# Roles of HIV Virion-associated Envelope Glycoprotein in Modulating Various Cellular Pathways and Facilitating Viral Replication and Its Pathogenicity

**By**

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A Thesis Submitted to the Faculty of Graduate Studies of the University of Manitoba

in Partial Fulfillment of the Requirements of the Degree of

DOCTOR OF PHILOSOPHY

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**ABSTRACT**

HIV envelope glycoprotein, composed of gp41 and gp120 subunits, is a key viral protein in HIV infection and replication. The role of HIV envelope glycoprotein in viral entry and membrane fusion has been well studied. However, how HIV envelope glycoprotein modulates downstream gene expression to enhance viral replication and the underlying mechanism of this action are poorly understood.

In order to better understand the role of HIV envelope glycoprotein in HIV expression and pathogenesis, my study focused on the characterization and role of the virion-associated envelope glycoprotein (vEnv) in viral transcription of HIV infected cells. My results showed that the vEnv binding to CD4 receptor and coreceptor (CCR5 or CXCR4) activates HIV transcription in HIV infected CD4+ T lymphocytes, including HIV infected cell lines and peripheral blood mononuclear cells (PBMCs) isolated from HIV aviremic patients.

Through transcriptome analysis, I found that numerous cellular gene products modulated by vEnv were involved in various signaling pathways, including cellular transcription, T cell receptor signaling, cell cycle, and actin skeleton organization. Among these modulated genes, I further identified a cellular microRNA, microRNA 181A2 (miR-181A2), which is associated with the transcriptional activation by vEnv. My results revealed that miR-181A2 is downregulated upon vEnv treatment, resulting in an increase of cellular p300/CBP associated factor (PCAF) expression, thereby allowing an increased HIV LTR histone H3 acetylation and HIV transcriptional activation.

Further, I also identified another vEnv-modulated cellular gene, histone deacetylase 10 (HDAC10), which is related to viral replication. The results showed the downregulation of HDAC10 benefits viral replication through promoting viral integration. Furthermore, the co-immunoprecipitation assay demonstrated that HDAC10 interacts with viral integrase by binding to integrase amino acid 55-165. Interestingly, this interaction does not alter the lysine acetylation state of integrase but specifically decreases the binding of HIV integrase to host factor LEDGF/p75, which leads to the inhibition of viral integration. In addition to regulating viral integration, the results also revealed that the progeny viral infectivity is inversely correlated with cellular HDAC10 level.

In conclusion, this thesis revealed the function of vEnv during HIV replication and provided new potential targets for HIV therapy.

**ACKNOWLEDGEMENTS**

How times fly! I remember the first time when I came to the University of Manitoba, everything was so fresh to me. For example, the language, the people and the environment are so different from the country where I lived before. At the beginning, everything was so hard for me but with the help from the others, I overcame the troubles and achieved the success in my academic career.

First, I would like to deeply appreciate my supervisor Dr Xiaojian Yao for his efforts in teaching me how to improve my academic career. I am very delighted to work with him for these years and I will remember the trainings I received in his lab.

Secondly, I would like to be grateful to my committee members, including Dr Keith Fowke, Dr Sam Kung, Dr Gary Kobinger and Dr Jason Kindrachuk for their advice and support in my PhD study. Besides, I also appreciate the external committee member Dr Andrés Finzi for reviewing this thesis.

Then I would like to thank the members in Dr Yao's lab during my PhD study. It is my pleasure to work with them. Here I will especially appreciate Dr Zhujun Ao for her technical support and guidance in my experiments. And I also express thanks to my best lab mate Xu Zhang for his help and support in these years. My thanks extend to my lab members Bihe Chen, Tian Tian, Tan Jun, Huang Jin, Bin Liu, Chidi Onyejiegbu, Titus Olukibiti and Lijun Wang.

I also especially would like to thank the members of the department of medical microbiology and infectious diseases at the University of Manitoba. Especially, I will express my great thanks to Angela Nelson and Adriana Trajtman for their patient and

kind help and support. I also appreciate the PhD candidate Monika Kowatsch for revising my thesis.

I am grateful for the funding support from Research Manitoba PhD scholarship and Canadian Institute of Health Research. With the scholarship, I finally finished my PhD study.

Last but the most important, I will greatly appreciate my family members and friends. I will remember their support and trust to me. Wherever I am, I think they always stand with me.

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**List of Abbreviations**

| Full Name | Abbreviation |
|---|---|
| Acquired immunodeficiency syndrome | AIDS |
| Zidovudine | AZT |
| APOBEC3G | A3G |
| Arginine-rich motif | ARM |
| Actin nucleating protein actin related proteins 2/3 | Arp2/3 |
| Aldrithiol-2 | AT-2 |
| AP-1 complex subunit sigma-2 | AP1S2 |
| AP-3 complex subunit mu-1 | AP3M1 |
| Rho guanine nucleotide exchange factor 1 | ARHGEF1 |
| Barrier-to-autointegration factor | BAF |
| Bone Marrow Stromal Cell Antigen-2 | BST-2 |
| Cluster of differentiation 4 | CD4 |
| C-C chemokine receptor type 5 | CCR5 |
| C-X-C chemokine receptor type 4 | CXCR4 |
| Capsid | CA |
| Cyclophilin A | CypA |
| Cis-acting repressive sequences | CRS |
| Carboxyl terminal domain | CTD |
| C-terminal heptad repeat | CHR |
| CAMP response element-binding protein | CREB |
| CREB-binding protein | CBP |
| Chromosome Region Maintenance 1 | CRM1 |
| Cullin5 | Cul5 |
| Cyclin dependent kinase 9 | CDK9 |
| Cyclin T1 | CycT1 |
| Cell Division Cycle 42 | Cdc42 |
| 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate | CHAPS |
| Cluster of differentiation 2 | CD2 |
| CD40 ligand | CD40L |
| Cell division protein kinase 8 | CDK8 |
| Chromatin immunoprecipitation assay | CHIP |
| Co-immunoprecipitation | Co-IP |
| Dimer initiation site | DIS |
| Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin | DC-SIGN |
| Diacylglycerol | DAG |
| Dulbecco's modified Eagle's medium | DMEM |
| Differential expression gene | DEG |
| DNA methyltransferase 1-associated protein 1 | DMAP1 |
| Death receptor 5 | DR5 |

