博士論文

Study on degradation of MCM10 by primate lentivirus accessory proteins Vpr/Vpx
（レンチウイルスアクセサリータンパク質 Vpr/Vpx による MCM10 の分解制御に関する研究）

常　浩

Distinct MCM10 proteasomal degradation profiles by primate lentiviruses Vpr/x proteins

(レンチウイルスアクセサリータンパク質 Vpr/Vpx による MCM10 の分解制御に関する研究)

Ph.D. Thesis

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March 2020

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Abbreviations

AIDS: Acquired immunodeficiency syndrome

HIV: Human immunodeficiency virus

SIV: Simian immunodeficiency virus

SIVdeb: Simian immunodeficiency virus DeBrazza' s Monkey

SIVsyk: Simian immunodeficiency virus Sykes Monkey

SIVlst: Simian immunodeficiency virus L'Hoest ' s Monkey

SIVmus: Simian immunodeficiency virus Mustached Monkey

SIVmon: Simian immunodeficiency virus Mona Monkey

SIVrcm: Simian immunodeficiency virus Red-capped Mangabey

SIVagm: Simian immunodeficiency virus African Green Monkey

SIVmac: Simian immunodeficiency virus Rhesus Monkey

SIVcol: Simian immunodeficiency virus Colobus'Monkey

LTR: long-terminal repeat

ENV: envelope

PR: polymerase

MA: matrix

CA: capsid

RT: reverse transcriptase

SU: surface envelope glycoprotein

TM: transmembrane envelope glycoprotein

PIC: pre-integration complex

Vpr: viral protein R

Vpx: viral protein X

Vpu: viral protein U

CCR5: C-C chemokine receptor type 5

CXCR4: C-X-C chemokine receptor type 4

MCM10: mini chromosome maintenance protein 10

DCAF1: DDB1 and CUL4 Associated Factor 1

DSB: Double strand Breaks

IP: immunoprecipitation

IFA: immune fluorescence assay

WB: western blotting

PBS: phosphate buffered saline

PBST: phosphate buffered saline supplemented with Tween 20

mAb: monoclonal antibody

pAb: polyclonal antibody

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PVDF: polyvinyldene difluoride

HRR: horseradish peroxidase

HA: Hemagglutinin

RIPA: Radioimmunoprecipitation assay buffer

Abstract

Background

During long term evolution of viruses driven by natural selection pressure and their antagonism against hosts, not just structural and enzymatic proteins play important roles contributing to infection and pathogenesis, various accessory proteins are also derived for increasing requirements. There is to exception to primate lentiviruses. Viral protein R (Vpr) is one of multifunctional accessory proteins among all primate lentivirus, including human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and different kinds of simian immunodeficiency viruses (SIVs).

As a small accessory protein, HIV-1 Vpr facilitates viral replication during various stages in host cells. Once encapsidated Vpr released into cytoplasm, it initiates multiple functions which are consistent with well-organized virus replication circle. HIV-1 Vpr promotes accuracy of viral genomic RNA reverse transcription, pre-integration complex (PIC) formation and nuclear localization, transcription regulation of viral and host genes, disorder of splicesome complex processing, dysregulation of cell cycle, selective inhibition of cellular pre-mRNA splicing both in vivo and in vitro and positive and negative regulation of apoptosis, activation of DNA damage response pathways. Of note, Vpr was found to hijack DCAF1 (DDB1)-Cul4-E3 ubiquitin ligase complex to lead numerous cellular targets for ubiquitination and proteasome-dependent degradation.

Minichromosome maintenance protein 10 (MCM10) is newly identified cellular target by HIV-1 Vpr, which is recruited to DCAF1-Cul4-E3 ligase for proteasome-dependent degradation. As a conserved component of eukaryotic replisome, MCM10 contributes to continuous replication process, including initiation of DNA replication, replication fork stability, DNA damage control. The multivalent properties of MCM10 are concordant with cell cycle

surveillance and also taken as a common molecular maker in cancer. Previous researches also suggested Vpr hijacked DCAF1-Cul4-E3 ligase for MCM10 degradation to induce G2/M arrest.
Objective

As mentioned above, in terms of sequence homology and functional conservation among various primate lentivirus Vpr, we wonder whether or not such properties are associated with MCM10 proteasome-dependent degradation. Therefore, firstly, we demonstrate whether MCM10 degradation also happens and is correlated with lineages of virus types. Secondly, we examine if there are other roles of Vpr on cellular target regulated by MCM10 degradation. Finally, we clarify this hijack of Vpr on E3 ubiquitin ligase for MCM10 proteolysis are also shared among human relative species such like cell lines derived from monkeys.

Materials and Methods

Phylogenetic analysis and Vpr alleles preparation: 96 full length HIV/SIV Vpr amino acid sequences were obtained from Los Alamos HIV Database and multiple alignments were performed with MUSCLE algorithm; Plasmids:11 HIV/SIV Vpr and Vpx were synthesized according to nucleotide sequences collected as mentioned above and subcloned into pcDNA 3.1 or pME18neo vectors; specific mutants of HIV-1 Vpr were conducted with standard site-directed mutagenesis kit; Immunofluorescence staining: HeLa cells or HEK293 cells were seeded on cover glasses in a 12-well plate and were transfected with HA-MCM10 either without/with lentiviruses FLAG-Vpr. They were stained with anti-FLAG rabbit or anti-MCM10 rabbit antibody and visualized with confocal fluorescence microscope; Co-immunoprecipitation assay: HEK293T cells were co-transfected with HA-MCM10 together with lentiviruses FLAG-Vprs, harvested and lysed. Total protein was incubated with anti-FLAG M2 affinity gel in binding/wash buffer. The gel was collected by centrifugation, washed and collected for Western Blot analysis; Cell cycle analysis: HeLa cells were seeded in a 6-well plate and transfected with FLAG-HIV-1 Vpr wild type and a panel of mutants mentioned above. After 48h, cells were stained with propidium iodide and performed with standard cell cycle

analysis protocol.

Results and discussion

Lentiviruses Vpr alleles preparation and sequences characterization

From HIV Database, 96 full-length Vpr amino acid sequences from divergent HIV/SIV lineages were obtained and global alignments were conducted. To cover most HIV/SIV lineages and minimum selection bias, HIV/SIV Vpr proteins derived from 10 lentivirus strains were chosen. Prototype viruses (Vpr+Vpx-Vpu-) include SIVdeb, SIVsyk, SIVlst, SIVagm and SIVcol Vprs. HIV-1, SIVmus and SIVmon Vprs present as HIV-1 type viruses (Vpr+Vpx-Vpu+). HIV-2 type viruses (Vpr+Vpx+Vpu-) were covered by SIVmac and SIVrcm.

A nuclear magnetic resonance structural analysis revealed that full length Vpr forms three amphipathic alpha helices surrounding a hydrophobic core (α-helix 1, 2 and 3). Interestingly, all lentiviruses Vpr proteins show potential zinc-binding motif (H33, H71, H76 and *78) located in α-helix 2 and 3, which is similar with conserved HIV-2 Vpx zinc-binding motif (HHCC). It was possible that zinc-binding motif was essential for function maintenance of both Vpr and Vpx.

MCM10 down-regulation profiling by primate lentiviruses Vpr/Vpx

To verify how MCM10 expression level changed under various HIV/SIV Vpr/Vpx proteins, we carried out co-transfection with HA-MCM10 and FLAG-Vpr/Vpx in HEK293T cells and monitored MCM10 expression.

The expression of HA-MCM10 was decreased by co-transfection with HIV-1, SIVmus and SIVrcm Vpr proteins while other strains failed to induce similar down-regulation. Through triplicate assays, relative intensity of MCM10 expression was calculated and normalized by

internal control Tublin. About 62% of intensity of MCM10 remained in the present of HIV-1 Vpr protein compared with that of only MCM10 control. In addition, MCM10 expression decreased to 40% and 54% in the presence of SIVmus and SIVrcm Vpr proteins, respectively. In addition, endogenous MCM10 expression level was also susceptible by HIV-1, SIVmus and SIVrcm Vpr proteins. However, because of less obvious sequence features, we speculated more than one site or site contributed to the role of MCM10 degradation.

MCM10 degradation by Vpr proteins is proteasome-dependent

To verify whether MCM10 degradation resulted from identical pathways by distinct Vpr proteins, HIV-1, SIVmus and SIVrcm Vpr proteins, MG-132, a reversible proteasome inhibitor was used to monitor the effects on MCM10 expression. At 43 h of post co-transfection of MCM10 and Vpr proteins, MG-132 or DMSO was treated into 293T and cell samples were harvest for Western Blot analysis after 48 h. For only-MCM10 control, MG-132 had little effect MCM10 expression. In contrast, HIV-1, SIVmus and SIVrcm Vpr proteins reduced MCM10 expression in DMSO-treated cultures but not MG-132-treated cultures. The results were confirmed with another proteasome irreversible inhibitor, lactacystin, which also targets the proteasome 20S. Compared to MG 132, lactacystin treatment led to a greater increase of MCM10 expression.

MCM10 interaction with HIV-1, SIVmus and SIVrcm Vpr

It is important to determine whether the MCM10 degradation by Vpr proteins mediate by the interaction between Vpr proteins and MCM10. Therefore, to demonstrate an interaction between Vpr proteins, including HIV-1, SIVmus and SIVrcm Vpr proteins, which induced MCM10 degradation, and MCM10, we performed co-immunoprecipitation using anti-FLAG beads in HEK293T cells. All the 3 FLAG tagged Vpr proteins were immunoprecipitated with HA-MCM10.

To confirm this interaction, we examined an immuno-fluorescence assay in HeLa cells. HA-MCM10 was found to be concentrated predominantly in the cellular nucleus as form of discrete replication foci. HIV-1, SIVmus and SIVrcm Vpr expression also accumulated mostly in nucleus of HeLa cells. On the other hand, distribution of Vpr proteins was observed to aggregate in dispersed form around the nucleoli. Furthermore, with merge results, the Vpr proteins partially co-localized with the MCM10 foci in the nucleus. The results suggested Vpr proteins form complex with HA-MCM10 in vivo. The interaction showed Vpr proteins interfered the MCM10 function during normal physical environment.

MCM 2-7 interaction domain of MCM10 is susceptible to degradation by Vpr

To investigate the determinant domain of MCM10 susceptible to degradate by Vpr proteins, a panel of truncation mutants of MCM10 was constructed. Recently, amino acids 530-655 of MCM10 was identified to mediate MCM10 interaction with MCM 2-7, which is essential for MCM10 nuclear localization. Here, MCM10 mutants were transiently co-transfected with 3 Vpr proteins, HIV-1, SIVmus and SIVrcm Vpr proteins, respectively. After 48 h, expression of MCM10 mutants was detected with Western Blotting and related intensity was calculated. MCM10 mutant (1-655), losing most parts of CTD but still maintaining MCM2-7 interaction domain, still were susceptible to HIV-1, SIVmus and SIVrcm Vpr. This suggested CTD of MCM10 expression maybe was not affected by Vpr proteins engagement. Collectively, region of 530-655 is a key region induced for MCM10 proteolytic consequence by Vpr.

Moreover, to verify subcellular localization alternation of HA-MCM10 mutants related to their degradation susceptibility, HEK293 cells were transfected with MCM10 mutants and subsequent IFA was performed after 48 h. MCM10 wildtype aggregated in nucleus and formed typical replication foci. In contrast, MCM10 (1-655) mainly distributed in the cytoplasm possibly resulted from lack of unidentified nuclear localization signals (NLSs) in CTD. Besides, a small fraction of MCM10 (1-655) still localized in nucleus but typical foci form disappeared. For mutants MCM10 (1-530), MCM10 (1-427) and MCM10 (1-145), localized predominantly

in cytoplasm. This suggested probably cellular distribution of MCM10 might be required for subsequent degradation

MCM10 degradation is specifically associated with HIV-1 Vpr G2/M arrest

We wonder whether other roles of Vpr also were involved positively or negatively in MCM10 degradation. Typical six kind of HIV-1 Vpr mutants were summed up and characterized. Through co-expression of HIV-1 mutants and HA-MCM10 in HEK293T cell and Western Blot analysis, we found that mutants K27M, C76A and R80A, which all lack of cell cycle blocking, reversed the MCM10 degradation compared to HIV-1 wildtype. However, P35A, W54R and R77Q, playing distinct roles but for G2/M arrest, didn't turn back MCM10 proteasome-dependent degradation. Combining quantitative data of MCM10 degradation profiling and G2/M:G1 ratio rendered by Vpr mutants, high correlation (R2=0.8589) between each function was revealed. Therefore, the correlation data demonstrated that G2/M arrest function of HIV-1 Vpr is specifically correlated with MCM10 degradation. In contrast, R77Q, as an apoptosis induction-deficient mutants, still down-regulated MCM10 expression, which showed MCM10 expression level is not affected by apoptosis function of HIV-1 Vpr. These results showed importance of relationship between MCM10 degradation and cell cycle arrest.

Uniform MCM10 degradation pattern by Vpr proteins in COS-1 cells

Moreover, we wondered whether MCM10 degradation by primate lentiviruses Vpr proteins is also conserved among different species such as monkeys. COS-1 cell, a fibroblast-like cell line, derived from African green monkey was used. Accordingly, MCM10 and Vpr proteins were co-transfected in COS-1 cells for MCM10 degradation profiling. Similarly, HIV-1, SIVmus and SIVrcm Vpr suppressed expression of MCM10 (Figure 8), which is as same as MCM10 degradation profiles in HEK293T cells. In contrast, Vpr proteins from other lineages lost ability of MCM10 degradation. This implicated E3 ligase hijacking by Vpr proteins are functionally conserved among primate species.

Conclusion

This study revealed that distinct MCM10 degradation profiles by primate lentiviruses Vpr/Vpx proteins through E3 ubiquitin proteasome-degradation pathway. Particularly, HIV-1, SVImus and SIVrcm Vpr, curbed MCM10 expression, while Vpr derived from other 8 Vpr/Vpx failed. Interestingly, co-localization and interaction of MCM10 and Vpr proteins also were observed. And MCM10 530-655 region was susceptible to degradation through proteasomal degradation pathway. For HIV-1, G2/M interruption was directly related with MCM10 degradation but other Vpr function defects did not. It's noteworthy that similar human MCM10 degradation profiles by such panel of Vpr proteins were observed in COS-1 cells. This result prompts that capacity of hijacking E3 ligase complex by Vpr proteins in both human and monkey cells is a conversed property. Our unpublished data also showed human MCM10 and Vpr proteins keep the similar co-localization pattern. However, it's still questionable whether such set of DNA damage response proteins exert synergistically a role to react by accessory protein Vpr and it's a proposed direction worth working on.

