]. In the case of ATR, the primarily recognition signal are long stretches (50-75 nucleotides) of ssDNA coated with RPA. ATR does not function as an independent unit but instead in association with its cofactor ATRIP (ATR-interacting protein). ATRIP mediates the recruitment of ATR to RPA-coated ssDNA by direct interactions with both proteins. This localizes ATR to the sites of DNA damages or replication stalling but it does not activate it. In order to activate ATR, the 9-1-1 clamp complex must first be loaded at the 5'DNA junction by the clamp loader Rad17-RFC in a RPA-dependent manner. Then, recruitment of TopBP1 by 9-1-1 permits the activation of ATR by contacting regions within ATRIP and ATR [519]. The order in which TopBP1 and 9-1-1 are recruited is controversial and might be damage type-dependent. Indeed, in the context of stalled replication TopBP1 along with polymerase alpha appear to be necessary for the recruitment of 9-1-1 and not the inverse [520]. Additionally, ATR-ATRIP might be recruited to sites of damages in an ssDNA-RPA-independent manner. In presence of replication stress, Cdc6 appears to recruit directly ATR-ATRIP to chromatin and some mismatch repair proteins might also recruit ATR-ATRIP to bulky adducts during DNA replication. Paradoxically however, most

DNA damages, including bulky adducts and DSBs do not directly induce formation of long stretches of RPA-coated ssDNA but can nevertheless activate ATR. Base damages such as oxidation (e.g. 8-oxo-guanine) by reactive oxygen species (ROS) or spontaneous deamination are directly recognized and repaired by the base excision repair (BER) pathway [521,522]. Bulkier base damages including intra-strand base crosslinks such as pyrimidine dimers caused by UV or chemically induced inter-strand crosslinks are resolved by the nucleotide excision repair (NER) pathway [523,524]. Both of these repair pathways generate short stretches of single-strand ssDNA (a few nucleotides in the case of BER and up to 35 nucleotides for NER) as repair intermediates. These might have served as platform to recruit RPA and subsequently ATR, but they appear to be too short in length. Indeed, a recent study by the Chen's laboratory has demonstrated that ATR is recruited to sites of UV damages and activated only after DNA replication has been initiated [200]. It is therefore the RPA-coated stalled replication forks or stalled DNA polymerases themselves that would be the primary signal for ATR recruitment and activation by bulky adducts. In S- and G2-phases, DSBs can arise, as in G1, when high-energy ionizing rays hit DNA. In addition, unresolved DBSs from G1 and partially repaired damages from G1 (gaps or nicks) lead to replication fork collapses and generate DSBs in the replicating strands [514]. As in G1, the DSB ends are initially recognized by the Ku heterodimers and by the MRN complex, activating ATM. However, phosphorylation of the MRN-associated exonuclease subunit CtIP by S- and G2-phase CDKs, and its subsequent ubiquitination by BRCA1-BARD1 (BRCA1 associated RING domain 1) induce the resection of DSBs, generating a long stretch (>50 nucleotides) of ssDNA. The generation of these ssDNA overhangs induces the dissociation of MRN from the DSBs and attenuates ATM activation. The ssDNA overhangs are then coated with RPA, finally recruiting ATRIP-ATR. [525,526]

Simple activation of ATR and ATM is insufficient to promote repair and to activate the checkpoints. Further amplification of the signal is warranted. After activation, ATM catalyzes the phosphorylation of the histone variant H2AX on S139, allowing recruitment of MDC1. MDC1 increases the activation status of ATM by promoting accumulation of MRN. In turn, ATM phosphorylates the N-terminus of

MDC1. Phosphorylated MDC1 then serves as a platform for the recruitment of the E3 ubiquitin ligase RNF8, which induces K63-linked polyubiquitination of H2A and H2AX. Ubiquitinated histones are subsequently recognized by another E3 ligase, RNF168, which further ubiquinates histones and other yet-unknown cellular factors to amplify the signal. These ubiquitinated proteins are finally recognized by the RAP80 subunit of the BARD1-BRCA1-ABRA1-RAP80 complex. BARD1-BRCA1 is itself another E3 ubiquitin ligase but most of its substrates are still unknown. Its role is however essential in checkpoint activation, in stimulating DSB repair, and for the induction of apoptosis. How ubiquitination events by RNF168 would allow recruitment of 53BP1 is still not known but they would presumably promote the association of 53BP1 with the dimethylated lysine 20 of histone 4 [412,527]. The exact role of 53BP1 in promoting repair is not clear but might involved increased motility of DSB ends. A recent study shows that the presence of 53BP1 at DSB repair foci stimulates the MRN-ATM activation loop and is essential for the recruitment of KAP-1/TRIM28, suggesting a role for 53BP1 in the relaxation of heterochromatin to promote repair [528]. In G1, in absence of sister chromatids, NHEJ (non-homologous end-joining) is the first mode of repair for DSBs and involves the religation of the free DSB ends. This repair mechanism is coordinated by the PIKK DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which maintain both ends in proximity of each other. Recruitment and activation of Ligase4–XRCC4 by DNA-PKcs finally induces the ligation of both ends. Repair of DSBs by NHEJ can sometimes be error-prone and can result in small insertions or deletions [529]. In the case of ATR, the mechanisms for signal amplification are not as clear [530]. Although ATR induces the phosphorylation of H2AX and MDC1, the recruitment of 53BP1 to sites of UV-induced lesions is largely RNF8-independent [531]. The choice of repair that ATR promotes is context-dependent: homologous recombination (HR) repair for DSBs and damage-bypass mechanisms for stalled replication forks. In S- and G2 phases, the favoured mode of repair for DSBs is homologous recombination (HR) because an identical sister chromatid can be used as a template to promote error-free repair [532]. The transition from ATM to ATR signalling is the critical event leading to HR-mediated repair instead of NHEJ. When activated at DSBs, ATR phosphorylates RPA to promote its

displacement by RAD51. ATR-mediated activation of Chk1 leads to phosphorylation of RAD51 and initiates HR [525,526]. ATR activation also permits the recruitment and phosphorylation of several factors including BRCA1, the FA complex, as well as the WRN (Werner syndrom protein) and BLM (Bloom syndrom protein) proteins to promote homologous recombination repair [514,533,534]. HR is a complex process that involves RAD51-mediated strand invasion and displacement to generate templates that are used by polymerases to re-generate continuous DNA strands [535]. At stalled replication forks, ATR phosphorylate numerous cellular factors associated with the replication complex. These include the MCM2-7 complex, MCM10, and several DNA polymerases to stabilize stalled forks and promote their recovery. Phosphorylation of MCM2 recruits PLK1 and might promote the CDC45-dependent firing of neighbouring origins to complete replication in the problematic region [530,536]. Additionally, ATR can promote two distinct mechanisms to bypass damages in order for replication to proceed. The first mechanism, called translesion synthesis (TLS), involves DNA replication across the genotoxic lesion by a highly processive translesion polymerase. This repair process is fast but is generally error-prone. The second mechanism is called template switching (TS) and involves re-priming downstream of the lesion and replacement of the damaged DNA portion. TS mechanistically resembles HR and is mediated by several cellular factors involved in HR [537]. How ATR chooses one repair mechanism over the other is unclear but appears to rely on the ubiquitination status of PCNA. Monoubiquitination of PCNA on K164 induces translesion synthesis whereas K63-polyubiquitination leads to template switch damage-bypass. The factors and mechanisms upstream and downstream of PCNA ubiquitination are however not well established but implicate Chk1 and the ubiquitin ligases Rad5 and Rad18 [396,538,539].

Because some DNA damages and DNA replication hurdles like DSBs or stalled replication fork can take multiple rounds of trials and errors, cell cycle progression has to stop to allocate sufficient time to repair these damages. Both activated ATM and ATR arrest cell cycling by targeting CDK activity via activation of the checkpoint kinases 1 and 2 (Chk1 and Chk2). ATM-mediated phosphorylation of Chk2 on T68 and

ATR-mediated phosphorylation of Chk1 on S317/345 induce their kinase activity. Chk1/2-mediated phosphorylation of Cdc25, at various stages of the cell cycle, induces CRL1(β-TrCP)-mediated ubiquitination and proteasomal degradation, leading to Wee1-induced inhibition of CDK activity. This results in the inhibition of CDK2/4/6 in G1, resulting in inhibition of E2F transcriptional activity, and inhibition of CDK2 in S-phase, inhibiting origin firing. In G2, phosphorylation of PLK1 (inhibitory) by ATM and/or ATR and of Wee1 (activating) by Chk1 additionally contributes to the inactivation of CDK1-Cyclin B [530,540,541]. Recent data also show that APC/$C^{Cdh1}$is re-activated following checkpoint activation and induces the degradation of PLK1 [542]. Finally, phosphorylation of p53 by ATM, ATR, Chk1, and Chk2 activates the CKI p21CIP and results in the inhibition of cell cycle progression in a CDK-independent manner. Activation of p53, p21, and BRCA1 by ATM and ATR eventually induce apoptosis following prolonged stimulation [543]. When damages are finally repaired, the checkpoint has to be inactivated and cell cycle has to be re-activated. This process involves the resorption of DNA repair foci by an absence of activating signal as well as by the activity of numerous protein phosphatases (PP) including PP2A, PP4, and PP6 which will directly inhibit checkpoint and repair factors [544,545]. In this context, Claspin appears to play a fundamental role in recovery from the G2 checkpoint by modulating Chk1 activity. During damage response, the association of Claspin with Chk1 is essential to induce and maintain Chk1 activity. During recovery from checkpoint response, reactivation of PLK1 by a yet-unknown mechanism activates Claspin to induce its CRL1(β-TrCP)-mediated ubiquitination and proteasomal degradation. Claspin proteolysis is essential for the rapid inactivation of Chk1 and the re-establishment of CDK2-cyclin B activity [546].

3.4. Mitotic checkpoint

The separation of each copy of a pair of sister chromatids into their respective daughter cell is crucial for the maintenance of genetic material. Defects in chromosome segregation leads to aneuploidy or hyperploidy, which are major causes of

developmental abnormalities. Proper separation of sister chromatids relies on the kinetochore, which monitors appropriate microtubule attachment as well as the bi-directionality of chromosome segregation. These monitoring mechanisms are globally called the mitotic checkpoint or the spindle checkpoint [494].

During S-phase, factors present in the replication complex such as claspin, RFC, and Ctf4 (chromosome transmission fidelity protein 4) promotes attachment of the replicating DNA strands (soon-to-be sister chromatids) via formation of cohesin complexes. Failure to maintain sister chromatids cohesion would be catastrophic during metaphase. During prophase, cohesin is hydrolyzed along the length of the chromosome except at the centromere, where sister chromatids attachment is maintained [547]. Centromeres are complex structures composed of CENP proteins (called CCAN, constitutive centromere associated network) and are the sites of the kinetochore assembly. Kinetochore proteins including the NDC80 (NDC80 homolog, kinetochore complex component) and spc105 (spindle complex 105) complexes accumulates at the centromere forming the inner and outer kinetochores [548]. The Ndc80 complex on the outer kinetochore must first properly capture the +end of the microtubule. This step is mediated by Aurora B, which, by phosphorylating Ndc80 and other kinetochore components, keeps the attachment labile until the end of the microtubule is properly oriented on the Ndc80 complex. It is postulated that Aurora B detects correct microtubule attachment by sensing tension. If each sister kinetochores are attached to microtubules from opposite microtubule-organizing centres (centrioles), it creates a tension on the kinetochores. On the other hand, if only one kinetochore is attached to a microtubule (monotelic) or if each sister kinetochores are attached to microtubules from the same microtubule-organizing centre (syntelic), there is no opposing mechanical forces to create tension between the sister centromere/kinetochore complexes. Tension presumably increases the distance between Aurora B and its kinetochore substrates, permitting the stabilization of the attachment. Aurora B appears to function in a similar manner in respect to its role in maintaining activation of the mitotic checkpoint [549].

The mitotic checkpoint (also called spindle checkpoint) is composed of several kinetochore-associated kinases, including Bub1 (budding uninhibited by benzimidazoles 1 homolog), Bub3 (budding uninhibited by benzimidazoles 3 homolog), BubR1 (budding uninhibited by benzimidazoles 1 homolog beta), and Mps1 (monopolar spindle 1). The relationship between these enzymatic elements and structural components of the checkpoint is still controversial. However, recent data have begun to unravel some of the interaction networks and underlying mechanisms. When microtubules are not properly attached, Aurora B can trigger a phosphorylation cascade involving Bub1, Bub3, and Mps1 [550]. This cascade results in the phosphorylation of Mad1(mitotic arrest deficient 1)-bound Mad2 (mitotic arrest deficient 2) and somehow induces a change in conformation in Mad2, allowing its activation and its dimerization. In G2, soluble BubR1 binds to Cdc20 and inhibits the activity of APC/C by blocking the premature recruitment of the substrates cyclin B and securin. When Mad2 is in its active conformation, it recruits the BubR1-Cdc20-APC/C complex to the kinetochore and locks it in an inactive conformation [494,551]. Upon microtubule attachment, Aurora B is unable to phosphorylate its substrates. This event would somehow activate the phosphatase PP1, which will rapidly dephosphorylate checkpoint components, ultimately activating the APC/C^{cdc20} complex. Other mechanisms such as inhibition of Mad2 by $p31^{comet}$ and direct inactivation of BubR1 upon microtubule attachment are likely to be involved as well [552]. Once activated, APC/C^{cdc20} will ubiquitinate securin, resulting in the activation of separase, in the removal of cohesin and ultimately in separation of the sister chromatids [547].

4. OBJECTIVES OF THE PRESENT STUDY

As exposed in the introduction to this thesis, the HIV-1 accessory protein Vpr induces a cell cycle arrest in the G2/M phase by a poorly defined mechanism. While Vpr is known to trigger the canonical DNA damage checkpoint mediated by the sensor ATR, the proximal causes of this activation remain unknown.

HIV-1 accessory proteins do not possess enzymatic activities but generally perform their role by usurping the function of cellular proteins, notably those involved in the ubiquitin-proteasome system. We therefore hypothesised that Vpr would activate ATR and promote the establishment of a G2/M cell cycle arrest by hijacking host proteins. Although several cellular proteins had already been shown to interact with Vpr, none had been convincingly implicated in Vpr-mediated G2 arrest. The first objective of the present study was to identify new cellular partners of Vpr and demonstrate their potential role in cell cycle perturbation (Chapter 1). The identification, in Chapter 1, of a Vpr-interacting cellular E3 ubiquitin ligase complex called CRL4A(VprBP) and the characterization of its involvement in G2 arrest suggested indirectly that Vpr might act as a connector between the ubiquitin-proteasome system and target proteins. The second objective of this study was therefore to investigate the mechanisms by which Vpr was utilizing this E3 ligase to promote G2 arrest (Chapter 2). Finally, the last objective was to establish in what subcellular compartments Vpr was recruiting CRL4A (VprBP) and to correlate this localization with the induction of G2 arrest (Chapter 3).

CHAPTER 1: HIV-1 VPR-MEDIATED G2 ARREST INVOLVES THE DDB1-CUL4^{VPRBP} E3 UBIQUITIN LIGASE

RÉSUMÉ

La protéine Vpr (Viral protein R) du VIH-1 cause un arrêt de cycle cellulaire en phase G2 en induisant l'inactivation ATR-dépendante de p34cdc2. Cependant, les facteurs cellulaires directement impliqués dans ce processus demeurent inconnus. Nous avons utilisé la purification d'affinité en tandem pour isoler des complexes natifs contenant Vpr. Nous avons trouvé que DDB1, VprBP et CUL4A, tous des composantes du complexe E3 ubiquitine ligase DDB1-CUL4A^{VprBP}, s'associaient à Vpr. La diminution de l'expression de VprBP à l'aide d'ARN d'interférence a inhibé l'induction de l'arrêt de cycle par Vpr. Il est important de noter, qu'en absence de Vpr, la diminution de l'expression de VprBP n'affecte pas la progression du cycle cellulaire ni l'activation de point de contrôle d'ATR, suggérant ainsi que le role de VprBP dans l'arrêt de cycle en G2 est spécifique à Vpr. De plus, des mutants de Vpr dans le domaine riche en leucines et isoleucines, atténués pour leur interaction avec VprBP et DDB1, sont aussi atténués pour l'induction de l'arrêt de cycle. En revanche, des mutations dans le domaine C-terminal inhibent l'arrêt de cycle mais n'affectent pas l'interaction avec ces deux protéines, suggérant que l'interaction de Vpr avec VprBP et DDB1 est nécessaire mais non suffisante pour bloquer la progression du cycle cellulaire. Globalement, ces résultats suscitent l'élaboration d'un modèle dans lequel Vpr agirait comme connecteur entre le complexe ubiquitine ligase DDB1-CUL4A^{VprBP} et un facteur cellulaire inconnu. L'ubiquitination de ce facteur induirait sa protéolyse ou modulerait son activité, ce qui mènerait à l'activation d'ATR et à un arrêt de cycle en G2.

HIV-1 Vpr-Mediated G2 Arrest Involves the DDB1-Cul4VprBP E3 Ubiquitin Ligase

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Running Title: Vpr recruits DDB1-Cul4^{VprBP} to induce G2 arrest

Author contributions: JPB, GD and EAC conceived and designed the experiments. JPB, GD, JM, NR and AF performed the experiments. JPB, GD, and EAC analyzed the data. JPB, GD, and EAC wrote the paper.

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ABSTRACT

HIV-1 viral protein R (Vpr) has been shown to cause G2 cell-cycle arrest in human cells by inducing ATR-mediated inactivation of p34cdc2, but factors directly engaged in this process remain unknown. We used tandem affinity purification to isolate native Vpr complexes. We found that DDB1, VprBP and Cul4, which are components of a Cul4 E3 ubiquitin ligase complex, DDB1-Cul4^{VprBP}, were able to associate with Vpr. Depletion of VprBP by siRNA impaired Vpr-mediated induction of G2 arrest. Importantly, VprBP knockdown alone did not affect normal cell cycle progression or activation of ATR checkpoints, suggesting that the involvement of VprBP in G2 arrest was specific for Vpr. Moreover, leucine/isoleucine-rich domain Vpr mutants impaired in their ability to interact with VprBP and DDB1 also produced strongly attenuated G2 arrest. In contrast, G2 arrest-defective C-terminal Vpr mutants were found to maintain their ability to associate with these proteins, suggesting that the interaction of Vpr with the DDB1-VprBP complex is necessary but not sufficient to block cell cycle progression. Overall, these results point towards a model in which Vpr could act as a connector between the DDB1-Cul4^{VprBP} E3 ubiquitin ligase complex and an unknown cellular factor whose proteolysis or modulation of activity through ubiquitination would activate ATR-mediated checkpoint signaling and induce G2 arrest.

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) viral protein R (Vpr) accessory protein is a small 96-amino acid protein that plays several roles during virus infection (reviewed in [1,2]). In particular, the protein mediates cell cycle arrest at the G2/M transition in various mammalian cells [3-6], a cytostatic ability that is well-conserved among the primate lentiviruses [7]. Its biological significance is not fully understood but may be related to general activation of virus expression [8] and/or induction of apoptosis [9]. Vpr suppresses cell proliferation by preventing the activation of the p34cdc2/cyclin B complex [3,4]. Accumulating evidence indicate that Vpr-mediated cell

cycle arrest depends on DNA damage response, but precise mechanisms of its induction remain obscure.

Phosphatidylinositol 3 kinase-like ATM (ataxia telangiectasia mutated) and ATR (ataxia and telangiectasia mutated and Rad3 related) are key components of the G2/M checkpoint. In addition, ATR also activates S-phase checkpoint in replication stress response resulting from stalled replication [10]. Depending on the type of stress, ATR- or ATM-mediated checkpoints are fully activated by the coordinated activity of Rad9-Rad1-Hus1 (9-1-1), Rad17-RFC, BRCA1, and 53BP (reviewed in [11-13]). ATR- or ATM-dependent phosphorylation of the histone variant H2AX triggers the formation of γ-H2AX/BRCA1 or 53BP foci. These foci are presumed to help in the recruitment and/or retention of DNA repair machinery and checkpoint effectors at the damaged DNA sites, thus promoting checkpoint signal amplification [12]. Downstream activation of Chk1 or Chk2 kinases by ATM and ATR results in the inactivation of Cdc25 phosphatase and increased expression of both Wee1 kinase and the 14-3-3 family of proteins. Inactivation of cdc2/cyclin by hyperphosphorylation and cytoplasmic retention prevents entry into mitosis before the completion of DNA repair [10,12].

Several groups have reported that Vpr expression induces Rad17- and Hus1-dependent activation of ATR but not of ATM, and induces the formation of nuclear γ-H2AX/BRCA1 foci [14-16]. However, the mechanism by which Vpr triggers ATR activation is still not well understood. Some authors proposed that Vpr would interfere with normal DNA replication leading to stalled replication forks [15,17] while others suggested that the protein may promote the formation of DNA double-strand breaks by recruiting unknown endonucleases to the chromatin [18]. Both models imply direct Vpr interactions with host chromatin [15,18]. On the other hand, blockade of the proliferation might rely on the mislocation of key cell cycle regulators, due to alterations in the nuclear envelope induced by membrane-anchored Vpr [19].

To identify Vpr-interacting cellular proteins responsible for the initial events leading to the induction of G2 arrest, we used the proteomic Tandem Affinity

Purification (TAP) procedure followed by mass spectrometry. Native complexes containing TAP-Vpr were purified from human cells by two consecutive affinity chromatographic steps under mild conditions. Here, we identify a novel protein complex comprising Vpr, the damaged DNA binding protein 1 (DDB1), the newly identified DDB1-Cul4-associated factor (DCAF), VprBP [20-22], and the E3 ubiquitin ligase scaffold protein Cullin 4 (Cul4), and provide functional evidence indicating that Vpr interaction with this E3 ubiquitin ligase complex is involved in induction of G2 cell cycle arrest.

RESULTS

Vpr interacts with DDB1 and VprBP

In order to purify cellular protein complexes interacting with HIV Vpr, Vpr was fused to a TAP tag containing two immunoglobulin-binding domains of protein A from Staphylococcus *aureus*, a cleavage site for the tobacco etch virus (TEV) protease, and the calmodulin-binding peptide (CBP). Since Vpr C-terminal modifications have been reported to alter its cytostatic abilities [23], the bipartite tag was introduced N-terminally. Purification of TAP-Vpr containing complexes was then conducted in HEK293T cells, although the tagged protein induced cell-cycle arrest less efficiently than wild-type Vpr (data not shown). After electrophoresis and silver staining, two bands corresponding to high-molecular weight proteins were repeatedly observed (data not shown). MALDI-TOF mass spectrometric analysis revealed that the upper band corresponded to VprBP, a 180-kDa protein that had been isolated as Vpr-binding factor a decade ago, but whose function still remained obscure [21,22]. The other 120-kDa protein was identified as damaged DNA binding protein 1 (DDB1). DDB1 was initially considered as part of a heterodimeric complex containing damaged DNA binding protein 2 (DDB2), involved in a cellular response to UV-induced DNA damages [24,25]. However, the protein is now emerging as a central scaffolding factor in the DDB1-Cul4-ROC1 E3 ubiquitin ligase complex associated with the COP9 signalosome [26]. Importantly, the WD40 protein VprBP has been recently demonstrated to interact

with DDB1 and most probably serves as an adapter to confer substrate specificity to the DDB1/Cul4/ROC1 E3 ubiquitin ligase complex [20].

We sought to confirm the interaction of Vpr with DDB1 and VprBP in HEK293T cells transfected with TAP or TAP-Vpr expression plasmids. TAP pull-down experiments were performed on cell lysates using IgG-coated sepharose beads. Co-precipitated endogenous DDB1 and VprBP were detected by western blot using specific antibodies. As shown in Fig. 1A, endogenous DDB1 and VprBP could be pulled-down when co-expressed with TAP-Vpr (lane 3) but not when the protein was in presence of the native TAP tag (lane 2), thus indicating that DDB1 and VprBP binding was specific to TAP-Vpr. These interactions could be detected in conditions containing 1% NP-40 (data not shown) as well as 0.5% Triton X-100 (Fig. 1A).

In order to confirm the specificity of the interaction between Vpr and DDB1, we performed pull-down assays in cells co-transfected with TAP-Vpr and HA-DDB1-encoding plasmids (Fig. 1B). We were able to observe that HA-DDB1 could be co-precipitated specifically in presence of TAP-Vpr (lane 6), but not in presence of the empty plasmid (lane 2) or a TAP-expressing plasmid (lane 5). We also constructed TAP-DDB1 as well as GFP-DDB1 expression plasmids to verify whether the interaction could be observed in the reversed orientation. However, immunoprecipitation using endogenous, TAP-tagged, HA-tagged or GFP-fused DDB1 as bait and wild-type or HA-tagged Vpr yielded inconsistent results (data not shown). These discrepancies between HA- and TAP-Vpr abilities to bind to DDB1 are reminiscent of the versatile association between DDB1 and the DNA replication licensing factor Cdt1. In that case, detection of DDB1/Cdt1 complexes in absence of chromatin was dependent on the amount of antibody used for the immunoprecipitation [20]. Given that Cdt1 has been shown to interact indirectly with DDB1 *via* the adapter protein Cdt2 [27,28], it would be tempting to hypothesize that Vpr would likewise interact indirectly with DDB1 through an adapter protein, perhaps VprBP, and that the TAP/IgG bead complexes may somehow stabilize the interaction.

To confirm the specificity of interaction between Vpr and VprBP in our system, we performed co-immunoprecipitation experiments in presence of endogenous VprBP and over-expressed HA-Vpr (Fig. 1C). We could specifically detect co-immunoprecipitated VprBP in presence of HA-Vpr (lane 2) but not in presence of the empty plasmid (lane 1). Finally, we could also observe an interaction between over-expressed HA-Vpr and Myc-tagged VprBP (Fig. 1D), confirming the specificity of the interaction between Vpr and VprBP.

DDB1 appears to bind to Vpr indirectly

To further investigate the apparent association between TAP-Vpr and DDB1, Vpr, Tap and TAP-Vpr sequences were subcloned in yeast two-hybrid expression constructs. *Saccharomyces cerevisiae* cells were co-transformed with each combination of plasmids. Interaction affinities were determined by measurement of the β-galactosidase activity. As expected, Vpr appeared to form homodimers, leading to a strong activity of the reporter gene (Fig. 2A). Surprisingly, no binding could be detected between Vpr and DDB1, a result consistent with the lack of interaction observed with some co-immunoprecipitation experiments. We found that dimerization of TAP-Vpr was 3-fold weaker compared to that of the wild-type protein. Hence, N-terminal addition of large peptide appears to disturb the tertiary structure of Vpr, explaining at least in part the reduction of TAP-Vpr cytostatic abilities. Importantly, we found that β-galactosidase activity remained undetectable in cells co-expressing TAP-Vpr and DDB1. This lack of stable interaction between Vpr and DDB1 did not result from lack of DDB1 expression in yeast, since we could observe DDB1 expression, by western blot in Vpr-expressing yeast cells (Fig. 2A). Finally, an association between Vpr and DDB1 could not be detected in co-immunoprecipitation experiments using *in vitro*-translated T7-Vpr and HA-DDB1 proteins (Fig. 2B) in conditions which have demonstrated an association between Vpr and VprBP [21], suggesting that the binding observed in human cells might be indirect and may involve a bridging factor.

Vpr associates with a DDB1-Cul4^{VprBP} E3 ubiquitin ligase complex

One possible explanation for the apparent association between Vpr and DDB1 is that Vpr would associate with the DDB1/Cul4/ROC1 ubiquitin ligase complex through perhaps a direct interaction with VprBP. Indeed, the WD40 protein VprBP has been recently demonstrated to interact with DDB1 and most probably serves as an adapter to confer substrate specificity to the DDB1/Cul4/ROC1 E3 ubiquitin ligase complex [20]. To assess the possibility that the Vpr/VprBP/DDB1 complex might be part of an ubiquitin E3 ligase complex, we investigated whether Vpr could associate with the E3 ligase scaffold protein Cul4. HEK293T cells were transfected with plasmids expressing TAP and TAP-Vpr, and pull-down assays were performed on cell extracts containing endogenous Cul4. These assays demonstrated that endogenous Cul4 could specifically associate with TAP-Vpr but not with native TAP (Fig. 3A, lane 3), thus suggesting that Vpr does indeed associate with a Cul4-scaffolded E3 ligase complex Moreover, anti-Cul4 immunoprecipitation experiments on cells that had been transfected with an empty plasmid or an HA-Vpr-expressing plasmid revealed that HA-Vpr co-immunoprecipitated with a Cul4-containing complex (Fig. 3B, lane 2), thus confirming that Vpr can associate with a E3 ligase complex with potential ubiquitinating activities. Importantly, VprBP could be co-immunoprecipitated along with Cul4 (Fig. 3B). Therefore, given the mutual association of Vpr (Fig. 1A, 1C, and 1D therein and [21,22]) and Cul4 (Fig 3B therein and [20]) with VprBP, these results suggest that Vpr interacts with the DDB1-Cul4^{VprBP} E3 ubiquitin ligase in a single complex and that this association might occur via the intermediary of VprBP.

The interaction between DDB1-Cul4^{VprBP} and Vpr is required for the induction of G2 arrest

We then sought to study the potential role of DDB1-Cul4^{VprBP} E3 ubiquitin ligase in the induction of G2 cell cycle arrest by Vpr. DDB1 depletion by siRNA has been reported to induce the accumulation of cells in G2/M due to DNA re-replication [28] and, consequently, this strategy could not be used to demonstrate the involvement of DDB1-Cul4^{VprBP} in the induction of Vpr-mediated G2 arrest. As an alternative strategy, we analyzed the effect of siRNA-mediated VprBP knockdown on Vpr

cytostatic properties. Cells transfected with VprBP siRNA displayed a major reduction of VprBP at the mRNA level (Fig. 4A) as well as at the protein level (Fig. 4B) compared to scrambled siRNA-transfected cells. We thus transfected HEK293 cells with siRNA against VprBP and, 24 hours later, transduced these cells with a lentiviral vector co-expressing GFP and native Vpr. We observed that cells transfected with VprBP siRNA displayed strongly attenuated Vpr-mediated G2 arrest as compared to cells that received scrambled control siRNA (Fig. 5A). This difference in the induction of G2 arrest was not due to a defect in infectivity potentially resulting from VprBP knockdown because the levels of transduction were equivalent in all the conditions tested (data not shown). To verify that this defect in the induction of G2 arrest in VprBP-depleted cells was specifically the result of the abrogation of the Vpr-VprBP interaction rather than a defect in cell growth, we treated these cells with nocodazole with the rationale that properly cycling cells should accumulate in mitosis due to the effect of the drug on microtubule polymerization (Fig. 5B). In response to nocodazole, cells transfected with VprBP siRNA alone or with the concomitant expression of Vpr accumulated at the G2/M phase, indicating that knockdown of VprBP specifically impaired Vpr-mediated G2 arrest functions. Moreover, the knockdown of VprBP did not produce any observable defects in the activation of the ATR-mediated checkpoints, since treatment with low concentrations of aphidicolin, a DNA replication inhibitor known to produce DNA double-strand breaks at fragile chromosomal sites and to activate the ATR-mediated intra-S checkpoint [29,30,31], blocked cell cycle progression at the S phase in scrambled as well as VprBP siRNA-transfected cells (Fig. 5C). Finally, we analyzed the role of VprBP in the induction of G2 arrest by Vpr in the context of viral infection. SiRNA-transfected cells were infected with VSV-G-pseudotyped fully infectious isogenic viruses defective (NL4-3ΔVpr-GFP, *vpr*-) or proficient (NL4-3-GFP, *vpr*+) for Vpr expression. Forty-eight hours after infection, cells were analyzed for their cell cycle profile. As expected, cells transfected with scrambled control siRNA accumulated in G2 after infection with the *vpr*+ but not with the *vpr*- virus (Fig. 5D). Knockdown of VprBP almost completely abrogated the accumulation of cells in G2 in response to *vpr*+ virus infection but did not significantly affect the cell cycle profile of *vpr*- virus-infected cells. Again, these differences in G2

arrest were not the result of differential infectivity (data not shown). Overall, these results indicate that VprBP is necessary for Vpr-induced G2 arrest, thus suggesting that the DDB1-Cul4^{VprBP} E3 ligase complex might be involved in this Vpr biological activity.