| | |
|---|---|
| Polycomb group embryonic ectoderm development protein | EED |
| Alpha subunit of eukaryotic initiation factor 2 | eIF2 α |
| Eukaryotic initiation factor | eIF-5A |
| Elogin C | EloC |
| Elogin B | EloB |
| Endoplasmic reticulum | ER |
| Endosomal sorting complexes required for transport | ESCRT |
| Extracellular signal-regulated kinase 1/2 | ERK1/2 |
| Envelope on defective particle | Env-VLP |
| Ebola virus | EBOV |
| Estrogen receptor beta | ESR2 |
| Elongation factor 1-delta | EEF1D |
| Enzyme-linked immunosorbent assay | ELISA |
| Transcription factor E2F2 | E2F2 |
| Fusion peptide region | FPPR |
| Fetal bovine serum | FBS |
| Fibronectin 1 | FN1 |
| Fc fragment of IgA receptor | FCAR |
| HIV envelope glycoprotein gp120 | Gp120 |
| HIV envelope glycoprotein gp41 | Gp41 |
| Group-specific antigen | gag |
| Guanine nucleotide exchange factor | GEF |
| Green fluorescent protein | GFP |
| Acid alpha-glucosidase | GAA |
| General Transcription Factor IIA Subunit 2 | GTF2A2 |
| Histone deacetylase 6 | HDAC6 |
| Histone deacetylase 7 | HDAC7 |
| Histone deacetylase 10 | HDAC10 |
| Human immunodeficiency virus | HIV |
| Highly active antiretroviral therapy | HAART |
| Histone acetyltransferase | HAT |
| Heat shock protein 70 | Hsp70 |
| High Mobility Group Protein A1 | HMGA1 |
| Histidine rich protein 2 | HRP2 |
| Heat-shock protein 60 | HSP60 |
| Human embryonic kidney HEK 293T cell | HEK 293T cell |
| Hours | Hours |
| Hsp70-binding protein 1 | HSPBP1 |
| Integrase interactor 1 | INI1 |
| Integrase | IN |
| Internal ribosome entry site | IRES |
| Inhibitory/Instability RNA sequences | INS |
| Inositol triphosphate | IP3 |
| Interleukin-2 | IL-2 |

| | |
|---|---|
| Janus kinase/Signal Transducer and Activator of Transcription | JAKs/STATs |
| Long terminal repeat | LTR |
| Lens epithelium-derived growth factor | LEDGF |
| lipoprotein receptor-related protein | LRP |
| LIM kinase | LIMK |
| Firefly luciferase | Luc |
| Laminin subunit gamma-1 | LAMC1 |
| Major histocompatibility complex-1 | MHC-I |
| Major histocompatibility complex-2 | MHC-II |
| Mitogen-Activated Protein Kinase Kinase 1 | MAP2K1 |
| myosin light chain phosphatase | MLCP |
| Moloney Murine Leukemia Virus | M-MLV |
| Micro mol/L | mM |
| Mediator of RNA polymerase II transcription subunit 21 | MED21 |
| Glycoprotein endo-alpha-1,2-mannosidase | MANEA |
| MAP kinase-interacting serine/threonine-protein kinase 2 | MKNK2 |
| Negative factor | Nef |
| Amino terminal domain | NTD |
| N-terminal heptad repeat | NHR |
| Nuclear factor kappa-light-chain-enhancer of activated B cells | NF- κB |
| Nuclear export signal | NES |
| Nuclear localization signal | NLS |
| Nuclear factor of activated T-cells | NFAT |
| Nano meter | nm |
| Nucleocapsid | NC |
| Nucleosome-1 | NUC-1 |
| Neuraminidase | NA |
| Next-generation sequencing | NGS |
| Nucleoporin 54 | NUP54 |
| NF-kappa-B inhibitor beta | NFKBIB |
| Nuclear protein of the ATM locus | NPAT |
| Open reading frame | ORF |
| Primer binding site | PBS |
| Polypurine tract | PPT |
| Psi element | PE |
| Preintegration complex | PIC |
| Positive transcription elongation factor | P-TEFb |
| Phosphofurin acid cluster sorting protein 1 | PACS-1 |
| Phospholipases C-c | PLC-c |
| Phosphatidylinositol-4,5-biphosphate | PIP2 |
| Protein kinase C | PKC |
| Phosphatidylinositide 3-kinases | PI3K |
| Protein kinase B | PKB |
| Proline-rich tyrosine kinase | Pyk2 |