Introduction

Characterization and Replication Cycle of Primate Lentiviruses

Primate lentiviruses group, including human immunodeficiency virus type-1 and 2 (HIV-1 and HIV-2) and other originated simian immunodeficiency viruses (SIVs), belongs to the genus lentivirus(Evans and Desrosiers 2001, Clapham and McKnight 2002, Qian, Le Duff et al. 2015). Unique various strains of primate lentiviruses have been found to derived from a range of African monkey species under natural environment. During long-term historic evolution progress, primate lentiviruses has adapted with individual host and species, although viral infection not producing overt exacerbation in the hosts from most cases(Lowenstine and Lerche 1988, Kirchhoff 2009, Kirchhoff 2010, Compton, Malik et al. 2013). However, there are still exceptions that specific primate lentiviruses could lead severe diseases. For instance, SIVmac, mainly spreading within colonies of captive macaques, leads to acquired immunodeficiency syndrome (AIDS) -like pathogenically infected disease as well as HIV-1 does(Haase 2010, Patel, Borkowf et al. 2014).

According to such interesting and important properties, overall investigation and studies of primate lentiviruses enlighten novel and helpful viewpoint and understanding for HIV-1 treatment and prevention. Besides, a major bottleneck in HIV-1 vaccine development is accessible to exploit such SIV research system to test as many as possible strategies for vaccine development(Burton, Desrosiers et al. 2004, Koff, Johnson et al. 2006, Burton, Ahmed et al. 2012). Accordingly, studies of HIV-1 could undoubtedly benefit from examination of possible infection mechanisms among relevant SIVs. Following continuous cross-species transmission events, how different primate lentiviruses manipulate host factors and intercellular mechanisms to adapt of viruses into new various species, counteract host restriction factors and innate and adapt immune responses, is still unrevealed and intriguing(McMichael and Rowland-Jones

2001).

Accordingly, to understand the lifecycle of HIV-1 and related SIV is crucial for ensuing exploration of new therapeutic methods and means. Once binding onto cellular CD4 receptor and co-receptors CCR5 or CXCR4, HIV-1 fuses into the host cell surfaces. Continuously, attached HIV RNA, reverse transcriptase, integrase and other viral proteins are released into cell cytoplasm(Sattentau and Weiss 1988, Bleul, Wu et al. 1997, Rottman, Ganley et al. 1997, Tavassoli 2011). Then, viral DNA is formed by reverse transcription and formed viral DNA is transported across the nucleus and integrated into host genomic DNA. When weak immune state or other external irritates activing, latent viral DNA is transcribed as mainly two different parts: viral genomic RNA and structural or auxiliary proteins(Jeang, Xiao et al. 1999). In the end, mature virion particle is released and viral protease cleaves new polyproteins to create mature infectious virus.

Viral protein R

During the viruses evolving under natural selection pressure and host immune defense, not only structural and enzymatic proteins are required for the viral fitness maintenance and replication process, various auxiliary proteins are also derived to meet increasingly intensive needs of viruses. Typically, Human immunodeficiency virus type 1 (HIV-1) exploits auxiliary proteins for optimizing the viral particle production and escaping from the surveillance of innate and adaptive immunity by hosts, and even manipulating multiple cellular metabolism pathways for their purpose. Viral protein R (Vpr), as one of auxiliary proteins, was firstly reported via sera of HIV-1 seropositive patients(Wong-Staal, Chanda et al. 1987). Vpr is encapsidated into the virions, which implicates it may be involved in the HIV-1 early infection establishment. Although viral infectivity and cytopathic effects not interrupted totally, the CD4+ T lymphocytes and primary macrophages replications are significantly decrease compared with

the wild type(Ogawa, Shibata et al. 1989, Cohen, Dehni et al. 1990, Balliet, Kolson et al. 1994). CD4+ T lymphocytes are the main targets of HIV-1 and help Acquired immunodeficiency syndrome (AIDS) persistence and progression. On the other hand, Macrophages are regarded as the reservoir during the chronic infection of HIV-1 pathogenesis. Interestingly, Vpr deletion in HIV-2 also results in viral propagation decrease in primary macrophages(Hattori, Michaels et al. 1990, Bergamaschi, Ayinde et al. 2009). In addition to HIV-1 and -2, Vpr function among other primate lentivirus lineages are evolutionarily conserved. For example, replication of SIVagm Vpr, isolated from African green monkeys (SIVagm), are found to be required for infection of macaque primary macrophages according to the mutagenesis assay(Park and Sodroski 1995, Fletcher, Brichacek et al. 1996, Campbell and Hirsch 1997).

Once target cells were invaded by HIV-1, encapsidated Vpr is conferred to initiate its multifunction to facilitate the favorable circumstances shaping in various stages of viral life cycle (Figure 1). The main function of Vpr includes initiation and modulation of accuracy of genomic RNA reverse transcription (Mansky 1996, Stark and Hay 1998, Mansky, Preveral et al. 2000), contribution to nuclear localization of viral genomic DNA(Heinzinger, Bukrinsky et al. 1994, Mahalingam, Collman et al. 1995), transcription regulation of viral and host gene expression(Wang, Mukherjee et al. 1995, Felzien, Woffendin et al. 1998), disturbance of splicesome complex processing(Kuramitsu, Hashizume et al. 2005, Zhang and Aida 2009), induction of cell G2/M arrest and apoptosis(Rogel, Wu et al. 1995, Stewart, Poon et al. 1997), enhancement of DNA damage response (DDR)(Roshal, Kim et al. 2003, Tachiwana, Shimura et al. 2006, Iijima, Kobayashi et al. 2018). Especially, Vpr binds DCAF1 (DDB1)-Cul4-E3 ubiquitin ligase, bridged by adaptor DCAF1, thus hijacking ubiquitin conjugated degradation system for diverse host factors to final destination(Le Rouzic, Belaidouni et al. 2007). Consequently, multi-layered cellular metabolism pathways are interposed and virus-host interplays are carried out.

For one of naturally infected cell types, MDM (monocytes derived macrophage). HIV-1 Vpr also down-regulates MCM10 expression in MDM cells. However, the further study of

MCM10 in MDM or other naturally infected cells, such as CD4 cells and other subset of immunocytes.

In spite of the fact that how Vpr contributes to the viral pathogenesis are increasingly elucidated in all its bearings, the authentic responsibility during infection course is still mysterious. Nowadays, emerging cellular targets or host factors, which are degraded via DCAF1-Cul4-E3 ubiquitin ligase manipulated by HIV-1 Vpr, become a study 'hotpot' to further Vpr function research and be linked to the virus-host interactivities(Thieu, Morrow et al. 2009, Kogan and Rappaport 2011, Nodder and Gummuluru 2019). Based on the study advances of multiple targets and their dysregulation on cell metabolism and effects on viral replication, it will better our understanding of real role of Vpr and help us find novel therapeutic methods for viral intervention.

Addition to integration of latest studies of versatile function of Vpr, this review will specifically focus on the how HIV-1 Vpr affects numerous biologic processes, mainly through engaging cellular ubiquitination proteasome dependent degradation pathway. Undoubtedly, some roles of Vpr are strengthened or inhibited by DCAF1-CUL4 E3 ubiquitin ligase system. Furthermore, relying on such roles transition, it improves our acknowledgement how Vpr reinforces HIV-1 prosperous propagation in cellular targets escaps from the host surveillance.

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Mini-chromosome Maintenance Protein 10

Mini-chromosome maintenance protein 10 (MCM10) is an evolutionarily conserved protein comprising the eukaryotic replication machinery. Although lack of catalytic subunits, MCM10 associates with replication complex and facilitates their activation and initiation and becomes part of the replisome complex(Thu and Bielinsky 2013, Thu and Bielinsky 2014, Baxley and Bielinsky 2017). Firstly, MCM2-7 duplicate hexamer is loaded onto DNA and mean the formation of replication origin. Then, MCM10 binds to MCM2-7 complex in S phage and start the replication(Ricke and Bielinsky 2004, Warren, Vaithiyalingam et al. 2008). At the same time, MCM10 also stabilizes DNA and is replaced by RPA later. On the other hand, MCM10 also loads DNA polymerase alpha and this is required for generation of RNA/DNA primers for lagging Okazaki fragment synthesis.

Moreover, the important roles of MCM10 during replication forks initiation also provide a clue involved in activated DNA repair mechanisms under stress from internal and external irritates. Some studies showed MCM10 deficient cells require DSB repair. Once DSB induced, MRX complex is one of the responding proteins located in the DSB sites and initiates resultant downstream effective adaptors or scaffold proteins and Mre11/Sae2 and Sgs1/Exol also facilitate DNA repair process(Izumi, Yatagai et al. 2001). On the other hands, MCM10 also interacts with such panel of protein clusters implying MCM10 mediating DSB repair. However, more experimental evidences are needed for roles of MCM10 in DSB repair(Araki, Kawasaki et al. 2003, Szuts, Christov et al. 2005).

Recently, HIV-1 Vpr was found to enhance degradation of MCM10 via E3 ligase complex dependent proteasome degradation pathway. This implies possible unidentified roles of MCM10 involved cross-talk between MCM10 and viral pathogens(Kaur, Khan et al. 2012). In addition, VprBP (Vpr binding protein), namely DCAF1, is the substrate recognition sub-component that target MCM10 for ensuing proteasome system dependent degradation. And such E3 ubiquitin ligase complex consists of DDB1, Cullin, Cul4 and ring finger protein Roc1.

Aim of the Study

The current study firstly investigated whether MCM10 degradation by primate lentiviruses Vprs occurs and whether it was correlated with viral lineages. Accordingly, we synthesized 11 representative primate lentiviruses Vpr/Vpx based on HIV database and performed phylogenetic analyses. Furthermore, distinct MCM10 degradation profiles by primate lentiviruses Vpr/x were mapped through proteasome dependent pathway. Moreover, our finding also indicated that the MCM2-7 interaction domain of MCM10 was a determinant domain susceptible to degradation by Vpr. However, MCM10 did not alleviate DNA damage response induced by Vpr proteins. Taken together, the study characterized interaction pattern of MCM10 and various primate lentiviruses Vpr/x through E3 ligase complex hijacking.

Materials and Methods

Phylogenetic analysis of multiple alignment of primate lentiviruses Vpr

96 full length HIV/SIV Vpr amino acid sequences were obtained from Los Alamos HIV Database and multiple alignments were performed with MUSCLE algorithm implemented in MEGA 7 [22]. According to alignment results, phylogenetic trees were constructed via neighbor-joining methods with 1000 replicates bootstrap value (cut-off value≧50%). SIVcol Vpr strain was taken as a reference group. In terms of phylogenetic trees, 10 representative HIV/SIV Vpr alleles were chosen as follows: HIV-1 NL 4-3 Vpr (accession number, P12520); SIVdeb Vpr (accession number, AAT68805); SIVsyk Vpr (accession number, AAA74709); SIVlst Vpr (accession number, AAF07319); SIVmus Vpr (accession number, ABO61047); SIVmon Vpr (accession number, AAR02379); SIVrcm Vpr (accession number, AAK69677); SIVagm Vpr (accession number, AAA64260); SIVmac Vpr (accession number, AAA47636) and SIVcol Vpr (accession number, AAK01035). HIV-2 Rod10 Vpx (accession number, AYA94987) was selected as the outgroup control for alignment.

Pair-wise alignment of amino acids of both human and green monkey MCM10

The amino acids of full-length human MCM10 (reviewed) and green monkey MCM10 (unreviewed) were downloaded from the UniProt database (https://www.uniprot.org/) and pair-wise alignment was performed by Muscle Methods implemented in MegAlign and subsequent identity was also showed.

Gene Synthesis and Plasmid Construction

In order to generate 11 HIV/SIV Vpr/x expression vectors, HIV-1, SIVdeb, SIVsyk, SIVlst, SIVmus, SIVmon, SIVrcm, SIVagm, SIVmac, and SIVcol, as well as the HIV-2 Vpx, were synthesized (GENEWIZ) according to the nucleotide sequences collected as stated above and subcloned in pcDNA3.1, which contained a N-terminally linked 3 x FLAG tag (pcDNA3.1/3 x FLAG). To generate specific mutant expression vectors of HIV-1 Vpr, namely, Vpr K27M, P35A, W54R, C76A, R77Q and R80A, specific mutants were introduced into the pME18neo-internal ribosomal entry site (IRES)-zsGREEN with FLAG-Vpr (pME18neo/FLAG-Vpr-IRESZsGreen1 using a standard site-directed mutagenesis kit (TAKARA, Kusatsu, Japan) [23]. To generate N-terminally HA tagged MCM10, cDNA of MCM10 was amplified from total mRNA of HeLa cells and subcloned into pcDNA 3.1 backbone (pcDNA 3.1/HA-MCM10). Domain-deficient mutants of MCM10, namely, HA-1-165, HA-1-427, HA-1-530, and HA-1-655 were also constructed from the pcDNA 3.1/HA-MCM10.

Cell Culture, Transfection and inhibitor treatment

Human embryonic kidney HEK293T cells, HEK293 and Human cervical HeLa, COS-1 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, Beijing, China) supplemented with 10% fetal calf serum in a 5% CO2 incubator at 37°C. Plasmid transfection was performed using FuGENE HD (Promega, Madison, WI, USA). For the experiment involving proteasome reversible inhibitor MG132 (Sigma-Aldrich, St. Louis, MO, USA) and irreversible inhibitor Lactacystin (EMD Millipore, Darmstadt, Germany), cells were transfected with the indicated plasmids for 43 h before addition of the inhibitor and cultured for a further 5 h.

Co-immunoprecipitation

HEK293T cells (4 x 106) were seeded into a 100 mm dish at the day prior to transfection and transiently transfected with either 10 μg of pcDNA3.1/HA-MCM10 or 10 μg of control pcDNA3.1 together with 10 μg of pcDNA3.1/3 x FLAG-Vpr/x. At 48 h of post-transfection, cells were harvested and lysed with NP-40 lysis buffer (50 mM Tris (pH7.5)–150 mM NaCl–0.5% NP-40-1 nM DTT with 1x protease inhibitors (Roche, Mannheim, Germany)) for 30 min on ice. Next, 100 μg total protein was incubated with 20 μL anti-FLAG M2 affinity gel in 500 μL binding/wash buffer (10 mM Tris-HCl (pH 7.5)-150 mM NaCl-1% NP-40-1 mM ethylenediamine tetraacetic acid (EDTA)) overnight at 4°C with rotation (10% mix was kept for input control detection). The gel was collected via centrifugation, washed, and subjected to western blotting.