To examine whether the association of Vpr to the E3 ligase complex is required, we tested the ability of several TAP-tagged Vpr mutants to associate with VprBP and DDB1 and their effect on Vpr-induced G2 arrest. HEK293T cells were transfected with plasmids expressing mutants of TAP-Vpr and TAP pull-down experiments were performed on these transfectant cellular extracts. As previously observed, TAP-Vpr was able to pull-down endogenous VprBP and DDB1 (Fig. 6A, lane 3). Interestingly, the classical Vpr mutants S79A and R80A that are attenuated for the induction of G2 arrest (Fig. 6C and [32,33]) could still associate with VprBP and DDB1 at levels comparable with the wild type protein (Fig. 6A, compare lane 3 with lanes 5 and 6), suggesting that the association between Vpr and the DDB1-VprBP complex per se is not sufficient to block cell cycle progression. Moreover, W54R, a mutant of Vpr that was previously shown to be defective for the interaction and degradation of uracil-DNA glycosylase (UNG2) [34], was still capable of associating with the DDB1-Cul4^{VprBP}complex (Fig. 6A, lane 4) and mediate G2 arrest (Fig. 6C), suggesting that UNG2 and VprBP bind to two distinct domains on Vpr. Zhao et al. had previously mapped the domain of interaction of VprBP to the leucine/isoleucine (LR)-rich domain of Vpr [22]. We thus analyzed whether the L64A and Q65R mutations in this domain of Vpr could abrogate binding to VprBP. Using our TAP-Vpr pull-down assay, we observed a very strong reduction of binding to VprBP and DDB1 with the Q65R mutant (under longer exposure VprBP could be detected) (Fig. 6B, compare lanes 3 and 4). A residual association with DDB1 was observed under these conditions, most likely reflecting difference in antibody affinities. Additionally, the reduction of TAP-Vpr binding to VprBP and DDB1 could be observed with the L64A mutant, albeit it was less pronounced (Fig. 6B, compare lanes 5 and 6). Interestingly, with both mutants, the reduced affinity for VprBP was accompanied by a concomitant decrease in the association with DDB1, again suggesting that VprBP and DDB1 are likely to form a single complex with Vpr

(Fig. 6B, compare lane 3 with lane 4 and lane 5 with lane 6). Importantly, we found that Vpr (L64A) and Vpr (Q65R) were strongly attenuated for the induction of G2 arrest (Fig. 6C). Indeed, the residual level of G2 arrest observed with these two mutants was comparable to the attenuated G2 arrest produced by the R80A mutation. Taken together these results suggest that the interaction of Vpr with the DDB1-Cul4^{VprBP} E3 ubiquitin ligase complex is necessary but not sufficient to induce G2 arrest.

DISCUSSION

The induction of G2 arrest by the HIV-1 accessory protein Vpr has been described more than 10 years ago [3-6] however, the mechanism by which Vpr can accomplish this function has remained elusive. Several recent reports have demonstrated that expression of Vpr leads to activation of ATR as well as the formation of DNA repair foci containing BRCA1 and γ-H2AX [14-16]. Nonetheless, the initial events leading to ATR checkpoint signaling are not known. Herein, we have used the TAP method to isolate cellular protein complexes interacting with HIV-1 Vpr in order to identify cellular factors that would be involved in Vpr-mediated ATR activation and subsequent G2 arrest. Using this strategy, we have identified DDB1 and VprBP as cellular factors forming a complex with Vpr.

DDB1 is part of the DDB1/Cul4/ROC1 E3 ubiquitin ligase complex that targets proteins for degradation via the COP9 signalosome [26]. In this complex, DDB1 serves as a scaffold protein presenting substrate to the E3 ubiquitin ligase. The protein is structurally complex, and contains three seven-bladed β propellers (βPA, βPB and βPC) [35]. The βPB propeller is involved in the interaction with Cul4 whereas the βPA-βPC double-propeller fold has been shown to be responsible for substrate presentation via interaction with WD40-repeat proteins [35]. Over 15 different WD40-containing substrate receptors, including Ddb2, CSA, Det-Cop1 and Cdt2, have been shown to interact with DDB1 and are thought to confer substrate specificity [20,35-37]. However, only a few cellular proteins have been found to date to be regulated by the

DDB1/Cul4/ROC1 complex. In human cells, DDB1-Cul4^{DDB2} promotes the ubiquitylation of histone 2A [38], histone 3, histone 4 [39], and the xeroderma pigmentosum group C protein (XPC) to regulate their activity [40]. In contrast, DDB1-Cul4^{CSA} and DDB1-Cul4$^{Det1\text{-}Cop1}$ promote proteolysis of Cockayne syndrome type B gene product (CSB) [26] and c-JUN [41] . Recently, DDB1, *via* the WD40 adapter Cdt2, has been shown to prevent DNA re-replication during normal S-phase progression or in response to S-phase accumulation of DNA lesions by regulating the degradation of the replication licensing factor Cdt1 [27,28].

Interestingly, DDB1 also forms complexes with two other viral proteins, namely HBV X protein [42-44] and simian paramyxovirus SV5 V protein [45,46]. Whereas the mechanisms underlying the DDB1-dependent cytotoxicity induced by HBV X protein remain poorly understood, it has been established that SV5 V protein facilitates the ubiquitination and subsequent signalosome-mediated degradation of STAT1 [45]. Several data argue in favor of a functional involvement of DDB1/Cul4/ROC1 in Vpr functions. Cullin 4A has been implicated in Vpr-induced degradation of UNG2 and SMUG1 proteins [47] and Vpr was shown to interact with VIP/mov34/CNS6, one of the subunits of Cul4-associated signalosome [48].

In our system, an interaction between Vpr and DDB1 was observed when Vpr was fused to a TAP tag (TAP experiments, Fig. 1A, and B). However, co-precipitations in the reverse direction with DDB1 fused to other tags (TAP, HA or GFP) only yielded inconsistent interaction results (data not shown). The lack of apparent interaction between DDB1 and Vpr in that context may be due to our experimental conditions or to the type of association that engages Vpr and DDB1. In that regard, the lack of interaction between Vpr and DDB1 in the yeast two-hybrid system (Fig. 2A) as well as in *in vitro* co-precipitation experiments (Fig. 2B) argued for an indirect interaction between these two proteins, although definite demonstration of this will require a more thorough analysis of this association in the future. These results were in contrast to SV5 V protein, which can directly interact with the DDB1 βPA-βPC domain [35,49]. Through the use of TAP procedures (data not shown), immunoprecipitation experiments

(Fig.1) as well as several Vpr mutants (Fig. 6), we identified VprBP as a cellular factor also in a complex with Vpr and DDB1 and possibly permitting the recruitment of a DDB1-Cul4 E3 ubiquitin ligase complex. The cellular function of VprBP has recently been uncovered through different proteomic approaches. It is a WD40 protein linked to the DDB1/Cul4/ROC1 complex and probably serves as an adapter presenting protein substrate for degradation [20]. Interestingly, no VprBP ortholog has been identified in yeast [21], explaining perhaps why we did not observe an interaction between Vpr and human DDB1 in S. cerevisae (Fig. 2A) and why the DDB1 ortholog was not found among the putative Vpr-interacting proteins isolated by TAP assay in *S. pombe* (data not shown).

To further characterize the possibility that Vpr might interact with a DDB1-Cul4^{VprBP} E3 ligase complex, we investigated whether the scaffold protein Cul4 could associate with Vpr. Interestingly, we could demonstrate the formation of a complex containing Vpr and Cul4 using two different approaches: by TAP-Vpr pull-down (Fig. 3A) and by anti-Cul4 immunoprecipitation (Fig. 3B). Therefore, it appears that Vpr can indeed recruit an E3 ligase complex with potential ubiquitinating activity. In these experiments, VprBP was found in respective association with Vpr (TAP experiments, Fig. 1A, C, and D), as well as with Cul4 (Fig. 3B). Therefore, taking into account these interaction results and previous reports demonstrating the association between Vpr and VprBP [21,22] as well as the association between Cul4 and VprBP [20], it is thus most likely that Vpr interacts with the DDB1-Cul4 E3 ligase complex via the intermediary of VprBP, although direct proof of that will require further analysis of the protein complex architecture.

Importantly, we also observed that VprBP but also the formation of a Vpr-DDB1-Cul4^{VprBP} complex was required for Vpr-mediated induction of G2 arrest. Indeed, siRNA targeting VprBP strongly impaired the induction of G2 arrest in the context of a lentiviral vector expressing Vpr and of a fully infectious provirus (Fig. 5A and 5D). Knockdown of VprBP did not produce cell cycle aberrations (Fig. 5B and 5C)

nor did it reduce viral/vector infection efficiency, suggesting that the observed defect in G2 arrest was specific to the association of Vpr with VprBP.

It has been extensively shown that Vpr C-terminal residues 78-96 are important for G2 arrest [23,50-52], but we found that TAP-tagged Vpr mutants S79A and R80A attenuated for cell cycle arrest were nevertheless competent for association with DDB1 (Fig. 6). This was not surprising given that Zhao et al. previously showed that VprBP binding required the leucine/isoleucine-rich domain of Vpr [22]. In fact, L64A and Q65R, two mutations in the leucine/isoleucine-rich domain reduced to different extent the binding of Vpr to VprBP (Fig. 6B). We also observed a concomitant decrease of affinity between Vpr and DDB1 as a result of these mutations, suggesting that a single complex comprising VprBP and DDB1 is binding to the leucine/isoleucine-rich domain of Vpr. Importantly, these two mutations strongly attenuated Vpr-induced G2 arrest (Fig. 6C). Overall, these results suggest that recruitment of DDB1-Cul4^{VprBP} by Vpr is necessary but not sufficient to induce G2 cell cycle arrest.

One possible model to explain how Vpr induces G2 arrest *via* the DDB1-Cul4^{VprBP} complex would be that Vpr could cause generalized defect in the activity of the whole DDB1 complex In this context, other investigators have shown that abrogation of DDB1 function using siRNA resulted in the failure to degrade Cdt1, thus leading to the accumulation of re-replicated DNA fragment and the induction of an ATR-dependent G2 arrest [28]. Therefore, Vpr, through a potential sequestration of DDB1, might be capable of inducing the defective regulation of Cdt1 or other DDB1-Cul4 E3 ligase complex substrates leading to ATR-mediated G2 arrest. However, we did not observe any obvious effect of Vpr on the S-phase degradation of Cdt1 (data not shown), suggesting that the DDB1/Cul4/ROC1 complex was fully functional. Another possible explanation would be that Vpr could block the proper ubiquitination of natural substrates of DDB1-Cul4^{VprBP} thereby affecting their biological activities or preventing their degradation. However, this is unlikely because siRNA-mediated knockdown of VprBP did not produce G2 arrest (Fig. 5). The most probable model would be that Vpr would trigger the degradation of a yet-unknown modulator of cell cycle progression by

targeting it to the substrate receptor VprBP, itself linked to the DDB1/Cul4/ROC1 E3 ligase complex. This situation would be highly similar to the mechanism by which Vpu induces the degradation of CD4 by recruiting directly CD4 to the $SCF^{\beta\text{-}TRCP}$ E3 ubiquitin ligase complex [53]. However, we cannot exclude the possibility that the Vpr-VprBP interaction might modulate the activity of substrates through ubiquitination, as was described for XPC and histones [38-40]. Therefore, the formation of a complex between Vpr and DDB1-$Cul4^{VprBP}$ through the Vpr leucine/isoleucine-rich domain could permit the hypothetical interaction of Vpr, through its C-terminal domain, with other cellular factors whose ubiquitination would induce the initial events in Vpr-mediated cell cycle arrest.

In conclusion, we presented biochemical and functional evidence suggesting that Vpr is likely the third protein encoded by HIV-1, besides Vif and Vpu, to interact with the ubiquitination machinery. Vpr was found to associate with DDB1 and Cul4, which are components of the DDB1/Cul4/ROC1 E3 ligase complexe through possibly the intermediary of the WD40 substrate receptor VprBP. Importantly, the interaction between Vpr and the VprBP-DDB1 complex was shown to be required for the induction of G2 arrest. Vpr could act as a connector to the DDB1-$Cul4^{VprBP}$ E3 ubiquitin ligase and recruiting via its C-terminal residues an unknown cellular factor whose proteolysis or modulation of activity through ubiquitination would activate ATR-mediated checkpoint signaling and cause G2 arrest.

MATERIALS AND METHODS

Cells and antibodies

Human embryonic kidney (HEK) 293 and HEK293T cells were maintained as described elsewhere [54]. The anti-HA tag and anti-Myc tag monoclonal antibodies were, respectively, clones 12CA5 and 9E10. Rabbit polyclonal antibody against VprBP was distributed by Accurate Chemical and Scientific Corporation (Westbury, NY). The mouse monoclonal antibody against DDB1 was obtained from BD Biosciences

(Mississauga, Ontario, Canada). The goat polyclonal antibody against Cul4 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Vpr was detected using a rabbit polyclonal antibody directed againt a Vpr N-terminal peptide [55].

Plasmid constructions

For the construction of mammalian expression plasmid psvCMV-TAP, N-terminal TAP tag was first PCR amplified from the pBS1479 plasmid [56] using primers 5'-TCTAGACATATGGCAGGCCTTGCGCAAC-3' and 5'-GGATCCTCACTACTCGAA-TCGTCATCATCAAGTGCC-3', the last one containing two stop codons between the XhoI and BamHI restriction sites. The XbaI/BamHI-digested PCR fragment was then inserted into psvCMV plasmid [54] linearized with XbaI/BglII. For the construction of the psvCMV-TAP-Vpr expression plasmid, wild-type and mutant Vpr genes were PCR-amplified from the respective psvCMV-HA.Vpr plasmids [54] using oligonucleotides 5'-CTCGAGATGGAACAAGCCCCAG-3' and 5'-GGATCCCTAGGATCTACTGGCT-3'. XhoI/BamHI Vpr fragments were finally fused to the XbaI/XhoI TAP sequence within psvCMV by 3-fragment ligation. Mammalian expression plasmids pSRAS-3HADDB1 and pSRAS-GFP.DDB1 [44] were kindly provided by M. Strubin (University of Geneva, Switzerland). The TAP-DDB1 expression plasmid was constructed by inserting the XbaI/XhoI TAP fragment in Xba/SalI linearized pSRAS-3HA.DDB1 plasmid. The Myc-VprBP expression plasmid was constructed by PCR amplification of the VprBP cDNA from Image clone ID 5547856 (American Type Culture Collection, Manassas, VA). The resulting PCR product was then subcloned in pCMV-Myc (Clontech, BD biosciences, Mississauga, Ontario, Canada) at the SalI and NotI sites. For the construction of the yeast expression plasmids, TAP and TAP-Vpr sequences were subcloned after PCR amplification in the BamHI sites within pEG202. The DDB1 sequence was extracted from pSRAS-3HADDB1 by SalI/NotI digestion, and introduced in pJG4-5. Vpr-expressing pJG4-5 and pEG202 plasmids have been described previously [57].

The second-generation self-inactivating lentiviral vector pWPI and pWPXL, as well as the packaging plasmid psPAX2 were obtained from D. Trono (School of Life

Sciences, Swiss Institute of Technology, Lausanne, Switzerland). A lentiviral vector tranducing Vpr and GFP (pWPI-Vpr) was generated from pWPI. Vpr was PCR-amplified from psvCMV-Vpr [54], using primers: 5'AAGGATCCATGGAACAAGCCCCAGAAGACC-3' and 5'-TACGACTAGTCTAGGATCTACTGGCTCCATTT-3', containing a BamHI site at the 5'end and a SpeI site at the 3'end, respectively. The BamH1/SpeI cleaved- PCR product was then ligated into pWPXL linearized at the same sites. A fragment containing the Vpr-coding sequence and the EF1-alpha promoter was excised with SpeI (Klenow-treated) and NotI and then ligated into pWPI at the PmeI and NotI sites to yield pWPI-Vpr.

Tandem affinity purification

Purification was done according to previously published procedures [56]. Briefly, HEK293T cells were seeded onto five to ten 150mm-diameter plates. The day after, cells were transfected with 10 μg of psvCMV TAP-Vpr plasmid. Forty hours later, cells were collected, washed and lysed in IPP150 buffer (10mM Tris-Cl pH 8.0, 150mM NaCl, 0.1% nonidet NP40) supplemented with EDTA-free complete protease inhibitors (Roche Diagnostics Canada, Laval, Qc, Canada). Cell debris were removed by low-speed centrifugation, and cleared supernatants were loaded onto IgG sepharose columns (Amersham BioSciences Canada, Baie d'Urfe, Qc, Canada). After extensive washes with IPP150 buffer, and a final wash with TEV cleavage buffer (10mM Tris-Cl pH8.0, 150mM NaCl, 0.1% NP40, 0.5mM EDTA, 1mM DTT), TAP-containing complexes were eluted by overnight digestion using 100 units of TEV protease (Invitrogen Canada, Burlington, Ontario, Canada) diluted in 1 ml of TEV buffer. Columns were eluted and washed with 3 ml of IPP150-calmodulin buffer (10mM β-mercaptoethanol, 10mM Tris-Cl pH8.0, 150mM NaCl, 1mM Mg-acetate, 1mM imidazole, 2mM $CaCl_2$, 0.1% NP40) supplemented with 3 ml of 1M $CaCl_2$ and EDTA-free complete protease inhibitors. Fractions were passed through calmodulin columns (Stratagene, La Jolla, CA), washed in IPP150 buffer and finally eluted with a minimal volume of IPP150 elution buffer (10mM β-mercaptoethanol, 10mM Tris-Cl pH8.0, 150mM NaCl, 1mM Mg-acetate, 1mM imidazole, 2mM EGTA, 0.1% NP40). Recovered proteins were

resolved by denaturing 12.5% SDS-PAGE. Bands detected after silver staining were cut and sent to Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA, USA) for MALDI-TOF mass spectrometry.

Transfection and immunoprecipitation

Cells were transfected using the calcium phosphate precipitation method. Two days 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 EDTA-free complete protease inhibitors). TAP pull-down assays were performed using 10 µl of IgG-coupled beads. After extensive washes in Triton lysis buffer, beads were resuspended in 100 µl of TEV cleavage buffer with 2 units of TEV and incubated for 16 hours at 4ºC. Cleaved proteins were diluted in Laemmli buffer, heat-denatured and loaded onto 12.5% acrylamide gels for SDS-PAGE. After protein transfer onto Hybond-ECL membrane (Amersham BioSciences Canada, Baie d'Urfe, Qc, Canada), proteins were detected by western blotting using specific antibodies. Immunoprecipitation experiments were performed on cell extracts lysed in the Triton lysis buffer as described previously [58].

In vitro translation

T7-Vpr and HA-DDB1 were *in vitro* translated using the Active Pro™ *In vitro* translation kit (Ambion, Austin, TX, USA) according to manufacturer instructions.

Cell cycle analyses

Cell cycle analysis of HEK293 cells transduced by lentiviral vectors was performed as previously described in [54]. The mathematical model MODFIT was used to calculate the proportions of cells in the G2/M phases and G1 phase of the cell cycle. For analysis of cell cycle profile in the context of TAP-Vpr, HEK293T cells were co-transfected with 1 µg of a GFP-expressing and 15 µg of TAP-Vpr expressing plasmids. Forty-eight hours after transfection, cells were fixed with 1% paraformaldehyde for 15 min followed by fixation/permabilization with 70% ethanol for 10 min. The rest of the procedure was as described previously [54] except that flow cytometry analysis was performed on the GFP-positive cell population.

Yeast two-hybrid assay

The yeast 'bait' strains were made by transforming the EGY48 yeast strain with a URA3 lacZ (beta-galactosidase) reporter plasmid and the different bait plasmids (expressing the HIS3 gene) by the lithium acetate method. The yeast 'bait' strain harboring the bait and reporter plasmids were transformed with different prey plasmids and selected for the tryptophan autotrophy phenotype (in addition to the His and Ura nutritional markers for the bait and LacZ reporter plasmids, respectively). Determination of the respective interactions was performed as previously described [57].

Lentiviral vector production, titration, and transduction

VSV-G-pseudotyped viral particles were produced by transient transfection of 40 µg of vector (pWPI or pWPI-Vpr), 30 µg of the packaging construct psPAX2, and 12 µg of the VSV-G-expressing plasmid psvCMV-IN-VSV-G in five millions HEK293T cells using the calcium phosphate precipitation method. The vector-containing supernatant was harvested 48 and 72 hours post-transfection, 0.45 micron-filtered, and concentrated by ultracentrifugation on a sucrose cushion. Concentrated vectors were resuspended in culture medium and stored at –80ºC. Vectors were titered as described previously with some modifications [59]. Briefly, $5X10^4$ HEK293T cells were transduced with serial dilutions of the vector preparations in absence of polybrene. Twenty-four hours later, cells were fixed with 2% PFA for 30 min and the percentage of GFP-expressing cells was determined by flow cytometry. The vector titer was calculated as the number of transduction unit (TU) in the linear range of transduction (5-10% of GFP-positive cells). For transduction experiments, $1X10^5$ HEK293 cells seeded in the wells of a 6-well plate were incubated for 24 hours with WPI or WPI-Vpr vectors (at a multiplicity of infection (MOI) of 1) in presence of 8 µg /ml polybrene, typically achieving a transduction efficiency of 90-95%. Twenty-four hours later, cells were harvested for flow cytometry analysis of cell cycle profiling and GFP expression.

Viral clones and infection

The infectious molecular clones, pNL4.3-GFP and pNL4-3ΔVpr-GFP, were obtained from Dr Juan Lama, Department of Medicine, University of California, San

Diego and described in [60]. Virus was produced by transfecting $5X10^6$ HEK293T cells with 40 µg of proviral DNA and 10 µg of the VSV-G expressing plasmid psvCMV-IN-VSV-G as described previously [54]. Virus-containing supernatant were titered by a standard RT assay as previously described [54]. Virions, at a concentration of 100 cpm/cell, were used to infect HEK293 cells in 1 ml of culture medium, in absence of polybrene. Forty-eight hours after infection, cells were harvested for flow cytometry analysis of cell cycle profiling and GFP expression.

SiRNA-mediated protein knock-down

SiRNA targeting VprBP (siGenome SMARTpool M-021119-00) and scrambled control siRNA (non-targeting siRNA #2) were obtained from Dharmacon Inc, Chicago, IL, USA. SiRNA were transfected using Oligofectamine (Invitrogen Canada, Burlington, Ontario, Canada), according to the manufacturer instructions. Briefly, 300 pmol of siRNA was pre-incubated with 15 µl of Oligofectamine and overlayed on cells at 50% confluence (the final concentration of siRNA was 125nM). Twenty-four hours after transfection, cells were transduced with lentiviral vectors. In some experiments, cells were subjected to two sequential transfections of 300 pmol of siRNA, 24 hours apart.

RT-PCR

RNA was extracted from siRNA-transfected cells at given time using the Qiagen RNeasy kit (Qiagen, Mississauga, Ontario, Canada), according to the manufacturer instructions. After elution, RNA was stored at -80ºC .All reagents for RT-PCR were purchased from Invitrogen Canada (Burlington, Ontario, Canada). RNA was reverse transcribed into cDNA using five hundred ng of RNA, which in a final volume of 12 µl supplemented with 1 µl of oligo $(dT)_{12-18}$ (500 µg/ml), and 1 µl of 10mM dNTP mix were incubated at 65ºC for 5 min. The mixture was cooled on ice and supplemented with 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 units/µl RNaseOUT recombinant ribonuclease inhibitor, and 10 units/µl of M-MLV RT. Finally, the reverse transcription reaction mixture was incubated at 37ºC for 50 min and heat-inactivated at 70ºC for 15 min. One µl of the reverse transcription reaction was

subsequently used for PCR amplification of β-actin and VprBP sequences. Briefly, the PCR amplification mix was composed of 20mM Tris-HCl pH 8.4, 50mM KCl, 1.5 mM $MgCl_2$, 50 µM dNTPs, 1 µM of sense and antisense primers, and 0.05 units/µl of Taq DNA polymerase. Primers 5'-GCTCGTCGTCGACAACGG-CTC-3' and 5'-CAAACATGATCTGGGTCATCTTCTC-3' were used for β-actin amplification and primers 5'-AGGCCATCCACAAGTTTGAC-3' and 5'-TCATCTGCCTGCAACATAGC-3' were used for VprBP amplification. The PCR amplification conditions for VprBP were as follow: 94ºC for 2 min; 25 cycles of (94ºC for 45 sec; 57ºC for 30 sec; 72ºC for 1 min); 72ºC for 5 min. The conditions of amplification for β-actin were the same except that 18 cycles of amplification were used.

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Competing interests. The authors have declared that no competing interests exist.

Note added in proof. During the final revision of this manuscript, two other reports demonstrating the involvement of DDB1 or VprBP in Vpr-induced G2 arrest were published by Le Rouzic et al. [61] and Schrofelbauer et al. [62].

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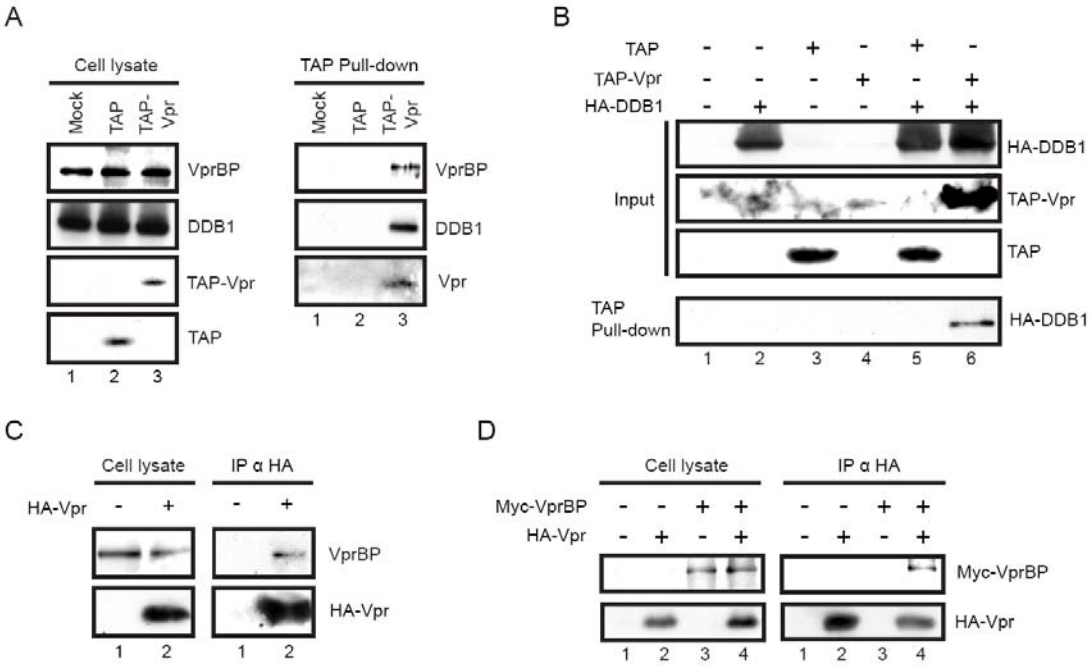
FIGURES

Figure 1. Immunoprecipitation of DDB1/Vpr and VprBP/Vpr complexes. (**A**) HEK293T cells were mock transfected (lanes 1) or transfected with either TAP (lanes 2) or TAP-Vpr expressing plasmids (lanes 3). Two days later, immunoprecipitations of TAP tag were performed on cell lysates using IgG-coupled beads and purified complexes were eluted by cleavage with TEV protease. The levels of endogenous VprBP and DDB1 were monitored in crude lysates and pulled-down fractions by western blot using specific antibodies. TAP, TAP-Vpr and cleaved Vpr were detected using a polyclonal rabbit antibody directed against a Vpr N-terminal peptide. (**B**) HEK293T cells were mock transfected (lanes 1-2) or transfected with either TAP (lanes 3 and 5) or TAP-Vpr expressing plasmids (lanes 4 and 6). Cells were transcomplemented with the empty vector (lanes 1, 3, and 4) or HA-DDB1- encoding plasmid (lanes 2, 5, and 6). (**C**) HEK293T cells were mock transfected (lanes 1) or transfected with HA-Vpr expressing plasmid (lanes 2). Immunoprecipitations using anti-HA antibodies were performed on cells extracts using protein A-sepharose beads. The levels of HA-Vpr and endogenous VprBP were monitored in cell extracts as well as immunoprecipitated fractions by western blotting using specific antibodies. (**D**) HEK293T cells were mock transfected (lanes 1 and 3) or transfected with a HA-Vpr expressing plasmid (lanes 2 and 4). Cells were transcomplemented with the empty vector (lanes 1 and 2) or Myc-VprBP encoding plasmid (lanes 3 and 4). Anti-HA immunoprecipitations were performed as described above.