| | |
|---|---|
| Phosphatidylinositol-3,4,5-Trisphosphate Dependent Rac Exchange Factor 1 | PREX1 |
| P21-activated kinase | PAK |
| P300/CBP-Associated Factor | PCAF |
| Peripheral blood mononuclear cells | PBMCs |
| Plastin 1 | PLS1 |
| Public Health Agency of Canada | PHAC |
| Phytohemagglutinin | PHA |
| Regulator of expression of virion protein | Rev |
| Rev response element | RRE |
| Rev-binding element | RBE |
| RAs-related Nuclear protein-GTP | Ran-GTP |
| Reverse transcription complex | RTC |
| RNA polymerase II | RNAP II |
| Ras-Related C3 Botulinum Toxin Substrate | Rac |
| Rhodopsin | Rho |
| RAS Like Proto-Oncogene | Ral |
| Rho kinase | ROCK |
| Reverse transcription polymerase chain reaction | RT-PCR |
| Reads per kilobase per million reads | RPKM |
| Recombinant gp120 | r-gp120 |
| E3 ubiquitin-protein ligase RING2 | RNF2 |
| REST Corepressor 2 | ROCR2 |
| Stromal cell derived factor 1 | SDF-1 |
| Soluble gp120 | Sgp120 |
| Syndecan-2 | SDC2 |
| SET And MYND Domain Containing 1 | SMYD1 |
| TTTTTT slippery site | SLIP |
| Transcription factor Sp1 | Sp1 |
| Src-Associated In Mitosis 68 KDa Protein | SAM68 |
| Suppressor of cytokine signaling box proteins | SOCS-box |
| Sodium dodecyl sulfate polyacrylamide gel electrophoresis | SDS-PAGE |
| SAM domain and HD domain-containing protein 1 | SAMHD1 |
| Transactivator | Tat |
| Target sequence for transactivation | Tar |
| Transmembrane | TM |
| T-Cell Lymphoma Invasion And Metastasis 1 | TIAM1 |
| Trichloroacetic acid | TCA |
| TATA box binding protein (TBP)-associated factor | TAF-9 |
| Tight junction protein-1 | TJP1 |
| Tumor necrosis factor receptor superfamily member 10D | TNRFR10D |
| Tripartite motif-containing protein 5 | TRIM5α |
| Transcription initiation factor TFIID subunit 9B | TAF9B |
| Tumor necrosis factor-related apoptosis-inducing ligand | TRAIL |

| | |
|---|---|
| Viral protein R | Vpr |
| Virion infectivity factor | Vif |
| Viral protein U | Vpu |
| Variable loop 1-3 | V1-3 |
| Vorinostat | VOR |
| Valproic acid | VPA |
| Vesicular stomatitis virus glycoprotein | VSV(G) |
| World Health Organization | WHO |
| Wiskott-Aldrich syndrome family protein | WASP |
| Western blot | WB |
| United Nations Program on HIV/AIDS | UNAIDS |
| Ubiquitin-conjugating enzyme E2 D1 | UBE2D1 |
| Untranslated region | UTR |
| Zinc finger protein 143 | ZNF143 |

# Chapter 1

# Introduction

## 1.1 Overview

### 1.1.1 HIV Epidemiology

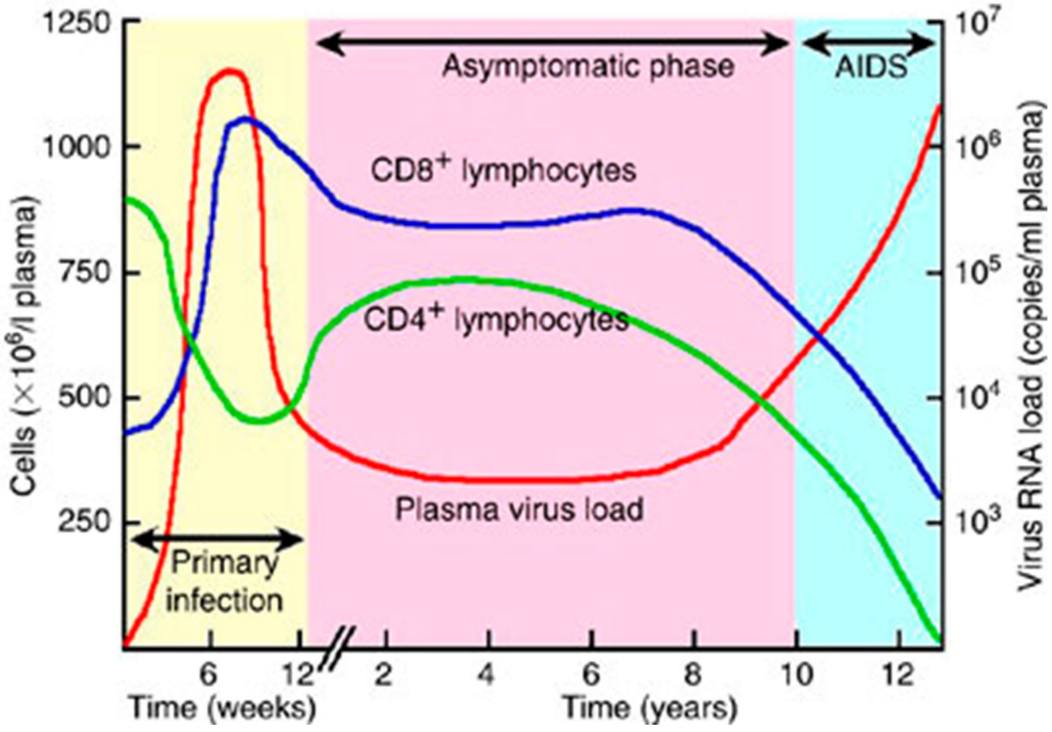
Human immunodeficiency virus (HIV) is a lentivirus that can infect cells of the human immune system, impair the human immune system and finally cause AIDS (acquired immunodeficiency syndrome) (1). It was discovered by Dr Luc Montagnier in 1983 and Dr Jay Levy in 1984, both of whom isolated HIV from AIDS patients (2-4). AIDS is characterized by the failure of the immune system allowing opportunistic infection by pathogens (bacteria, virus, fungi or protozoa), which usually do not cause diseases in normal conditions (5, 6). According to a recent report from UNAIDS (United Nations Program on HIV/AIDS), there were 36.9 million people living with HIV globally at the end of 2017. In the same year, 1.8 million people got newly infected and 0.94 million people died of HIV (7). The HIV epidemic varies throughout different countries. The report from UNAIDS showed that sub-Saharan Africa is the most severe HIV-influenced region, where 25.5 million people are believed to be infected with HIV (7). Although Canada is not the most severe HIV-affected region, HIV infection in Canada is still a big problem. According to the report (SUMMARY: MEASURING CANADA'S PROGRESS ON THE 90-90-90 HIV TARGETS) published by Public Health Agency of Canada (PHAC), there were 65,040 people in Canada living with

HIV and 26,400 people had died of HIV until the end of 2014 (8). What's worse, up to date, the number of the people newly infected with HIV in Canada is still rising (9). Therefore, it is still important to develop efficient therapeutic approaches for preventing and curing HIV.