Immunofluorescence Assay

HeLa cells (2.5 x 105) or HEK293 cells (2.5 x 105) were seeded on cover glasses in a 12-well plate and transfected with 1 μg of pcDNA 3.1/HA-MCM10, with or without 1 μg of pcDNA3.1/3 x FLAG-Vpr. Following 48 h of transfection, immunofluorescence staining was performed as described previously [24]. In brief, cells on a cover slip were fixed with 4% paraformaldehyde for 10 min at room temperature. Paraformaldehyde was then replaced with cold methanol and the cells were maintained at –20°C for 20 min. The cells were then washed with PBS and incubated with anti-FLAG rabbit mAb (MBL), anti-HA mouse mAb (MBL), or anti-gamma H2AX mouse mAb (Abcam, Cambridge, UK) for 1 h at room temperature. Following further washing with PBS, Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Waltham, MA, USA) or Alexa Fluor 594 goat anti-mouse IgG (Invitrogen) was added for 1 h at room temperature in the dark. Nucleus was stained with Hoechst 33342 (Thermo Fisher Scientific) for 5 min in the dark. Coverslips were then rinsed with PBS and mounted on glass

slides. Processed samples were visualized using a confocal fluorescence microscope (IX81-FV1000-D/FLUOVIEW System, Olympus, Tokyo, Japan).

Cell-cycle Analysis

HeLa cells (1 x 105) were seeded in a 6-well plate and transfected with 2 μg of pME18neo/FLAG-Vpr-IRESZsGreen1 Vpr wild type and the panel of mutants stated above. After 48 h, the cells were harvested and fixed using 70% ethanol. After being washed twice with PBS, the cells were resuspended in RNase A (Invitrogen; 100 μg/ml) at 37°C for 20 min and stained with propidium iodide (PI; Sigma; 50 μg/ml) at room temperature for 10 min. Stained cells were analyzed using a BD AccuriTM C6 Plus with a Sampler flow cytometer (Becton-Dickinson, Franklin lakes, NJ, USA). The data were analyzed using FlowJo v10 (FlowJo, LLC, Ashland, OR, USA).

Western Blotting

HEK293T cells (2.5 x 105 cells) were seeded into a 12-well-plate at the day prior to transfection, then were transfected with 1 μg of pcDNA 3.1/HA-MCM10 with/without 10 μg of pcDNA3.1/3 x FLAG-lentiviruses Vpr/x. At 48 h following transfection, cells were harvested and lysed for western blotting. Each protein sample was boiled in 4 x SDS sample buffer and electrophoresed via 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA) using a Trans-Blot Turbo apparatus (Bio-Rad, Hercules, CA,USA). The membrane was blocked in 5% skimmed milk (in PBST [phosphate-buffered saline (PBS)/0.1% Tween-20]) for 1 h and incubated overnight at 4°C with primary antibodies, such as anti-FLAG rabbit/mouse

monoclonal antibodies (mAb; MBL Nagoya, Japan), anti-Tubulin mouse mAb (Sigma-Aldrich, Saint Louis, MO, USA) or anti-MCM10 rabbit polyclonal antibodies (pAb; Proteintech,Posemont, IL. USA), washed with PBST, and incubated with the appropriate secondary antibodies, such as anti-HRP-conjugated goat anti-mouse IgG (Amersham Biosciences, Uppsala, Sweden) or anti-HRP-conjugated goat anti-rabbit-IgG (Amersham Biosciences) at room temperature for 1 h. The chemiluminescent signal was developed using the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) and imaged using a FluorChem 5500 (Alpha Innotech, San Leandro, CA, USA). Band densities were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA)

Real-Time qRT-PCR Analysis of Human MCM10 mRNA Expression

Total RNA was extracted using the RNeasy mini kit with DNase digestion, according to the manufacturer's instructions (QIAGEN). RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher) and stored at −80 °C until use. Reverse transcription was performed using High Capacity RNA-to-cDNA (Thermo Fisher), according to the manufacturer's manual. qRT-PCR was performed using a Prism 7500FAST sequence detection system (Applied Biosystems). Samples were run in triplicate and all data were normalized to GAPDH mRNA expression as an internal control.

Statistical analysis

All data were expressed as mean ± standard deviation, based on at least 3 independent experiments. Statistical significance was evaluated using Student's t-test. Differences were

estimated to be significant at $p<0.05$(*), and strongly significant at $p<0.01$(**) and $p<0.001$(***).

Correlation efficient was analyzed with linear regression and significance was calculated with Pearson correlation analysis.

Results

Phylogeny, multiple alignments and expression of Vpr/x from representative strains

Firstly, 96 full-length Vpr amino acid sequences from divergent HIV/SIV lineages were obtained from the Los Alamos HIV Database (https://www.hiv.lanl.gov), and global alignments were conducted. Subsequently, a phylogenetic analysis was performed via neighbor-joining methods (Figure 3 A). To cover most HIV/SIV lineages and ensure minimum selection bias, HIV/SIV Vpr proteins derived from 10 lentiviruses strains were chosen as follows: Prototype viruses (Vpr+Vpx-Vpu-) were covered by SIVdeb, SIVsyk, SIVlst, SIVagm and SIVcol Vprs, and HIV-1 type viruses (Vpr+Vpx-Vpu+) including HIV-1, SIVmus and SIVmon Vprs, and HIV-2 type viruses (Vpr+Vpx+Vpu-) were covered by SIVmac and SIVrcm.

Nuclear magnetic resonance (NMR) and crystal structural analysis revealed that revealed that full length Vpr forms 3 amphipathic alpha helices surrounding a hydrophobic core (α-helix 1, 2 and 3) [25-27]. It also has a flexible, negatively-charged N terminal domain flanking the helices, while its C-terminal domain is also flexible, positively charged and rich in arginine residues. Based on the phylogeny of primate lentiviruses and virus type classification, amino acid sequences of 10 Vpr proteins from each group were analyzed using multiple alignments via the MEGA 7 program (HIV-2 Rod10 Vpx was added as the external reference). Then structure alignment was re-generated by ESPript 3.0 with HIV-1 Vpr structure as the criterion (Figure 3 B). Sequence alignments indicated that all Vpr/Vpx proteins shared conserved tertiary structures. For example, residues framed by a blue-line box depicted similarities in both sequence and structure. Additionally, such similarities were mainly enriched in 3 α-helices, including the residues 18-34, 38-49 and 54-77. Interestingly, all lentiviruses Vpr proteins displayed potential zinc-binding motifs (H33, H71, H76 and *78) located in α-helix 2 and 3, which were similar to the conserved HIV-2 Vpx zinc-binding motif (HHCC). It was suggested

that a zinc-binding motif was essential for maintaining both Vpr and Vpx. The potential zinc-binding motif of Vpr/Vpx also engaged E3 ubiquitin ligase complex formation and hijacked it to induce cellular factor degradation [28]. By contrast, flexible C-terminal domains exhibited sequence diversity compared to the central region of Vpr/Vpx proteins. Typically, HIV-2 Vpx was characterized by a poly-proline motif (PPM) in the C-terminal domain (residues from 91 to 97) whereas other lentiviruses Vpr proteins possessed few such properties.

Next, in order to compare the expression and function of these Vpr/x proteins, 11 HIV/SIV Vpr and Vpx were synthesized according to nucleotide sequences collected as stated above. Subsequently, HEK293T cells were transfected with pcDNA3.1 that encoded 3 x FLAG-tagged HIV/SIV Vpr/x proteins, namely, 3 x FLAG-HIV-1, SIVdeb, SIVsyk, SIVlst, SIVmus, SIVmon, SIVrcm, SIVagm, SIVmac and SIVcol Vprs, and HIV-2 Vpx, or the control, pcDNA3.1/3 x FLAG and examined expression via western blotting with the FLAG-mAb. Specific expression levels of all of 11 HIV/SIV Vpr and Vpx were detectable via western blotting (Figure 3 C).

MCM10 down-regulation by primate lentiviruses Vpr/x proteins

In order to verify the changes in MCM10 expression caused by various HIV/SIV Vpr/Vpx proteins, co-transfection with pcDNA 3.1/HA-MCM10 and pcDNA3.1/ 3 x FLAG-HIV/SIV Vpr/x was carried out in HEK293T cells, and HA-MCM10 expression was monitored via western blotting (Figure 4 A). Densities of the HA-MCM10 band were normalized with those of tubulin. The relative density of HA-MCM10 was decreased by co-transfection of HIV-1, SIVmus and SIVrcm Vpr proteins (Figure 4 A under panel), while other strains failed to induce similar down-regulation of MCM10. MCM10 expression due to the presence of HIV-1 Vpr protein decreased to approximately 62% compared to only-MCM10 control. In addition, MCM10 expression decreased to 40% and 54% due to the presence of SIVmus and SIVrcm Vpr, respectively. In contrast, HIV-2 Vpx did not downregulate MCM10.

To confirm whether only HIV-1, SIVmus, and SIVrcm Vpr proteins are able to induce MCM10 degradation among 11 primate lentiviruses Vpr/x, HEK293T cells were transiently transfected with 1.0 μg of pcDNA 3.1/HA-MCM10 together with 0, 0.3, 0.5, or 1.0 μg of pcDNA3.1/ 3 × FLAG-HIV/SIV Vpr/x. As predicted, HIV-1, SIVmus, and SIVrcm downregulated MCM10 expression in a dose-dependent manner (Figure 4B), while levels of MCM10 degradation by other Vpr proteins, such as SIVdeb, SIVsyk, SIVlst, SIVmon, SIVagm, SIVmac and SIVcol Vprs, and HIV-2 Vpx, were not effective in each concentration

Furthermore, we investigated whether endogenous MCM10 expression levels were also susceptible to HIV-1, SIVmus, and SIVrcm Vpr proteins. Alikely, endogenous MCM10 degradation were validated (Figure 4 C). MCM10 expression was downregulated to 62%, 79% and 63% compared to only-MCM10 control, respectively.

Next, we performed real-time qRT-PCR analysis of MCM10 mRNA expression in HEK293T cells, which were transiently transfected with either pcDNA3.1/3 × FLAG-HIV-1, SIVmus, or SIVrcm Vprs and found that MCM10 mRNA expression was detected in similar levels among transfections of HIV-1, SIVmus, and SIVrcm Vpr proteins. This result indicated that down-regulation of endogenous MCM10 by HIV-1, SIVmus, and SIVrcm were induced at protein level (Figure 4D).

Overall MCM10 down-regulation profiles and primate lentiviruses classification were comprehensively analyzed. Interestingly, all Vpr proteins belonging to prototype viruses did not enhance the downregulation of MCM10, while some HIV-1 (HIV-1 Vpr) and HIV-2 (SIVmus and SIVrcm Vpr) type viruses curbed MCM10 expression by varying degrees. However, no characteristic residues affecting MCM10 down-regulation were found, implying that maybe more than one residue from different sites corporately contributed to decreasing MCM10.

MCM10 degradation via proteasome dependent pathway

In order to verify by which pathway MCM10 degradation exploited by distinct HIV-1, SIVmus and SIVrcm Vpr proteins, MG-132, a reversible proteasome inhibitor, was used to monitor the effects on MCM10 expression. At 43 h following co-transfection of pcDNA3.1/HA-MCM10 together with pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs or the control pcDNA3.1/3 x FLAG, HEK293T cells were treated with 10 μM MG-132 or DMSO. At 48 h after transfection, HEK293T cells were harvested for western blotting. HIV-1, SIVmus and SIVrcm Vpr proteins recovered MCM10 expression in MG-132-treated cultures, as compared with that of DMSO-treated cultures (Figure 5 A). By contrast, MG-132 exerted only a minor effect on MCM10 expression in cells transfected with only the control, pcDNA3.1/3 x FLAG. This result was confirmed with another irreversible proteasome inhibitor, lactacystin, which also targets the 20S proteasome resulting in proteasomal degradation inhibition. Treatment with lactacystin (20 μM) resulted in an increase in MCM10 expression that was higher than the increase caused by MG 132 (Figure 5 B).

Interaction between MCM10 and HIV-1, SIVmus and SIVrcm Vprs

To investigate the interaction between MCM10 and Vprs, including HIV-1, SIVmus and SIVrcm Vprs, we performed co-immunoprecipitation using anti-FLAG beads in HEK293T cells transfected with either pcDNA3.1/HA-MCM10, together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus or SIVrcm Vprs (Figure 6 A). All 3 x FLAG tagged Vpr proteins were immunoprecipitated by HA-MCM10. By contrast, no specific HA-MCM10 band was detected in cells transfected with the control pcDNA3.1/ 3 x FLAG.

To confirm this interaction, we performed an immunofluorescence assay in HeLa cells. HeLa cells were transiently transfected with pcDNA 3.1/HA-MCM10 together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs, or the control pcDNA3.1/3 x FLAG,

and cells were stained with anti-FLAG mAb followed by Alexa Fluor 488 goat anti-rabbit IgG to detect Vpr (green), with anti-HA mAb followed by Alexa Fluor 594 to detect MCM10 (red), and with Hoechst 33342 to detect nucleus (blue) (Figure 6 B). HA-MCM10 was predominantly concentrated in the nucleus as a form of discrete replication foci. HIV-1, SIVmus and SIVrcm Vpr expression also accumulated mostly in nucleus of HeLa cells. On the other hand, Vpr distribution was observed to aggregate in a dispersed form around the nucleoli. Vpr proteins partially co-localized with the MCM10 foci in the nucleus in merged images (orange) (Figure 6 B).