A
Cell lysate
TAP Pull-down
Mock
TAP
TAP-Vpr
VprBP
DDB1
TAP-Vpr
TAP
Vpr
1 2 3
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TAP
TAP-Vpr
HA-DDB1
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HA-DDB1
TAP-Vpr
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TAP Pull-down
HA-DDB1
1 2 3 4 5 6
C
Cell lysate
IP α HA
HA-Vpr
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HA-Vpr
1 2 1 2
D
Cell lysate
IP α HA
Myc-VprBP
HA-Vpr
Myc-VprBP
HA-Vpr
1 2 3 4 1 2 3 4

Figure 2. Absence of direct Vpr binding to DDB1 in yeast. The EGY48 reporter strain containing LexA-TAP, LexA-Vpr, or LexA-TAP-Vpr (« bait ») was transformed with B42, B42-DDB1 or B42-Vpr-expressing plasmid (« prey »). (**A**) The binding affinity between the different proteins was assessed by assaying beta-galactosidase activity using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) method. Histograms represent averaged data from 2-4 different clones, and are representative of two independent assays. Western blot analysis of induced and non-induced B42-HA-DDB1 expression in the B42 and B42-DDB1-transformed EGY48/LexA-Vpr reporter strain is shown below. (**B**) *In vitro*-translated T7-Vpr was immunoprecipitated with an anti-T7 antibody in presence or absence of *in vitro* translated HA-DDB1. Amounts of protein initially added to the assay (input) are shown in the left panel. * Represents non-specific proteins immunoprecipitated by the ani-T7 antibody.

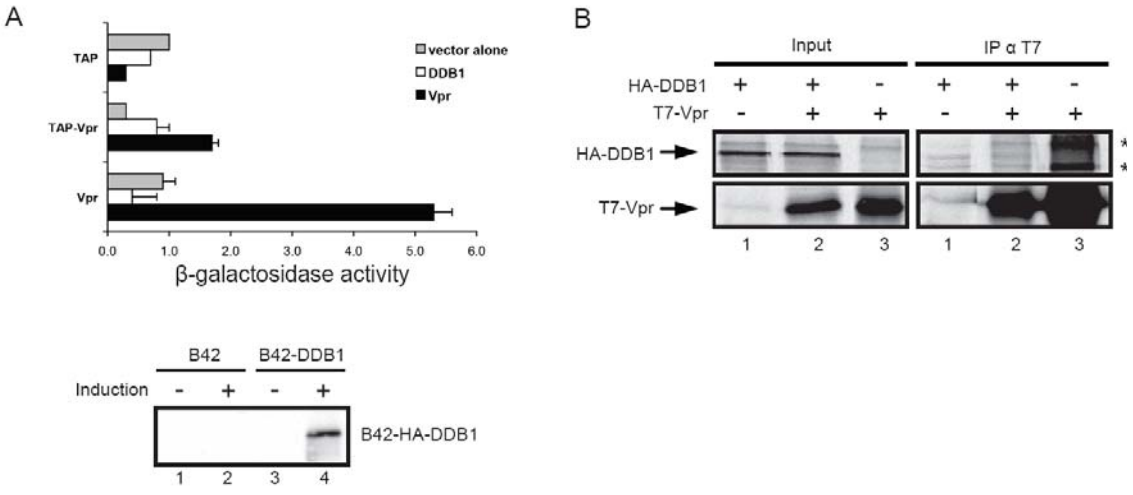

A
TAP
TAP-Vpr
Vpr
vector alone
DDB1
Vpr
0.0
1.0
2.0
3.0
4.0
5.0
6.0
β-galactosidase activity
B42
B42-DDB1
Induction
- + - +
B42-HA-DDB1
1 2 3 4
B
Input
IP α T7
HA-DDB1 + + - + + -
T7-Vpr - + + - + +
HA-DDB1
T7-Vpr
1 2 3 1 2 3

Figure 3. Vpr associates with the ubiquitin ligase scaffold protein Cul4. (**A**) Five million HEK293T cells were transfected with 40 μg of empty (lanes 1), TAP-expressing (lanes 2) or TAP-Vpr-expressing plasmids (lanes 3). Forty-eight hours after transfection, TAP pull-downs were performed on cell lysates using IgG-coupled beads and purified complexes were eluted by cleavage with TEV protease. The levels of endogenous Cul4 were monitored in crude lysates and pulled-down fractions by western blot using a polyclonal goat anti-Cul4 antibody. TAP, TAP-Vpr and cleaved Vpr were detected using a polyclonal rabbit antibody directed against a Vpr N-terminal peptide. (**B**) Ten million HEK293T cells were transfected with 80 μg of empty plasmid (lanes 1) or with HA-Vpr expressing plasmid (lanes 2). Immunoprecipitation of endogenous Cul4 was performed using a goat polyclonal anti-Cul4 antibody and protein A-sepharose beads. The levels of endogenous Cul4, VprBP and over-expressed HA-Vpr were monitored in crude lysates and immunoprecipitated fractions by western blot using, respectively, a polyclonal goat anti-Cul4 antibody, a polyclonal rabbit anti-VprBP antibody and a monoclonal mouse anti-HA antibody. * represents a non-specific protein detected by the anti-Cul4 antibody. The anti-Cul4 antibody generally recognized a doublet of Cul4 when the gel resolution was sufficiently high. In the TAP pull-down fractions only the upper band of Cul4 was detected.

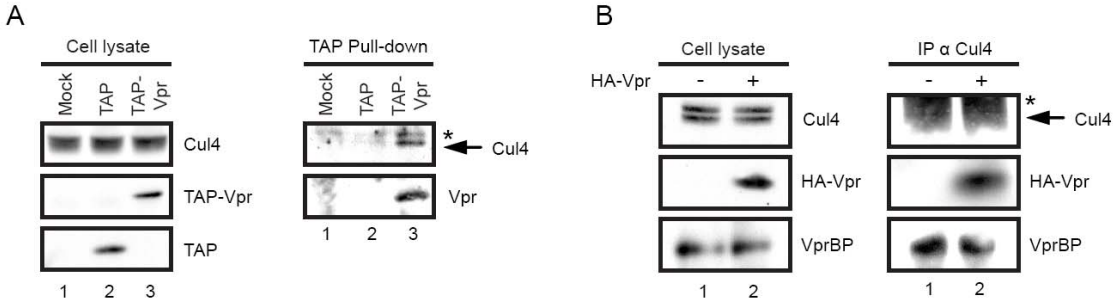

A
Cell lysate
Mock
TAP
TAP-Vpr
Cul4
TAP-Vpr
TAP
1 2 3
TAP Pull-down
Mock
TAP
TAP-Vpr
*
Cul4
Vpr
1 2 3
B
Cell lysate
HA-Vpr
-
+
Cul4
HA-Vpr
VprBP
1 2
IP α Cul4
-
+
*
Cul4
HA-Vpr
VprBP
1 2

Figure 4. Depletion of VprBP using siRNA. HEK293 cells were transfected with 300 pmol of VprBP-targeting siRNA or control scrambled siRNA using Oligofectamine. (**A**) At twenty-four, forty-eight and seventy-two hours after transfection, RNA was extracted and analyzed by RT-PCR to determine the extent of VprBP down-regulation at the mRNA level. PCR products were analyzed in the exponential phase of amplification. Actin levels were used as a control for RNA quality and reverse transcription efficiency. (**B**) Forty-eight hours after siRNA transfection, cells lysates were harvested and analyzed by western blot using a polyclonal rabbit anti-VprBP antibody to demonstrate the downregulation of VprBP at the protein level. Actin levels were used as a control for protein loading.

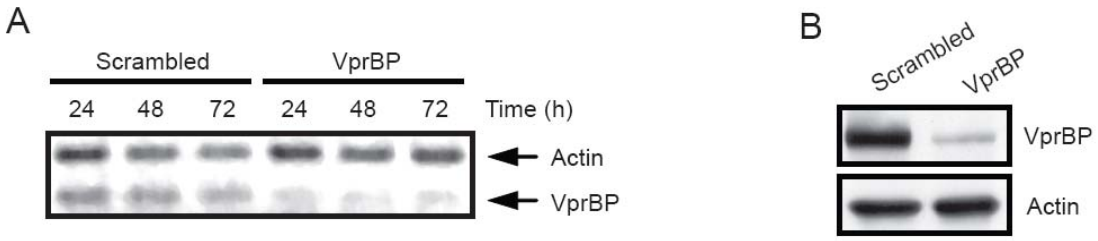

A
Scrambled
VprBP
24
48
72
24
48
72
Time (h)
Actin
VprBP
B
Scrambled
VprBP
VprBP
Actin

Figure 5. Effect of VprBP depletion on Vpr-induced G2 arrest. HEK293 cells were transfected with 300 pmol of VprBP-targeting siRNA or control scrambled siRNA using Oligofectamine, followed by the same transfection twenty-four hours later. Twenty-four hours after the second siRNA transfection, cells were transduced at a MOI of 1 with lentiviral vectors expressing Vpr (WPI-Vpr) or the empty vector (WPI). Cell cycle profiles were analyzed twenty-four hours after transduction by flow cytometry using propidium iodide staining (**A**). To determine if cell growth (**B**) or checkpoint activation (**C**) were affected by VprBP knockdown, HEK293 cells were transfected once with siRNA, as described above, and treated respectively with 1 μg/ml nocodazole and 0.5 μM aphidicolin twenty-four hours later. Cell cycle profiles were analyzed twenty-four hours after drug treatment. To determine if VprBP knockdown could also abrogate the induction of G2 arrest in the context of viral infection, siRNA-transfected cells were infected with NL4.3-GFP and NL4-3ΔVpr-GFP at a concentration of 100 cpm/cell and cell cycle profiles were analyzed forty-eight hours later (**D**). Percentages of G1 and G2/M cell populations were determined using the ModFit software. These results are representative of the data obtained in at least two independent experiments.

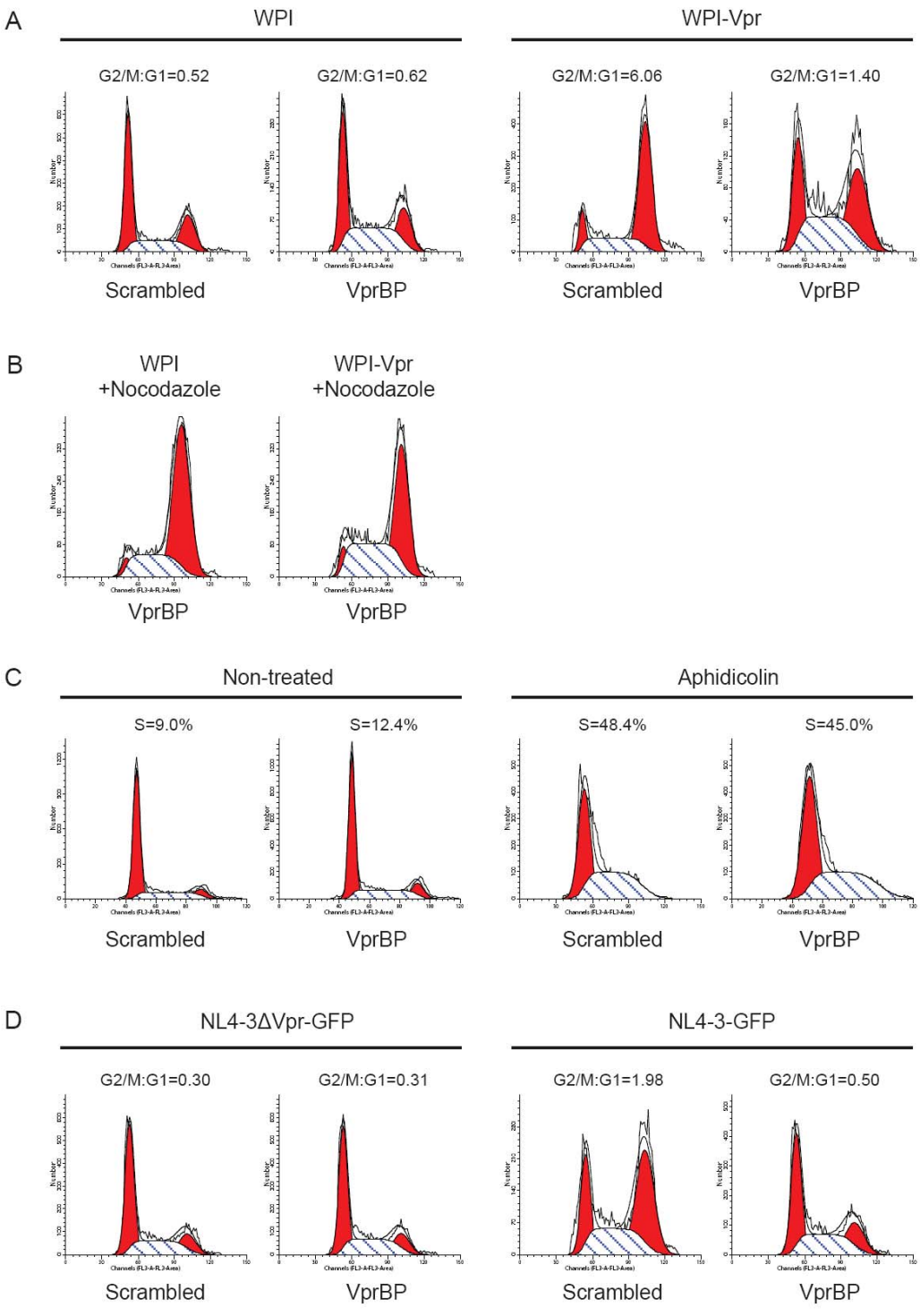
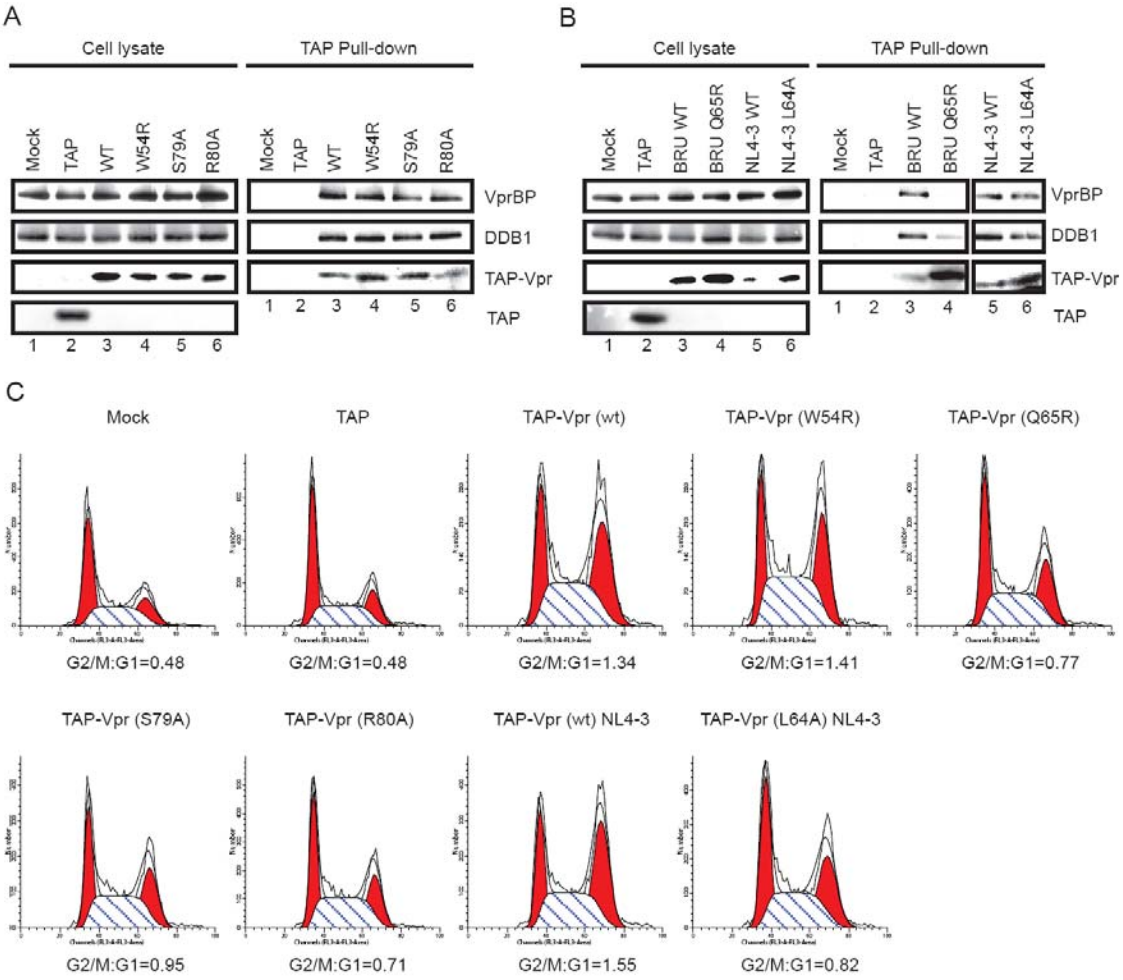

A
WPI
WPI-Vpr
G2/M:G1=0.52
G2/M:G1=0.62
G2/M:G1=6.06
G2/M:G1=1.40
Scrambled
VprBP
Scrambled
VprBP
B
WPI
+Nocodazole
WPI-Vpr
+Nocodazole
VprBP
VprBP
C
Non-treated
Aphidicolin
S=9.0%
S=12.4%
S=48.4%
S=45.0%
Scrambled
VprBP
Scrambled
VprBP
D
NL4-3ΔVpr-GFP
NL4-3-GFP
G2/M:G1=0.30
G2/M:G1=0.31
G2/M:G1=1.98
G2/M:G1=0.50
Scrambled
VprBP
Scrambled
VprBP

Figure 6. DDB1 and VprBP binding affinities of TAP-tagged Vpr mutants. (**A**) HEK293T cells were transfected with TAP-Vpr plasmids encoding for wild-type Vpr (lanes 3), or Vpr mutants W54R (lanes 4), S79A (lanes 5) and R80A (lanes 6). As control, cells were mock transfected (lanes 1) or transfected with a TAP-expressing plasmid (lanes 2). Following TAP pull-down using IgG-coupled beads, the levels of endogenous VprBP and DDB1 were monitored in crude lysates and pulled-down fractions by western blot using specific antibodies. TAP, TAP-Vpr and cleaved Vpr were detected using a polyclonal rabbit antibody directed against a Vpr N-terminal peptide. (**B**) HEK293T cells were transfected with TAP-Vpr plasmids encoding for wild-type Bru Vpr (lanes 3) and wild-type NL4-3 Vpr (lanes 5), or Vpr mutants Bru Q65R (lanes 4), and NL4-3 L64A (lanes 6). As control, cells were mock transfected (lanes 1) or transfected with a TAP expressing plasmid (lanes 2). TAP pull-downs and immunodetection of VprBP, DDB1, TAP, and Vpr were performed as described for panel A. (**C**) HEK293T cells were co-transfected with 1 μg of GFP-expressing plasmid and 15 μg of TAP-Vpr plasmids expressing wild-type or mutant proteins. Cell cycle analysis was performed using propidium iodide staining on the GFP+ cell population as described in Materials and Methods. Percentages of G1 and G2/M cell populations were determined using the ModFit software.


A
Cell lysate
TAP Pull-down
Mock
TAP
WT
W54R
S79A
R80A
VprBP
DDB1
TAP-Vpr
TAP
1 2 3 4 5 6
B
Cell lysate
TAP Pull-down
Mock
TAP
BRU WT
BRU Q65R
NL4-3 WT
NL4-3 L64A
VprBP
DDB1
TAP-Vpr
TAP
1 2 3 4 5 6
C
Mock
TAP
TAP-Vpr (wt)
TAP-Vpr (W54R)
TAP-Vpr (Q65R)
G2/M:G1=0.48
G2/M:G1=0.48
G2/M:G1=1.34
G2/M:G1=1.41
G2/M:G1=0.77
TAP-Vpr (S79A)
TAP-Vpr (R80A)
TAP-Vpr (wt) NL4-3
TAP-Vpr (L64A) NL4-3
G2/M:G1=0.95
G2/M:G1=0.71
G2/M:G1=1.55
G2/M:G1=0.82

CHAPTER 2: HIV-1 VPR INDUCES THE K48-LINKED POLYUBIQUITINATION AND PROTEASOMAL DEGRADATION OF TARGET CELLULAR PROTEINS TO ACTIVATE ATR AND PROMOTE G2 ARREST

RÉSUMÉ

La protéine Vpr (Viral protein R) du VIH-1 induit un arrêt de cycle cellulaire en phase G2/M par un mécanisme impliquant l'activation du senseur de dommage à l'ADN ATR. Certaines recherches incluant les nôtres ont récemment démontré que Vpr remplissait cette fonction en détournant l'activité de la E3 ubiquitine ligase DDB1-CUL4A (VprBP). Vpr pourrait donc agir comme connecteur entre la E3 ligase et un facteur cellulaire inconnu. L'ubiquitination de ce facteur induirait un arrêt de cycle cellulaire. Cependant, ce modèle est uniquement basé sur l'observation indirecte que certains mutants de Vpr sont incapables d'induire un arrêt de cycle tout en conservant leur interaction avec la E3 ligase. En utilisant une approche de purification d'affinité en tandem, nous avons observé que Vpr interagissait avec des protéines ubiquitinées. Ces interactions requéraient une E3 ligase active car la diminution de l'expression de VprBP à l'aide d'ARN d'interférence ainsi que la surexpression d'un mutant dominant-négatif de CUL4A réduisaient l'association aux protéines ubiquitinées. De plus, des mutants C-terminaux de Vpr, inactifs pour l'induction de l'arrêt de cycle, ont une association réduite aux protéines ubiquitinitées. Nous avons aussi démontré que l'inhibition de l'activité protéosomale augmentait cette association et que les chaînes d'ubiquitine était en partie constituées de liens classiques K48. L'inhibition de la polyubiquitination de type K48 a inhibé spécifiquement la phosphorylation de H2AX induite par Vpr mais n'a pas affecté celle induite par les rayons ultraviolets. Globalement, nos résultats apportent des preuves directes que l'association de Vpr à la E3 ubiquitine ligase DDB1-CUL4A (VprBP) induit la polyubiquitination de type K48 de facteurs cellulaires encore inconnus, menant à leur dégradation protéosomal et à l'activation d'ATR.

HIV-1 VPR INDUCES THE K48-LINKED POLYUBIQUITINATION AND PROTEASOMAL DEGRADATION OF TARGET CELLULAR PROTEINS TO ACTIVATE ATR AND PROMOTE G2 ARREST

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Running Title: Vpr-Induced Ubiquitination of Substrates

Author contributions: JPB and EAC conceived and designed the experiments. JPB, JR and NR performed the experiments. YX contributed novel reagents. JPB and EAC analyzed the data. JPB and EAC wrote the paper.

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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 [1-3]. 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 [4]. 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) [5-11].

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 [12,13]. 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 [14]. Mass spectrometric analyses of CUL4A-DDB1 complexes have revealed physical interactions to at least 30 different WDxR substrates receptors [15-18], 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 [19,20], of xeroderma pigmentosum group C protein (XPC) [21] and probably of xeroderma pigmentosum group A protein (XPA) [22] to facilitate UV damage repair. CRL4A (CSA) and CRL4A (DET1-COP1) induce the proteolysis of respectively Cockayne syndrome type B gene product (CSB) [23] and c-JUN [24]. 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 [25,26] as well as of the CDK inhibitor p21 [27,28]. 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 [29,30]. 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 [31]. Moreover, depletion of VprBP reduced the rate of DNA replication, blocked cells in S-phase, and impeded cellular proliferation [10,32]. Consequently, genetic ablation of VprBP in mouse [32] and in the evolutionary distant *Arabidopsis thaliana* [33] 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 [34-36]. Notably, abnormal accumulation of infected cells in G2 can be observed in tissues from patients infected by HIV-1 [36]. 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 [36-40]. 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) [36-38,40] and by activation of the CHK1 kinase [39]. 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 [5-11]. 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 [5-11]. These later mutants also displayed trans-dominant negative activity [11], 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 [41]. 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 [6,42]. Vpr-induced degradation of UNG2 was found to be mediated by DDB1 [6] but surprisingly did not require VprBP [9]. 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) [5,43-45]. 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 [4]. 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 [43-46].

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 [6] or overall increased activity of CRL4A (VprBP) [10]. 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.

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 [7] 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 [47,48]. 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 [7]). 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 [5,7,8,11], 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 [5]. 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 [49]. 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 [50]. 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 [47,48], this HA-Ub (K48R) construct has been previously shown to potently block ubiquitin chain elongation through lysine 48 [50]. 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 [8,11]. 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 [37,38]. Following exogenously induced DNA damages, phosphorylation of H2AX occurs in absence of any ubiquitination events [51], 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 [52,53]. 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 [47,48] coupled with a highly specific tandem affinity purification procedure [7] 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 [49], 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 [5]. It is arguably unlikely that this minimal domain, which also contains the WDxR motif responsible for the association with DDB1 [5,18], 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) [10] given that recruitment of substrates as well as substrate adaptors to CRL complexes, including CRL4A ligases, were shown to markedly promote neddylation [54].

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 [55,56].

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 [8,11], 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 [51]. 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 [36]. Importantly, Vpr did not appear to cause DNA double-strand breaks in cycling cells in conditions where ATR was activated [37]. 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 [36]. 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 [37], ultimately leading to ATR activation [39] 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 [37]. 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 [57]. 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.

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 [59]. 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 [7]. 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) [60] 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 [7]. Plasmids expressing Myc-His-tagged ubiquitin K48R [48] and HA-tagged ubiquitin (WT, K0, and K48R) [50] 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 [9] 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

[61]. 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 [7].

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 [7]. In some experiments, where indicated, IgG pulldown assays were performed instead of the full tandem affinity purification, as described previously [7]. 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 [7,60]. 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 [62], 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 [60]. 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 [63].

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 [7]. 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.

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FIGURES

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.

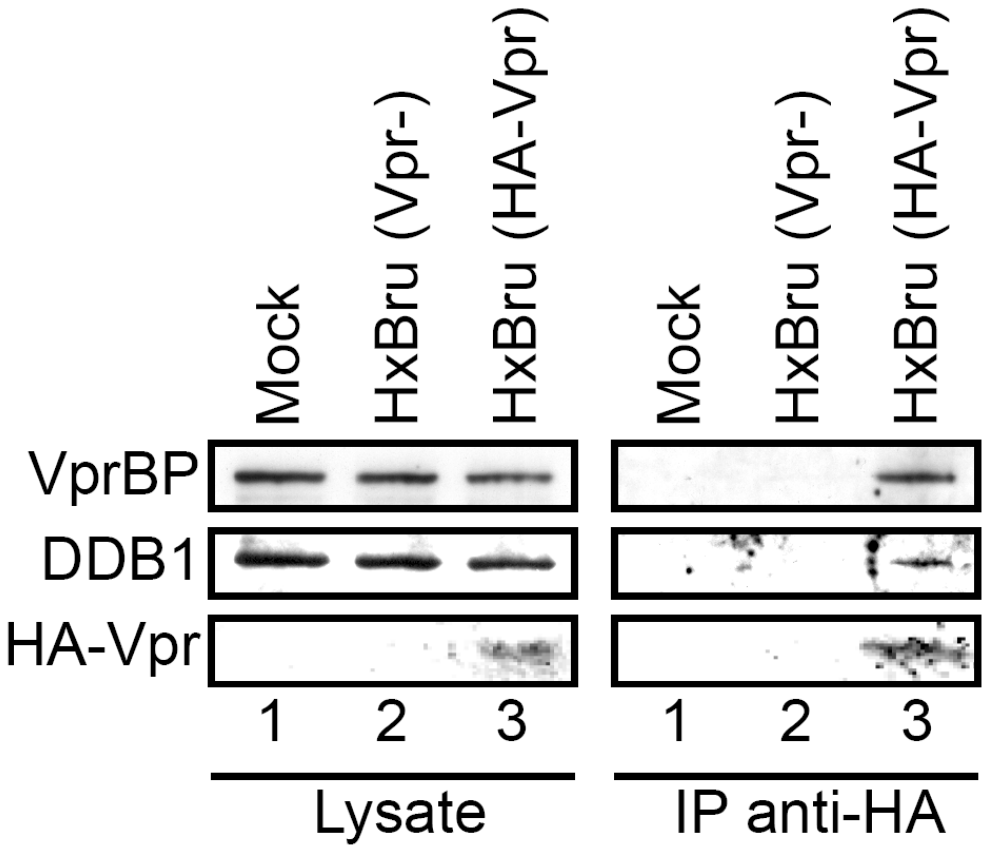

Mock
HxBru (Vpr-)
HxBru (HA-Vpr)
Mock
HxBru (Vpr-)
HxBru (HA-Vpr)
VprBP
DDB1
HA-Vpr
1
2
3
1
2
3
Lysate
IP anti-HA

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.

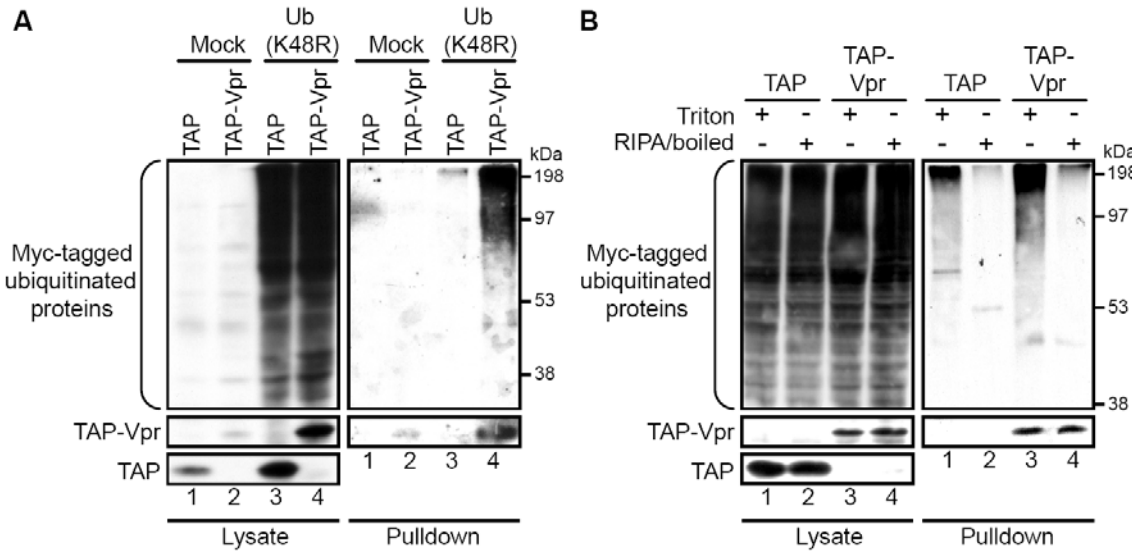

A
Mock
Ub (K48R)
Mock
Ub (K48R)
TAP
TAP-Vpr
TAP
TAP-Vpr
TAP
TAP-Vpr
TAP
TAP-Vpr
kDa
198
97
53
38
Myc-tagged ubiquitinated proteins
TAP-Vpr
TAP
1 2 3 4
1 2 3 4
Lysate
Pulldown
B
TAP
TAP-Vpr
TAP
TAP-Vpr
Triton
RIPA/boiled
+ - + - + - + -
- + - + - + - +
kDa
198
97
53
38
Myc-tagged ubiquitinated proteins
TAP-Vpr
TAP
1 2 3 4
1 2 3 4
Lysate
Pulldown

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.