### 1.1.2 HIV transmission and disease progress

HIV can be transmitted by several routes, including sexual intercourse, blood transfusion and mother-to-child transmission (10). It can be spread only by certain body fluids contact, including blood, semen, rectal fluid, breast milk, and vaginal fluid. These body fluids must either come in contact with the host mucous membrane or directly enter the bloodstream, thereby allowing HIV gain access to the human circulatory system (11, 12). Once HIV enters the bloodstream, it disseminates with the assistance of the circulatory system and is transmitted into the lymph nodes within days, and finally spreading throughout the patient's body (13, 14). In the human body, HIV's mainly targets are primary immune cells, including macrophages, dendritic cells and especially CD4+ T cells (15-17).

According to the appearance of viral as well as host markers in the plasma, the progress of HIV infection and development is divided into three stages, including acute infection (also called primary infection), chronic infection (also called asymptomatic phase) and AIDS (Fig. 1.1 (18)).




(Source from Munier ML et al, 2007, Immunol Cell Biol)

Figure 1.1 **The schematic of three stages of HIV infection**. The changes of CD4+ and CD8+ cell counts in peripheral blood as well as plasma viral load during the three stages of HIV infection and development.

**Primary Acute Infection**: Following HIV crossing the mucosal barrier through sexual transmission or mother-to-baby transmission, a number of viruses ( 50% infective dose ranging from 2-65,000 copies of viral RNA) enter the circulatory system and disseminate into the secondary lymphoid tissues in the gut, spleen and peripheral nodes (17, 19-21). These lymphoid tissues are the main site of virus production, storage, persistence and pathogenesis and also is the site where massive CD4+ T lymphocytes depletion subsequently occurs (22). In these lymphoid tissues, billions of progeny viruses are produced from these infected activated CD4+ T cells. A previous publication showed that basic reproductive number (the basic reproductive number refers to the number of newly infected cells that were infected by the progeny virus from a single HIV-infected cell ) is 19.3, which means one HIV virus can produce 19 fold in progeny virus (23). At the same time, the massive depletion of CD4+ T cells occurs in these secondary lymphoid tissues as a result of viral mediated cell death or Fas-mediated apoptosis or pyroptosis (24-26). Especially, since gut is the largest immunologic organ in the body. Within the gut, more than 60% of total lymphocytes are harbored within the GALTs (gut-associated lymphoid tissues) (27). During HIV infection, the massive depletion of intestine CD4+ T cells occurs in a short period as a result of HIV infection (27% decrease in only 4 days), which is essential for HIV pathogenesis (28, 29).

This massive CD4+ T cell depletion is largely responsible for the immunodeficiency seen in HIV patients and animal models (30). Early studies have demonstrated that CD4+ T cell count is a marker of HIV infection progress and that low CD4+ T cell count results in immunodeficiency, leaving patients more susceptible

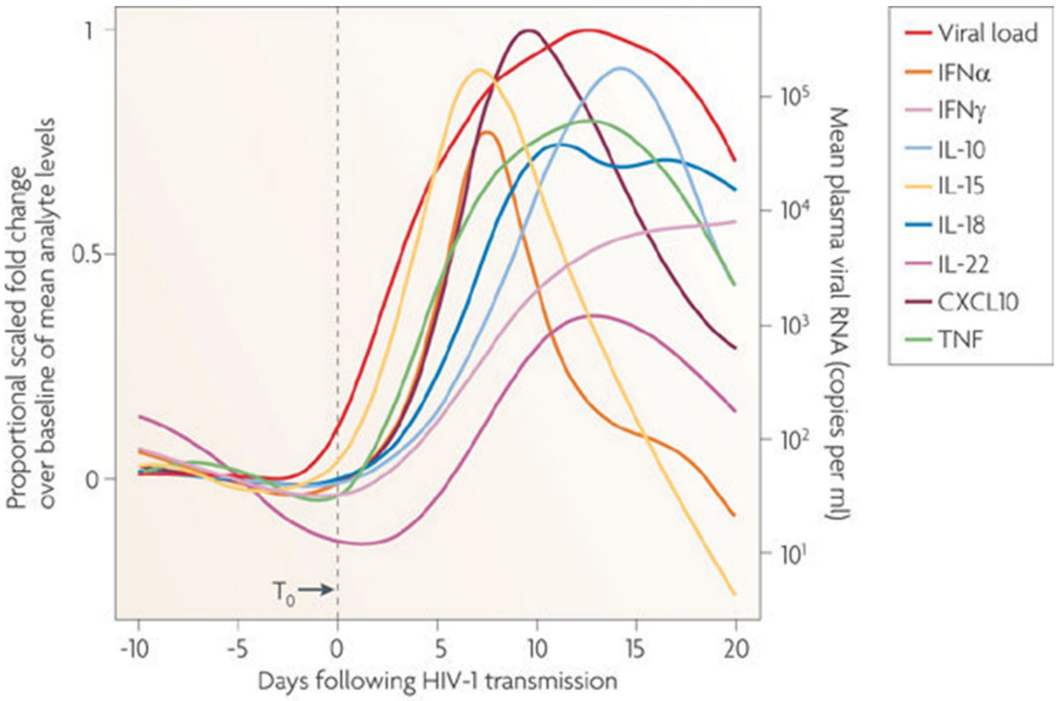
to opportunistic infections and cancers (31, 32). In support of this, Nishimura Y et al have reported that SHIV (a virus combining parts of the HIV and SIV genomes) can cause massive CD4+ T cell depletion in monkeys, which consequently results in death for immunodeficiency in a short time (33, 34). Without appropriate anti-viral treatment, the massive and rapid immune cell depletion finally results in the development of HIV-related illness or even death(30).