MCM 2-7 interaction domain of MCM10 susceptible to degradation by Vprs

MCM10 is composed of different domains as follows: N-terminal domain (NTD, amino acids 1-145) responsible for MCM10 self-oligomerization, Internal domains (ID, amino acids 230-427) interacting with proliferating cell nuclear antigen (PCNA) and DNA polymerase-α (Pol-α), C-terminal domain (CTD, amino acids 596-860) that interacts with DNA and polymerase-α, MCM 2-7 interaction domain (amino acids 530-655) mediating MCM10 interaction with MCM 2-7 complex, which is also essential for MCM10 nuclear localization (Figure 7 A) [21]. To investigate the determinant domain of MCM10 susceptible to degradation by Vpr proteins, pcDNA3.1 expression vectors encoding HA tagged domain-deficient mutants of MCM10, namely, HA-1-165, 1-427, 1-530 and 1-655 were constructed (Figure 7 A) and transfected into HEK293T cells. Next, the expression and distribution of these were examined by western blotting and immunofluorescence staining, respectively (Figure 7 B and 7 C). The expression of all domain-deficient mutants of MCM10 was specifically detectable via western blotting using anti-HA MAb (Figure 7 B). Wildtype MCM10 aggregated in the nucleus and formed typical replication foci (Figure 7 C). By contrast, MCM10 (1-655) distribution detected in the cytoplasm was possibly caused by a lack of unidentified nuclear localization signals (NLSs) in CTD. Besides, a small fraction of MCM10 (1-655) was still localized in the nucleus but typical

foci formation disappeared. Mutant MCM10 (1-530), MCM10 (1-427) and MCM10 (1-165) were localized in the cytoplasm.

Next, we investigated the determinant domain of MCM10 degradation by HIV-1, SIVmus and SIVrcm Vpr. HEK293T cells were transiently transfected with either pcDNA3.1/HA-MCM10 WT, 1-165, 1-427, 1-530 or 1-655 together with either pcDNA3.1/3 x FLAG-HIV-1, FIVmus or SIVrcm Vprs and the expression of each HA-MCM10 mutant was monitored via western blotting, wherein the densities of each band were normalized with those of tubulin (Figure 8 A). The MCM10 mutant, 1-655, missing most parts of CTD but still maintaining the MCM2-7 interaction domain, was still susceptible to degradation by HIV-1, SIVmus and SIVrcm Vpr. This suggested that the CTD of MCM10 expression may not be affected by being engaged by Vprs. By contrast, the MCM mutant, 1-530, missing both MCM 2-7 interaction and CTD domains, was resistant to degradation by Vpr mediation. Besides, the truncation mutant, 1-427 and 1-165, were both resistant to downregulation by Vprs. Collectively, the region, 530-655, appears to be a key region that is responsible for MCM10 proteolysis by Vpr.

In order to confirm above results, MCM10 1-655 or 1-530, was co-transfected with 3 Vprs, following which coherent results were obtained via western blotting (Figure 8 B), where MCM10 1-655 retained susceptibility to Vprs, while MCM 1-530 showed resistance to degradation by Vprs.

MCM10 failure to alleviate DDR inducted by primate lentiviruses Vprs

Previous studies showed that MCM10 prevented DNA damage during the replication process [20]. The speculation that DNA damage response (DDR) may be involved in the degradation process led to the need to explore possible consequences of MCM10 degradation by Vprs. Variant histone H2AX (H2AX), a DDR marker, is phosphorylated (γ-H2AX) in response to

DNA double strand breaks (DSBs) by intra- or inter-pathogens or other environmental irritants. Firstly, we investigated whether these 3 Vprs provoked a DNA damage response. HEK293 cells were transiently transfected with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs, or the control pcDNA3.1/3 x FLAG, following which transfected cells were examined for γ-H2AX foci formation, using immunofluorescence staining with anti-γ-H2AX mAb. As shown, HIV-1, SIVmus and SIVrcm Vpr induced γ-H2AX foci to aggregate in the nucleus of HEK293 cells (Figure 9 A). By contrast, HEK293 cells transfected with the control vector was negative for immunofluorescence of γ-H2AX. Similarly, γ-H2AX expression in all 3 groups of HEK293T cells transfected with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus or SIVrcm Vprs was increased, while γ-H2AX expression in cells transfected with the control pcDNA3.1/3 x FLAG vector was not (Figure 9 B).

To clarify whether MCM10 alleviates DNA damage induced by the 3 Vprs, HEK293T cells were co-transfected with pcDNA 3.1/HA-MCM10 and pcDNA3.1/3 x FLAG-HIV-1, SIVmus or SIVrcm Vprs, and γ-H2AX expression was monitored via western blotting with anti-γ-H2AX mouse mAb and anti-Tubulin mAb (data not shown), wherein band densities of γ-H2AX were normalized using those of tubulin (Figure 9 C). Interestingly, γ-H2AX expression levels increased by the 3 Vprs remained unaltered following the addition of MCM10. The relative intensity of γ-H2AX expression showed no significant differences with or without MCM10.

Correlation of MCM10 degradation with HIV-1 Vpr G2/M arrest

Previously, HIV-1 Vpr was found to enhance its G2/M arrest effect by increasing MCM10 degradation via proteasome-dependent pathway[18]. However, considering that HIV-1 Vpr performs multiple functions on cellular targets, a question arose as to whether other function of Vpr also played roles in the degradation of MCM10. Firstly, 7 typical HIV-1 Vpr mutants were summed up and characterized (Figure 10 A). HIV-1 Vpr K27M, C76A and R80A curbed the

cell cycle at the G2/M phage [15, 29, 30]; K27M, R77Q and R80A impaired the function of apoptosis induction [15]; P35A specifically lacked of Vpr oligolimerization [31]; and W54R failed to interact with the host factor UNG2 [32]. Secondly, we generated the expression vectors pME18neo/FLAG-Vpr-IRESZsGreen1 encoding the HIV-1 Vpr mutants, K27M, P35A, W54R, C76A, R77Q and R80A, and transfected HEK293T cells for cell cycle analysis. The expression of all Vpr mutants confirmed by western blotting (data not shown). Three mutants, K27M, C76A and R80A, actually decreased cell cycle arrest activity of G2/M phase compared with wildtype HIV-1 (Figure 10 B), indicating the 3 mutants failed to induce cell cycle blocking.

Next, we performed co-transfection with pcDNA 3.1/HA-MCM10 and pME18neo/FLAG-Vpr, encoding HIV-1 Vpr mutants, in HEK293T cells and monitored HA-MCM10 expression via western blotting (Figure 10 C, left panel). Densities of the HA-MCM10 band were normalized with those of tubulin. The expression of HIV-1 mutants, K27M, C76A and R80A reverse MCM10 degradation, compared with that of the HIV-1 wildtype (Figure 10 C, right panel). By contrast, P35A, W54R and R77Q failed to reverse MCM10 proteasome-dependent degradation.

Finally, quantitative data related to MCM10 degradation profilies and G2/M:G1 ratios, associated with Vpr mutants, indicated a high correlation ($R2=0.8589$; $P=0.0009$) with each of the functions tested (Figure 10 D). The G2/M arrest function of HIV-1 Vpr was specifically correlated with MCM10 degradation. By contrast, R77Q, an apoptosis induction-deficient mutant, downregulated MCM10 expression, demonstrating that MCM10 expression levels were not affected by the apoptosis function of HIV-1 Vpr. Another mutants P35A, which lost function of Vpr oligolimerization, also failed to reverse MCM10 degradation.

MCM10 degradation by primate lentiviruses Vpr proteins is highly conserved between human and green monkey

With unique MCM10 degradation profiling by various primate lentiviruses Vpr proteins, we also wondered if such E3 ubiquitin ligase complex hijacking by lentiviruses Vpr also exist in other human-like species. Then we co-transfected MCM10 and Vpr protein in COS-1 cells, one cell line derived from African Green Monkeys. Interestingly, we also found the same down-regulation profiles of MCM10 suffered from primate lentiviruses Vpr. HIV-1, SIVmus and SIVrcm Vpr down-regulated MCM10 expression with detection by Western Blotting at 48 h of post-transfection (Figure 11 A). Furthermore, according to our pair-wise alignment, we found MCM10 was high conserved between human and green monkey. With full-length MCM10 alignment, the identity of MCM10 between human and green monkey is 96.7%. Besides, we also dissected the newly found MCM2-7 interaction domain of MCM10 proteins is consistently conserved (Figure 11 B). The identity of MCM2-7 interaction domain of both MCM10 proteins is more than 95%. This results revealed E3 hijacking by Vpr proteins is high conserved among primate species, which also implied the importance of E3 ligase dependent degradation pathway manipulated by Vpr proteins, and also implicated E3 ubiquitin related host factors-Vpr may provide a new viewpoint explain the co-evolutionary process between hosts and viruses.

Discussion

Previous studies have indicated that HIV-1 Vpr increased MCM10 degradation by manipulating DCAF1-Cul4-E3 ubiquitin ligase for proteasome dependent degradation pathway and that such degradation was related to Vpr-mediated G2/M arrest. However, it was unclear whether various primate lentiviruses Vprs also complied with MCM10 proteasome dependent degradation. The current study reached 3 major conclusions regarding to MCM10 degradation pattern by various primate lentiviruses Vpr proteins. Firstly, the study revealed that MCM10 degradation resulting from identical proteasome pathways was caused by distinct SIVmus and SIVrcm Vprs, in addition to HIV-1Vpr. However, Vpr proteins derived from prototype virus lineages lost ability of MCM10 degradation, implying MCM10 degradation associated with species specificity of Vprs. Secondly, our results demonstrated that MCM10 interacted with HIV-1, SIVmus and SIVrcm Vprs. And the MCM2-7 interaction domain of MCM10 was the determinant region susceptible to degradation by these 3 Vprs. Thirdly, our data showed although the γ-H2AX expression levels increased by the 3 Vprs remained unaltered following overexpression of MCM10 in 293T cells, suggesting that MCM10 did not alleviated DNA damage response induced by the 3 Vprs.

In this study, according to phylogenetic outcomes, 10 representative Vpr proteins from different primate lentiviruses lineages were selected and synthesized. Interestingly, a potential zinc-binding motif (H33, H71, H76 and *78) [33] was found among α-helices 2 and 3 in the Vpr proteins from diverse virus strains. Multiple zinc-binding regions involved in viral proteins are indispensable for negotiating with host factors. For instance, the zinc-binding region (HX5CX17-18CX3-5H) of HIV-1 Vif mediated interaction with Cul5 E3 ligase to exert ubiquitination targeting APOBEC3G [34, 35]. Two other zinc-binding sites in the nucleocapsid (NC) of HIV-1 also play an important role in the interaction with nucleus acids of PSI RNA and the eventual promotion of HIV-1 genomic RNA packaging into virus particles [36, 37]. However, there is little evidence indicating whether such Vpr sequences mimic the full role

played by typical HHCC zinc-binding motifs. Recently, some studies have revealed that the HHCH motif of HIV-1 Vpr, which is in a position parallel to that of HIV-2 Vpx, may show capacity to interact with the E3 ligase complex [28]. However, whether the potential zinc-binding motif contributes to the function of Vprs remains unclear.

Actually, due to convenience of sequence obtaining, many Vpr or Vpr/x phylogenetic trees are found in may research works. However, according to the differences of sequences (amino acid or nucleotide sequences; number of sequences; full-length or partial sequences alignments); differences of methods (neighbor joining, max-likelihood or other methods; boot strap replication value setting); different softwares and algorithms, the results of alignment is still different. And our results also are similar but not same due to special handling methods.

Among Vprs from 10 lineages, HIV-1, SIVmus and SIVrcm Vprs were identified to curb specifically expression of MCM10 with varying capacities, while other strains failed to do so. Besides, MCM10 was co-localized with such Vprs in the nucleus and formed complexes with them. Interestingly, it is suggested that Vprs originating from prototype viruses are unable to induce downregulation of MCM10. Multiple alignment results indicated that there were no distinguishable sequences or point features that would enable differentiation of the capacity to degrade MCM10. Accordingly, it is speculated that more than one amino acid, or a combination of amino acids, in distinct virus strains may perform the function of MCM10 degradation. However, more proof is required to determine whether Vpr proteins of whole prototype virus lineages exhibit MCM10 degradation properties.

Vpr does not interact with E3 ligase complex directly. Their interaction needs some adaptor molecules, including DDB1 and DCAF1. However, whether direct interaction between Vpr and host factors depends individual situations. For MCM10, such evidence showed one of HIV-1 Vpr mutants Q65R, losing ability of Vpr binding to DCAF1, failed to interact with MCM10 in co-IP assay. It suggests DCAF1 is required of Vpr-MCM10 interaction. And the DCAF1-siRNA assay also confirm the results.

The region encompassing amino acids 530-655, also known as the MCM2-7 interaction domain, was mapped as a determinant domain of MCM10 degradation under induction by Vpr [20, 38]. Previously little was known about the 530-633 region of MCM10, identified as a newly identified functional domain, flanked by an ID and involved in parts of CTD. This region exhibits little sequence characterization or secondary structures, by way of sequence alignments and structure prediction, in spite of the compositional bias of hydrophobic amino acids [39, 40]. This flexible region is also partly responsible for the intrinsically disordered proteins (IDP). Some studies revealed that IDPs adopted multiple structures and were inclined to enfold, thus mediating binding with other targets of interest [41].

Once released into target cells, a virion-associated HIV-1 Vpr initiates multiple functions to facilitate its replication. MCM10 degradation by HIV-1 Vpr was found to induce G2/M cell arrest in Hela cells [18]. However, whether Vpr played other roles in MCM10 degradation remain unknown. Therefore, we constructed a series of functionally deficient Vpr mutants and performed MCM10 degradation profiling. Our mutagenesis assay indicated that the G2/M cell cycle, instead of apoptosis induction, oligolimerization or nuclear localization, was correlated with MCM10 expression. This finding supported the key role played by MCM10 in cell cycle modulation.

For the MCM10 alternation under pressure from exogenous viral pathogens or other stimulates, there is little evidence about their relationship. Therefore, I could provide a kind of viewpoints from the functions or roles of both to speculate possible mechanism. MCM10 was found to form complex or axis regulate DNA replication and repair (B Mitto 2014; YM Thu 2014). And Vpr plays an important role in MDM cell or other none dividing cells (MA Vodicka Genes & development; RI Conor Virology). According to such accumulated works, we think whether Vpr strengths DNA damage through antagonism against subset of DNA repaired proteins such as MCM10, UNG2, Exol1 to induce dysregulation of main target immunocytes to potentiate replication and reservoir maintenance.