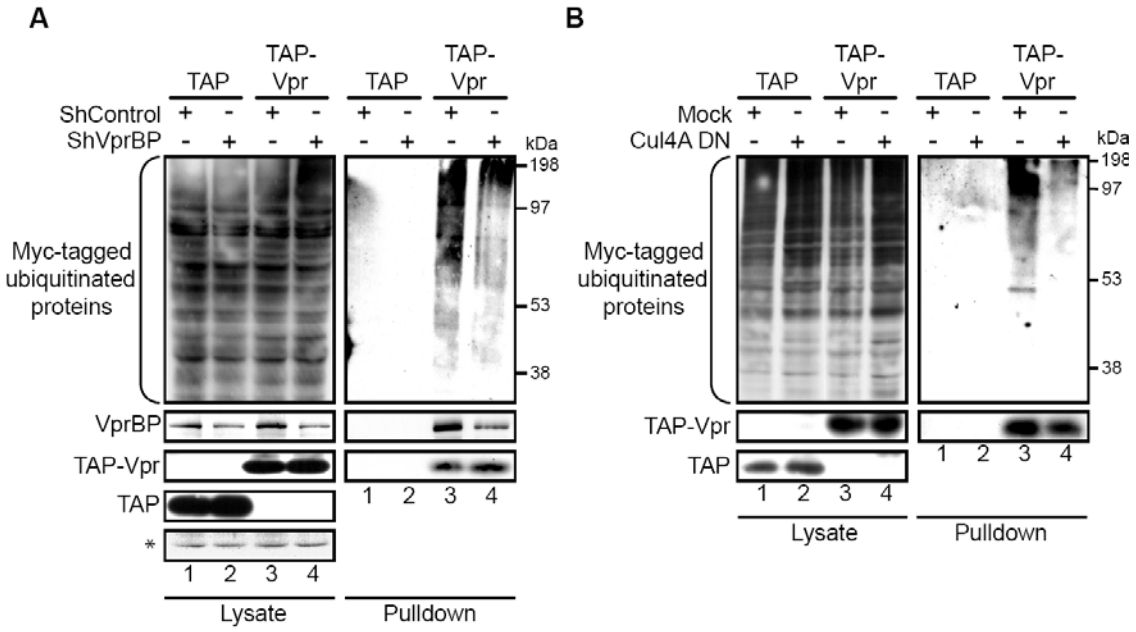

A
TAP
TAP-Vpr
TAP
TAP-Vpr
ShControl
ShVprBP
kDa
198
97
53
38
Myc-tagged ubiquitinated proteins
VprBP
TAP-Vpr
TAP
*
1 2 3 4
1 2 3 4
Lysate
Pulldown
B
TAP
TAP-Vpr
TAP
TAP-Vpr
Mock
Cul4A DN
kDa
198
97
53
38
Myc-tagged ubiquitinated proteins
TAP-Vpr
TAP
1 2 3 4
1 2 3 4
Lysate
Pulldown

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.

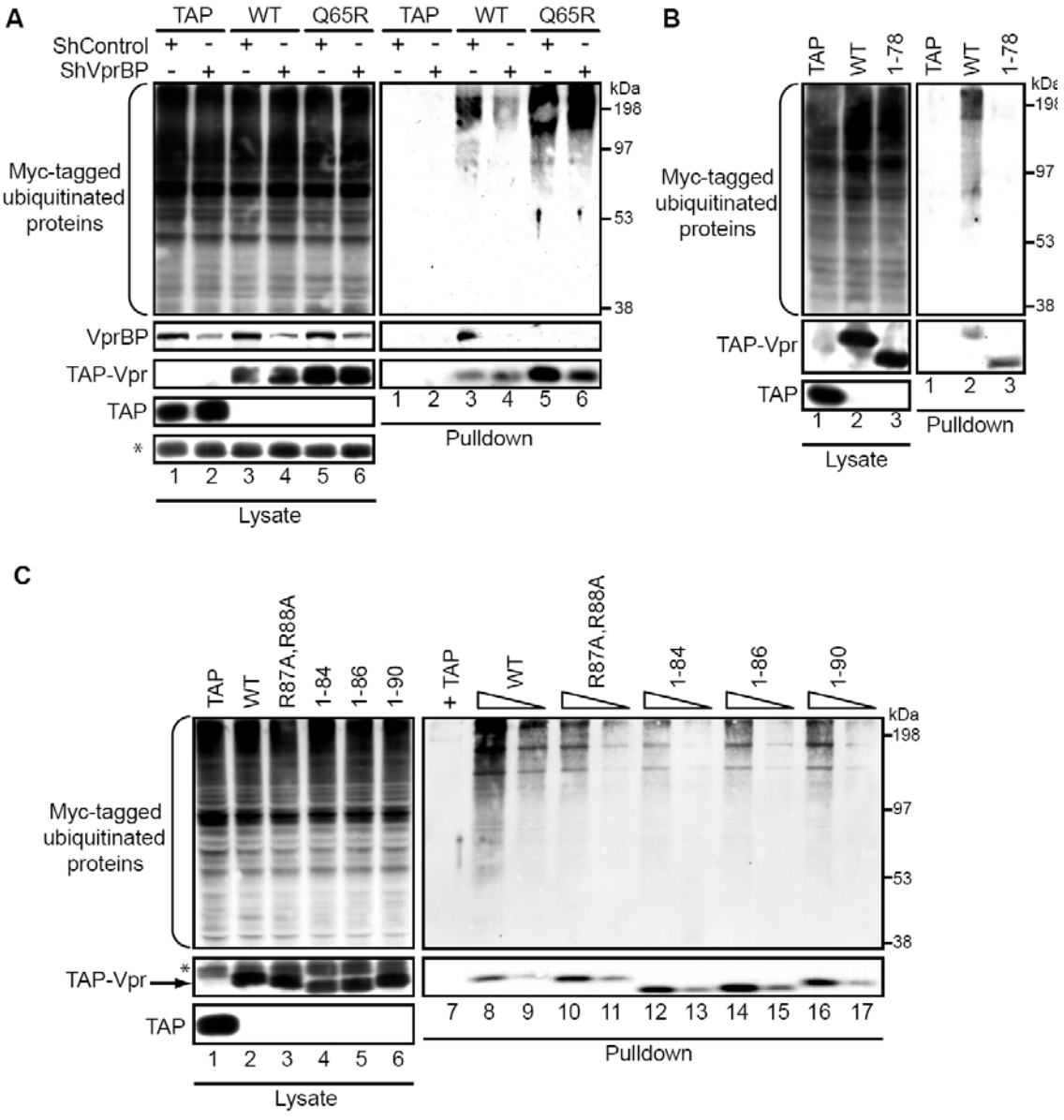

A
TAP
WT
Q65R
TAP
WT
Q65R
ShControl
ShVprBP
kDa
198
97
53
38
Myc-tagged ubiquitinated proteins
VprBP
TAP-Vpr
TAP
*
1 2 3 4 5 6
Pulldown
1 2 3 4 5 6
Lysate
B
TAP
WT
1-78
TAP
WT
1-78
Myc-tagged ubiquitinated proteins
TAP-Vpr
TAP
1 2 3
1 2 3
Pulldown
Lysate
C
TAP
WT
R87A,R88A
1-84
1-86
1-90
+ TAP
WT
R87A,R88A
1-84
1-86
1-90
kDa
198
97
53
38
Myc-tagged ubiquitinated proteins
TAP-Vpr
*
TAP
1 2 3 4 5 6
Lysate
7 8 9 10 11 12 13 14 15 16 17
Pulldown

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.

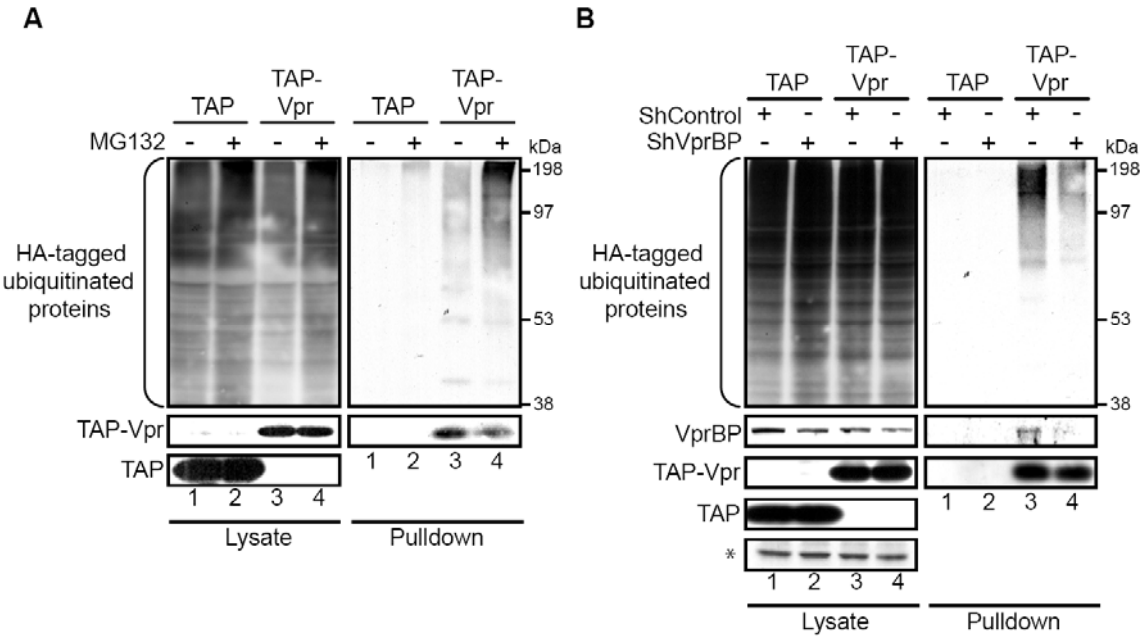

A
TAP
TAP-Vpr
TAP
TAP-Vpr
MG132 - + - + - + - +
kDa
198
97
53
38
HA-tagged ubiquitinated proteins
TAP-Vpr
TAP
1 2 3 4
1 2 3 4
Lysate
Pulldown
B
TAP
TAP-Vpr
TAP
TAP-Vpr
ShControl + - + - + - + -
ShVprBP - + - + - + - +
kDa
198
97
53
38
HA-tagged ubiquitinated proteins
VprBP
TAP-Vpr
TAP
*
1 2 3 4
1 2 3 4
Lysate
Pulldown

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.

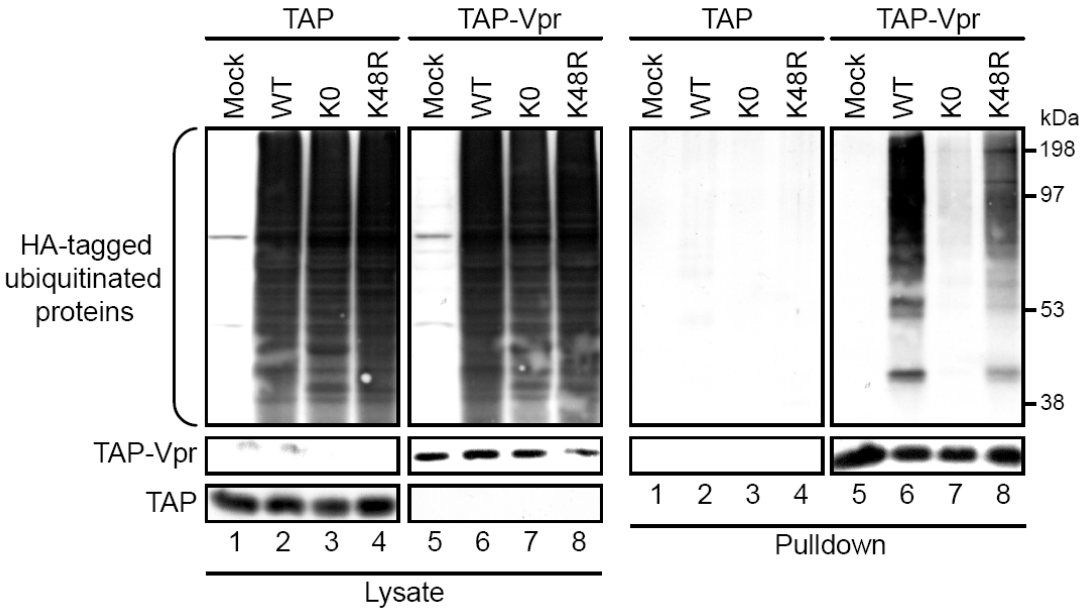
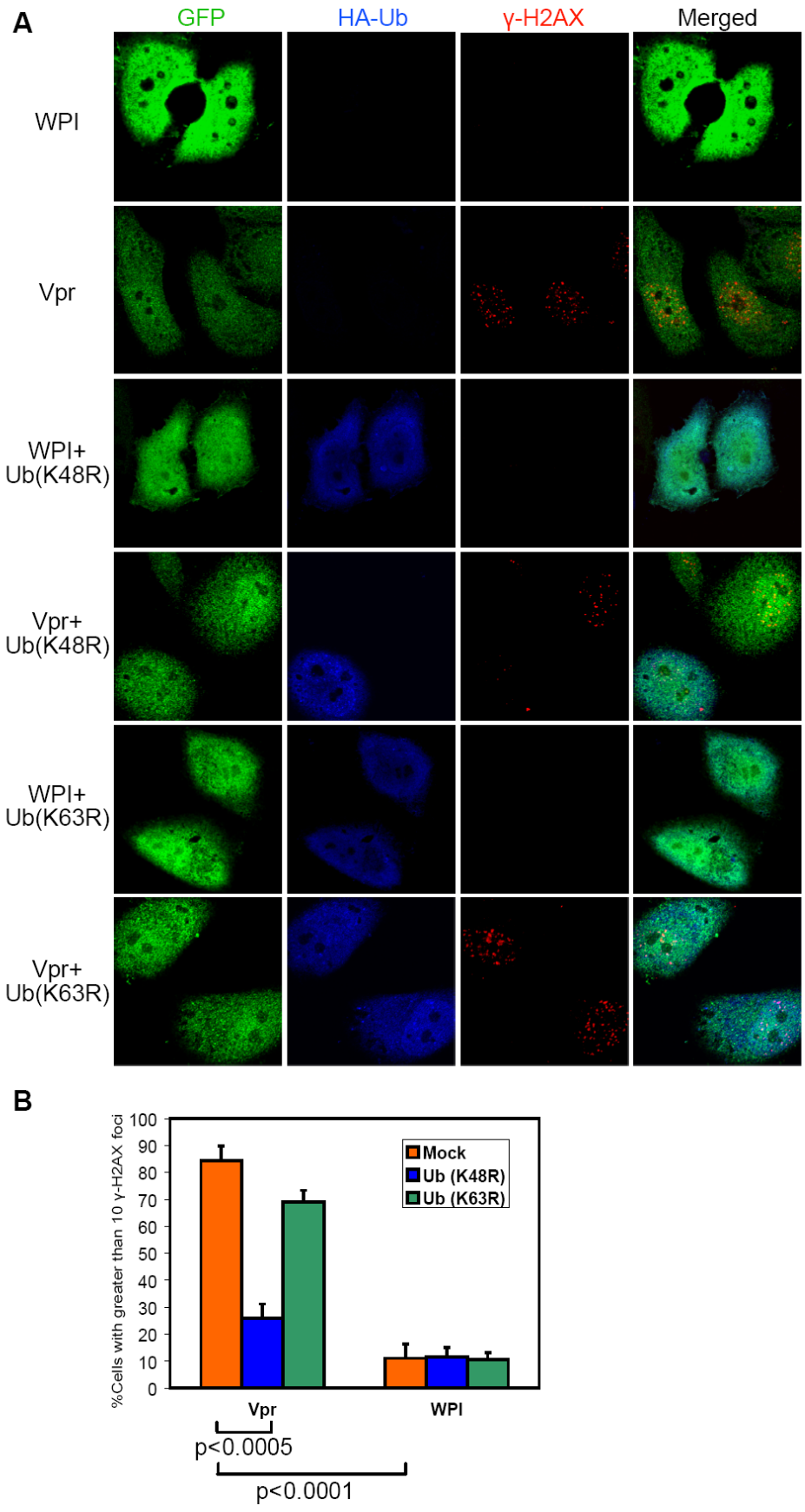

TAP
TAP-Vpr
TAP
TAP-Vpr
Mock
WT
K0
K48R
Mock
WT
K0
K48R
Mock
WT
K0
K48R
Mock
WT
K0
K48R
kDa
198
97
53
38
HA-tagged
ubiquitinated
proteins
TAP-Vpr
TAP
1 2 3 4 5 6 7 8
Lysate
1 2 3 4 5 6 7 8
Pulldown

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

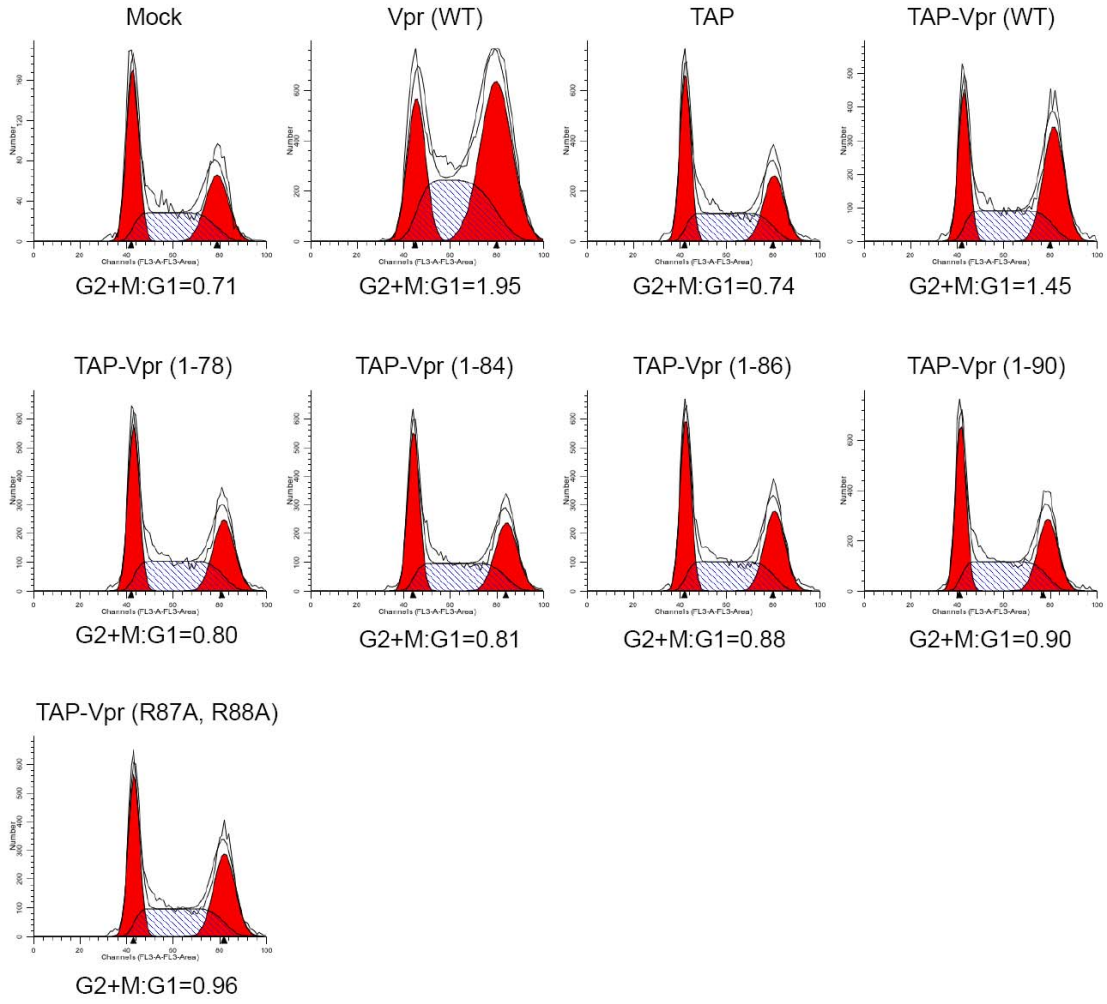
A) HeLa cells were transiently transfected with an empty plasmid or plasmids expressing HA-Ub (K48R) or HA-Ub (K63R). Twenty-four hours after transfection, cells were transduced with a lentiviral vector expressing GFP alone (WPI) or co-expressing Vpr and GFP (Vpr). Two days later, cells were fixed, permeabilized 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 ($p < 0.05$).


A
GFP
HA-Ub
γ-H2AX
Merged
WPI
Vpr
WPI+ Ub(K48R)
Vpr+ Ub(K48R)
WPI+ Ub(K63R)
Vpr+ Ub(K63R)
B
%Cells with greater than 10 γ-H2AX foci
100
90
80
70
60
50
40
30
20
10
0
Mock
Ub (K48R)
Ub (K63R)
Vpr
WPI
p<0.0005
p<0.0001

SUPPLEMENTAL MATERIAL

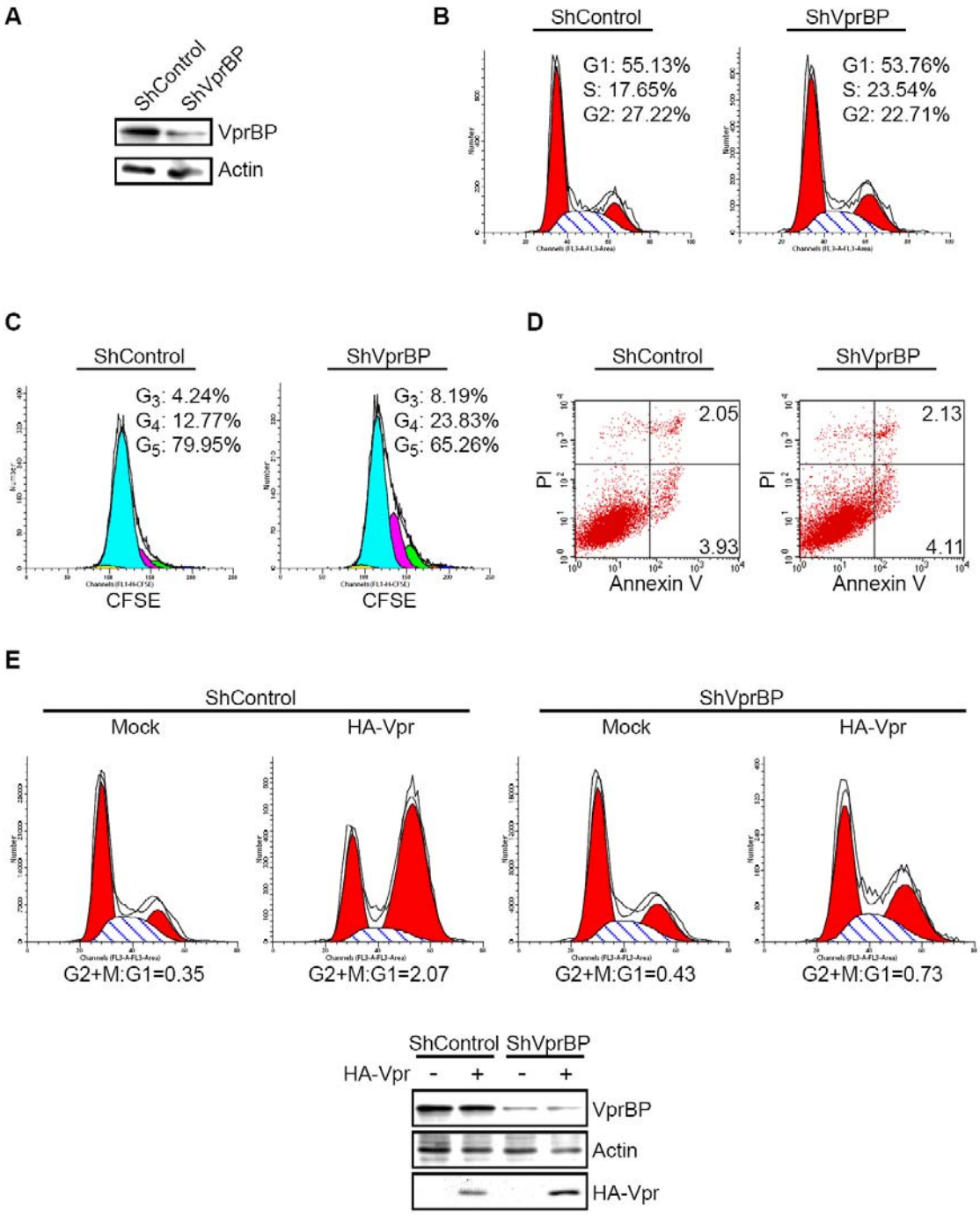
SUPPLEMENTAL FIGURE 1. Analysis of the induction of G2 arrest by TAP-tagged wild type Vpr and mutants.

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.


Mock
G2+M:G1=0.71
Vpr (WT)
G2+M:G1=1.95
TAP
G2+M:G1=0.74
TAP-Vpr (WT)
G2+M:G1=1.45
TAP-Vpr (1-78)
G2+M:G1=0.80
TAP-Vpr (1-84)
G2+M:G1=0.81
TAP-Vpr (1-86)
G2+M:G1=0.88
TAP-Vpr (1-90)
G2+M:G1=0.90
TAP-Vpr (R87A, R88A)
G2+M:G1=0.96

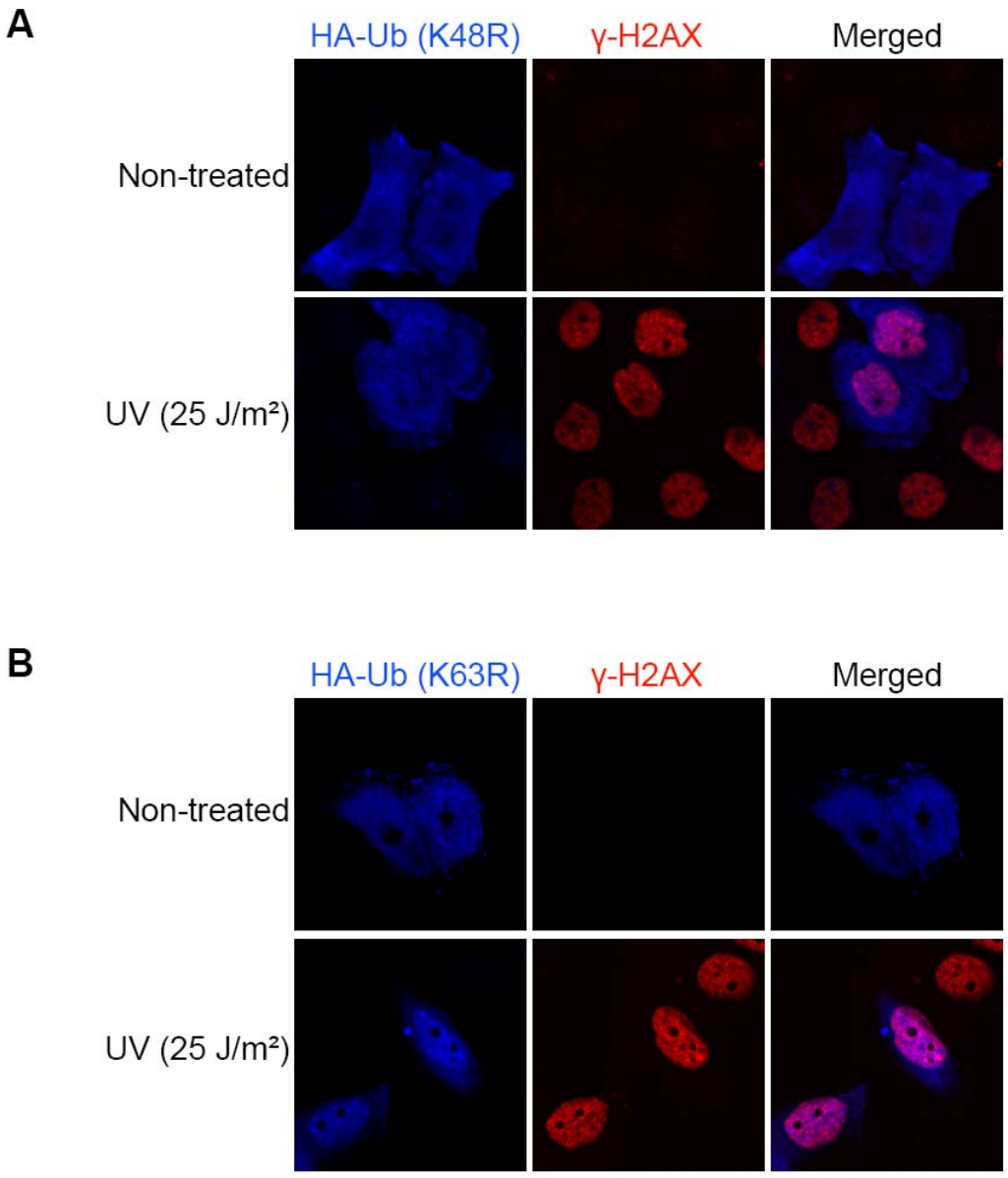
SUPPLEMENTAL FIGURE 2. Characterization of stable cell lines expressing control and VprBP-targeting shRNA.

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.


A
ShControl
ShVprBP
VprBP
Actin
B
ShControl
ShVprBP
G1: 55.13%
S: 17.65%
G2: 27.22%
G1: 53.76%
S: 23.54%
G2: 22.71%
C
ShControl
ShVprBP
G3: 4.24%
G4: 12.77%
G5: 79.95%
G3: 8.19%
G4: 23.83%
G5: 65.26%
CFSE
CFSE
D
ShControl
ShVprBP
2.05
3.93
2.13
4.11
PI
Annexin V
E
ShControl
ShVprBP
Mock
HA-Vpr
Mock
HA-Vpr
G2+M:G1=0.35
G2+M:G1=2.07
G2+M:G1=0.43
G2+M:G1=0.73
ShControl
ShVprBP
HA-Vpr - + - +
VprBP
Actin
HA-Vpr

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

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).