While CD4+ T cell depletion is an important factor in HIV-related illness, on the other hand, the initial immune response is also an important factor in mediating the development of the disease. This initial immune response occurs from 3 to 6 months after primary acute infection (35). The immune response during acute infection is very complex, including various cytokines/chemokines expression, DC and NK cell activation and CD4+ and CD8+ T cell response (36).

**Cytokines and chemokines expression**

HIV acute infection is characterized by a systemic increase of cytokine and chemokine expression (shown in Fig. 1.2 (36)). The cytokine and chemokine responses include the increase of IL-10, 15, 18, 22 (interleukin-10, 15, 18, 22), IFNα (interferon-α), CXCL10 (CXC-chemokine ligand 10) and TNF-α (tumor necrosis factor) (36). The expression of IL-15, CXCL10 and IFNα increase greatly but only retain for a short time while the expression of IL-18, 22, IFNγ and TNF-α increase fast and are retained at high levels (37). Some of these cytokines and chemokines have antiviral activity. For instance, Lapenta C et al have shown that type I interferons can inhibit HIV infection in U937-SCID mouse model (severe combined immunodeficiency mice transplanted

with human pro-monocyte cell line U937) (38).




(Source from McMichael AJ et al, 2010, Nat Rev Immunol)

Figure 1.2 **Relative expression changes of cytokines and chemokines in the plasma during HIV acute infection**.

**DC and NK cell activation**

During HIV acute infection, DCs are believed to be one of the first types of cells that encounter HIV (39). These DCs are rarely infected but they are important in transferring HIV to CD4+ T cells. The immature DCs capture HIV by expressing HIV attachment factor C-type Lectins, and then migrate to CD4+ T cells enriched lymphoid tissues, where the DCs carrying HIV can infect CD4+ T cells by synaptic transmission (39, 40). During the migration, the immature DCs can differentiate into mature DCs by HIV envelope gp120 induced production of type I interferons or HIV viral genomic RNA induced TLR-7 activation (41, 42). These activated DCs, which are exposed to infectious HIV virus or non-infectious virus, can activate naïve CD4+ and enhance HIV transmission efficacy (43, 44).

NK cells are important in controlling viral load during HIV acute infection, in which the activated NK cells effectively suppress HIV replication by cytolysis of infected cells as well as producing antiviral cytokines (45-48). NK cell activation is controlled by the interplay of activating and inhibitory signals, in which KIRs (killer-immunoglobulin-like receptors) play an important role (49). During HIV acute infection, the combination of KIR and HIV peptides present on HLAs (human leukocyte antigen) determines the activation and subsequent protective efficacy of NK cell responses (50, 51). For instance, Martin MP et al have shown that the combination of KIR3DL1 and HLA-B can significantly decrease HIV viral load in plasma and inhibit AIDS progression (51). Besides, NK cells suppress viral infection by inducing ADCC (antibody-dependent cellular cytotoxicity) in HIV infected cells. The NK cells

bind to the antibody bound to infected cells and release proteins, such as perforin and protease, which causes the lysis of infected cells (52). However, HIV can downregulate the expression of these ligands by triggering a NK cytotoxic response to escape from NK cell's antiviral effect (53-56).

**CD4+ and CD8+ cell responses**

As a result of the massive CD4+ T cell depletion during the acute HIV infection, the HIV specific CD4+ T cell response is greatly disrupted. The CD4+ T cell response is weak or even undetectable in HIV patients who had high viral loads (57). Conversely, the HIV specific CD4+ T cell response, particularly specific for Gag p24, can be detected in the patients with naturally sustained low persisting viral loads or who received early intervention with ART (57, 58).

Although the CD4+ T cell response is impaired, the CD8+ T cell response is very important for controlling viral load during the acute infection (59). The first HIV specific CD8+ T cell responses arise as viremia reaches its peak, which are specific for Env and Nef. The responses for Gag p24 and Pol arise in the later T cell response (60). As the CD8+ T cell response reaches its peak, due to selection by CD8+ T cells, the frequency of mutations that occur in the cytotoxic T lymphocytes epitopes increases. This can result in escape mutant virus's replacing the original virus to the majority (59, 61).

**Chronic Infection**: After the acute infection, chronic HIV infection occurs, which is characterized of the establishment of protective HIV antibodies and low viral load levels. If there is no anti-viral treatment, this stage of chronic infection can last for a

long period, varying from three years to ten years (62). At the beginning of this stage, there are few typical or no symptoms at all, and many people may experience some AIDS-like symptoms at the end of this stage, such as gastrointestinal problems, weight loss and fever (63). Clinical studies have shown that about 50-70% of the patients also experience the symptoms of enlarged painful lymph nodes, which can last more than six months (64, 65). The host immune system responds to the large amount of virus located in the follicular dendritic cells (FDC) and consequentially it causes the lymph nodes to become persistently swollen (66). In addition, if there is no antiviral treatment, HIV replication usually progresses in a short time into AIDS. Studies have found that HIV RNA level in blood determines the rate of disease progression. Therefore, patients with high level of HIV RNA in their blood exhibit a much faster progress to AIDS than the patients with low viral RNA levels (67).

Fortunately, due to advances of treatment in the past three decades, HIV has changed from a lethal disease into a chronic disease. Highly active antiretroviral therapy (HAART) has improved patients' health and greatly prolonged their life (68). HAART is a combination of different classes of inhibitors, which suppress different stages of viral life cycle, including viral entry, reverse transcription, viral integration and cleavage (69). In both high-income and low-income countries, the clinical studies have shown that the life expectancy of HIV patients who received successful HAART treatment might approach that of uninfected populations (68, 70). According to a report from UNAIDS, it was estimated that there were 21.7 million people receiving HAART at the end of 2017, though it is important to note that 41% of HIV patients still had no

access to antiretroviral therapy (7).

**AIDS**: In the absence of ART (antiretroviral treatment), the HIV infection progresses rapidly and results in AIDS within 3 to 10 years. AIDS is defined based on the patients' CD4+ T cell count dropping below 200 per μL or presence of opportunistic infection commonly associated with HIV (71). Usually, AIDS results in symptoms such as sweats at night, fevers, weight loss, and swollen lymph nodes. (72). It is crucial to note that death can occur from opportunistic infections caused by pathogens that cannot cause disease under normal condition (73).