Diverse viruses negotiate with and eliminate cellular factors, by exploiting host metabolism pathways to facilitate virus replication and escape host immune surveillance. Particularly, the ubiquitin dependent degradation pathway, was one of the attractive machineries manipulated by multiple accessory proteins, such as, Vif, Vpu and Vpx, of primate lentiviruses. Particularly, Vpr was found to invoke increasingly numerous host factors for proteasome dependent degradation, such as MCM10, MUS81, helicase-like transcription factor (HLTF), Exonuclease 1(Exo1) and histone deacetylases (HDACs) [42-46]. Interestingly, these cellular targets also respond to DNA damage from viruses or other environmental stimulants. For instance, HLTF labels the proliferating cell nuclear antigen (PCNA) with Lys-63 polyubiquitin chain to reverse leading strand replication with that of the lagging strand, rather not damaged template at replication level error correction. Exo1, another target of HIV-1 Vpr, was also depleted via the proteasomal degradation pathway. Vpr may possibly load Exo1 onto the E3 ligase complex and remodel the post-replication DNA repair machinery independently of PCNA bridging. However, the correlation between Exo1 depletion by Vpr and DNA damage response remains unknown [46, 47].

Little evidence or research focus on the viral accessory protein degradation. However, for Vpr, I did not think Vpr was affected by E3 ubiquitin ligase complex dependent degradation pathway. With our results of 20S proteasome inhibitors assay, including MG132 and lactacystin. 3 Vpr proteins, HIV-1, SIVmus and SIVrcm Vpr expression were not affected by both inhibitors targeting against proteasome degradation pathway. In conclusion, we suggest exogenous Vpr (with transfection) was not down-regulated or degraded by 20S proteasome inhibitors or proteasome degradation pathway involved.

We find 2 full-length MCM10 amino acid sequence including human (reviewed sequence meaning reliable sequence information; 875aa)and green monkey (not reviewed sequences and less reliable sequence information; 874aa). For the full-length alignments, percent identity is 96.7%. For the MCM 2-7 binding sites domain (530-655 aa), the identity is also more than 95% (120/126), and most substitution are similar ones. Therefore, we suggest MCM10 is highly

conserved among human and monkey species and MCM10 degradation by E3 ligase complex hijacked by Vpr is common pathway existing in human and monkeys.

DNA damage and late S/G2 phase arrest are induced through MCM10 siRNA treated cells. During replication, MCM10 depletion supposedly blocks the synthesis of the lagging DNA strand, and the subsequent replication fork stalling also generates phosphorylated H2AX. The DSB signal cascade leads to cell cycle arrest finally [48, 49]. In addition, HIV-1 was found to enhance MCM10 degradation which initiated G2/M cell cycle blocking [18]. Mutagenesis and flowcytometry assays also revealed that the degree of MCM10 degradation was correlated with cell cycle arrest with HIV-1 Vpr. However, in our study, MCM10 alone does not alleviate the DNA damage induced by 3 Vpr proteins, suggesting complexity of DNA modulation exposing to viral pathogens. It is still unclear that wheth er primary role of Vpr on DNA damage exceeds resultant DNA repair machinery activation to favor the purpos e of virus replication. Taken together, the results of our studies highlight distinct interplay model of host factor MCM10 with various primate lentiviruses Vpr proteins and their ensuing roles on physiological alternation of cellular targets partly. This research enlightens model of primate lentiviruses Vprs antagonism against increasingly found host factors and “arm-race” of host-virus coevolution.

Conclusions

This study revealed that distinct MCM10 degradation profiles by primate lentiviruses Vpr/Vpx proteins through E3 u biquitin proteasome-degradation pathway. Particularly, HIV-1, SVImus and SIVrcm Vpr, curbed MCM10 expre ssion, while Vpr derived from other 8 Vpr/Vpx failed. Interestingly, co-localization and interaction of MCM 10 and Vpr proteins also were observed. And MCM10 530-655 region was susceptible to degradation through proteasomal degradation pathway. For HIV-1, G2/M interruption was directly related with MCM10 degradation but other Vpr function defects did not. It's noteworthy that similar human MCM10 degradation profiles by such panel of Vpr proteins were observed in COS-1 cells. This result prompts that capacity of hijacking E3 ligase complex by Vpr proteins in both human and monkey cells is a conversed property. Our unpublished data also showed human MCM10 and Vpr proteins keep the similar co-localization pattern. However, it's still questionable whether such set of DNA damage response proteins exert synergistically a role to react by accessory protein Vpr and it's a proposed direction worth working on.

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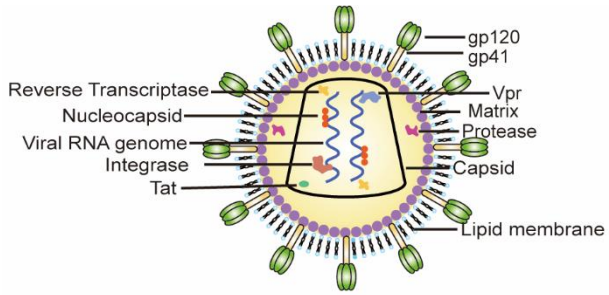
Figures and Tables

A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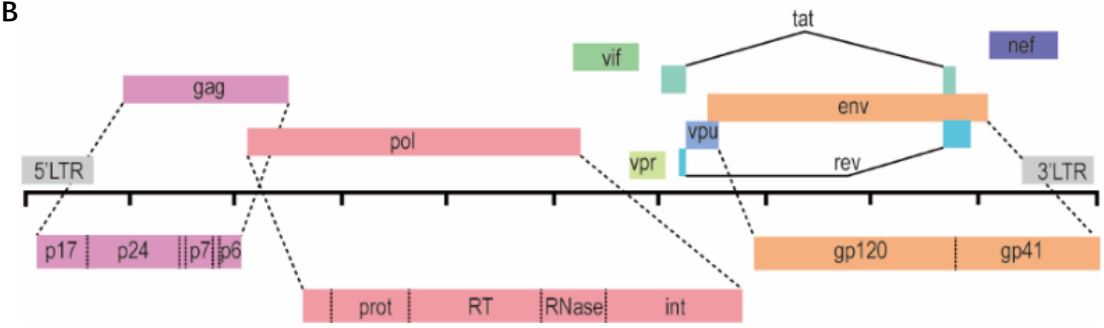
Nucleocapsid
Viral RNA genome
Integrase
Tat
Vpr
Matrix
Protease
Capsid
Lipid membrane

B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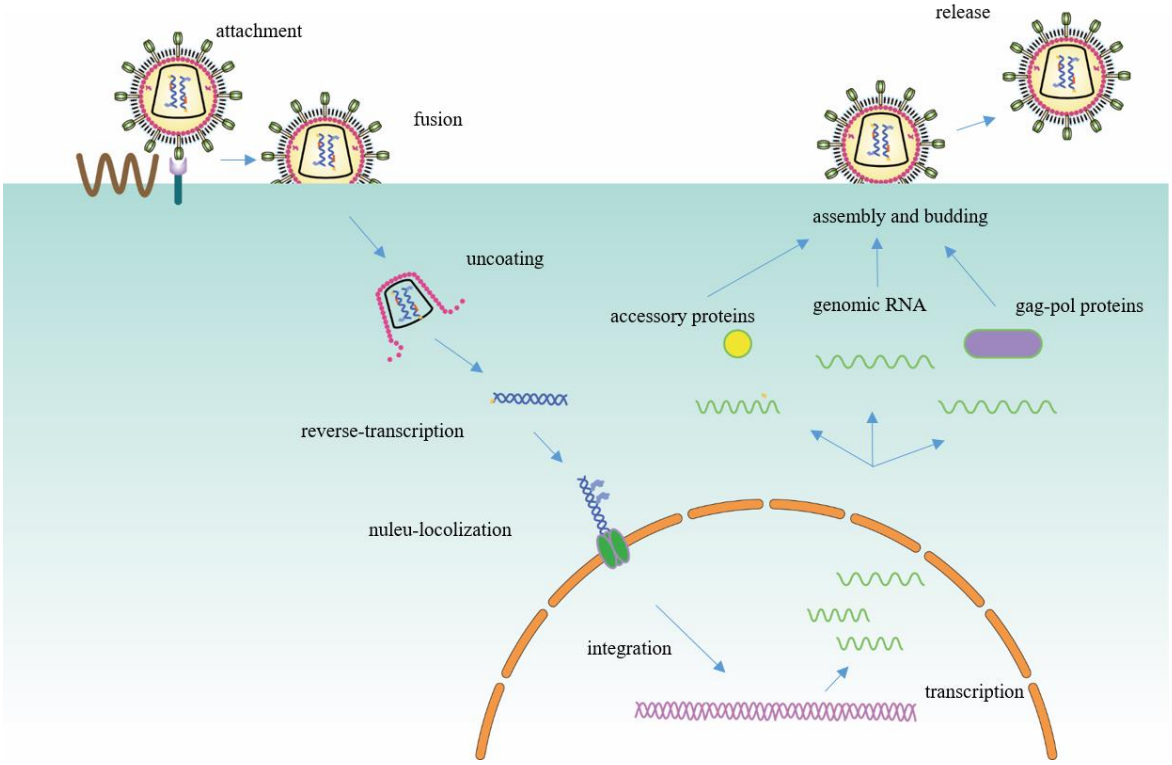
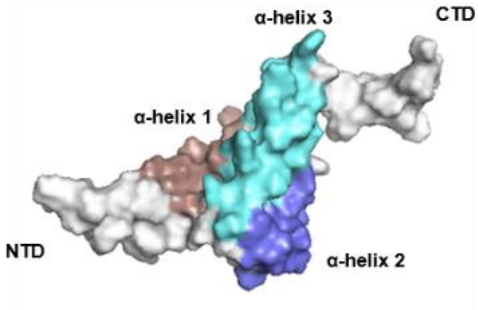

attachment
fusion
release
assembly and budding
uncoating
accessory proteins
genomic RNA
gag-pol proteins
reverse-transcription
nuleu-locolization
integration
transcription

Figure1 Structure and lifecycle of HIV-1 virion during infection. (A) (B). Schematic diagram of structure and ge nomic composition of HIV-1 virus particle. HIV-1 is composed of 2 positive-sense single strand RNA packaged in capsid proteins composed of the viral protein p24. Besides, such RNA sequences also bind onto nucleocapsid and other enzymatic proteins such as integrase and reverse transcriptase. Matrix proteins of HIV-1 composed of p17 surrounds the capsid. Out of matrix proteins, the envelope glycoproteins, containing gp120 and gp41, are on the surface of virus. (C) HIV-1 infection progress during the target cells. Complete HIV-1 virion binds to CD4+ receptor and co-receptors with its envelop gp120, then fuses into cellular membrane via gp41 engagement. Once entering into cellular environment, viral RNA pairs comes out of capsid of virus and are reverse transcribed into DNA and integrate into host genomic DNA. During exogenous irritates or weak immune situations, viral DNA is transcribed into complete viral RNA and essential structural/accessory proteins. Such combination aggregates in cellular membrane and packages into intact virion.

A


α-helix 3
CTD
α-helix 1
NTD
α-helix 2

B

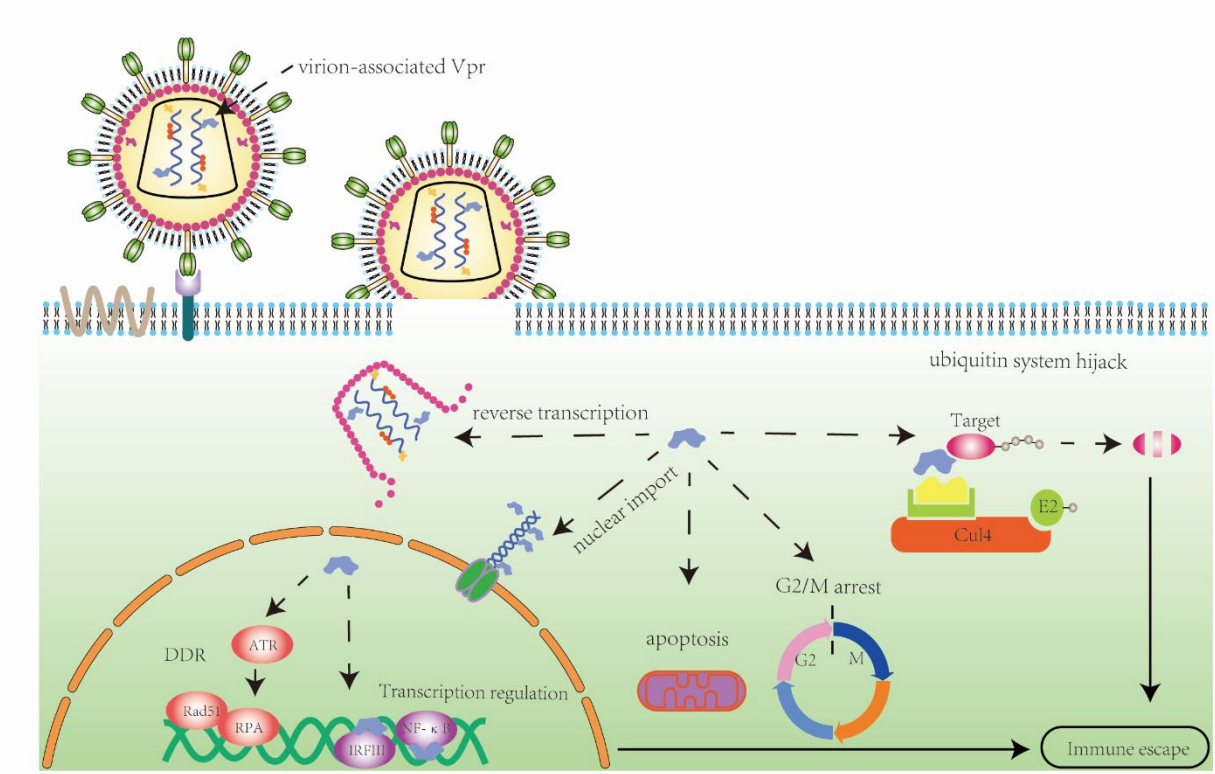
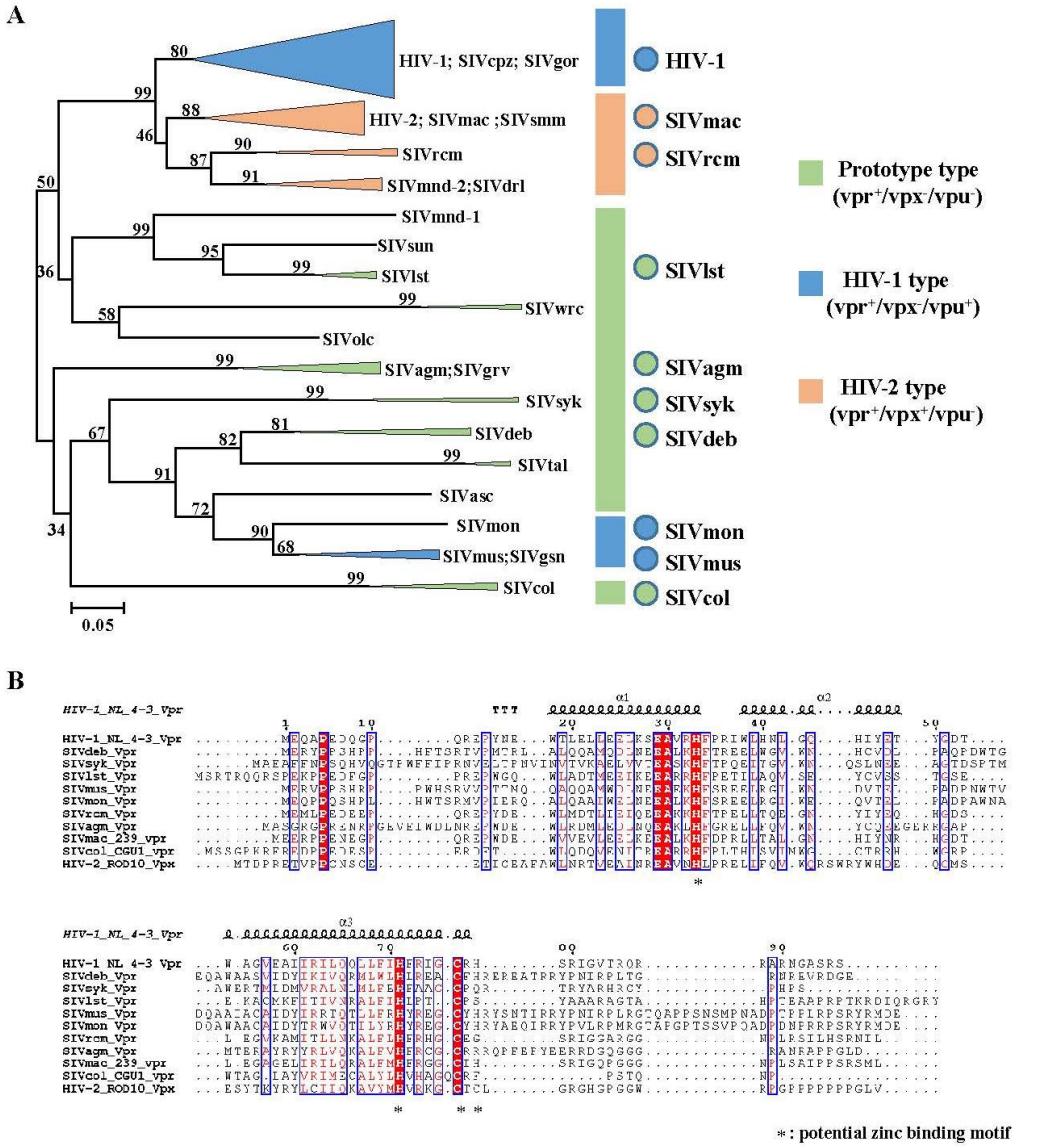