A
HA-Ub (K48R)
γ-H2AX
Merged
Non-treated
UV (25 J/m²)
B
HA-Ub (K63R)
γ-H2AX
Merged
Non-treated
UV (25 J/m²)

CHAPTER 3: FORMATION OF CHROMATIN-ASSOCIATED NUCLEAR FOCI CONTAINING HIV-1 VPR AND VPRBP IS CRITICAL FOR THE INDUCTION OF G2 CELL CYCLE ARREST

RÉSUMÉ

La protéine Vpr (Viral protein R) du VIH-1 induit un arrêt de cycle cellulaire en phase G2/M en activant le senseur de dommage à l'ADN ATR. Certaines recherches incluant les nôtres ont récemment démontré que Vpr remplissait cette fonction en recrutant la E3 ubiquitine ligase DDB1-CUL4A (VprBP). Cependant, le compartiment subcellulaire où le complexe se forme et où il agit demeurent inconnus. En utilisant des techniques d'immunofluorescence et de microscopie confocale nous avons démontré que Vpr forme des foyers nucléaires dans plusieurs types cellulaires incluant des cellules HeLa et des lymphocytes T CD4+ primaires. Nous avons découvert que ces foyers nucléaires co-localisaient avec VprBP ainsi qu'avec des facteurs de réparation d'ADN tels que 53BP1 et RPA32. Des traitements avec de la caféine, un inhibiteur non-spécifique d'ATR, ou avec des ARN d'interférence ciblant VprBP n'ont eu aucun effet sur la formation de ces foyers. En revanche, des mutations dans le domaine C-terminal de Vpr ainsi que la séquestration cytoplasmique de Vpr induite par la surexpression de Gag-Pol ont inhibé la formation des foyers et l'induction de l'arrêt de cycle. En accord avec ces résultats, nous avons aussi observé que la protéine Vpr du singe mangabé enfumé pouvait former ces foyers mais pas son paralogue Vpx, incapable d'induire un arrêt de cycle. Ainsi, ces résultats suggèrent que la formation de ces foyers de Vpr constitue un événement précoce important pour l'induction d'un arrêt de cycle en G2. En outre, nous avons découvert que Vpr pouvait s'associer à la chromatine via son domaine C-terminal et qu'il pouvait y former un complexe avec VprBP. Globalement, nos résultats suggèrent que Vpr induirait l'ubiquitination d'un substrat cellulaire associé à la chromatine, menant à la génération de stresses au niveau de la réplication de l'ADN, à l'activation d'ATR et finalement à un arrêt de cycle en phase G2.

FORMATION OF CHROMATIN-ASSOCIATED NUCLEAR FOCI CONTAINING HIV-1 VPR AND VPRBP IS CRITICAL FOR THE INDUCTION OF G2 CELL CYCLE ARREST

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

* Contributed equally to the work

Running Title: Role of Vpr nuclear foci in G2 arrest

Author contributions: JPB, LA, FCAG and EAC conceived and designed the experiments. JPB, LA, and FCAG performed the experiments. JPB, LA, and EAC analyzed the data. JPB and EAC wrote the paper.

This article in currently under review for publication

ABSTRACT

HIV-1 Viral protein R (Vpr) induces a cell cycle arrest at the G2/M phase by activating the ATR DNA damage/stress checkpoint. Recently, we and several other groups showed that Vpr performs this activity by recruiting the DDB1-CUL4A (VprBP) E3 ubiquitin ligase. While recruitment of this E3 ubiquitin ligase complex has been shown to be required for G2 arrest, the subcellular compartment where this complex forms and functionally acts is unknown. Herein, using immunofluorescence and confocal microscopy, we show that Vpr forms nuclear foci in several cell types including HeLa cells and primary CD4+ T-lymphocytes. These nuclear foci were found to co-localize with VprBP and the DNA repair factors 53BP1 and RPA32. While treatment with the non-specific ATR inhibitor caffeine or depletion of VprBP by siRNA did not inhibit formation of these foci, mutations in the C-terminal domain of Vpr and cytoplasmic sequestration of Vpr by overexpression of Gag-Pol resulted in impaired formation of these nuclear foci and defective G2 arrest. Consistently, we observed that G2 arrest-competent sooty mangabey Vpr could form these foci but not its G2 arrest-defective paralog Vpx, suggesting that formation of these foci represents a critical early event in the induction of G2 arrest. Indeed, we found that Vpr could associate to chromatin via its C-terminal domain and that it could form a complex with VprBP on chromatin. Overall, our results suggest that Vpr would target a chromatin-bound cellular substrate for ubiquitination in order to induce DNA damage/replication stress, ultimately leading to ATR activation and G2 cell cycle arrest.

INTRODUCTION

HIV-1 encodes several proteins termed accessory that have been implicated in the modulation of host cell environment to promote efficient viral replication and to impair innate and acquired immunity [1]. One of these accessory proteins, viral protein R (Vpr), is a small amphipathic protein of 96 amino acids. In addition to being expressed in infected cells, Vpr is packaged into virions through an interaction with the

p6 domain of the Gag polyprotein precursor [2-4]. The molecular structure of Vpr was recently resolved and found to consist of a hydrophobic core comprising three interacting alpha helices flanked by N- and C-terminal flexible domains [5]. Of note, the third alpha helix includes a leucine-rich region essential for the stability of the core and the flexible C-terminus comprises a functionally important stretch of positively charged arginine residues [6]. Several biological functions have been attributed to Vpr including transactivation of the viral long terminal repeat (LTR), enhancement of infection in macrophages, induction of apoptosis, and promotion of a cell cycle arrest at the G2/M phase [7].

Vpr-mediated G2 arrest likely plays an important role *in vivo* for viral replication or pathogenesis given that this activity is highly conserved among primate lentiviruses [8,9] and since abnormal accumulation of cells in G2/M can be observed in HIV-infected individuals [10]. Indeed, recent studies reported that Vpr upregulated the expression of ligands for the activating NKG2D receptor and promoted natural killer (NK) cell-mediated killing by a process that relied on Vpr ability to induce a G2 arrest, thus suggesting an immunomodulatory role for Vpr that may not only contribute to HIV-1-induced CD4+ T-lymphocyte depletion but may also take part in HIV-1-induced NK cell dysfunction [11,12]. Several investigators have reported that Vpr-induced cell cycle arrest involves the activation of the ATR (ataxia telangiectasia-mutated and Rad3-related)-mediated G2/M checkpoint [10,13,14]. ATR is a kinase of the phosphatidylinositol 3 kinase-like family and is involved in the activation of the G2/M checkpoint and in the coordination of DNA repair following the occurrence of DNA damages or DNA replication stress. Activation of ATR by exogenous DNA damaging agents such as UV leads to phosphorylation of several effector molecules, including Chk1 and H2AX (histone 2A, variant X), inducing the formation of DNA repair foci containing γ-H2AX (phosphorylated H2AX), MDC1 (mediator of DNA damage checkpoint 1), 53BP1 (p53 binding protein 1), BRCA1 (breast cancer 1), as well as the RPA (replication protein A), 9-1-1 (Rad9-Hus1-Rad1), and Rad17 complexes on the sites of DNA damage [15,16]. Activation of ATR by Vpr similarly leads to phosphorylation of Chk1 and to the formation of DNA repair foci containing γ-H2AX,

53BP1, RPA, Hus1, Rad17, and BRCA1 [13,14,17,18]. The immediate cause of the activation of ATR following Vpr expression has remained elusive but implicates in part the recruitment by Vpr of the host DDB1 (damage DNA binding protein 1)-CUL4A (cullin 4A) E3 ubiquitin ligase complex via a direct binding to the substrate specificity receptor VprBP (Vpr-binding protein, also known as DCAF1) [19-25]. Specifically, RNA interference-mediated depletion of VprBP or mutations in the hydrophobic leucine-rich core domain of Vpr impaired association to the E3 ligase complex and induction of G2 arrest. In contrast, G2 arrest-defective mutants of Vpr in the C-terminal arginine-rich domain, which maintained their association to the E3 ligase, nevertheless failed to induce G2 arrest [19-25]. These results indicate that association of Vpr to the E3 ligase complex is required but not sufficient to induce G2 arrest, thus supporting a model in which Vpr would act as a connector between a ubiquitin ligase complex and a yet-unknown cellular protein. We recently provided evidence that Vpr-induced K48-polyubiquitination and proteasomal degradation of this protein(s) would lead to DNA damage/stress, activation of ATR, and ultimately G2 cell cycle arrest [26]. HIV-2 and some species of simian immunodeficiency virus (SIV) encode a paralog of Vpr, called Vpx, which does not induce G2/M arrest but instead counteracts a putative restriction factor expressed in macrophages and dendritic cells that affects infection at a post-entry step [1]. Interestingly, Vpx also interacts with DDB1-CUL4A (VprBP) via its hydrophobic leucine-rich core domain. This association is required for the inactivation of the restriction factor and probably leads to its proteasomal degradation [27-29].

The subcellular localization of Vpr and its importance for the induction of G2 arrest has remained a source of controversy. Several investigators reported that Vpr expressed in absence of any other viral proteins primarily localized to the nucleus in a diffuse pattern [30-36] while others observed a significant accumulation at the nuclear envelope [37-42]. Of note, Sherman et al. showed that Vpr shuttles between the cytoplasm and nucleoplasm [43]. Moreover, Vpr has been shown to form punctuate structures in the nucleus [17] as well as to induce and co-localize with nuclear membrane herniations [44]. C-terminal mutations impairing G2 arrest did not alter localization of Vpr whereas other mutations, predominantly in the first alpha-helix,

impaired both nuclear import and G2 arrest, implying that nuclear/nuclear-envelope localization of Vpr would be required but not sufficient for this activity [33,38]. In agreement with this model, Lai et al showed that nuclear punctuate structures formed by Vpr were associated to chromatin and partially co-localized with γ-H2AX, suggesting that Vpr might target host cell DNA and interfere with DNA replication [17]. In contrast, the F34I, V57L, R62P, L68S, and I70S mutations in Vpr caused a re-localization of the protein to the cytoplasm without significantly affecting the induction of G2 arrest [30,36,37,41]. Although inconsistent results were also obtained for some of these mutants [38], these data would suggest instead that Vpr does not induce G2 arrest from the nucleus but from an extra-nuclear compartment.

Therefore, the spatial prerequisites for the induction of Vpr-mediated G2 arrest remain unclear. Additionally, while recruitment of the DDB1-CUL4A (VprBP) E3 ubiquitin ligase complex has been shown to be critical for G2 arrest, the subcellular compartment where this association occurs is still unknown. We thus sought to locate the Vpr-VprBP interaction and to determine the relevance of this localization for the induction of G2 arrest with the goal of furthering our understanding of the mechanism underlying Vpr activation of ATR and providing important information on the potential substrate targeted by Vpr. Herein, we show that Vpr forms foci that co-localizes with VprBP and the DNA repair proteins RPA32 and 53BP1 in the nucleus of cells. Moreover, we provide evidence that formation of these Vpr nuclear foci constitute a critical early event in the induction of G2 arrest. We also show that Vpr associates to chromatin via its C-terminal domain and that it binds VprBP on chromatin, suggesting that the Vpr-DDB1-CUL4A (VprBP) E3 ubiquitin ligase complex might target a chromatin-bound substrate in order to activate ATR and induce G2 arrest.

RESULTS

HIV-1 Vpr forms nuclear foci containing VprBP

The interaction between Vpr and VprBP was previously revealed to be required for the induction of a G2 cell cycle arrest [19-25]. However, the subcellular localization where this event might take place still remains to be determined. To this end, we performed laser-scanning confocal fluorescence immunohistochemistry to identify the respective subcellular localization and potential co-localization of Vpr and VprBP. HeLa cells were transduced with a lentiviral vector co-expressing HA-tagged Vpr (HA-Vpr) and GFP or a control lentiviral vector expressing GFP alone. Forty-eight hours after transduction, cells were fixed, permeabilized and stained with antibodies against HA, VprBP, and nucleoporin. The localization of HA-Vpr was mostly diffuse in the nucleus at standard amplification gain (data not shown). However, when the gain was reduced, we could observe that HA-Vpr formed small circular nuclear structures of variable relative sizes that we refer to thereafter as Vpr nuclear foci (VNF) (Fig 1A). The number of Vpr nuclear foci varied from cell to cell and from experiment to experiment but generally averaged 35 foci (SD ± 10) per cell. Formation of these foci was not due to the HA tag because we observed that native Vpr could also form nuclear foci (Fig S1A). Endogenous VprBP was found to be mostly localized to the nucleus in a punctuate pattern (Fig 1A). We observed that HA-Vpr colocalized with endogenous VprBP in the nucleus. Strikingly, a significant fraction but not all of Vpr nuclear foci co-localized with VprBP foci, suggesting that Vpr might be able to recruit the E3 ubiquitin ligase complex to these discreet structures. Although the localization of endogenous VprBP was found to vary greatly between the different cell lines tested (HeLa, HEK293T, and HEK293) or different clones of the same cell line (HeLa), ranging from completely nuclear to mostly cytoplasmic, co-localization with Vpr was always observed in these nuclear foci (data not shown). Of note, in presence of Vpr, we also observed some nuclear membrane perturbations reminiscent of the previously described Vpr-induced membrane herniations [44]. Importantly, transduction of activated primary CD4+ T-lymphocytes with a lentiviral vector expressing HA-Vpr also resulted in the formation of Vpr nuclear foci that co-localized with VprBP (Fig 1B),

indicating that these foci are not solely the result of overexpression of Vpr in transformed cell lines but that their formation also occurs in a physiological cellular host of HIV. Infection of HeLa cells with a VSV-G- pseudotyped virus expressing HA-Vpr (HxBru HA-Vpr) also resulted in the formation of Vpr nuclear foci in a minor fraction of cells (Fig S1B). However, the majority of cells displayed a relocalization of HA-Vpr to cytoplasmic compartments (Fig S1B), suggesting that formation of these foci would be a dynamic process, regulated over time during the infection cycle.

Vpr nuclear foci co-localize with DNA repair proteins

To investigate the nature and composition of these Vpr nulear foci we first evaluated whether these would correspond to known well-defined nuclear micro-domains with similar sizes and numbers. We did not however find any significant co-localization with the canonical nuclear speckle marker SC35 (also known as SFRS2) or with PML (promyelocytic leukemia) bodies (data not shown). Lai et al. previously reported formation and partial co-localization of Vpr nuclear foci with the DNA repair protein γ-H2AX [17]. We thus evaluated if the Vpr nuclear foci described herein where the same foci that Lai et al reported. Interestingly, we observed a co-localization between a significant fraction of HA-Vpr nuclear foci and 53BP1 (Fig 2A). Indeed, expression of HA-Vpr induced the re-localization of 53BP1 from its sites of residence in the nucleus to HA-Vpr-containing foci. We also observed a co-localization between some HA-Vpr nuclear foci and phosphorylated RPA32 (replication protein A2, 32kDa) (Fig 2B). Interestingly, in foci where co-localization was observed, the Vpr signal overlapped with 53BP1 foci while it generally concurred with RPA32 foci. Finally, even though we could detect a significant increase in γ-H2AX foci in response to Vpr expression [26], only a weak co-localization was observed between Vpr and phosphorylated H2AX (data not shown).

Formation of Vpr nuclear foci represents a critical early event in Vpr-mediated G2 arrest

Co-localization of Vpr with DNA repair factors suggest that formation of these foci by Vpr might represent an early event in the induction of G2 arrest that would be

responsible for the generation of DNA replication stress or DNA damage. Conversely, those might simply reflect the re-organization of the nuclear compartment following the activation of the ATR checkpoint by Vpr. To distinguish between these two possibilities, we transduced HeLa cells with a lentiviral vector expressing HA-Vpr and concomitantly treated the cells with caffeine, a non-specific inhibitor of ATR and ATM (ataxia telangiectasia mutated). In these conditions, the addition of caffeine inhibited Vpr-induced cell cycle arrest (Fig S2). However, we did not detect significant changes in the number of Vpr nuclear foci (Fig 3A, 33±10 for non-treated cells vs 32±9 for caffeine-treated cells), suggesting that formation of these foci would take place independently of the activation of ATR. Moreover, consistent with the observation that not all Vpr nuclear foci co-localized with VprBP (Fig 1A), depletion of VprBP by siRNA in HeLa cells (Fig 3B) did not significantly alter the number of foci (36±10 for control siRNA vs 33±8 for VprBP siRNA), indicating that VprBP is dispensable for the formation of Vpr nuclear foci. Similar results (data not shown) were obtained in a HEK293T monoclonal cell line stably depleted of VprBP [26]. These results indicate that Vpr forms these foci prior to activation of ATR and G2 arrest and suggest that it is Vpr that recruits VprBP to these foci and not the inverse.

To evaluate the potential role of these Vpr nuclear foci in the induction of G2 arrest, we monitored the capacity of several G2 arrest-defective Vpr mutants to form these foci. HeLa cells were transfected with plasmids expressing HA-tagged Vpr mutants and formation of nuclear foci was evaluated by fluorescence immunohistochemistry and confocal microscopy (Fig 4). We found that Vpr (R80A), which still interacts with the E3 ligase but is strongly attenuated for the induction of G2 arrest, was defective for the formation of nuclear foci (2.4±1.1). Deletion of the C-terminus of Vpr (Vpr 1-78), which also maintains the association with the E3 ligase [22] but impairs the induction of G2 arrest [45], similarly resulted in a defect in the formation of nuclear foci (Fig 4). Similar results were also obtained with the C-terminal mutants Vpr (S79A) and Vpr (1-86) (data not shown). Vpr (Q65R), which is unable to associate with the E3 ligase and is consequently defective for G2 arrest, was found to be defective for the formation of nuclear foci and also accumulated in cytoplasmic aggregates. The

results obtained with the Q65R mutation are in contrast with the siRNA-mediated depletion of VprBP which did not block the formation of Vpr nuclear foci, suggesting that this mutant protein might have additional defects besides an impaired interaction with VprBP. These results thus suggest that the putative C-terminal substrate-interacting domain of Vpr, which is required for the induction of G2 arrest, is also critical for the formation of Vpr nuclear foci.

The observation that C-terminal, G2 arrest-defective mutants of Vpr are compromised in their capacity to form nuclear foci suggest that these nuclear foci might constitute an important early event in the induction of G2 arrest by Vpr. To directly address this possibility, we first evaluated the functional effect of artificially sequestering Vpr in the cytoplasm by overexpression of Gag-Pol. Co-transfection of HeLa cells with HA-Vpr- and Gag-Pol-expressing plasmids produced a re-localization of HA-Vpr from the nucleus to p24-positive cytoplasmic compartments (Fig 5A). This relocalization abrogated Vpr nuclear foci formation (Fig 5A). Similar results were obtained in HEK293T cells (data not shown). To evaluate the functional effect of this cytoplasmic sequestration of Vpr, HEK293T cells were co-transfected with plasmids expressing HA-Vpr and Gag-Pol or with adequate empty plasmid controls. Two days later, the cell cycle profile of transfected cells was evaluated by flow cytometry (Fig 5B and 5C). Expression of HA-Vpr alone produced an accumulation of cells in G2/M (G2+M:G1=1.81 vs 0.66 for mock-transfected cells). Interestingly, expression of Gag-Pol completely abrogated HA-Vpr-induced G2 arrest (G2+M:G1=0.67) in absence of any significant effect on the cell cycle when expressed alone (G2+M:G1=0.77). Although overexpression of Gag-Pol led to a reduction of the affinity between HA-Vpr and endogenous VprBP, the overall increase in the expression of HA-Vpr resulted in an increase in the levels of Vpr-bound VprBP (Fig 5D), excluding the possibility that overexpression of Gag-Pol inhibited G2 arrest by preventing the Vpr-VprBP interaction. These results thus imply that nuclear localization of Vpr and possibly the formation of nuclear foci would be required for the induction of G2 arrest. These data also suggest that the stability of Vpr may be increased when in complex with Gag.

To further show that the formation of these Vpr nuclear foci is critical for the induction of G2 arrest, we evaluated the capacity of SIV Vpr and its paralog Vpx to form these foci. Both of these proteins are able to associate with the E3 ligase complex but in contrast to Vpr, Vpx does not induce G2 arrest but counteract a putative restriction factor in macrophages and dentritic cells [27-29]. HeLa cells were transfected with plasmids expressing either HA-tagged sooty mangabey Vpr (HA-Vpr sm) or Vpx (HA-Vpx sm). Two days after transfection, cells were fixed, permeabilized, and stained with antibodies against HA, nucleoporin and VprBP (Fig 6). Consistent with its ability to induce G2 arrest (data not shown and [9]), we found that Vpr sm could accumulate into nuclear foci (16±4 foci per cell) in contrast to the G2-arrest incompetent Vpx that did not form any foci despite being present in the nucleus (Fig 6). Overall, these results indicate that formation of Vpr nuclear foci is an early event that is required to induce G2 arrest. These results also indicate that nuclear localization of Vpr is not sufficient to induce formation of foci.

Vpr oligomerization is not sufficient to induce foci formation

Given that these foci constitute an early event in the induction of G2 arrest, we sought to determine how they would form. These foci are likely the results of a local observable accumulation of Vpr either through oligomerization of the protein or following its recruitment by a locally abundant tethering factor. To distinguish between these two possibilities, we first monitored the dimerization efficiency of the Vpr mutants Q65R and R80A, which are defective for foci formation. HEK293T cells were co-transfected with plasmids expressing enhanced yellow fluorescence protein (eYFP) fused to the N-terminus of wild type Vpr and *renilla* luciferase (Rluc) fused to the N-terminus of wild type Vpr and mutants. Two days after transfection, self-affinity was assessed by bioluminescence resonance energy transfer (BRET). Figure 7A reveals that all Vpr fusion proteins were efficiently expressed. In this system, we observed a specific energy transfer between eYFP-Vpr (WT) and Rluc-Vpr (WT) (Fig 7B). The maximum energy transfer at saturation ($BRET_{max}$) was 0.983 and the concentration of acceptor at 50% of $BRET_{max}$ ($BRET_{50}$) was 0.397. In contrast, co-expression of eYFP and Rluc-Vpr did not lead to any significant energy transfer, demonstrating the

specificity of the eYFP-Vpr/Rluc-Vpr interaction. The Q65R mutant, showed a significant decrease in its affinity for wild type eYFP-Vpr ($BRET_{50}$ = 0.791, 50% self-affinity) as well as a drastic decrease in $BRET_{max}$ (0.314 for Q65R vs 0.983 for wild type Vpr), suggesting that in addition to a reduction in dimerization efficiency, formation of higher-order complexes (multimerization) would also be synergistically decreased. In contrast, the R80A mutant displayed an affinity for wild type Rluc-Vpr that was at least comparable to wild type Vpr ($BRET_{50}$ = 0.326, 121% self-affinity relative to wild type). Similar results were obtained when eYFP-Vpr R80A and Rluc-Vpr R80A were co-expressed (data not shown). Thus, these results suggest that the ability of Vpr to oligomerize does not directly correlate with nuclear foci formation and does not explain the defect in foci formation observed in the context of C-terminal mutants.

Association of Vpr to chromatin correlates with formation of nuclear foci

Since oligomerization does not fully account for the ability of Vpr to form foci, Vpr could thus be tethered to specific sites by a cellular factor. Co-localization of Vpr nuclear foci with chromatin-bound DNA repair factors suggests that this tethering factor could be a chromatin-bound protein or DNA itself. To assess this possibility, HeLa cells were first transiently transfected with an empty plasmid or a plasmid expressing HA-Vpr and cells were lysed with 0.5% Triton X-100, resulting in the release of soluble proteins. Treatment of Triton-insoluble cellular material, including chromatin, with micrococcal nuclease resulted in the solubilization of chromatin-bound cellular proteins including RPA70 (replication protein A1, 70 kDa) (data not shown) and histone 3 (Fig 8A). These proteins were not detected when cell extracts were incubated in buffer without micrococcal nuclease. Importantly, chromatin extracts were not contaminated with cytoplasmic proteins as revealed by the absence of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Fig 8A). Using this system, we found that a fraction of HA-Vpr was released in extracts treated by micrococcal nuclease but not with buffer alone, indicating that Vpr associates with chromatin directly or indirectly via other proteins (Fig 8A). A specific association of a fraction of endogenous VprBP with chromatin was also observed in presence and in absence of Vpr (Fig 8A). To determine whether the

defects of foci formation observed with C-terminal mutants of Vpr would correlate with a defect in chromatin association, we analyzed the capacity of several Vpr mutants to associate to chromatin in HeLa cells. Interestingly, both Vpr (R80A) and a C-terminal deletion mutant (Vpr 1-78) showed a drastic reduction in their association to chromatin (Fig 8B). Of note, Vpr (Q65R) also failed to associate with chromatin (Fig 8B), possibly explaining its unexpected incapacity to form foci. Therefore, the ability of Vpr to form foci correlates with its ability to associate with chromatin.

Vpr and VprBP interact on chromatin

Co-localization of Vpr nuclear foci with VprBP and the association of both proteins to chromatin suggest that they might interact on chromatin. To evaluate this possibility, we transfected HeLa cells with an empty plasmid or a plasmid expressing HA-Vpr and performed anti-HA immunoprecipitations on proteins released from chromatin by microccocal nuclease (Fig 9). Interestingly, we could detect co-immunoprecipitation of endogenous VprBP specifically in cells extracts containing HA-Vpr, in the soluble fraction as well as in the chromatin fraction (Fig 9). These data suggest that Vpr interacts with VprBP on chromatin. Importantly, histone 3 did not co-immunoprecipitate with HA-Vpr in the chromatin fraction, thus excluding the possibility that the observed Vpr-VprBP interaction was mediated by incompletely digested chromatin fragments. Overall, our results suggest that Vpr would bind to chromatin via its C-terminus where it would contact a yet-unknown cellular factor, triggering accumulation of Vpr and formation of nuclear foci. These would serve as a scaffold to recruit the DDB1-CUL4A (VprBP) E3 ubiquitin ligase to induce the ubiquitination and degradation of a chromatin-bound substrate, resulting in DNA damage or replication stress.

DISCUSSION

Our results show that Vpr mainly localizes to the nucleus in transformed epithelial cells, such as HeLa and HEK293T cells, as well as in primary CD4+ T-lymphocytes (Fig 1 and data not shown). We noticed that the localization of Vpr in

HeLa cells closely resembles that observed in primary CD4+ T-lymphocytes, prompting us to select this cellular model for most of our study. Moreover, we found that both ectopically and virally expressed HA-tagged Vpr had a subcellular localization similar to that of the native protein (Fig S1). Our localization data show that Vpr can form nuclear punctuate structure that we termed Vpr nuclear foci (Fig 1), as was reported previously by Lai and colleagues [17]. It is noteworthy that these foci are not readily apparent and require careful calibration of gain to be observed (data not shown). Importantly, we observed a strong co-localization of Vpr with VprBP in the nucleus, particularly in these foci, suggesting that Vpr might interact with the E3 ubiquitin ligase at the levels of these punctuate structures. In contrast to the observations of other investigators [37-42], we did not observe a significant accumulation of Vpr at the nuclear membrane in these cell types. Several technical reasons might explain these discrepancies, including cell types, levels of expression, fixation and permeabilization conditions, or the tag used. We did however observe some enrichment at the nuclear membrane in another clone of HeLa cells (data not shown). Given that this phenotype is not generally observed in primary CD4+ T-cells, it is probably not physiologically relevant for the induction of G2 arrest.

We obtained several lines of evidence demonstrating that Vpr nuclear foci are involved in Vpr-mediated G2 arrest. First, we observed a partial colocalization between these foci and DNA repair factors such as RPA32, 53BP1 and γ-H2AX (Fig 2 and data not shown). Similar results were obtained by Lai and colleagues in the case of γ-H2AX [17]. Secondly, C-terminal mutants of Vpr defective for G2 arrest failed to induce formation of Vpr foci despite their nuclear localization (Fig 4). Thirdly, cytoplasmic sequestration of Vpr by overexpression of Gag inhibited G2 arrest as well as foci formation (Fig 5). Fourthly, only Vpr from sooty mangabey SIV but not its G2 arrest-defective paralog Vpx was able to form these foci (Fig 6). Lastly, the reduced number of foci formed by sooty mangabey Vpr in comparison to HIV-1 Vpr correlated with reduced G2 arrest activity in human cells (data not shown and [9]). All these results suggest that formation of foci is linked to G2 arrest. Moreover, these results also suggest that nuclear localization of Vpr is required but not sufficient to induce formation

of these foci. Our results and conclusions are in contrast with previous reports, including one of ours, describing cytoplasmic mutants of Vpr that retain their G2 arrest activity [30,36,37,41]. We had reported over a decade ago that the V57L and R62P mutations induced the relocalization of Vpr to the cytoplasm, while these mutants were still able to induce G2 arrest [36]. However, careful re-examination of the localization of these mutants showed that both mutants could localize to the nucleus to some degree. While, the V57L mutant had a reduced capacity to form foci, the R62P mutant was completely defective for foci formation (Fig S3A). The reduced capacity of V57L mutant and the defect of the R62P mutant in foci formation correlated, respectively, with attenuation and abrogation of G2 arrest (Fig S3B). These differences between our present localization data and our previously published results can probably be explained by improved imaging sensitivity, whereas the discrepancies in G2 arrest activity are unclear. Nevertheless, these results highlight an important technical limitation in these types of localization experiments: lack of detection in a subcellular compartment does not necessarily indicate an absence of protein.

Correlation between G2 arrest and formation of Vpr nuclear foci implies that the formation of these foci could either be an early event leading to G2 arrest or could be a consequence of this G2 arrest. We observed that treatment with the ATR/ATM inhibitor caffeine (Fig 3A) did not abrogate formation of Vpr foci, thus indicating that these foci likely constitute an early event in the induction of G2 arrest by Vpr. In fact, formation of Vpr foci were not affected by knockdown of VprBP indicating that their formation is independent of the recruitment of the E3 ligase complex and would therefore precede ubiquitination and degradation of the putative G2 arrest substrate (Fig 3B). In contrast, we found that the Q65R mutant of Vpr was unable to form foci. In addition to a reduced affinity for VprBP [19,20,22], this mutation also leads to other defects including accumulation of Vpr in the cytoplasm (Fig 4), reduced dimerization efficiency (Fig 7), and absence of binding to chromatin (Fig 8B), indicating that the Q65R mutation has pleitropic effects on the functions of Vpr. Cautions should thus be used when interpreting results obtained with this mutant.

Given that Vpr foci containing VprBP co-localize with chromatin-bound protein such as RPA32 and that Vpr associates with DNA *in vitro* [46] and *in vivo* (Fig 8A and [17]), we propose that Vpr might be able to target a chromatin-bound cellular factor. In support of this hypothesis, Lai et al. showed that *in situ* nuclease treatment of Vpr-expressing cells eliminates Vpr nuclear foci [17], suggesting that Vpr nuclear foci are anchored to chromatin. Deletion of the putative substrate-interacting C-terminal domain of Vpr drastically reduced foci formation (Fig 4) and its chromatin association (Fig 8B). Similar results were obtained by Lai and colleagues [17] . Moreover, mutation of the arginine at position 80 did not affect direct binding to nucleic acids *in vitro* [46] but nevertheless impaired association to chromatin *in vivo* (Fig 8B), implying that a cellular factor rather than a direct binding to DNA would be implicated in association to chromatin. Importantly, we also observed protein-protein interaction between Vpr and VprBP on chromatin (Fig 9), suggesting that Vpr would be able to recruit the E3 ligase DDB1-CUL4A (VprBP) onto chromatin. Therefore, Vpr could bind to its cognate chromatin-bound substrate via its C-terminus. This event would induce the local accumulation of Vpr (formation of Vpr nuclear foci) because of large amount of the putative substrate on these genomic loci and would trigger the recruitment of DDB1-CUL4A (VprBP). Although possible, it is however unlikely that Vpr multimerization (Fig 7)[30,40] would play a role in this process given that the I70S mutation was previously shown to block dimerization without affecting the induction of G2 arrest [30]. It also remains unclear whether VprBP would bind Vpr pre- or post-foci formation, particularly when considering the important level of interaction observed in the Triton-soluble fraction (Fig 9). Finally, as was recently documented, the substrate would be covalently modified with classical K48-linked polyubiquitin chains in a DDB1-CUL4A (VRPBP)-dependent manner and degraded by the proteasome [26]. This model would imply that, although Vpr would initially require binding to the substrate to form foci, once formed, these structures would be sufficiently stable to outlast the degradation of the substrate. Alternatively, Vpr could interact with a chromatin-bound cellular cofactor via its C-terminal domain. This first event would then allow Vpr to contact a chromatin-bound substrate via a yet-undefined domain and target it for degradation. Nonetheless, our results also imply that Vpr might not target

the overall pool of substrate for degradation but only locally in these foci. Partial rather than complete co-localization of Vpr nuclear foci with the DNA repair factors 53BP1 (Fig 2A), RPA32 (Fig 2B), and γ-H2AX [17] further suggest that ubiquitination and degradation of this unknown cellular factor would induce a generalized genomic instability, affecting sites other than that of Vpr nuclear foci. However, given that Vpr nuclear foci might be dynamic structures, we cannot completely exclude the possibility that Vpr could induce DNA damage or replication stress *in situ* and that the absence of co-localization would be due to the disappearance of Vpr nuclear foci.