Clinical studies have shown that due to the deficiency of the immune system, people with AIDS have a much higher risk of being diagnosed with various viral-induced cancers than healthy individuals (74). For example, KS (Kaposi's sarcoma) is the most common cancer, which is found in about 40%-48% of AIDS patients (75, 76). Kaposi's sarcoma is linked with human herpesvirus 8, which can infect healthy individuals but does not cause any symptoms (77). With HAART, the incidence of KS decreases to 8% when compared to a non-HAART treated population (78). The second is Burkitt's lymphoma, which leads to the death of about 16%-20% of AIDS patients (79-81). Burkitt's lymphoma is associated with Epstein-Barr virus and its incidence rate is much higher (more than 57 times) in HIV positive population than HIV negative population (82, 83). However, contrast to KS, the incidence of Burkitt's lymphoma and the survival rate of these patients with Burkitt's lymphoma were not significantly improved by HAART (84).

### 1.1.3 Current HIV therapy, its side effect and limitation

At present, HIV still cannot be cured. However, with the development of HIV therapy, HIV is turning from "untreatable" to "functionally cured". Beginning with the first generation of HIV anti-viral drugs Retrovir® (zidovudine, AZT) in 1985, a series of inhibitors have been developed, targeting viral entry, reverse transcription, viral integration, viral cleavage and maturation (85, 86). Currently, the most popular HIV therapy is the HAART (highly active antiretroviral therapy), a combination of multiple antiretroviral drugs against HIV replication (87). After years of clinical testing, this treatment has proved to be very successful, which effectively decreases the viral load and maintains the immune system to prevent the progress of AIDS.

Although HAART can suppress HIV viral load into an undetectable level, the viral load rebounds rapidly after HAART is ceased (88). Due to the small number of latently HIV infected cells in patients' body (89). While most of the viruses infect activated CD4+ T lymphocytes, a small population of HIV-infected resting CD4+ T cells are generated and they are considered to be latently infected cells (89). These latently HIV infected cells harbor integrated viral DNA and can either produce a small amounts of virus or become completely silent in HIV production (89). Without stimulation, there is no effective method to distinguish latently infected CD4+ T cells and uninfected CD4+ T cells (90). When HAART treatment is ceased, these latently infected cells become activated by some stimuli and begin to produce a virus, which results in a rapid rebound of viral load in patients (89). Thus, this small population of latently infected cells becomes the main barrier to a full HIV cure. There are several mechanisms

contributing to the formation of HIV latency, including mutations of the viral genome (91), transcriptional interference (92, 93), changes of host chromatin structure (94, 95) and epigenetic silencing (94, 96). These mechanisms contribute to the prevention of HIV expression, thereby allowing latently infected cells to evade the immune system. Therefore, HAART cannot eliminate HIV latently infected cells.

HAART has several side effects. For instance, some protease inhibitors are toxic to the liver. As many HIV patients are co-infected with hepatitis C, the usage of these protease inhibitors may increase the risk of poor liver function (97). Another adverse systemic effect of HAART is an increased risk of hyperglycemia and dyslipidemia (98, 99). Hyperglycemia is a condition in which an excessive amount of glucose circulates in the blood plasma (100) and dyslipidemia is a condition resulting in abnormal lipid levels in the blood (101). Therefore, the usage of HAART may cause a higher risk for the patients who already have Type 2 diabetes mellitus or cardiovascular disease (102, 103).

### 1.1.4 HIV therapy research

In order to completely eradicate HIV latency, several research groups have developed different strategies and made some progress. One of the popular strategies is "shock and kill", in which HIV latently infected cells are activated by some stimuli and then eliminated by immune response or anti-HIV drugs (104). For example, by altering the balance of acetyltransferase and deacetylase activity, as such, several histone deacetylase (HDAC) inhibitors have been considered as a promising approach to

eradicate the HIV latent reservoirs *in vitro* (105). However, whether the HDAC inhibitors can efficiently reactivate HIV latency *in vivo* is still under investigation. In addition to HDAC inhibitors, recently Eric N. Borducchi et al reported that the combination of an HIV bNAb (broadly neutralizing antibody) PGT121 and TLR7 (Toll-like receptor 7) agonist GS-9620 can significantly reduce the number of latently infected cells and delay viral rebound in SHIV-infected rhesus monkeys, which received antiretroviral treatment for 2.5 years and harbor latently infected cells (106). However, whether this combination also can work in human is still unknown.

Another strategy is to fully or partially replace the immune system through genetic modification. In 2007, an HIV positive patient, Brown, received a hematopoietic stem cell transplantation in Berlin and these stem cells were donated from a CCR5 Δ 32 healthy individual, and after three years his viral load in the blood became undetectable and HIV antibody also declined (107). Since the case of the 'Berlin patient' was the only one successful cure of HIV-1, it suggests that CCR5 mutation can be a potential strategy to eliminate HIV latency (108). Up to date, to achieve this goal, several more advanced gene editing technologies have been utilized to generate CCR5 modified CD4+ T cells, including ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases) and CRISPR/CAS9 (109-111). However, these clinical trials using genetic CCR5-modification strategy are still under investigation.

## 1.2 HIV virology

### 1.2.1 A brief introduction of HIV structure

The HIV-1 virus is spherical and the diameter of a viral particle ranges from 100 to 120 nm (Fig. 1.3). On the outer surface of the virion, HIV envelope glycoprotein is inserted into the lipid bilayers and protrudes outside the surface. HIV envelope glycoprotein is composed of two subunits, including gp120 and gp41, which form a trimer. Gp120 is exposed on the surface of the virus, while gp41 is non-covalently bound to gp120 and its transmembrane domain and cytoplasmic domain are buried into the viral particle (112, 113). The structure of HIV envelope complex will be described in detail in section 1.3.1. The core of the viral particle is mainly composed of three structural proteins, including p17 (MA), p24 (CA) and p6. The matrix (MA) protein p17 is located inside lipid bilayer of the viral envelope and it is attached to the lipid bilayer through a bipartite membrane-binding domain (114). Inside, the cone-shaped core is composed of about 1,500 copies of structure protein p24 (115), while the NC protein p7 is encased in the viral core, which binds and protects the viral genome (116, 117).