virion-associated Vpr
ubiquitin system hijack
reverse transcription
Target
E2
Cul4
nuclear import
G2/M arrest
apoptosis
G2
M
DDR
ATR
Rad51
RPA
Transcription regulation
IRF III
NF-κB
Immune escape

Figure 2 Schematic illustrations of HIV-1 Vpr proteins and its multiple function consistent with viral life cycle (A). The complete structure of HIV-1 Vpr was firstly determinated by nuclear magnetic resonance (NMR) in 2003. This 96-amino acid small protein is composed of 3 centrally located α-helices, spanning among 17-33,38-50 and 55-77 residues. The amphiphilic helices are flanked by flexible terminal domains. (B). Once target cells were invaded by HIV-1, encapsidated Vpr is conferred to initiate its multifunction to facilitate the favorable circumstances shaping in various stages of viral life cycle The main function of Vpr includes initiation and modulation of accuracy of genomic RNA reverse transcription, contribution to nuclear localization of viral genomic DNA, transcription regulation of viral and host gene expression, disturbance of splicesome complex processing, induction of cell G2/M arrest and apoptosis, enhancement of DNA damage response (DDR). Especially, Vpr binds DCAF1 (DDB1)-Cul4-E3 ubiquitin ligase, bridged by adaptor DCAF1, thus hijacking ubiquitin conjugated degradation system for diverse host factors to final destination.



*: potential zinc binding motif

Figure 3. Phylogeny of 96 primate lentiviruses Vprs and multiple alignment and expression of Vpr/x selected from representative strains. (A) Phylogenetic tree were constructed from 96 full-length HIV/SIV Vpr amino acid sequences via neighbor-joining methods using 1000 bootstrap replicates. Scale bars depict genetic distance. Representative Vprs from 10 different lineages were selected as later alignment candidates. These originated from viruses belonging to 3 different groups containing HIV-1 type (HIV-1, SIVmus and SIVmon), prototype (SIVdeb, SIVsyk, SIVlst, SIVagm and SIVcol) and HIV-2 type (SIVmac, SIVrcm and HIV-2), which are shown in blue, green and orange, respectively. (B) Sequence alignments of candidate HIV/SIV Vprs and HIV-2 Vpx. HIV-1 Vpr was chosen as standard sequence, and HIV-2 Vpx as outgroup control. Alignments of HIV/SIV Vpr/x showed sequence and structural conservation, characterized by 3 α-helices and a potential zinc-binding motif among lentiviruses Vpr/x, indicated by the reference structure, HIV-1 NL 4-3, at the top of alignments. (C) Expression of 10 HIV/SIV Vprs and 1 HIV-2 Vpx. HEK293T cells were transfected with pcDNA3.1that encoded 3 x FLAG-tagged HIV/SIV Vpr/x proteins. Transfected cells were harvested at 48 h following transfection and lysates with the equal protein amounts were subjected to western blotting.

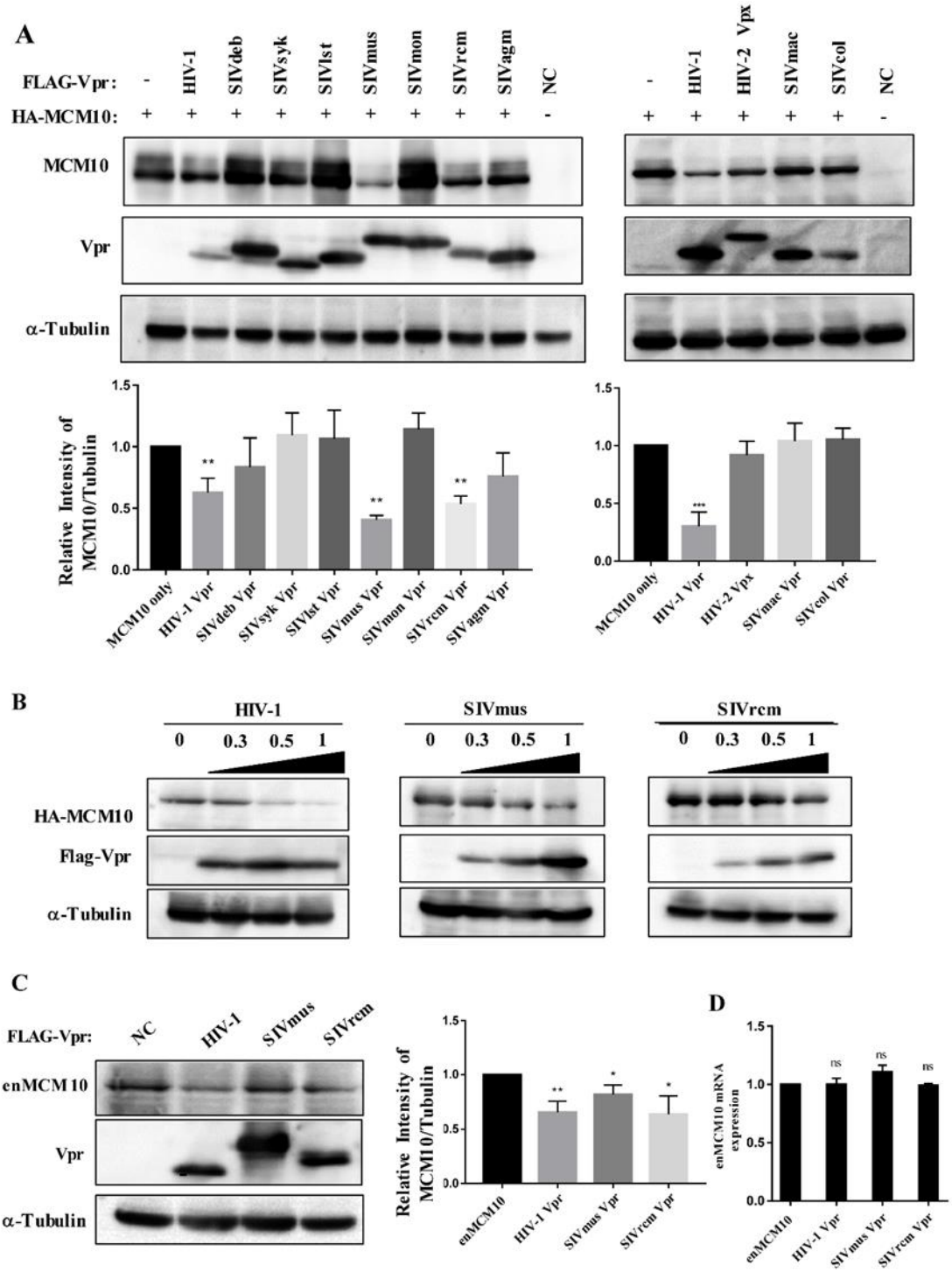

A
FLAG-Vpr:
HA-MCM10:
HIV-1
SIVdeb
SIVsyk
SIVlst
SIVmus
SIVmon
SIVrcm
SIVagm
NC
HIV-2 Vpx
SIVmac
SIVcol
MCM10
Vpr
α-Tubulin
Relative Intensity of MCM10/Tubulin
MCM10 only
HIV-1 Vpr
SIVdeb Vpr
SIVsyk Vpr
SIVlst Vpr
SIVmus Vpr
SIVmon Vpr
SIVrcm Vpr
SIVagm Vpr
HIV-2 Vpx
SIVmac Vpr
SIVcol Vpr
B
HIV-1
SIVmus
SIVrcm
0
0.3
0.5
1
HA-MCM10
Flag-Vpr
α-Tubulin
C
FLAG-Vpr:
NC
HIV-1
SIVmus
SIVrcm
enMCM10
Vpr
α-Tubulin
Relative Intensity of MCM10/Tubulin
enMCM10
HIV-1 Vpr
SIVmus Vpr
SIVrcm Vpr
D
enMCM10 mRNA expression
ns

Figure 4. Downregulation of exogenous and endogenous MCM10 by HIV-1, SIVmus, and SIVrcm among 11 primate lentiviruses Vpr/x. (A) Downregulation of exogenous MCM10 by 11 primate lentiviruses Vpr/x. HEK293T cells were transiently transfected with pcDNA 3.1/HA-MCM10 together with either pcDNA3.1/3 × FLAG-HIV/SIV Vpr/x, or the control pcDNA3.1/3 × FLAG (NC: negative control). Transfected cells were harvested at 48 h after transfection and lysates with the equal protein amounts were subjected to western blotting with anti-FLAG mouse monoclonal antibodies (mAb), anti-HA mouse mAb, anti-Tubulin mouse mAb (upper panel). The positions of 3 × FLAG-Vpr/x, HA-MCM10 andα-Tubulin are indicated. Band densities of HA-MCM10 and α-Tubulin were quantified by densitometry analysis using ImageJ software. The relative intensities were calculated as the ratio of density of MCM10 to density of α-Tubulin. Each column and error bar represents the mean ± SD of three independent experiments (under panel). (B) A dose-dependent manner of downregulation of exogenous MCM10 by HIV-1, SIVmus, and SIVrcm Vprs. HEK293T cells were transiently transfected with pcDNA 3.1/HA-MCM10 together with 0, 0.3, 0.5, or 1.0 μg of either pcDNA3.1/3 × FLAG-HIV-1, SIVmus or SIVrcm Vprs. The positions of 3 × FLAG-Vpr/x, HA-MCM10 and tubulin are indicated (upper panel). (C,D) Downregulation of endogenous MCM10 protein by HIV-1, SIVmus, and SIVrcm at protein level. HEK293T cells were transiently transfected with either pcDNA3.1/3 × FLAG-HIV-1, SIVmus, or SIVrcm Vprs, or the control pcDNA3.1/3 × FLAG (NC: negative control). After 48 h, endogenous MCM10 protein was examined using western blotting with anti-MCM10 rabbit polyclonal antibodies, anti-FLAG mouse mAb, and anti-Tubulin mouse mAb (C), and MCM10 mRNA expression was evaluated using Real-time qRT-PCR analysis (D). (C) The positions of 3 × FLAG-Vpr, endogenous MCM10 and α-Tubulin are indicated. Band densities of endogenous MCM10 and α-Tubulin were quantified by densitometry analysis using ImageJ software. The relative intensities were calculated as the ratio of density of MCM10 to density of α-Tubulin. Each column and error bar represents mean ± SD for three independent experiments (right panel). The asterisk indicates a statistically significant difference (* $p < 0.05$, ** $p < 0.01$). (D) Samples were run in triplicate and all data were normalized to GAPDH mRNA expression as an internal control.

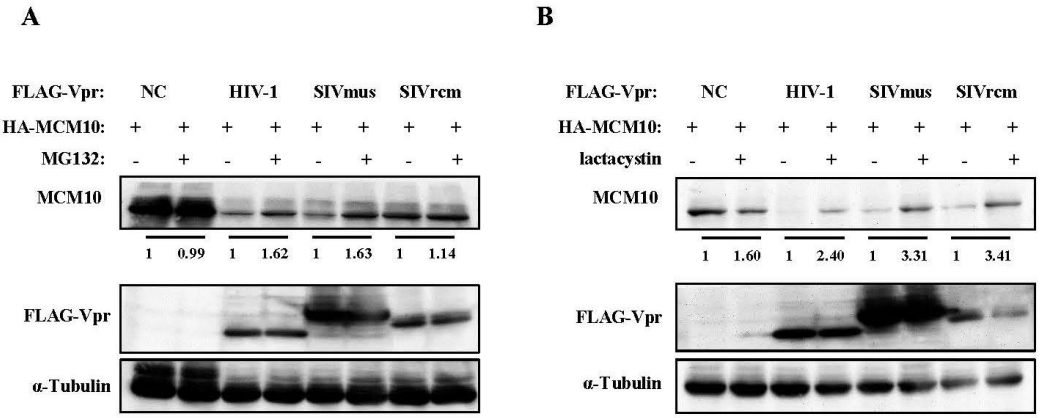


Figure 5. MCM10 degradation by HIV-1, SIVmus and SIVrcm Vprs via proteasome dependent pathway. HEK293T cells were transiently transfected with pcDNA 3.1/HA-MCM10 together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs, or the control, pcDNA3.1/3 x FLAG. After 43 h of transfection, cells were treated with 10 μM MG132 (a reversible proteasome inhibitor) or DMSO (A), and another irreversible proteasome inhibitor, lactacystin (20 μM) or DMSO (B). Cells were harvested at 48 h after transfection and lysates with equal protein amounts were subjected to western blotting. The molecular mass positions of 3 x FLAG-Vpr, HA-MCM10 and tubulin are shown.