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Overall, our results show that Vpr forms nuclear foci that co-localizes with VprBP and demonstrate that formation of these foci constitutes a critical early event in the induction of DNA damage/stress and G2 arrest. Our results further suggest that the putative G2 arrest substrate targeted by Vpr in these foci is likely to be a chromatin-associated protein.

MATERIALS AND METHODS

Cells, antibodies, and other reagents

HeLa and HEK293T cells were cultured as previously described [47]. Primary CD4+ T-lymphocytes were isolated and cultured as previously described [26]. Caffeine was purchased from Sigma-Aldrich (St. Louis, MO, USA). SiRNA targeting VprBP (siGenome SMARTpool M-021119-00) and scrambled control siRNA (non-targeting siRNA #2) were obtained from Dharmacon (Chicago, IL, USA). The anti-HA (clone 12CA5) and anti-p24 (catalog no. HB9725) monoclonal antibodies were produced from hybridomas obtained from the American Type Culture Collection (Manassas, VA, USA). Mouse anti-nucleoporin, mouse anti-RPA70, rabbit anti-53BP1, rabbit anti-GAPDH, and rabbit anti-H3 antibodies were purchased from Abcam (Cambridge, MA, USA), whereas the rabbit anti-phospho RPA32 (S4/S8) antibody was obtained from Bethyl Laboratories (Montgomery, TX, USA). The rabbit polyclonal antibody against VprBP and actin were respectively obtained from Accurate Chemical and Scientific

Corporation (Westbury, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA). The monoclonal antibody against Vpr (clone 8D1) was a kind gift of Y. Ishizaka (International Medical Center of Japan, Tokyo, Japan) [48]. All fluorochrome-conjugated secondary antibodies were obtained from Molecular Probes (Invitrogen, San Diego, CA, USA).

Plasmid construction

SVCMV-HA-Vpr (WT), SVCMV-HA-Vpr (V57L), SVCMV-HA-Vpr (R62P), SVCMV-HA-Vpr (Q65R), SVCMV-HA-Vpr (R80A), SVCMV-HA-Vpr (S79A), SVCMV-HA-Vpr (1-86), and SVCMV-HA-Vpr (1-78) were constructed by PCR as previously described [19,45]. Plasmids pCDNA3.1_eYFP-MCS(MB) and pCDNA3.1_Rluc-MCS(MB) for the expression of eYFP and renilla luciferease (Rluc) N-terminal fusion proteins were kind gifts of M. Baril and D. Lamarre [49]. Wild type Vpr was amplified by PCR from SVCMV-HA-Vpr (WT) and subcloned into pCDNA3.1_eYFP-MCS(MB) and pCDNA3.1_Rluc-MCS(MB) to generate respectively pCDNA3.1-eYFP-Vpr(WT) and pCDNA3.1-Rluc-Vpr (WT). Vpr (R80A) and Vpr (Q65R) were subcloned into pCDNA3.1_Rluc-MCS(MB) to generate pCDNA3.1-Rluc-Vpr (R80A) and pCDNA3.1-Rluc-Vpr (Q65R) using the same strategy. The lentiviral vector pWPI as well as the packaging plasmid psPAX2 were obtained from D. Trono (School of Life Sciences, Swiss Institute of Technology, Lausanne, Switzerland). The lentiviral vector pWPI-HA-Vpr (WT) tranducing HA-tagged Vpr and GFP was generated from the parental vector pWPI using a strategy described previously [19]. The plasmids expressing sooty mangabey HA-tagged Vpr and Vpx were obtained from S. Benichou (Institut Cochin, Paris, France) [4]. The infectious molecular clones HxBru (Vpr-) and HxBru (HA-Vpr) were described previously [26,50]

Production and titration of viruses and lentiviral vectors

The production and titration of VSV-G-pseudotyped HIV particles and lentiviral vectors were performed as described previously [19,45].

Transfection, transduction and infection

HeLa cells were transfected using the Lipofectamine 2000 reagent (Invitrogen Canada, Burlington, Ontario, Canada) according to the manufacturer's instructions. HEK293T cells were transfected by a standard calcium phosphate precipitation protocol. SiRNA were transfected using Oligofectamine (Invitrogen Canada, Burlington, Ontario, Canada), according to the manufacturer's instructions and as previously published [19]. HeLa cells were transduced with the lentiviral vectors WPI and WPI-HA-Vpr in presence of 8μg/ml polybrene at a multiplicity of infection of 0.5 or 2.5, as indicated for each experiment. Primary CD4+ T-lymphocytes were transduced by spinoculation at a multiplicity of infection of 1. Briefly, cells were mixed with lentiviral vector particles in presence of 8μg/ml polybrene and centrifuged for 2 hours at 1200g. HeLa cells were infected with VSV-G-pseudotyped viruses at a concentration of 100 cpm/cell in presence of 8 μg/ml polybrene. For all experiments, cells were harvested two days after transfection, transduction or infection.

Fluorescence immunohistochemistry

Fifty thousand HeLa cells were seeded on cover slips in 24-well plates. Cells were transfected, transduced, or infected as indicated for each experiment. Two days later, cells were processed for immunohistochemistry and laser-scanning confocal microscopy as previously described [51]. For analysis of CD4+ primary T-lymphocytes, $5X10^5$ cells were first adhered on poly-Lysine-treated coverslips for two hours in PBS and then processed as described [51]. Quantification of Vpr nuclear foci was performed in at least 30 randomly selected cells by manual counting.

Cell cycle analysis

Cell cycle analysis was performed using propidium iodide staining and flow cytometry as previously described [19].

Immunoprecipitation and westen blot

Immunoprecipitations using anti-HA-conjugated agarose beads were performed as previously described [26]. Analysis of proteins by western blot was performed as previously described [26].

Bioluminescence resonance energy transfer (BRET) assays

HEK293T cells were transfected in 24-well plates with 10 ng of the BRET donor plasmids pCDNA3.1_Rluc-MCS(MB), pCDNA3.1-Rluc-Vpr (WT), pCDNA3.1-Rluc-Vpr (R80A) or pCDNA3.1-Rluc-Vpr (Q65R) and increasing concentration (0 to 500 ng) of the BRET acceptor plasmids pCDNA3.1_eYFP-MCS(MB) or pCDNA3.1-eYFP-Vpr (WT) using Lipofectamine 2000. Two days after transfection, cells were harvested, washed twice in PBS, and aliquoted in two wells of a 96-well plate (Costar 3917). Total eYFP fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength at 520±10 nm. BRET was initiated by adding 5µM of the *renilla* luciferase substrate coelenterazine H (Prolume Ltd., Lakeside, AZ, USA). Luminescence was then measured 10 minutes later at 475±15 nm and BRET fluorescence was measured at 535±15 nm. All measurements were performed on a PheraStar microplate reader (BMG Labtech, Cary, NC, USA). BRET ratios were calculated using this formula: (emission at 535 nm/emission at 475 nm)-(background emission at 535nm/background emission at 475 nm), as previously described [52].

Chromatin binding assays

Cells were lysed in triton lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and complete protease inhibitors cocktail (Roche) for 15 minutes. Insoluble cell debris, including chromatin, was pelleted by centrifugation (2500g for 10 minutes). The supernatant was harvested and represented the soluble input control. Pellets were washed once with nuclease buffer (50 mM Tris pH 8.0, 5 mM $CaCl_2$, and 100 µg/ml BSA), split in two, and resuspended in nuclease buffer alone or nuclease buffer containing 200 U/ml microccocal nuclease (New England Biolabs, Ipswich, MA, USA). Pellets were incubated for 30 minutes on ice and then centrifuged at 12000g for 10 minutes. The supernatant was harvested and represented the chromatin-bound

fraction. The corresponding supernatant obtained in absence of nuclease was used to control for non-specific release.

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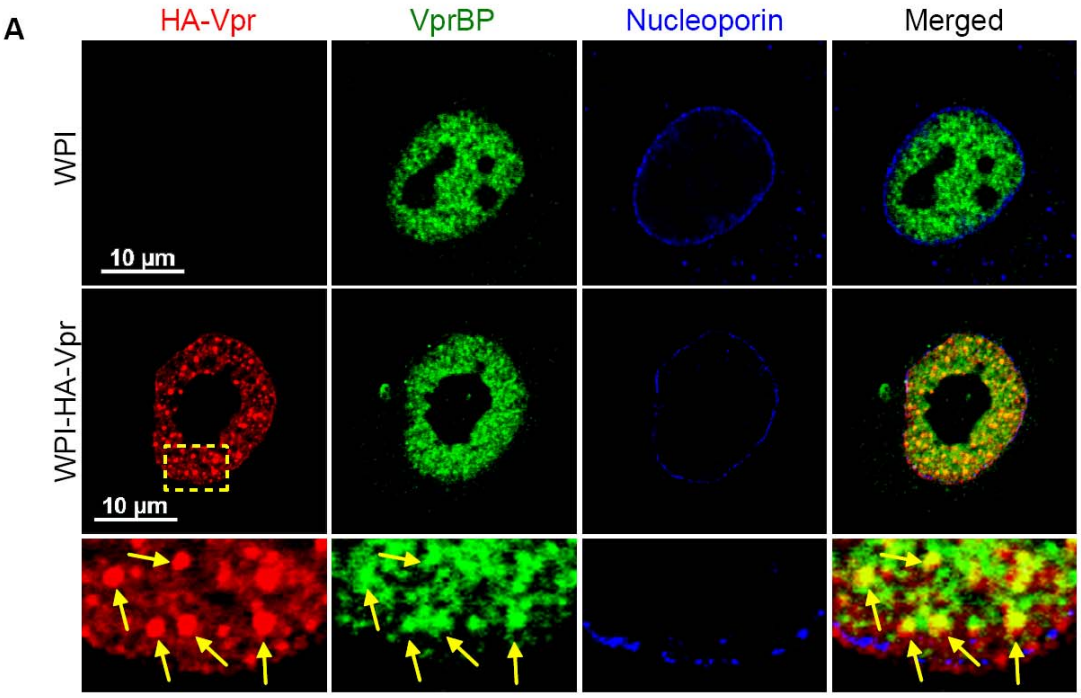
FIGURES

Figure 1. HIV-1 Vpr forms nuclear foci containing VprBP.
A) HeLa cells were transduced with lentiviral vectors expressing GFP (WPI) or co-expressing GFP and HA-tagged Vpr (WPI-HA-Vpr) at a multiplicity of infection of 0.5. **B)** Primary activated CD4+ T-lymphocytes were transduced by spinoculation with WPI or WPI-HA-Vpr at a multiplicity of infection of 2.5. For both panels, two days after transduction, cells were fixed, permeabilized, and stained with antibodies against HA (red), nucleoporin (blue) and VprBP (green). Images were acquired by confocal microscopy with a 63X objective. Images shown are representative of multiple fields. Enlarged (3X) images are shown below panels. Yellow arrows highlight examples of punctuate co-localization.


A
HA-Vpr
VprBP
Nucleoporin
Merged
WPI
WPI-HA-Vpr
10 µm
10 µm

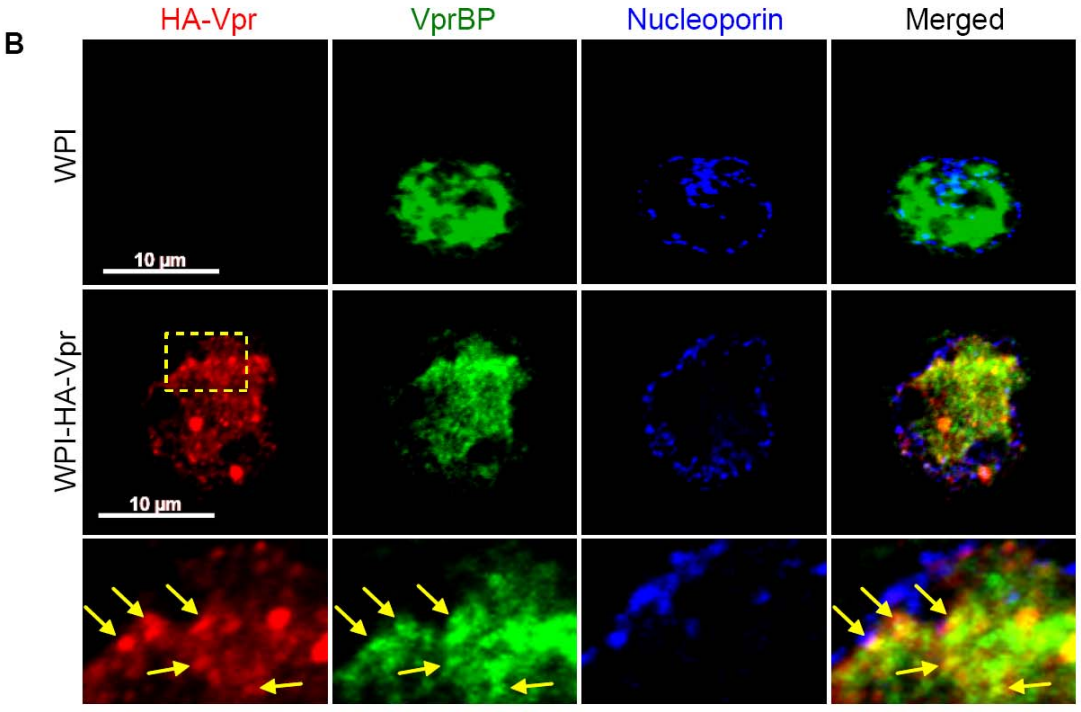
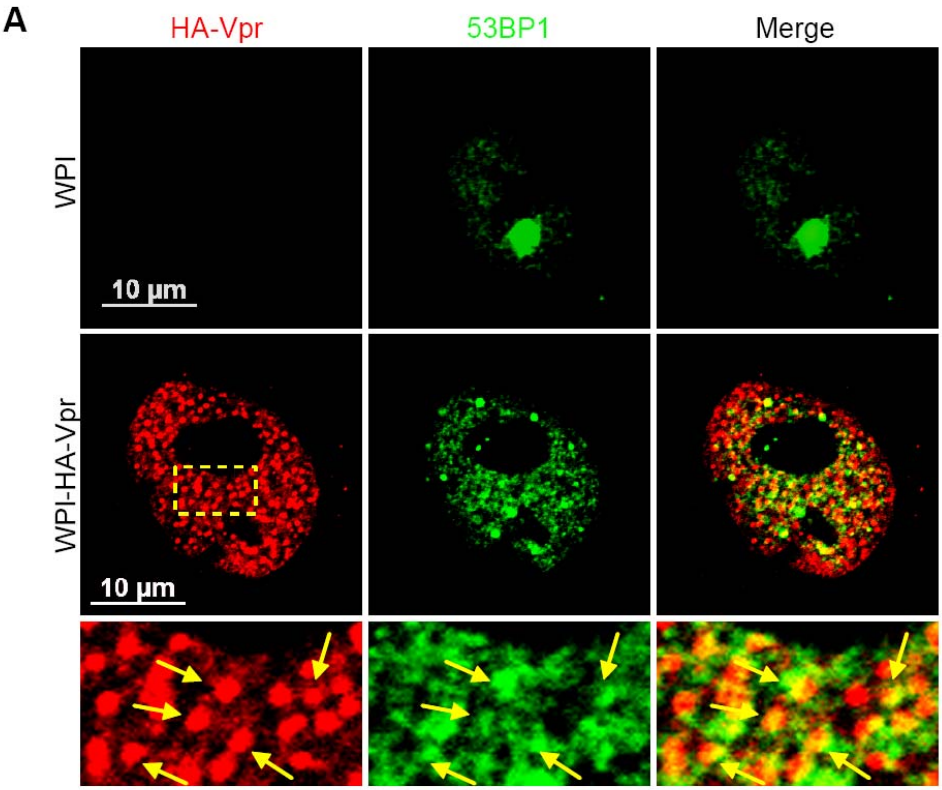

B
HA-Vpr
VprBP
Nucleoporin
Merged
WPI
WPI-HA-Vpr
10 µm
10 µm

Figure 2. Vpr nuclear foci co-localizes partially with DNA repair foci.

HeLa cells were transduced with lentiviral vectors expressing GFP (WPI) or co-expressing GFP and HA-tagged Vpr (WPI-HA-Vpr) at a multiplicity of infection of 0.5. Two days after transduction, cells were fixed, permeabilized, and stained with antibodies against HA (red) and with either rabbit polyclonal antibodies against 53BP1 (green) **(A)** or phospho-RPA32 (green) **(B)**. Images were acquired by confocal microscopy. Images shown are representative of multiple fields. Enlarged (3X) images are shown below panels. Yellow arrows highlight examples of punctuate co-localization.


A
HA-Vpr
53BP1
Merge
WPI
10 μm
WPI-HA-Vpr
10 μm

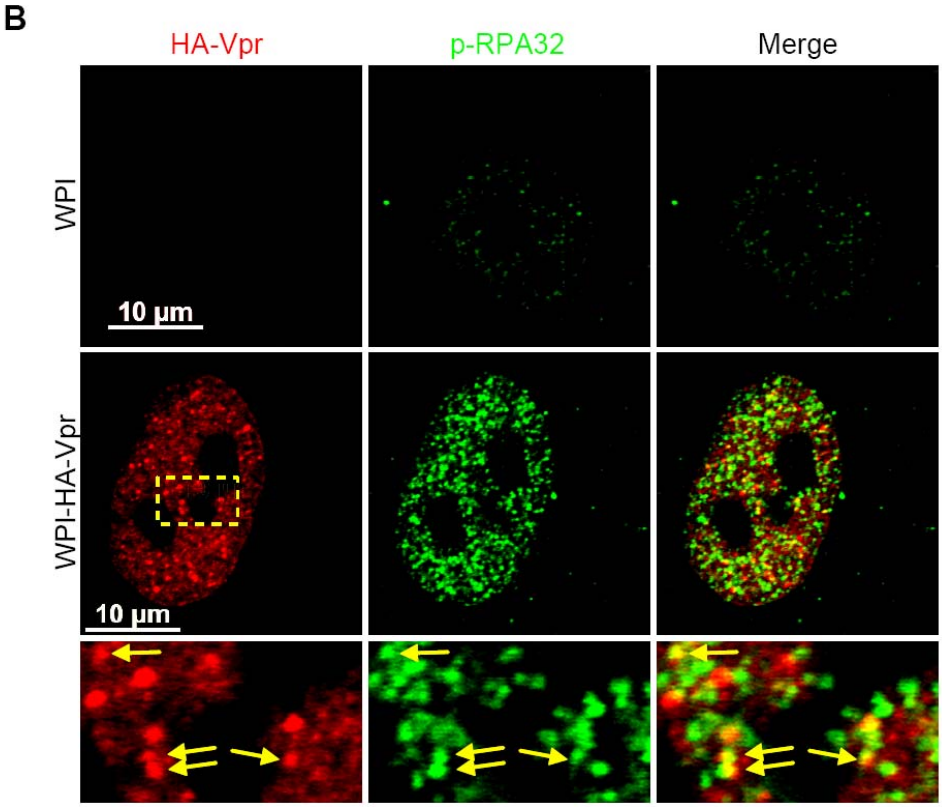

B
HA-Vpr
p-RPA32
Merge
WPI
10 μm
WPI-HA-Vpr
10 μm

Figure 3. Formation of Vpr nuclear foci is independent of ATR activation and of the recruitment of VprBP.

A) HeLa cells were pre-treated with 2.5 mM caffeine for 1 hour and then transduced with lentiviral vectors co-expressing GFP and HA-Vpr (WPI-HA-Vpr) or expressing GFP alone (WPI). **B)** HeLa cells were transfected with control scrambled siRNA or siRNA targeting VprBP. Twenty-four hours after transfection, cells were transduced with lentiviral vectors co-expressing GFP and HA-Vpr (WPI-HA-Vpr) or expressing GFP alone (WPI). Two days after transduction, cells were fixed, permeabilized, and stained with antibodies against HA (red), nucleoporin (blue) and VprBP (green). Images were acquired by confocal microscopy. Images shown are representative of multiple fields. Averages of the number of Vpr nuclear foci (VNF) per cell and corresponding standard deviations are shown.

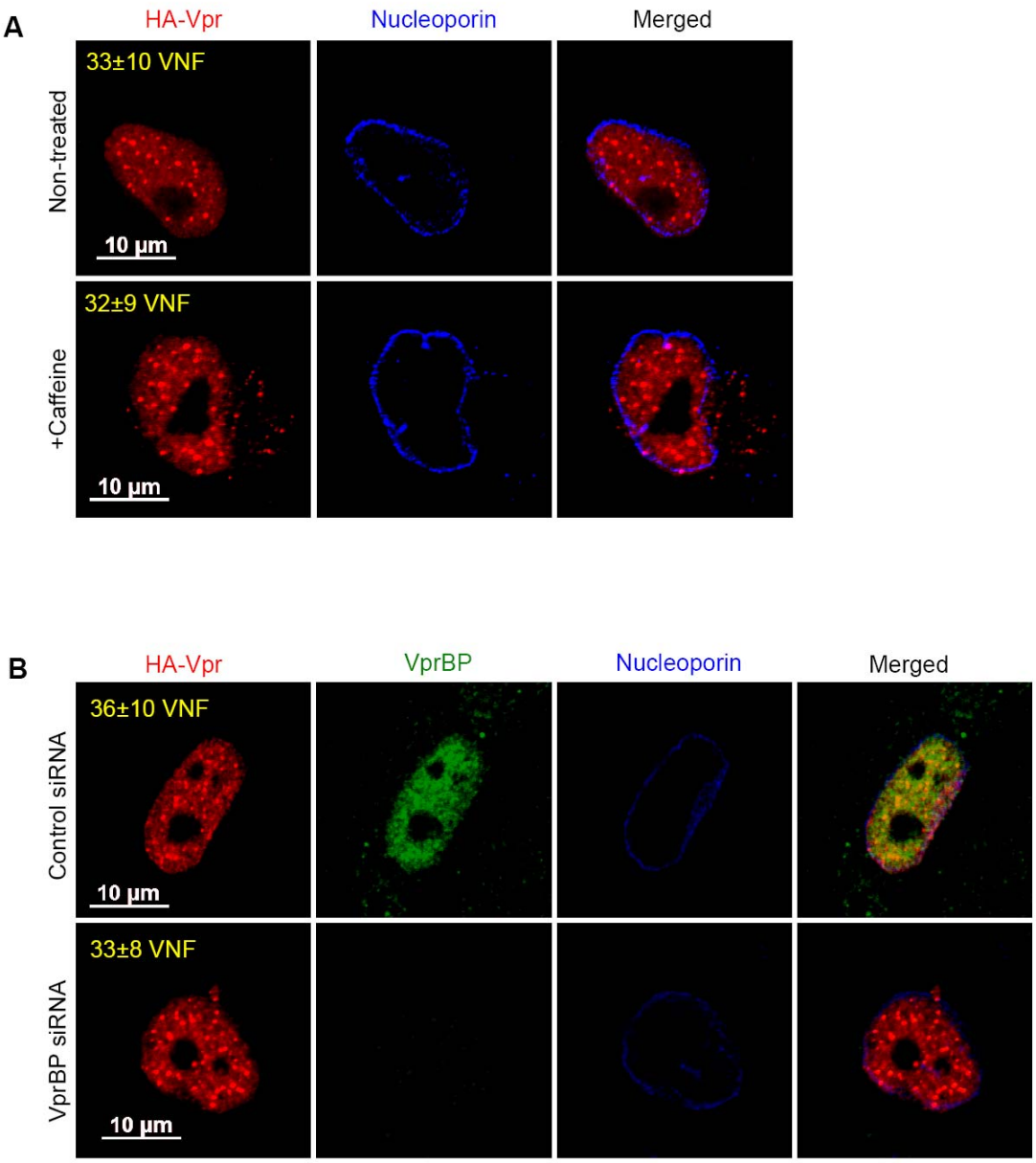

A
HA-Vpr
Nucleoporin
Merged
33±10 VNF
Non-treated
10 µm
32±9 VNF
+Caffeine
10 µm
B
HA-Vpr
VprBP
Nucleoporin
Merged
36±10 VNF
Control siRNA
10 µm
33±8 VNF
VprBP siRNA
10 µm

Figure 4. Analysis of the capacity of Vpr mutants to form nuclear foci.

HeLa cells were transfected with plasmids expressing HA-tagged Vpr (WT), Vpr (Q65R), Vpr (R80A), and Vpr (1-78). Forty-eight hours after transfection, cells were fixed, permeabilized, and stained with antibodies against HA (red), nucleoporin (blue) and VprBP (green). Images were acquired by confocal microscopy. Images shown are representative of multiple fields. Averages of the number of Vpr nuclear foci (VNF) per cell and corresponding standard deviations are shown.

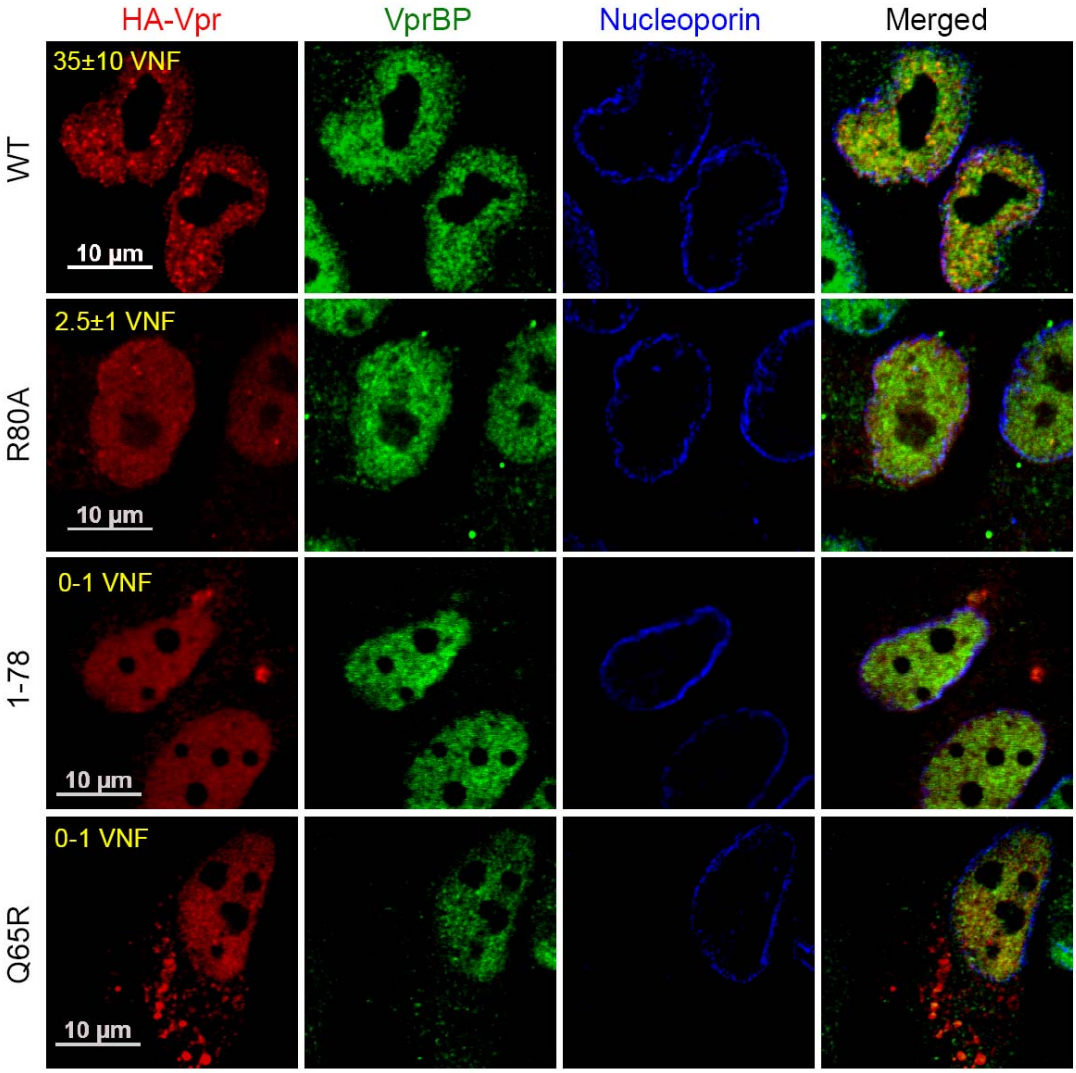

HA-Vpr
VprBP
Nucleoporin
Merged
WT
R80A
1-78
Q65R
35±10 VNF
2.5±1 VNF
0-1 VNF
0-1 VNF
10 µm
10 µm
10 µm
10 µm

Figure 5. Cytoplasmic sequestration of Vpr abrogates foci formation and G2 arrest.

A) HeLa cells were co-transfected with the packaging plasmid psPAX2 encoding Gag-Pol, Tat, and Rev and with an HA-Vpr-expressing plasmid or appropriate empty plasmid control. Two days after transfection, cells were fixed, permeabilized, and stained with antibodies against HA (red), nucleoporin (blue) and p24 (green). Images were acquired by confocal microscopy. Images shown are representative of multiple fields. **B)** HEK293T cells were cotransfected with plasmids expressing GFP, HA-Vpr and Gag-Pol (psPAX2) or with an empty plasmid control as indicated. Forty-eight hours after transfection, cell cycle analysis was performed by flow cytometry using propidium iodide staining. Percentages of G1 and G2/M cell populations were determined using the ModFit software. **C)** Expression of HA-Vpr and p24 was monitored by western blot using specific antibodies. HA-Vpr and p24 were detected using specific monoclonal antibodies. Actin was detected using a rabbit polyclonal antibody. **D)** HEK293T cells were transfected as in B). Two days after transfection, cells were lysed and subjected to anti-HA immunoprecipitation as described in Materials and Methods. HA-Vpr, p24 or VprBP levels were evaluated in cell lysates and immunocomplexes. HA-Vpr and p24 were detected using specific monoclonal antibodies. VprBP was detected using a rabbit polyclonal antibody.