In the core of the virion, there are two copies of single-stranded, unspliced, positive sense viral RNA (118). The RNA is composed of about 10,000 nucleotides, which includes a 5' cap , nine ORFs (open reading frame) and a 3' poly(A) tail (119). In addition to viral RNAs, there are several viral proteins and cellular proteins incorporated into the core. These viral proteins include p7 and p6, RT (viral reverse

transcriptase), IN (integrase), PR (protease) and Vpr (viral protein R) (120-123) and several cellular proteins including INI1 (integrase interactor 1), CypA (cyclophilin A), Hsp70 (heat shock protein 70), ubiquitin, Staufen, A3G (APOBEC3G, APOlipoprotein B mRNA-editing, Enzyme-catalytic, polypeptide-like 3G) and Ku70, which have specific roles in HIV pathogenesis (124-130)

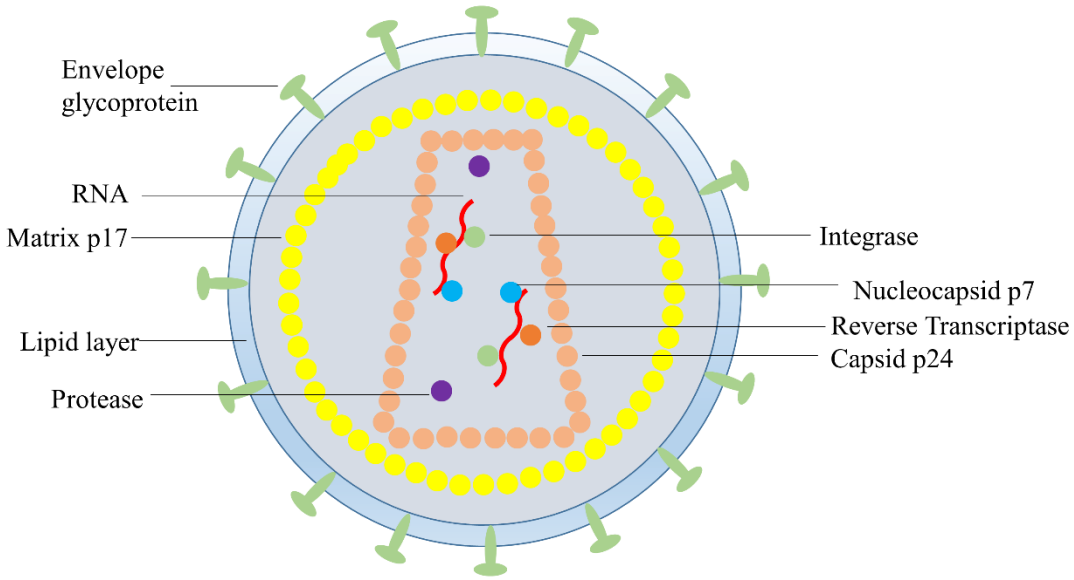



Figure 1.3 **Morphologic structure of HIV-1 viral particle**. HIV envelope glycoproteins are exposed on the outer surface and inserted into the lipid bilayers. HIV envelope glycoprotein consists of gp120 and gp41, which form a trimer present on the surface. The matrix protein p17 is located inside lipid bilayer of the envelope and is attached to the lipid bilayer through a bipartite membrane-binding domain. About 1,500 CA protein p24 forms the viral core. The NC protein p7 is encased in the core, which binds and protects the viral genome. In the core of the virus, there are two copies of single-stranded, positive-sense and unspliced RNA and several viral factors, including reverse transcriptase, integrase, and protease.

### 1.2.2 HIV genome and its encoded proteins

HIV genomic RNA is composed of 9749 nucleotides, which contains a 5' cap (Gppp), nine ORFs and a 3' poly(A) tail (119). Shown in Fig. 1.4, these ORFs encode HIV structural proteins, regulatory proteins, and accessory proteins. For the structural proteins, Gag, Pol and Env polyprotein precursors are encoded by three of these ORF respectively and are further cleaved into individual proteins by a viral or cellular protease. The Gag precursor is cleaved into the proteins p6, p17, p24, and p7 by PR (viral protease) (131). The pol precursor is cleaved into RT (reverse transcriptase), IN (integrase) and PR (protease) by PR (116). And the Env precursor is cleaved by cellular protease furin, generating gp120 and gp41, which form the envelope glycoprotein trimer on the surface (132). The other six ORFs are responsible for encoding regulatory or accessory proteins, including Tat (transactivator), Rev (regulator of expression of virion protein), Nef (negative factor), Vif (virion infectivity factor), Vpr (viral protein R) and Vpu (viral protein U) (133). The functions of these HIV encoded proteins are listed in the Table 1. Also, there are several essential elements located in the different sites of genome, including LTR (long terminal repeat), IRES (internal ribosome entry site), TAR (target sequence for transactivation), DIS(dimer initiation site), RRE (Rev response element), PE (Psi element), SLIP (a TTTTTT slippery site), PBS (primer binding site), CRS (Cis-acting repressive sequences), PPT (polypurine tract) and INS (Inhibitory/Instability RNA sequences) (134). These elements play important roles in HIV replication, as described in Table 2.