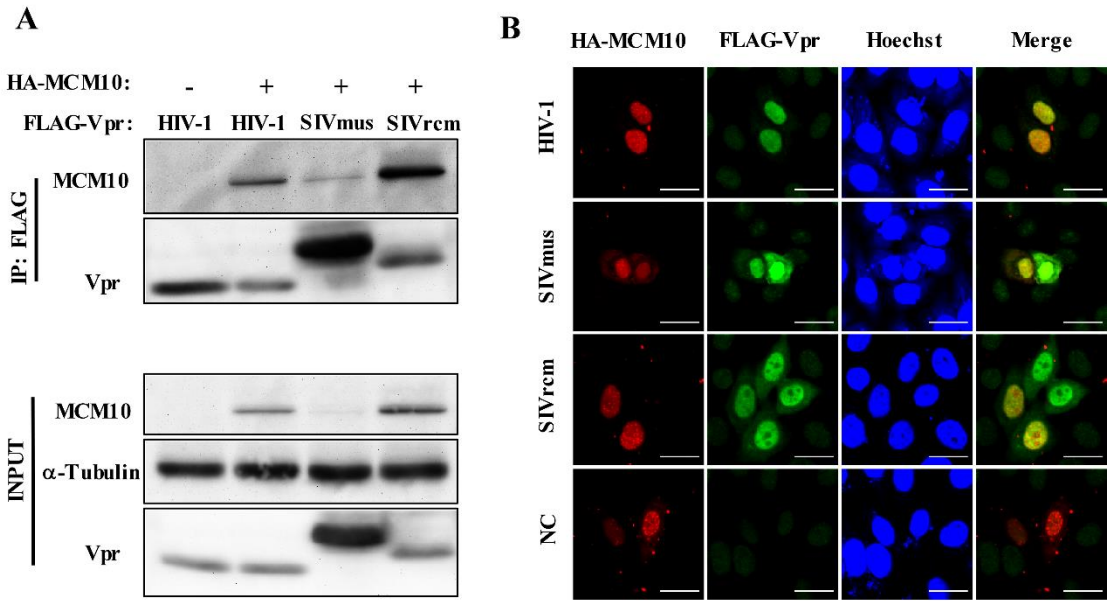
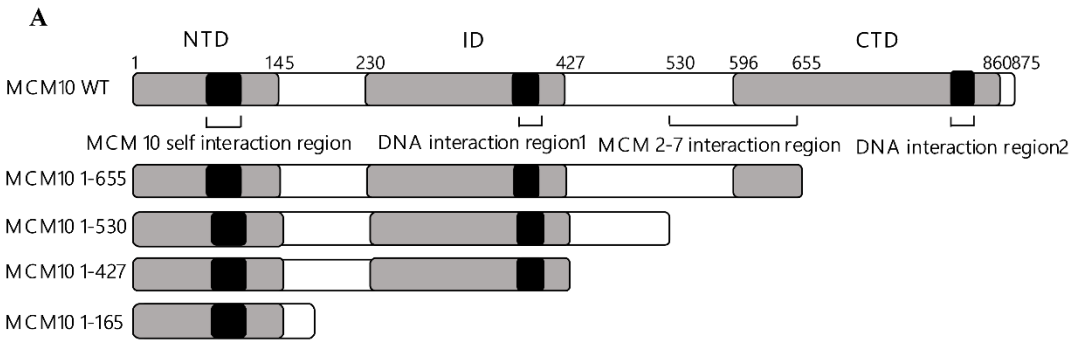


Figure 6. MCM10 interacts and co-localizes with HIV-1, SIVmus and SIVrcm Vprs. (A) HEK293T cells transiently transfected with either pcDNA 3.1/HA-MCM10, or the control, pcDNA 3.1, together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus or SIVrcm Vprs. At 48 h following transfection, cell lysates were collected and incubated overnight with anti-FLAG agarose beads. Subsequently, Cell lysate inputs and agarose beads were collected, washed and subjected to western blotting. Molecular mass positions of 3 x FLAG-Vpr, HA-MCM10 and tubulin are indicated. (B) HeLa cells on the cover glass were transiently transfected with pcDNA 3.1/HA-MCM10 together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs, or the control, pcDNA3.1/3 x FLAG. At 48 h following transfection, cells were stained with anti-FLAG rabbit mAb followed by Alexa Fluor 488 goat anti-rabbit IgG to detect Vpr (green), with anti-HA mouse mAb followed by Alexa Fluor 594 to detect MCM10 (red), and with Hoechst 33342 to detect nucleus (blue), and observed using a FV-1000 fluorescence microscope. Merged images (orange) indicate localization pattern of both proteins. Bar=20μm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NTD
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CTD
1
145
230
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530
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655
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MCM10 WT
MCM 10 self interaction region
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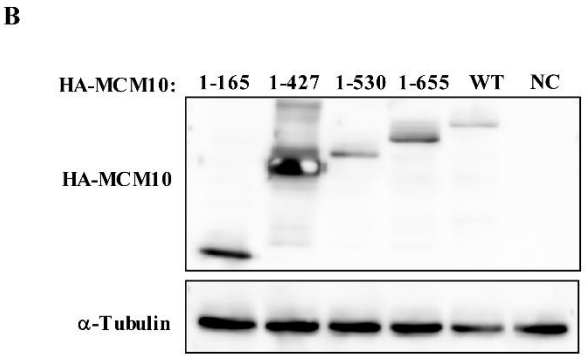
MCM10 1-165


B
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1-165
1-427
1-530
1-655
WT
NC
HA-MCM10
α-Tubulin

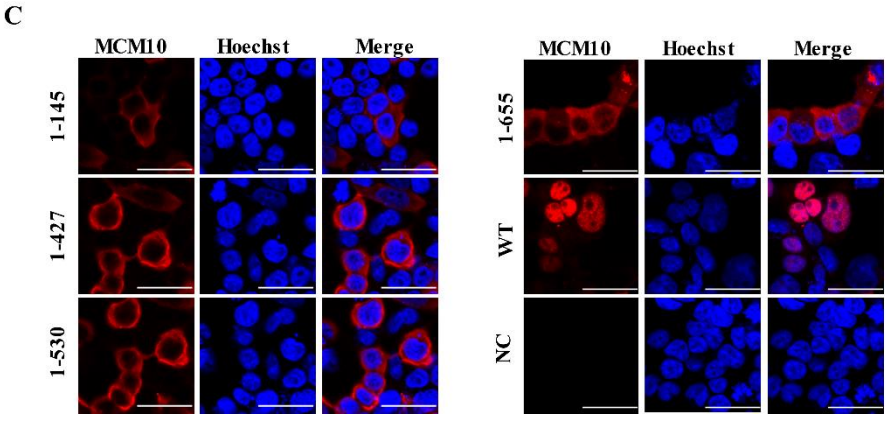

C
MCM10
Hoechst
Merge
1-145
1-427
1-530
MCM10
Hoechst
Merge
1-655
WT
NC

Figure 7. Expression and localization of MCM10 mutants. (A) Schematic diagram shows the domain structures of wildtype and MCM10 truncation mutants. Positions of the predicted N-terminal domain (NTD,1-165), internal domain (ID, 230-427), C-terminal domain (CTD, 596-860), recently identified MCM2-7 interaction domain (530-655). MCM10 self-interaction region involved in NTD and 2 DNA interaction regions located in ID and CTD are indicated. (B) Expression of MCM 10 mutants. HEK293T cells were transiently transfected with pcDNA3.1/HA-MCM10 WT, 1-165, 1-427, 1-530 or 1-655, respectively. Transfected cells were harvested at 48 h of post-transfection and lysates with the equal protein amounts were subjected to western blotting. Molecular mass positions of HA-MCM10 and tubulin are indicated. (C) Subcellular distribution of MCM 10 mutants. HEK293 cells were transiently transfected with either pcDNA3.1/ MCM10 HA-WT, HA-1-165, HA-1-427, HA-1-530 or HA-1-655 and the subcellular distribution of MCM 10 mutants was determined via immunofluorescence staining with anti-HA mouse mAb at 48 h post-transfection. The nucleus was stained with Hoechst 33342 and observed using an FV-1000 fluorescence microscope. Bar=20μm

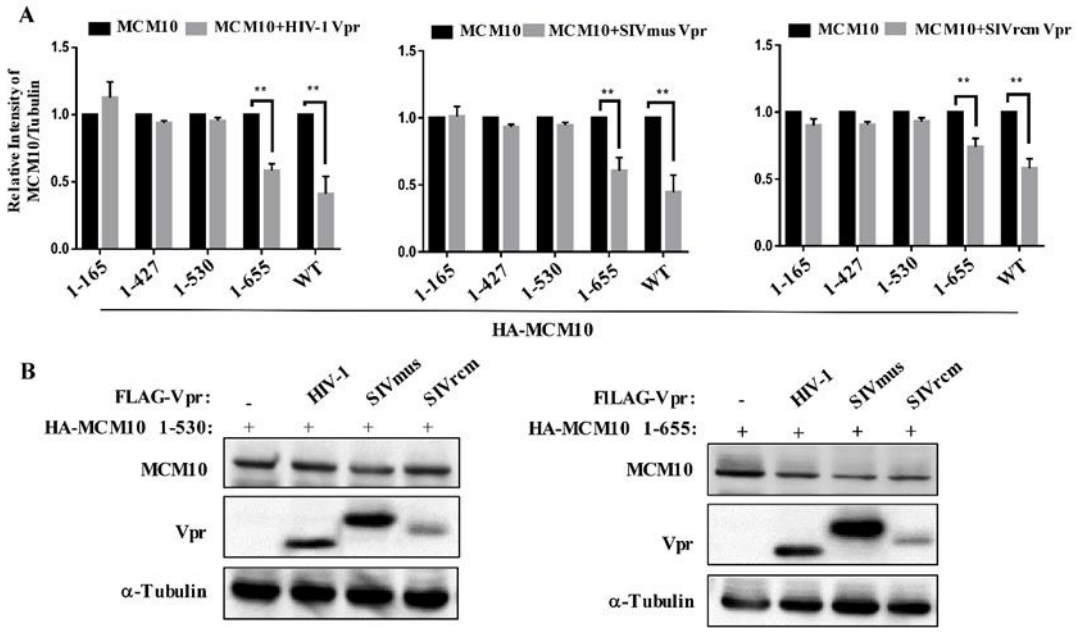


Figure 8. MCM 2-7 interaction domain of is susceptible to degradation by HIV-1, SIVmus and SIVrcm Vprs. (A) HEK293T cells were transiently transfected with either pcDNA3.1/HA-MCM10 WT, HA-1-165, HA-1-427, HA-1-530 or HA-1-655 together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus or SIVrcm Vprs. The cells were then harvested at 48 h after transfection and lysates with equal protein amounts were subjected to western blotting. Band densities of HA-MCM10 and tubulin were quantified using ImageJ software. Densities of HA-MCM10 were normalized with those of tubulin. Each column and error bar represents the mean + SD for three independent experiments (right panel). The asterisks indicate a statistically significant difference (**$p < 0.01$). (B) HEK293T cells were transiently transfected with either pcDNA3.1/HA MCM10-1-530 or 1-655 together with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs to analyze western blotting. Molecular mass positions of 3 x FLAG-Vpr, HA-MCM10 and tubulin are indicated.