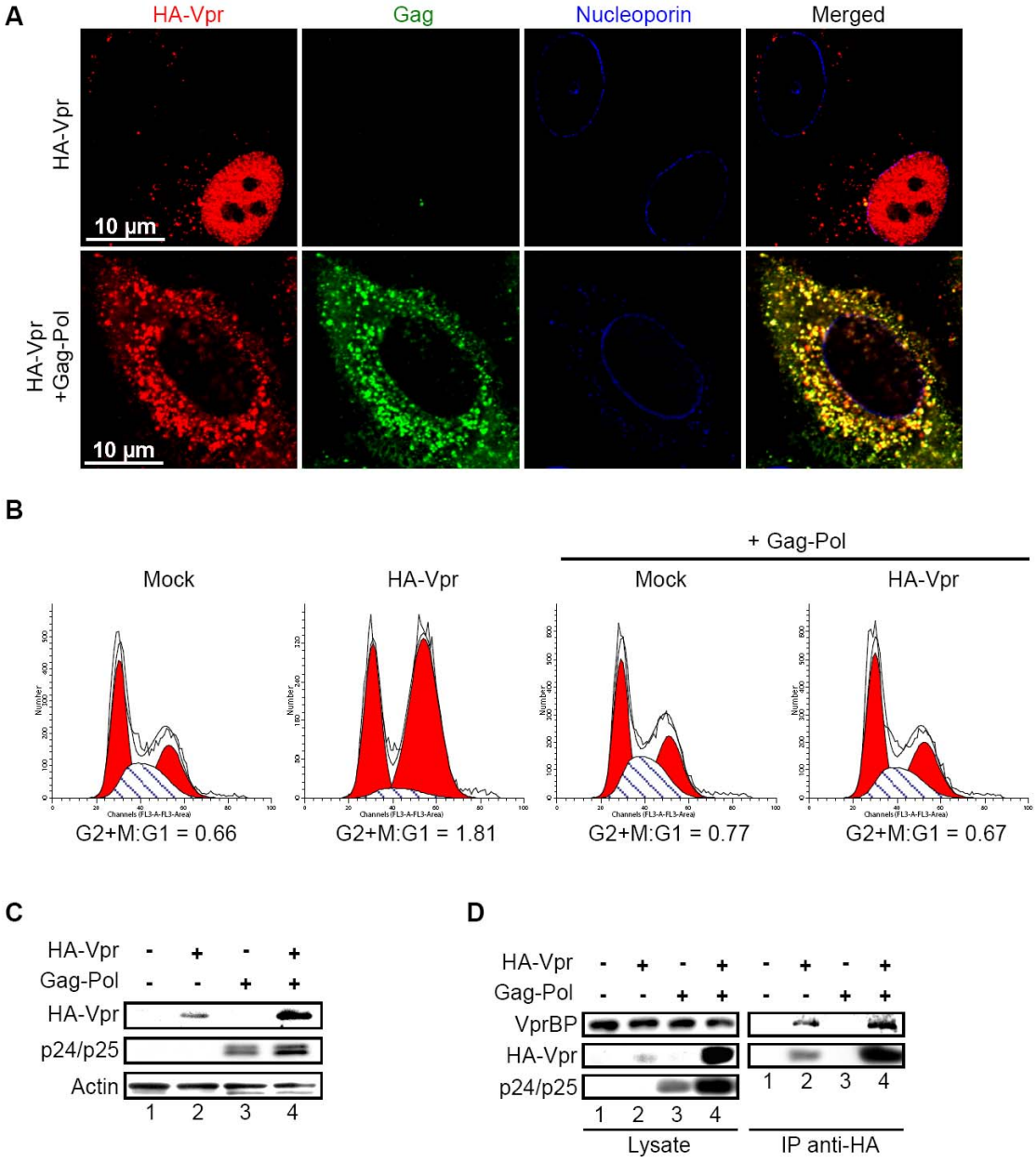

A
HA-Vpr
Gag
Nucleoporin
Merged
HA-Vpr
10 µm
HA-Vpr +Gag-Pol
10 µm
B
+ Gag-Pol
Mock
HA-Vpr
Mock
HA-Vpr
G2+M:G1 = 0.66
G2+M:G1 = 1.81
G2+M:G1 = 0.77
G2+M:G1 = 0.67
C
HA-Vpr - + - +
Gag-Pol - - + +
HA-Vpr
p24/p25
Actin
1 2 3 4
D
HA-Vpr - + - + - + - +
Gag-Pol - - + + - - + +
VprBP
HA-Vpr
p24/p25
1 2 3 4
1 2 3 4
Lysate
IP anti-HA

Figure 6. Sooty mangabey Vpr but not Vpx forms nuclear foci.

HeLa cells were transfected with plasmids expressing sooty mangabey HA-tagged Vpr (HA-Vpr sm) or Vpx (HA-Vpx sm). Two days after transfection, cells were fixed, permeabilized, and stained with antibodies against HA (red), nucleoporin (blue) and VprBP (green). Images were acquired by confocal microscopy. Images shown are representative of multiple fields. Averages of the number of Vpr nuclear foci (VNF) per cell and corresponding standard deviations are shown.

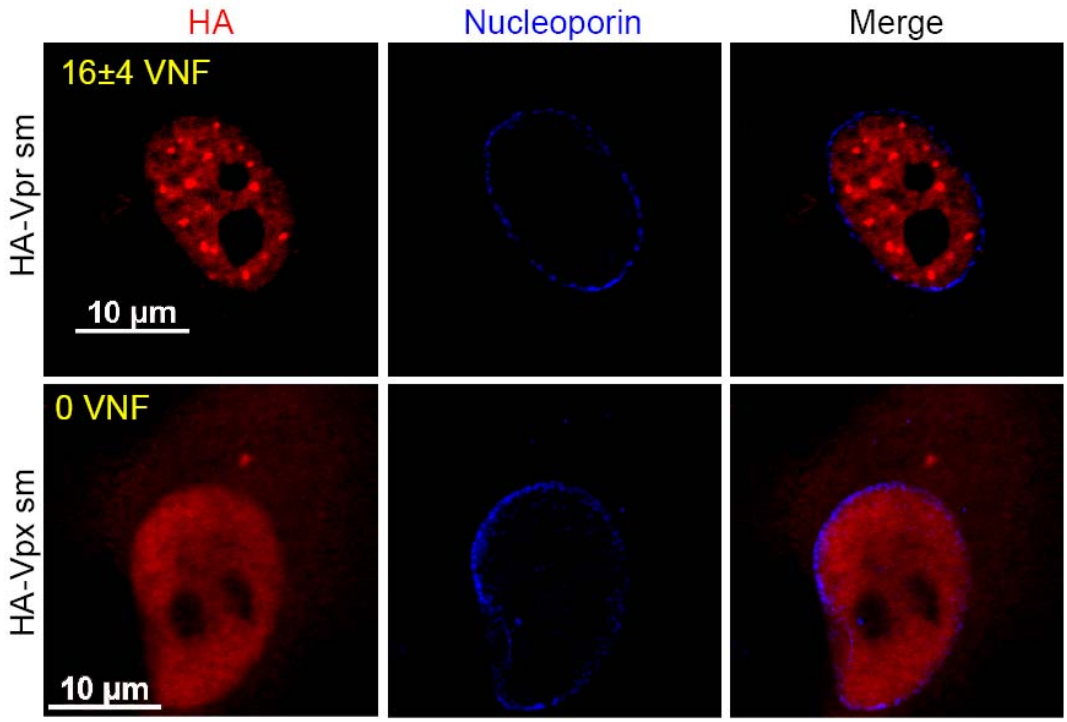
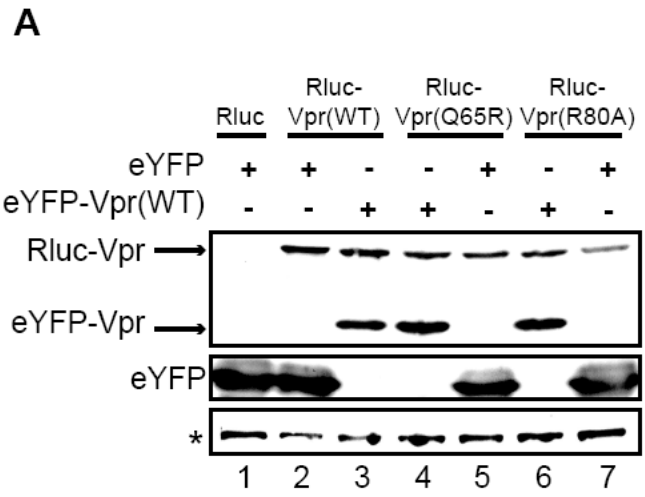

HA
Nucleoporin
Merge
16±4 VNF
HA-Vpr sm
10 µm
0 VNF
HA-Vpx sm
10 µm

Figure 7. Analysis of the self-affinity of wild type Vpr and mutants.

A) HEK293T cells were co-transfected with plasmids expressing Rluc-Vpr (WT), Rluc-Vpr (Q65R), Rluc-Vpr (R80A), eYFP-Vpr (WT) and eYFP fusion proteins. Two days later, cell lysates were resolved by SDS-PAGE and protein expression was determined by western blot using a rabbit polyclonal antibodies directed against Vpr and GFP. A non-specific band, depicted by the asterisk, was used as loading control. **B)** BRET saturation assays were performed with live HEK293T cells. A plasmid expressing Rluc-Vpr (WT), Rluc-Vpr (Q65R) or Rluc-Vpr (R80A) (BRET donor) was co-transfected with increasing concentration of a plasmid expressing eYFP-Vpr (BRET acceptor) or eYFP (non-specific control). Forty-eight hours post-transfection, energy transfer was initiated by addition of the cell-permeable *renilla* luciferase substrate coelenterazine H. Donor saturation curves were obtained by measuring BRET in presence of a fixed quantity of donor and increasing amounts of acceptor. The x-axis shows the ratio between the fluorescence (520 nm) of the acceptor (YFP-YFP_0, where YFP_0 is the fluorescence value in cells expressing the BRET donor alone) and the luminescence (475 nm) of the donor. BRET ratios (y-axis) were calculated as described in Materials and Methods. $BRET_{max}$ is the maximal BRET signal reached at saturation. $BRET_{50}$, which represent the concentration (fluorescence/ luminescence) of acceptor giving 50% of $BRET_{max}$, is a measure of the relative affinity of each fusion protein. Self- affinities relative to wild type are depicted in the graph. Curves shown represent the means ± standard deviations of results from one representative experiment performed in duplicate. The curves were generated by non-linear regression, in which a single binding site was assumed using the Sigma Plot software v.10.


A
Rluc
Rluc-Vpr(WT)
Rluc-Vpr(Q65R)
Rluc-Vpr(R80A)
eYFP
eYFP-Vpr(WT)
Rluc-Vpr
eYFP-Vpr
eYFP
*
1 2 3 4 5 6 7

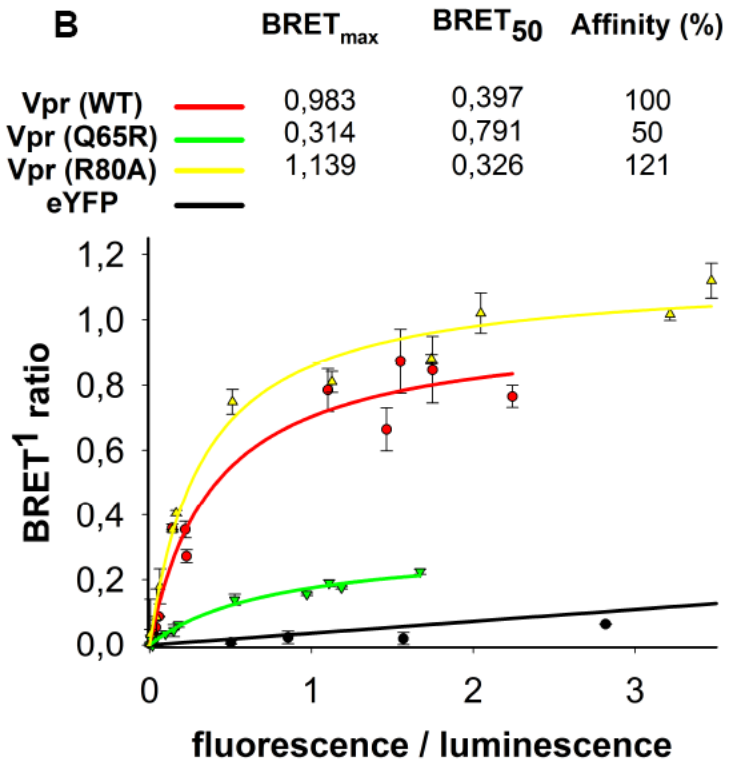

B
BRET max
BRET 50
Affinity (%)
Vpr (WT) 0,983 0,397 100
Vpr (Q65R) 0,314 0,791 50
Vpr (R80A) 1,139 0,326 121
eYFP
BRET1 ratio
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
1
2
3
fluorescence / luminescence

Figure 8. Association of Vpr to Chromatin correlates with the formation of nuclear foci.

A) HeLa cells were transfected with plasmids expressing HA-tagged Vpr (WT) or an empty plasmid used as negative control. Forty-eight hours after transfection, cells were harvested and lysed with 0.5% Triton X-100. The soluble fraction was used as input control (Sol.input). Insoluble debris containing chromatin was treated with micrococcal nuclease (+MNase) or with buffer alone (-MNase). The resulting solubilized fractions and input controls were resolved by SDS-PAGE and analyzed by western blot. Specific monoclonal antibodies were used to detect GAPDH (cytoplasmic marker) and HA-Vpr. Histone 3 (chromatin marker) and VprBP were detected using rabbit polyclonal antibodies. * Denotes a non-specific band detected with the anti-HA antibody. **B)** HeLa cells were transfected with plasmids expressing HA-tagged Vpr (WT), Vpr (Q65R), Vpr (R80A), and Vpr (1-78). Cell extracts were processed and analysed as in A).

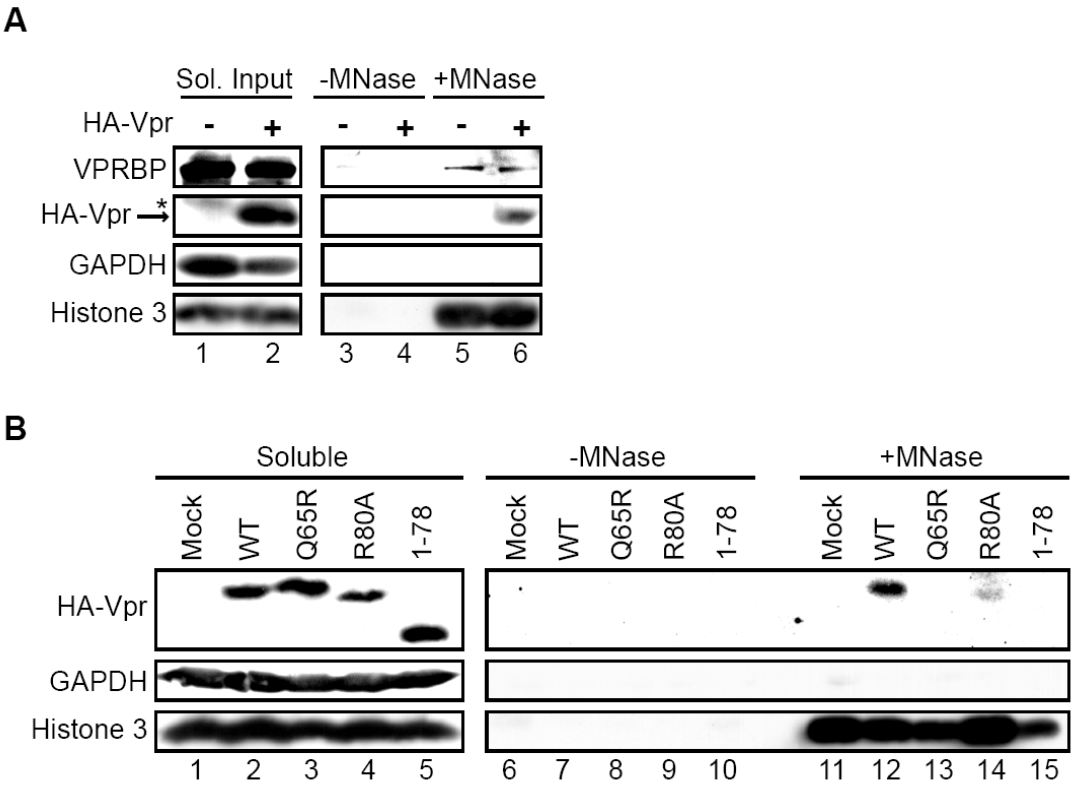
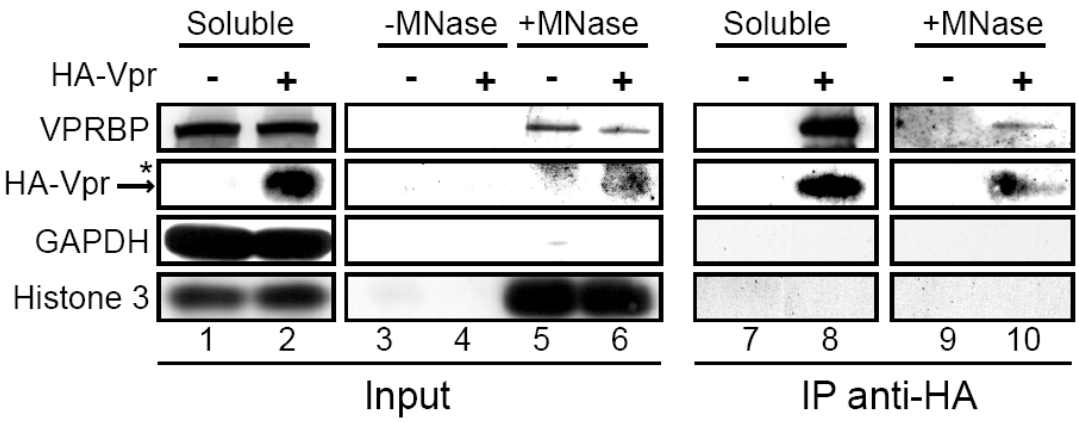

A
Sol. Input
-MNase
+MNase
HA-Vpr
-
+
-
+
-
+
VPRBP
HA-Vpr
*
GAPDH
Histone 3
1
2
3
4
5
6
B
Soluble
-MNase
+MNase
Mock
WT
Q65R
R80A
1-78
Mock
WT
Q65R
R80A
1-78
Mock
WT
Q65R
R80A
1-78
HA-Vpr
GAPDH
Histone 3
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 9. Vpr and VprBP associate on chromatin.

HeLa cells were transfected with a plasmid expressing HA-tagged Vpr (WT) or an empty plasmid used as negative control. Soluble and chromatin-bound fractions were isolated as before and were subjected to anti-HA immunoprecipitation as described in Materials and Methods. Input controls and immunoprecipitates were resolved by SDS-PAGE and analyzed by western blot. Specific monoclonal antibodies were used to detect GAPDH (cytoplasmic marker) and HA-Vpr. Histone 3 (chromatin marker) and VprBP were detected using rabbit polyclonal antibodies. * Denotes a non-specific band detected with the anti-HA antibody.


Soluble
-MNase
+MNase
Soluble
+MNase
HA-Vpr
- + - + - + - + - +
VPRBP
HA-Vpr *
GAPDH
Histone 3
1 2 3 4 5 6 7 8 9 10
Input
IP anti-HA

SUPPLEMENTAL MATERIAL

Figure S1. Native Vpr and virally encoded Vpr form nuclear foci.

A) HeLa cells were transfected with plasmids expressing native Vpr. Two days after transfection, cells were fixed, permeabilized, and stained with monoclonal antibodies against Vpr (clone 8D1) and analyzed by confocal microscopy. **B)** HeLa cells were infected with VSV-G-pseudotyped viruses defective for Vpr expression (HxBru Vpr-) or expressing HA-tagged Vpr (HxBru HA-Vpr) at 100 cpm/cell. Two days after infection, cells were fixed, permeabilized, and stained with antibodies against HA (red), nucleoporin (blue) and VprBP (green). Images were acquired by confocal microscopy. Images shown are representative of multiple fields that encompass minor and major phenotypes.

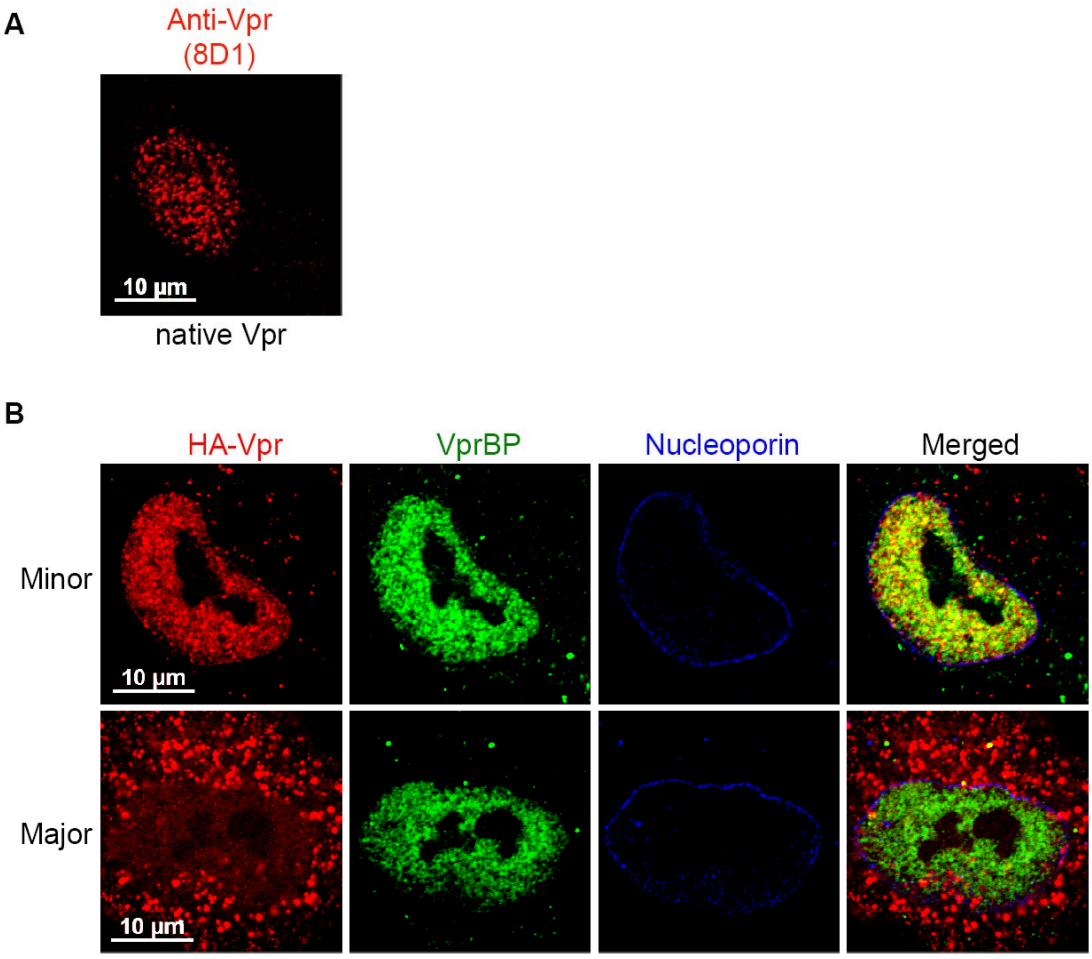

A
Anti-Vpr
(8D1)
10 µm
native Vpr
B
HA-Vpr
VprBP
Nucleoporin
Merged
Minor
Major
10 µm
10 µm

Figure S2. Caffeine inhibits the induction of G2 arrest by HA-Vpr.

HeLa cells were transduced with lentiviral vectors expressing HA-Vpr or with control lentiviral vectors. Cells were concomitantly treated or not with 2.5mM caffeine for the duration of the experiment. Twenty-four hours after transduction, cells were harvested, stained with propidium iodide and their cell cycle profile was monitored by flow cytometry. Percentages of cells in G1 and G2/M were determined using the ModFit software.

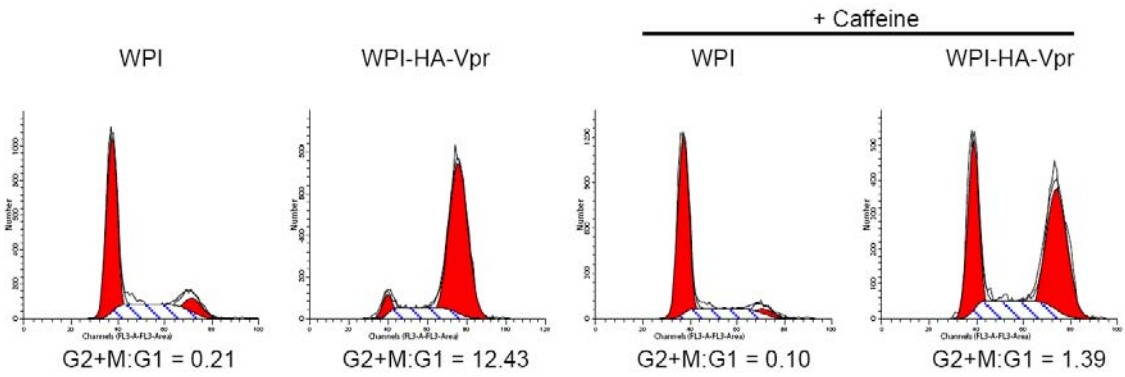
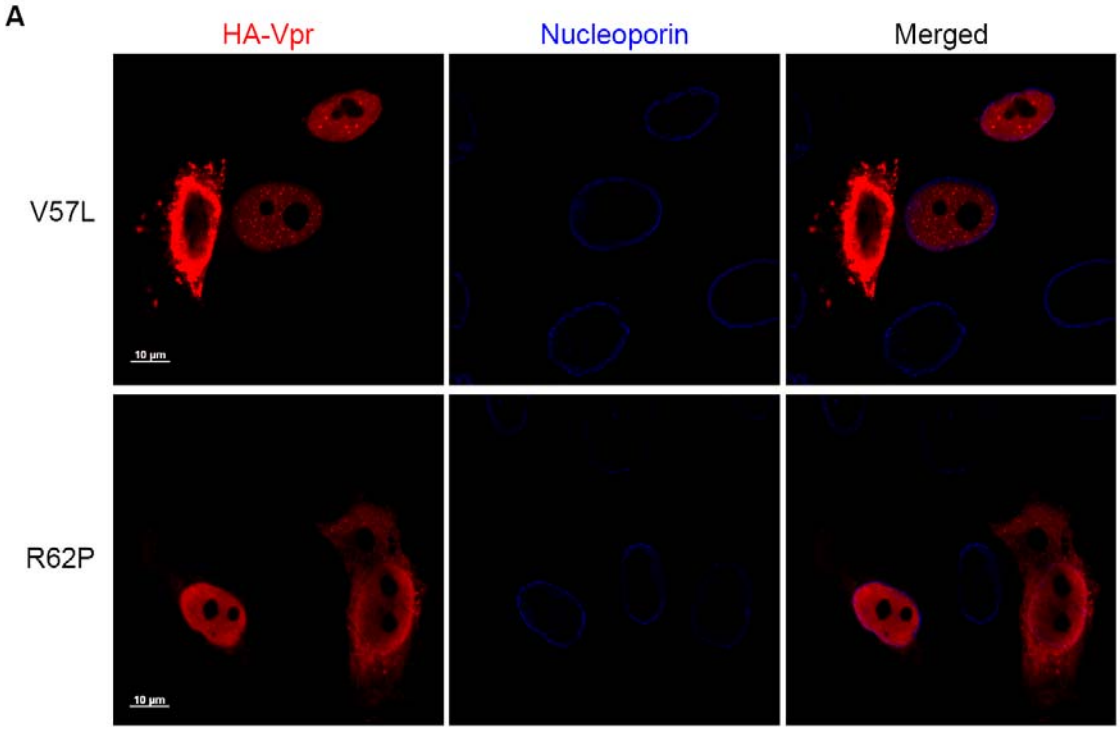
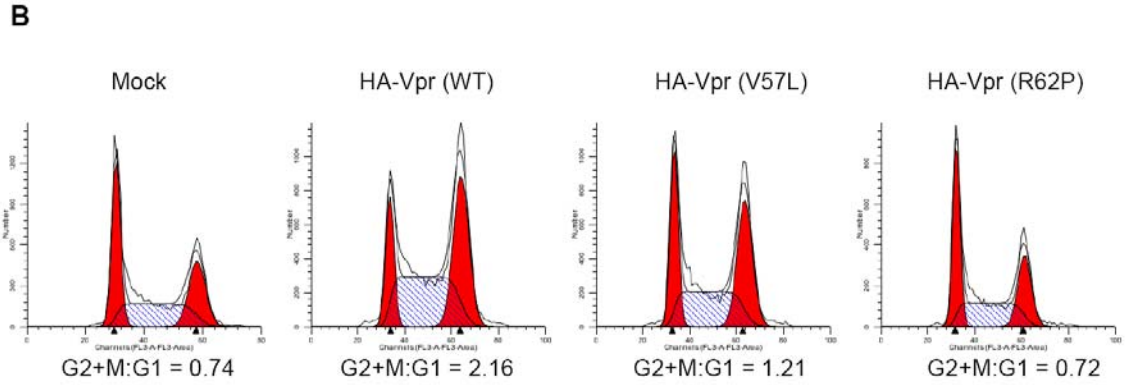

+ Caffeine
WPI
WPI-HA-Vpr
WPI
WPI-HA-Vpr
G2+M:G1 = 0.21
G2+M:G1 = 12.43
G2+M:G1 = 0.10
G2+M:G1 = 1.39

Figure S3. Localization and G2 arrest activity of the Vpr mutants V57L and R62P. **A)** HeLa cells were transfected with plasmids expressing HA-tagged Vpr (V57L) and Vpr (R62P). Forty-eight hours after transfection, cells were fixed, permeabilized, and stained with antibodies against HA (red) and nucleoporin (blue). Images were acquired by confocal microscopy. Images shown are representative of multiple fields. 60% of cells expressing HA-Vpr (V57L) could form nuclear foci while the remaining 40% displayed perinuclear accumulation with reduced or absence of nucleoporin staining. 20% of cells expressing HA-Vpr (R62P) displayed an exclusive nuclear localization while the remaining 80% of cells showed accumulation of Vpr in the cytoplasm. In all cases, HA-Vpr (R62P) did not form nuclear foci. **B)** HEK293T cells were co-transfected with a plasmid expressing GFP and a plasmid expressing HA-Vpr (WT), HA-Vpr (V57L), or HA-Vpr (R62P). An empty plasmid was used as negative control (mock). Forty-eight hours after transfection, cell cycle analysis was performed by flow cytometry using propidium iodide staining. Percentages of G1 and G2/M cell populations were determined using the ModFit software.