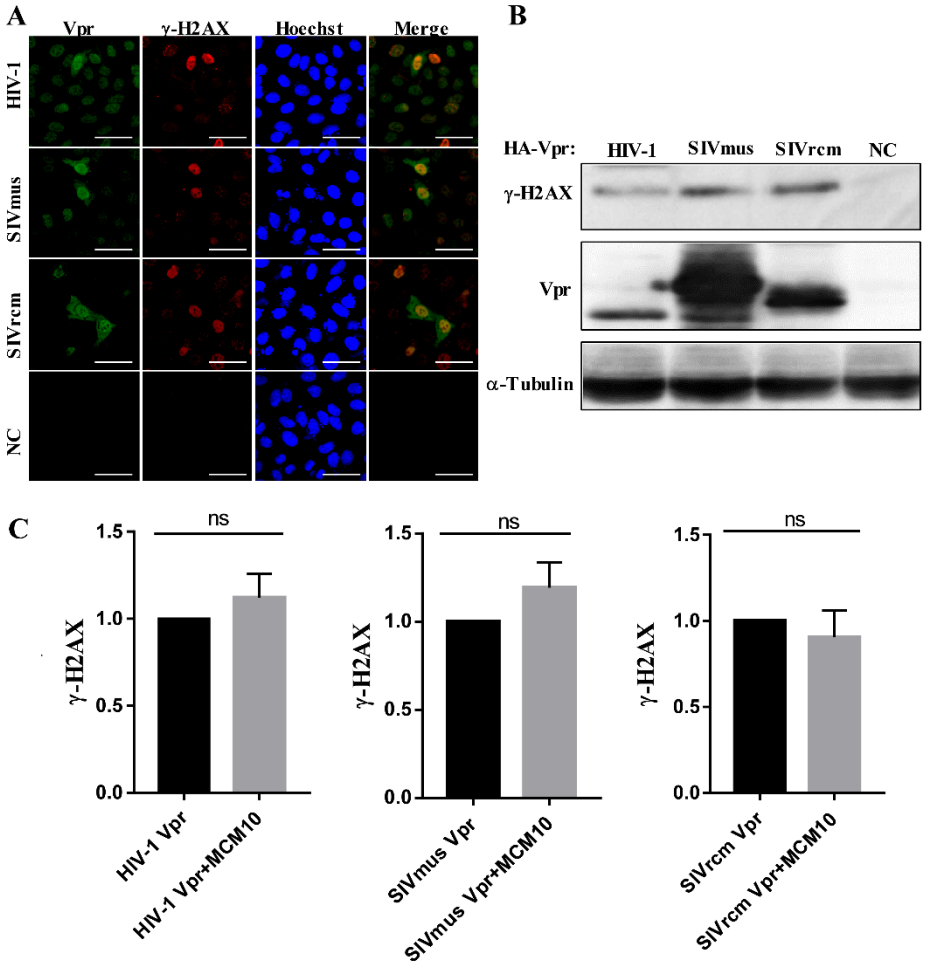


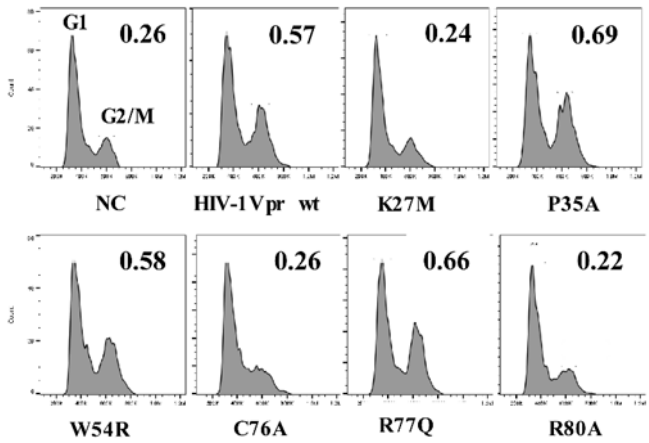
Figure 9. MCM10 did not alleviate DNA damage response (DDR) induced by HIV-1, SIVmus and SIVrcm Vpr proteins. (A and B) HEK293T cells were transiently transfected with either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs, or the control, pcDNA3.1/3 x FLAG. (A) Transfected cells were stained with anti-γ-H2AX mouse mAb followed by Alexa Fluor 594 to detect γ-H2AX at 48 h of post-transfection, and the nucleus was stained with Hoechst 33342 and observed using an FV-1000 fluorescence microscope. (B) Transfected cells were harvested at 48 h of post-transfection and lysates with equal protein amounts were subjected to western blotting. The molecular mass positions of 3 x FLAG-Vpr, γ-H2AX and tubulin are indicated. (C) HEK293cells were transiently transfected with pcDNA 3.1/HA-MCM10 together with

either pcDNA3.1/3 x FLAG-HIV-1, SIVmus and SIVrcm Vprs, or the control, pcDNA3.1/3 x FLAG. Transfected cells were harvested at 48 h of post-transfection and lysates with equal protein amounts were subjected to western blotting. Densities of γ-H2AX were normalized with those of tubulin. Each column and error bar represents the mean + SD for three independent experiments. Bar=20μm

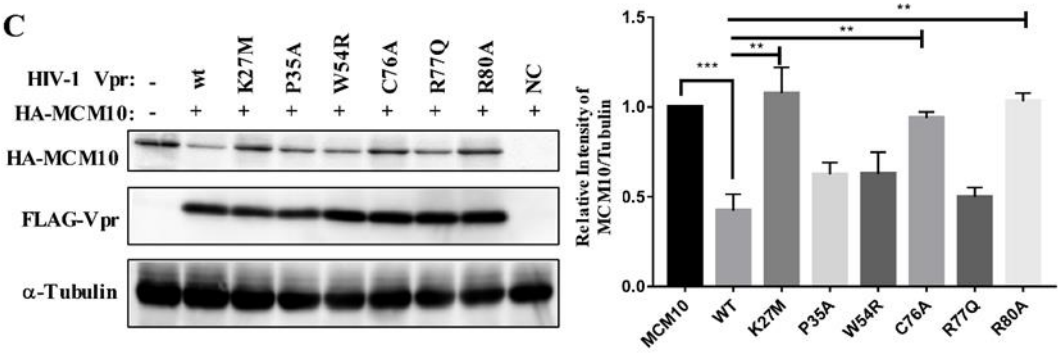
A

Functions \ Vpr mutants	WT	K27M	P35A	W54R	C76A	R77Q	R80A	References
G2/M arrest	+	-	+	+	-	+	-	[15,31,32]
Apoptosis induction	+	-	+	ND	+	-	-	[11,13,15]
Nuclear localization	+	-	+	+	+	+	+	[5,7,32]
Oligomerization	+	+	-	ND	-	+	+	[33]
Virion incorporation	+	+	+	ND	+	+	+	[33]
TNF activation	+	-	ND	+	±	+	-	[31]
Interaction interrupted		HCGI		UNG2			MUS81;PLK1	[34–37]

B



C



D

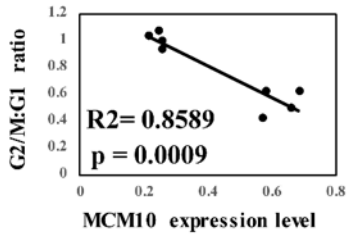
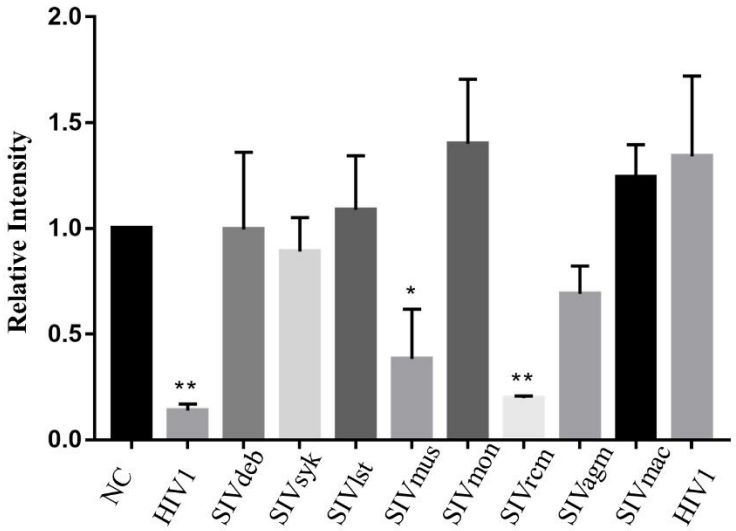


Figure 10. MCM10 degradation by HIV-1Vpr is positively correlated with G2/M arrest function of Vpr. (A) The table summarizes previous determined functional properties of various HIV-1 Vpr mutants. (B) HEK293 cells were transfected with pME18neo/FLAG-IRESZsGreen1 that encoded FLAG-tagged HIV-1 Vpr wild type and a panel of mutants stated above. At 48 h after transfection, cells were harvested to analyze DNA content and stained with propidium iodide. ZsGreen1-positive cells were analyzed using a BD AccuriTM C6 Plus with a Sampler flow cytometer. For each mutant, 10000 events were acquired and subsequent G2/M:G1 ratio was calculated using FlowJo software. (C) HEK293T cells were transiently transfected with either pcDNA3.1/HA-MCM10 together with either HIV-1 pME18neo FLAG-tagged HIV-1 Vpr, wild type and a panel of mutants. Transfected cells were harvested at 48 h after transfection and lysates with the equal protein amounts were subjected to western blotting (left panel), and band densities of HA-MCM10 and tubulin were analyzed using ImageJ software (right panel). Each column and error bar represents the mean + SD for three independent experiments. The asterisks indicate a statistically significant differences (**$p < 0.01$, ***$p < 0.001$). (D) Correlation between MCM10 degradation and G2/M arrest by HIV-1 Vpr mutants. The line represents the approximate curve. R= Pearson's correlation coefficient ($p = 0.0009$).

A


Relative Intensity
2.0
1.5
1.0
0.5
0.0
**
**
*
NC
HIV1
SIVdeb
SIVsyk
SIVlst
SIVmus
SIVmon
SIVrcm
SIVagm
SIVmac
HIV1

B

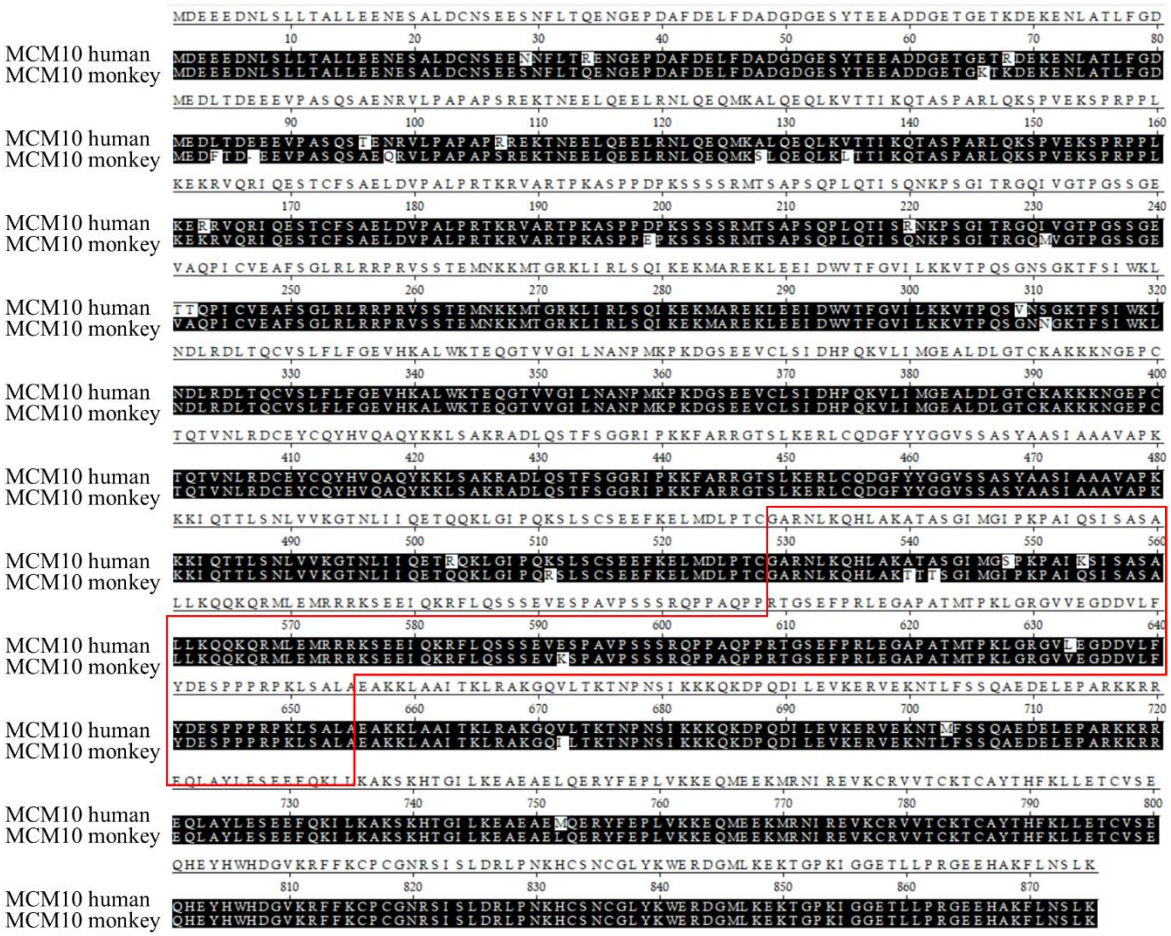

MCM10 human
MCM10 monkey

Figure 11. MCM10 down-regulation by primate lentiviruses Vpr proteins is highly conserved among human and green monkey (A). Downregulation of exogenous MCM10 by 11 primate lentiviruses Vpr/x. Cos-1 cells were transiently transfected with pcDNA 3.1/HA-MCM10 together with either pcDNA3.1/3 x FLAG-HIV/SIV Vpr/x, or the control pcDNA3.1/3 x FLAG. Transfected cells were harvested at 48 h after transfection and lysates with the equal protein amounts were subjected to western blotting. Each column and error bar represent the mean + SD for three independent experiments. The asterisks indicate a statistically significant differences (**$p < 0.01$, ***$p < 0.001$). (B). full-length MCM10 amino acid sequence including human (reviewed sequence meaning reliable sequence information; 875aa) and green monkey (not reviewed sequences and less reliable sequence information; 874aa). For the full-length alignments, percent identity is 96.7%. For the MCM 2-7 binding sites domain (530-655 aa), the identity is also more than 95% (120/126), and most substitution are similar ones.

Acknowledgement

This is the most wonderful time when I start writing the acknowledgement of Ph.D. thesis. The finishing of my Ph.D. work would have never been possible and easy without authentic help from so many people who provide their academic and life experiences with complete and true love.

Firstly, I want to express my appreciation to Prof. Aida, my supervisor. She provides a study platform to support my research and opportunities for academic exchanges. Besides, she also helps me with guidance of HIV-1 studies, especially directing how to get into a new scientific area. I have benefited from her a lot.

Moreover, I am grateful to the Japanese Government MEXT Scholarship. With such persistent financial support, I could learn so much from Japanese teachers and made so many friends with them and other students from different backgrounds. Besides, MEXT supports me to finish my whole Ph.D. life and diversities my life including personal development and study continuing.

Meanwhile, I am thankful to so many lab members although we come from different countries and cultures. Nopporn san guides me how to perform special experiments, how to design a specific test and fulfill it and recommends me for a future academic career. Matsuura san, my classmate, tutors me for my whole Ph.D. life and solves any questions I raise with undoubted enthusiasm. Bai san and Lu san, also my Chinese friends, help me both in my life and my work, with a wonderful open and clear heart. I also want to say my authentic “Thank you” to so many people even through some of them have left here, Sato san, Watanuki san, Wakamatsu san, Suzuki san and so on. Without your helpful and friendly behaviors, I could not make may study career so quickly and smoothly.

In addition, I want to show my gratefulness to professor Kaoru Uchimaru, professor Tetsuro Matano, associated professor Kazumi Nakano and associated professor Kei Sato. During my

preliminary Ph.D. defense, they provided so many precious and constructive academic suggestions, which helped me improve my experiment designing and scientific thinking. In addition, they are my advisors and I also benefited from their seriousness about research and persistence in their study field to keep moving on and never stopping.

After recalling before, I should focus the future right now. one famous Chinese philosopher said: At age thirty, I stood firm. Now I am thirty and about to graduate. I should stay firm and continue my research forward to some basic scientific questions benefit to social and natural world. My graduations is not end. In contrast, it is a beginning of my life forwarding the study in the future.