A
HA-Vpr
Nucleoporin
Merged
V57L
R62P
10 μm
10 μm


B
Mock
HA-Vpr (WT)
HA-Vpr (V57L)
HA-Vpr (R62P)
G2+M:G1 = 0.74
G2+M:G1 = 2.16
G2+M:G1 = 1.21
G2+M:G1 = 0.72

DISCUSSION

1. HIV-1 VPR HIJACKS A CELLULAR CULLIN-RING E3 UBIQUITIN LIGASE

The work presented in this thesis identifies the cellular E3 ubiquitin ligase CRL4A(VprBP) as an essential cellular partner of HIV-1 Vpr for the activation of ATR and induction of G2/M cell cycle arrest. Importantly, the involvement of CRL4A(VprBP) in Vpr-induced G2 arrest was corroborated by several other studies [332,553-556]. This interaction with CRL4A(VprBP) and its role in Vpr-induced G2 arrest is also conserved in other lentiviral lineages, including HIV-2 and SIVmac [553,554,556]. Vpx, a simian paralog of Vpr, also functionally interacts with CRL4A(VprBP)[557-560]. Vpx is present in the SIVmac/SIVsm/HIV-2 lentiviral lineage and probably arose as a result of a gene duplication event. In contrast to Vpr, Vpx does not induce G2 arrest but instead counteracts a putative restriction factor acting in the early steps of the infection of cells of the monocytic lineage. All these observations points towards an important role of the Vpr-CRL4A(VprBP) interaction in viral replication and dissemination throughout lentiviral evolution.

Vpr is the third HIV-1 protein known to hijack a CRL complex. Vpu and Vif respectively recruit CRL1(β-TrCP) and CRL5 to perform their functions [561]. Vpr and Vpx engage the CRL4A ubiquitin ligase similarly to the way Vpu interacts with CRL1. All three proteins interact directly with a substrate receptor: β-TrCP for Vpu and VprBP for Vpr and Vpx. In contrast, Vif does not need a substrate receptor and appears to itself act as a substrate receptor by interacting directly with cullin 5 and the heterodimeric adaptor Elongin B-C. HIV-1 is not the only virus to encode proteins usurping the functions of CRLs. HBV and parainfluenza viruses such SV5 hijack CRL4A via direct interactions between viral proteins (HBx and SV5 V) and DDB1. [562-564]. Other notable examples include the Epstein-Barr virus and adenovirus. The Epstein-Barr nuclear antigen 3C (EBNA3C) engages CRL1(SKP2) to induce the ubiquitination and proteasomal degradation of the tumor suppressor Rb [565]. The adenoviral proteins

E4orf6 and E1B55K functionally interact and form a complex with a cullin 5-based ligase to ubiquitinate and degrade several cellular proteins including p53, MRN components and DNA ligase IV (reviewed in [566]. Our results therefore add Vpr to a long list of viral proteins hijacking CRLs, suggesting that it is a very common strategy used by viruses.

Our study and the ones of other investigators [332,553-556] have highlighted the existence of at least two functional domains in Vpr. The first one is involved in engaging VprBP and was mapped to the third alpha helix. The second one is located at the C-terminus. The C-terminus of Vpr is not involved in engaging CRL4A(VprBP) but is nevertheless essential for the induction of G2 arrest. The simplest model to explain these results is that Vpr would recruit a cellular substrate via its C-terminus to forcibly induce its ubiquitination by the CRL4A(VprBP) complex (Figure 9, p.197). Our results presented in Chapter 2 provide direct evidence for this model. Indeed, we showed that Vpr could induce the K48-linked polyubiquitination and proteasomal degradation of interacting cellular proteins. Vpr-mediated K48-polyubiquitination and proteasomal degradation of these substrates was found to be necessary for the activation of ATR and for G2 arrest. Moreover, in Chapter 3, we demonstrate that this substrate is probably a chromatin-bound cellular protein. Specifically, we found that Vpr could associate to chromatin via its C-terminus and could form structures that we called Vpr nuclear foci. It is still unclear whether Vpr contacts its substrates directly on chromatin or via a potential cellular cofactor (Figure 9, p.197). The latter situation would explain why Vpr still associates with chromatin and why Vpr nuclear foci are still present in G2, when the substrate is supposedly degraded. The use of a chromatin-bound cofactor in Vpr might involve additional functional domains in the protein but not necessarily. Indeed, Vpr could interact with its chromatin-associated cofactor, which would be in proximity or even physically interacting with the substrate. This would allow Vpr to position the E2 in proximity to its substrate and would induce efficient transfer of ubiquitin. Similar mechanisms are used by SV5 V and by CRL4A(DDB2). SV5 V acts

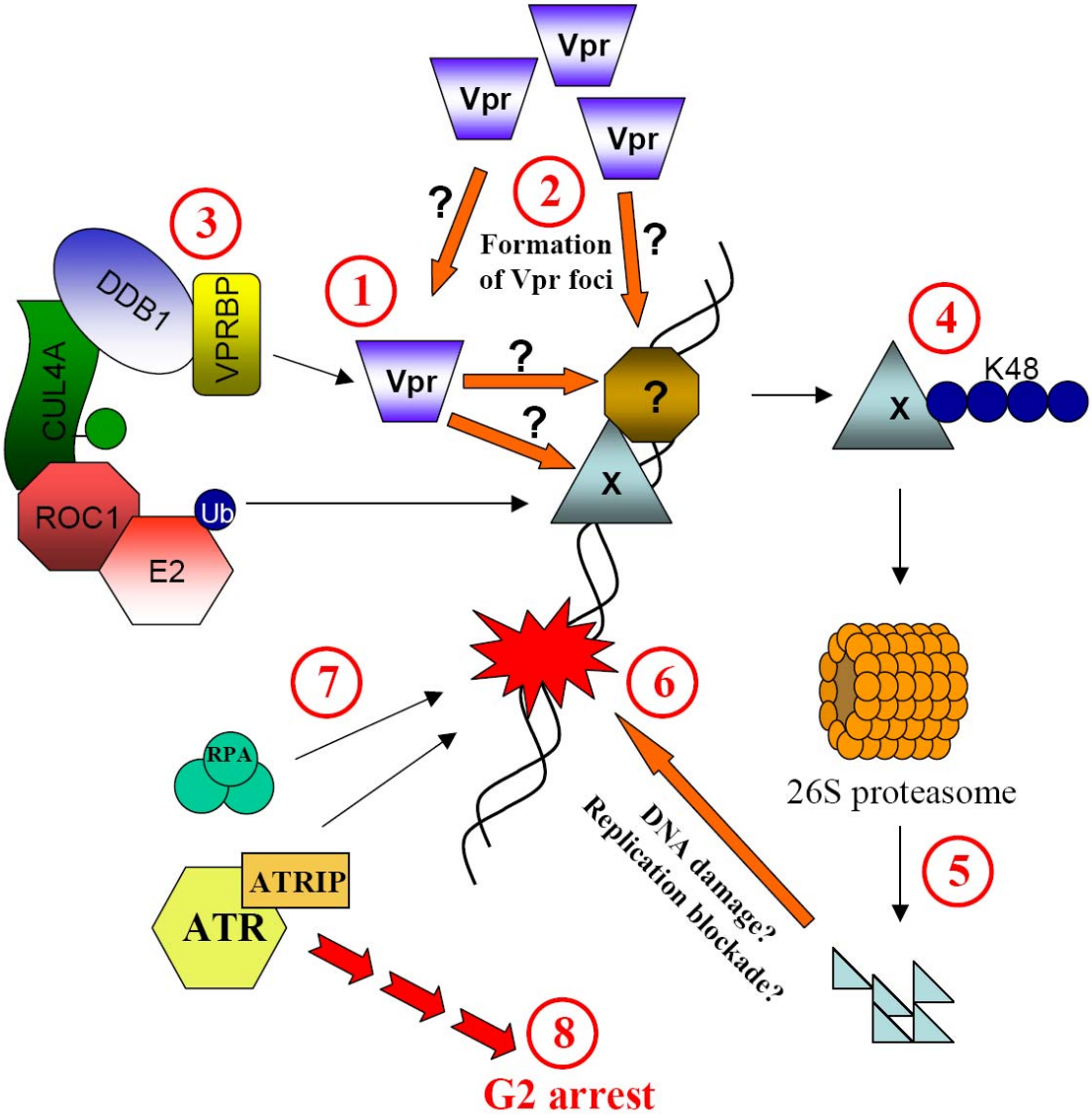


Figure 9. Proposed model for the mechanism of induction of G2 arrest by Vpr.

Vpr associates directly or via an unknown cofactor with a putative chromatin-bound cellular substrate X (1), triggering accumulation of Vpr via oligomerization of the protein or increased association to the substrate or cofactor (2). Vpr then recruits the CRL4A(VprBP) E3 ubiquitin ligase through an interaction with the substrate receptor VprBP (3) and induces the K48-linked polyubiquitination of the substrate X (4). Degradation of the ubiquitinated substrate by the proteasome (5) stalls DNA replication or induces other types of DNA damages (6). These genotoxic stresses are recognized by DNA damage sensors including RPA (7) and leads to the activation of the ATR-mediated DNA damage checkpoint, ultimately resulting in G2 arrest (8).

as a connector between DDB1 and STAT2, inducing the ubiquitination of STAT2-bound STAT1 [567]. Binding of DDB2 to UV-induced bulky adducts orients the E2 in a way that it can transfer its ubiquitin to nearby histones [568]. Conversely, Vpr could interact simultaneously with a cofactor and its substrate, both of which would be associated to chromatin. Such a mode of action is exemplified by CRL4A(Cdt2). CRL4A(Cdt2) needs to bind simultaneously to chromatin-bound PCNA and Cdt1 in order to induce Cdt1 ubiquitination [569]. Finally, because of a lack of knowledge on the normal functions of VprBP, we have not been able to determine whether Vpr induces ubiquitination of its own substrates or whether it forcibly increases the ubiquitination of the natural substrates of VprBP. It is noteworthy that most CRL4A complexes are spatially regulated, particularly at the level of chromatin [438]. Vpr could therefore bypass this regulation by binding to a cofactor on chromatin, thereby recruiting CRL4A(VRPBP) in proximity of its substrates. Constructing chimeras between Vpr and the G2-arrest-incompetent Vpx might address some of these possibilities by identifying additional functional domains in Vpr necessary for association to chromatin, formation of nuclear foci and induction of G2 arrest. A similar strategy has been used recently and identified the N-terminus of Vpx as an essential domain to facilitate infection of macrophages [570]. However, a complete comprehension of the mechanism by which Vpr uses CRL4A(VprBP) to induce G2 arrest will require the identification of the cellular proteins targeted by Vpr and VprBP.

2. IDENTIFICATION OF VPR'S G2 ARREST SUBSTRATE

The most important unresolved aspect of the mechanisms by which Vpr induces G2 arrest is of course the identity of the chromatin-bound cellular proteins targeted by Vpr. There are several approaches that can be pursued to identify these proteins. Ideally, these approaches should encompass both mechanistic possibilities: Vpr targets its own substrates or Vpr induces the ubiquitination of VprBP's substrates. Identification of the substrate by tandem affinity purification and mass spectrometry represents the most direct approach. This is the technique that we used in chapter 1 to

identify VprBP and DDB1 as cellular partners of Vpr. Because the substrate is degraded and because enzyme-substrate interactions are generally transient, special care has to be taken to stabilize the interaction between Vpr and its potential substrates [571,572]. As presented in Chapter 2, we have already optimized conditions to detect the interaction between Vpr and its ubiquitinated substrates. To further enrich Vpr-interacting ubiquitinated proteins, we improved the approach described in chapter 2 by adding an additional purification step (data not shown). The new procedure called TSAP-Ub (three-step affinity purification of ubiquitinated substrates) involves denaturation of purified TAP-Vpr complexes and isolation of Myc-Ub-conjugated proteins using anti-myc agarose. This procedure has to date yielded several interesting candidates. Since Vpr has a tendency to be a promiscuous binding protein [320], non-porous magnetic beads and short post-lysis incubation time could be used to decrease non-specific interactions and limit the number of candidates. Alternatively, the HEK293T cell line stably depleted of VprBP (chapter 2) can be used as an experimental system to block ubiquitination and degradation of Vpr's substrates. The caveat of this last approach is that it will only identify Vpr's own substrates and not VprBP's.

Moreover, strategies other than proteomic approaches can be used, not to directly identify the substrate, but to improve our knowledge of its cellular roles and functional interactions. In chapter 3, we described the formation of chromatin-associated Vpr nuclear foci and suggest that Vpr would contact its substrates on chromatin, either directly or indirectly. These discreet foci also imply that Vpr could associate to specific regions of chromatin. Identification of the sequence of these regions of chromatin might therefore yield important information about the processes targeted by Vpr and might even allow the identification of the substrate. We can therefore perform ChIP-chip or ChIP-seq experiments to determine if Vpr binds to chromatin on consensus sequences and to identify these sequences. Comprehensive work by the Planelles' laboratory described the activation by Vpr of the canonical ATR-mediated DNA damage checkpoint, suggesting that Vpr would induce the formation of RPA-coated ssDNA intermediates [285,286,289,290]. Our results (Chapters 2 and 3) support these conclusions and further demonstrate that Vpr nuclear foci partially co-

localize with these RPA-coated ssDNA intermediates. Extensive knowledge is now available on the different DNA damage repair mechanisms. Co-localization between Vpr-induced RPA foci and markers of BER (base excision repair), NER (nucleotide excision repair), DSBs (DNA double-strand breaks), stalled replication fork, and delayed origin firing could potentially identify what genotoxic stresses and which repair processes are induced by Vpr. Most proteins involved in DNA replication associate to chromatin in a cell cycle-dependent manner. Careful analysis of the timing of Vpr nuclear foci and association of Vpr to chromatin in synchronized cells would determine at what phase of the cell cycle the cofactor/substrate associate to chromatin. Similarly, temporal monitoring of H2AX phosphorylation in response to Vpr will determine if Vpr causes direct DNA damages (phosphorylation of H2AX in G1) or whether it interferes directly with origin firing or DNA replication fork progression (phosphorylation of H2AX in S-phase). These experiments would unlikely identify the substrate but would indicate in what cellular pathways it is involved. These data would be very useful to restrict the number of candidates identified by other means such as mass spectrometry. A pre-characterization of the restriction factor counteracted by Vpu has similarly contributed to the later identification of Tetherin [155]. Indeed, the initial observations by Neil and colleagues that the restriction factor was endogenously expressed in some cell lines, that it could be up-regulated by IFN-alpha and that it was acting at the cell surface greatly helped to restrict the number of candidates that had to be individually tested [152-154].

3. ROLE OF G2/M ARREST DURING INFECTION

The identification of the substrates of Vpr will likely be necessary to our understanding of the function of ATR activation and G2 arrest in viral replication and pathogenesis. Activation of the G2/M checkpoint could be an end in itself or could be the unavoidable consequence of the inhibition of a cellular restriction factor. In support of the former, expression from the HIV-1 LTR is modestly up-regulated (2 to 4 folds) when cells are in G2, leading to increased viral production [321]. Recent evidences show that UV-induced G2 arrest abolishes the requirement for P-TEFb and SKIP to

overcome a restriction on transcriptional elongation from the LTR [573]. It is reasonable to assume that Vpr could function in a similar manner. Other potential benefits of a G2/M arrest include increased proviral integration [574] and active translation from the HIV-1 IRES [575]. Many viruses induce cell cycle perturbation to optimize viral replication [468-470]. However, they unusually curb checkpoint responses [576]. For instance, adenoviruses and HSV-1 induce a pseudo-S-phase to increase viral DNA replication but inhibit ATR and ATM activation by respectively sequestering the MRN complex and by inhibiting RNF8 and RNF168 [577,578]. The fact that HIV-1 does not inhibit ATR activity points to potential roles of the checkpoint response during infection. These could include immunomodulatory functions important for viral persistence *in vivo* and for pathogenesis. Our group and others have recently shown that Vpr-induced activation of ATR leads to the up-regulation at the cell surface of ligands of the activating NK cell receptor NKG2D and results in increased NK cell cytolytic activity [340,341]. This up-regulation could act at the level of T-cell depletion but could also be involved in NK cell exhaustion. Interestingly, recent studies show that HBV-induced NKG2D ligand up-regulation in hepatocytes is causing extensive liver damage by producing an acute immune response mediated by NK and NKT cells [579-581]. Moreover, preliminary data show that MCMV mutants defective for NKG2D ligands down-modulation, although initially attenuated in replication, were ultimately causing a persistent infection lasting longer than wild type viruses [582]. A precise regulation of NKG2D ligands expression might therefore be required for immunosurveillance. Vpr could contribute to viral pathogenesis and persistence by upsetting this balanced NKG2D response. A reassessment of the role of Vpr in the pathogenic lentiviral infection of simian models, particularly at the level of immune function, would probably be required to confirm this hypothesis.

4. IMPLICATION OF CRL4A(VprBP) IN OTHER FUNCTIONS OF VPR

In this thesis, we have mostly studied the involvement of CRL4A(VprBP) in Vpr-induced G2 arrest. As mention in the introduction, Vpr also performs other

functions in the viral replication cycle: it facilitates infection of macrophages, induces apoptosis, and perturbs immunomodulatory functions. Moreover, as mentioned above, the simian paralog of Vpr, Vpx, also usurps CRL4A(VprBP) to block a restriction factor in cells of the monocytic lineage [557-560]. This conserved functional interaction suggest that, besides G2 arrest, CRL4A(VprBP) could be involved in other functions of Vpr. Like Vpx, Vpr has been implicated in facilitating infection of non-dividing cells, notably macrophages. However, Vpr and Vpx probably target different processes since Vpx can drastically increase the infection of HIV-1, even in presence of Vpr [557,560]. It is therefore possible, even probable, that Vpr could hijack CRL4A(VprBP) to inactivate a distinct cellular restriction factor. Interestingly, the C-terminus of Vpr appears to be important for both G2 arrest and infection of macrophages, suggesting that Vpr could be targeting the same substrate responsible for G2 arrest to somehow facilitate infection of macrophages. Moreover, Vpr has been shown to induce apoptosis independently of G2 arrest by interacting with ANT [302,326,327]. Is Vpr somehow inducing the ubiquitination and degradation of ANT or other anti-apoptotic factors by recruiting CRL4A (VprBP)? A likely role of Vpr in viral pathogenesis is to modulate the activity of the immune system. In vitro, several mechanisms have already been proposed, including defects in DC and NK cell activity [336]. Some of these functions, such as up-regulation of NKG2D ligands, require activation of ATR and therefore involves the recruitment of CRL4A(VprBP) [340,341]. However, the immunomodulatory role of Vpr has also been correlated to its expression in non-dividing cells such as macrophages and dendritic cells. Is Vpr using the same E3 ligase complex to target modulator of immune functions in these cell types? Thorough analyses of the involvement of CRL4A(VprBP) in the other functions of Vpr would therefore be very informative and would help resolves the paradox of how such a small protein can perform so may different functions.

5. ARCHITECTURE AND COMPOSITION OF THE E3 LIGASE COMPLEX RECRUITED BY VPR

Several questions also arise in respect to the composition and architecture of the E3 ubiquitin ligase complex recruited by Vpr. First, our observation that Q65R, the bona fide mutant of Vpr defective for the interaction VprBP, also displays aberrations in its localization and defects in oligomerization, raise the question as to whether the 3rd alpha-helix of Vpr is the domain of interaction or whether lack of binding merely reflects misfolding of the compact hydrophobic ternary structure of Vpr. Given that most mutations in the third alpha helix of Vpr have pleiotropic effects [257], it is unlikely that a mutagenesis approach will convincingly characterize its VprBP interaction domain. The most appropriate way to map the VprBP-interaction domain would by obtaining the crystal structure of the Vpr-VprBP heterodimer. Another interesting question is the functional implication of the C-terminus of Vpr, specifically the role of phosphorylation at position S79 [272]. Why does Vpr need to be phosphorylated in order to perform its functions? In the case of Vpu, its phosphorylation mimics a phosphodegron, the recognition domain of Cullin 1-Skp1-associated F-Box substrate receptors [561]. In the case of Vpr, phosphorylation is not involved in the interaction with the E3 ubiquitin ligase (Chapter 1). Is phosphorylation necessary to interact with phospho-specific cellular factors such as those involved in DNA replication and DNA repair signalling or does it induce a change in formation in Vpr allowing the proper positioning of the E3 ligase?

The domain of VprBP involved in the interaction with Vpr is also unclear. The minimal binding domain was mapped to a large C-terminal fragment of the protein [553]. This region comprises the dual WDxR motifs characteristic of the DDB1 substrate receptor family and is thought to form a seven-bladed β-propeller. This β-propeller was originally thought to constitute the binding interface for DDB1 [583-585]. However the recently characterized crystal structure of the DDB2-DDB1 heterodimer in complex with damaged DNA shows that it is rather involved in binding to DNA [568]. A protruding alpha-helix in DDB2 is contacting the hydrophobic groove formed

between beta-propellers A (BPA) and C (BPC) in DDB1. The same binding interface is used by alpha-helices in SV5 V and HBx to interact with DDB1 [568,583,586], suggesting that it is the way WDxR substrate receptors interact with DDB1. Intriguingly, mutating the WDxR motifs of VprBP abrogated both its interaction with Vpr and with DDB1 [553]. In DDB2, the WDxR motif is not directly involved in either binding to DNA or DDB1. Instead, it is presumed to be involved in the proper folding of the β-propeller [568]. Therefore, we can assume that the interaction domains of both DDB1 and Vpr on VprBP reside in the β-propeller or in its close vicinity. However, the exact residues involved remain to be identified. Does VprBP possess a protruding alpha-helix involved in its interaction with DDB1? Is the β-propeller in VprBP involved in its binding to chromatin? Is Vpr somehow mimicking the recognition structure of the VprBP β-propeller to forcibly recruit it to Vpr nuclear foci? Again, a mutagenesis approach might give some clues on these interaction domains and their functions, but given the highly organized architecture of these proteins, the full characterization of the structure of the Vpr-VprBP-DDB1 complex is likely to be necessary to understand its architecture.

The composition of the E3 ubiquitin ligase complex recruited by Vpr is also unknown. The functional involvement of VprBP, DDB1, CUL4A, ROC1, and DDA1 has been previously described (Chapter 1 and [332,553-556]). However, although DDB1, VprBP, and DDA1 could be readily identified by mass spectrometry as Vpr-associated factors, CUL4A and ROC1 were never detected by these large-scale approaches. Moreover, only very limited amount of CUL4A could be co-immunopurified with Vpr when using western blot as a detection method (Chapter 1). These data suggest that the interaction between the Vpr-VprBP-DDB1 subcomplex and the rest of the E3 machinery is very transient or is spatially restricted to a minor fraction of these complexes. Moreover, studies by Hrecka and colleagues showed that, in absence of Vpr, VprBP could form complexes, ranging from 250 kDa to over 1MDa, with presumably other cellular proteins [554]. Subunits of the signalosome (CSN) could also be co-immunopurified with VprBP complexes. As mentioned in the introduction to this thesis, the CSN complex appears to sequesters in its inactive form subsets of

CRL4A ligases, including CRL4A(VprBP) [461]. In presence of Vpr however, CSN subunits did not associate anymore with CRL4A(VprBP) [554], suggesting that Vpr would activate CRL4A(VprBP) by uncoupling CSN. A similar mechanism was described for CRL4A(DDB2). Upon UV irradiation, CSN dissociates from CRL4A (DDB2), allowing the E3 ligase to bind damaged DNA and induce ubiquitination of its substrates [462]. There are other functional implications to the uncoupling of CSN from CRLs. The absence of CSN would likely limit the activity of CRL4A(VprBP) to one cycle of substrate recognition and ubiquitination by preventing deneddylation of Cullin 4A. In support of this, increased neddylation of CRL4A(VprBP) has been observed in presence of Vpr [554]. Another implication of uncoupling of CSN would be the possible autoubiquitination and degradation of Vpr and VprBP. Results by our group and others have shown that interfering with the recruitment or activity of CRL4A(VprBP), either by using mutants or small interfering RNA, results in an increased levels of Vpr (Chapter 2 and [556]) . If Vpr truly dissociates CSN from the ubiquitin ligase, it is therefore paradoxical that the molecular weights of CRL4A(VprBP) complexes associated with Vpr are higher in molecular weights than their native counterparts [554]. This implies that the interaction of Vpr with CRL4A(VprBP) would induce a change of complex architecture such as formation of a dimer or would recruit additional cellular factors. The latter might be constituents of the chromatin-associated foci formed by Vpr (Chapter 3 and [290]). Large-scale proteomic approaches using milder extraction conditions would probably be necessary to identify these factors.

6. INTERACTIONS BETWEEN VPR AND OTHER COMPONENTS OF UPS

The association between Vpr and CRL4A(VprBP) is not the first instance of an interaction between Vpr and the ubiquitin-proteasome system (UPS). Indeed, two other cellular proteins implicated in UPS, Mov34/hVIP/CSN6 [296] and hHR23A (human homologue of Rad23) [297,298], have been previously reported to interact with Vpr. Although the interaction between Vpr and CSN6 has been to date primarily implicated

in glucocorticoid signalling [587], the function of CSN6 suggests other potential roles in Vpr functions. Indeed, CSN6 is a core subunit of the signalosome (CSN). It contains a non-catalytic metalloenzyme JAMM domain involved in maintaining the structural integrity of the whole CSN complex [588]. CSN6 interacts directly with ROC1 and likely assists in the docking of the CSN complex to CRLs [589,590]. Could it be that Vpr needs first to dissociate CSN6 from CRL4A(VprBP) in order to activate the ligase? Could it account for the apparent lack of association between CRL4A(VprBP) and CSN subunits in presence of Vpr? Could it result in increased neddylation of CUL4A? The hHR23A protein represents an interesting functional paradox. It has a role in NER repair via its interaction with the damage sensor XPC but it is also one of the ubiquitin receptors on the regulatory subunit of the proteasome [591]. Vpr interacts directly with an hydrophobic loop region within the ubiquitin-binding domain of hHR23A [592,593]. This interaction was first implicated in Vpr-mediated G2 arrest [297,298], but a later mutagenesis study using the yeast two-hybrid system showed that binding to hHR23A was neither required nor sufficient to induce G2 arrest [301]. The Vpr mutants defective for the interaction with hHR23A spanned all three helices and showed additional defects in UNG2 and Gag interactions. Surprisingly, they were nevertheless active for G2 arrest [301]. In spite of these negative results, it is tempting to speculate that recruitment of the proteasome to the site of ubiquitination of its substrates could allow Vpr to efficiently induce their degradation by evading the regulatory activity of cellular deubiquitinases. It is possible that Vpr would form foci containing multiple units of Vpr to coordinate the activation of the E3 ligase, the polyubiquitination of substrates and the efficient proteasomal degradation of these substrates. Alternatively, given the role of hHR23A in NER, perhaps Vpr targets it to inactivate damage recognition by NER. During infection, inactivation of NER leads to a modest but significant increase in 2-LTR circle formation, in integrated provirus, and in viral replication [594]. These phenotypes are very similar to the effect of Vpr on nuclear import of the pre-integration complex and on viral replication [236,270,314,317]. Therefore, the binding of Vpr to CSN6 and to hHR23A should probably be revisited to reassess their potential involvement in the functions of Vpr using modern tools such as RNA interference. A

confocal analysis to monitor the presence or absence of hHR23A and CSN6 in Vpr nuclear foci would also be informative.

7. DEVELOPMENT OF DRUGS TARGETING THE VPR-VprBP BINDING INTERFACE

The Vpr-VprBP interaction represents an interesting target for the development of protein-protein interaction inhibitors. Targeting Vpr activity is unlikely to generate much commercial or therapeutic interest given the non-essential role of the protein in viral replication. However, such an inhibitor would constitute a very interesting tool to study the functional involvement of CRL4A(VprBP) in the functions of Vpr other than G2 arrest and would likely help in the characterization of the substrates of Vpr. In addition, this inhibitor could be used to characterize the function of Vpr in pathogenesis by blocking Vpr's activity at different stages of infection in rhesus macaques. The development of small molecule inhibitors targeting protein-protein interaction has not been intensively pursued by the pharmaceutical industry, particularly in regard to HIV-1 treatment. Notable exceptions include a series of entry inhibitors targeting gp41 fusion intermediates, the gp120-CD4 interaction, and the gp120-CCR5 interaction [595]. However, recent work by Debyser's group and collaborators describes the identification of a small inhibitor disrupting the integrase-LEDGFp75 interaction [596]. De Luca and colleagues used software-assisted modeling to design a pharmacophore that would block this interaction. Virtual screening of chemical database and further optimization of the candidates led to the discovery of a potent inhibitor able to interfere with the integrase-LEDGF/p75 interaction at micromolar concentration. The lack of structural information about the Vpr-VprBP interaction interface precludes the use of computer-assisted rational design. However, BRET- or FRET-based high-throughput *in vitro* screening of chemical libraries constitutes an interesting alternative. This strategy has been used successfully to develop inhibitors targeting IL-2, Bcl-XL, MDM2, TNF, and human papillomavirus E2 [597]. The development of small inhibitor targeting the Vpr-VprBP interaction will likely contribute to a better understanding of the functions of Vpr in pathogenesis.

CONCLUSION

The mechanism by which the HIV-1 accessory protein Vpr induces G2 arrest is a complex one. A first aspect of it was solved a few years ago by the discovery that Vpr activates the canonical ATR-mediated DNA damage checkpoint. The studies presented in this thesis resolve another piece of the puzzle by identifying CRL4A(VprBP) as an essential cellular partner of Vpr. However, much work remains to fully understand how Vpr induces G2 arrest and its functional implications for viral replication and pathogenesis.

In summary, the major scientific contributions of this thesis are:

1- **Vpr engages the cellular E3 ubiquitin ligase complex CRL4A(VprBP) through an interaction with the substrate receptor VprBP.**
2- **The interaction between Vpr and CRL4A(VprBP) is required but not sufficient for the induction of G2 arrest.**
3- **Vpr hijacks CRL4A(VprBP) to induce the K48-linked polyubiquitination and proteasomal degradation of as-yet-unknown cellular proteins.**
4- **Vpr-mediated K48-polyubiquitination is necessary for ATR activation.**
5- **Vpr forms chromatin-associated foci that co-localize with VprBP and DNA repair factors.**
6- **Formation of Vpr nuclear foci represents a critical early event in the induction of G2 arrest.**
7- **Vpr associates with chromatin via its C-terminal putative substrate recognition domain and interacts with VprBP on chromatin.**

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