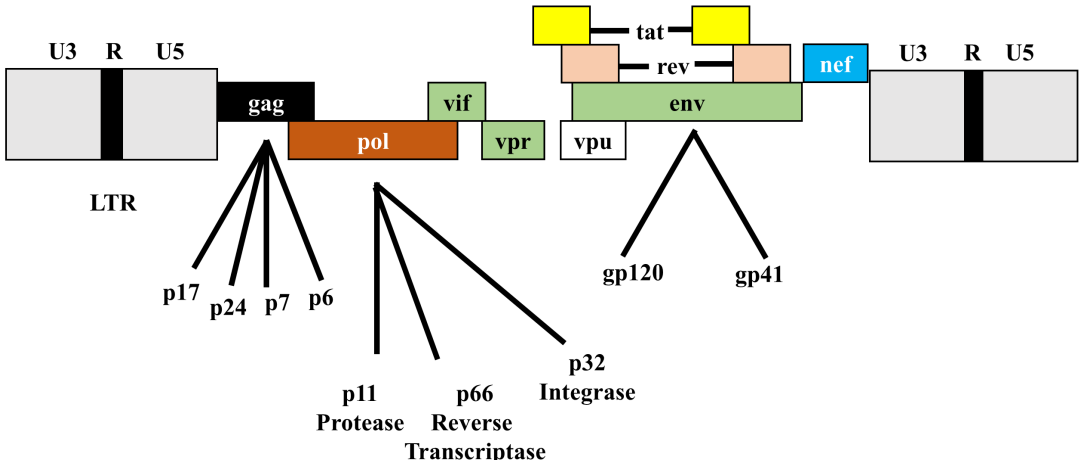



Figure 1.4 **HIV-1 Genomic organization**. HIV-1 genome contains two LTRs and 9 ORFs. These ORFs encode structure protein precursors of Gag, Pol, and Env; and regulatory protein Rev and Tat; and accessory protein Vif, Vpu, Vpr, and Nef. Gag and Pol polyproteins are cleaved by viral protease into MA, CA, NC, p6, PR, RT and IN, respectively. Envelope glycoprotein precursor gp160 is cleaved into gp120 and gp41 by cellular furin.

Table 1 Major functions of HIV encoded proteins

| Gene | Protein | Major Functions |
|---|---|---|
| gag | MA | MA mainly functions in viral assembly and viral budding (135-137). |
| | | MA also is involved in viral entry, uncoating, and RNA transport through the cellular cytoplasm and nuclear import (138-142). |
| | CA | CA participates in uncoating (143) and viral assembly (144, 145) |
| | NC | NC remodels the structure of nucleic acid to maintain stable conformation, which subsequently influences viral reverse transcription (146, 147) |
| | | NC is required for viral RNA packaging as well as genomic RNA dimerization, which greatly influences HIV progeny virus infectivity (148-151). |
| | p6 | P6 is can engage cellular Tsg101 and ALIX, which links Gag to ESCRT (Endosomal sorting complex required for transport) and enhances viral particle release (152). |
| | | P6 is an essential requirement for the incorporation of Vpr into viral particles (153). |
| pol | RT | RT mainly catalyzes the process of reverse transcription in the cytoplasm, in which it converts a single-stranded RNA into a double-stranded linear DNA (154). |
| | IN | IN mainly catalyzes the insertion of newly synthesized viral cDNA into host chromatin (155). |
| | | IN is also involved in reverse transcription, nuclear import and chromatin targeting (155). |
| | PR | PR participates in the cleavage of the Gag and Gag-Pol polyproteins during viral maturation (156) |
| env | Gp120 | gp120 interacts with CD4 and coreceptor (CCR5 or CXCR4)(157). |
| | Gp41 | gp41 mediates viral membrane fusion with plasma membrane(157). |

| | | |
|---|---|---|
| tat | Tat | Tat binds to HIV LTR promotor and enhances HIV transcription (158) |
| rev | Rev | Rev binds to viral mRNA and exports mRNA from the nucleus to the cytoplasm (159) |
| vif | Vif | Vif directly binds the newly synthesized A3G and causes the degradation of A3G through ubiquitin-dependent proteasomal pathway (160-163) |
| nef | Nef | Nef mainly functions in T cell activation and downregulates the expression of cell surface receptor, including CD4, MHC-I and chemokine receptors (164) |
| vpu | Vpu | Vpu mainly acts in cellular CD4 degradation and inhibiting BST-2's (bone marrow stromal antigen 2) function to enhance progeny virus release (165, 166) |
| vpr | Vpr | Vpr plays an important role in multiple steps of HIV infection, including reverse transcription, PIC nuclear import and incorporation into progeny virus (167, 168) |

Table 2 Functions of HIV genome elements

| Element | Function |
|---|---|
| LTR | LTR impacts on viral transcription (169) and participates in viral reverse transcription and integration (154, 170, 171) |
| IRES | IRES recruits 40S ribosomal subunit to initiate viral translation (172); IRES becomes activated in G2/M phase of the cell cycle (173) |
| TAR | TAR interacts with Tat, which is required for viral transcription (174); TAR encoded miRNA prevents infected cells from apoptosis by modulating cellular proteins (175, 176) |
| DIS | DIS initiates HIV genomic RNA dimerization for viral RNA package (177) |
| RRE | Rev binds to RRE to form a RNP complex to mediate nuclear export of viral RNA (178) |
| PE | PE participates in viral genomic RNA packaging (179) |
| SLIP | SLIP mediates the programmed-1 ribosomal frameshift from gag reading frame into pol reading frame (180) |
| PBS | $tRNA_3^{Lys}$ binds to PBS to initiate reverse transcription (181) |
| CRS | CRS regulates nuclear retention of HIV env mRNA (182) |
| PPT | PPT participates in reverse transcription (183) |
| INS | INS decreases the efficacy of viral expression (184, 185) |

### 1.2.2 An overview of the HIV life cycle

HIV-1 replication is complex, in which many viral and cellular factors participate. According to the timeline, the life cycle of HIV can be divided into two phases: the early stage that is defined as the period from viral entry to viral DNA integration; the late stage that is defined as the period from viral transcription to virus maturation (review in (186)). As shown in Fig. 1.5, each step of the HIV life cycle will be briefly introduced.

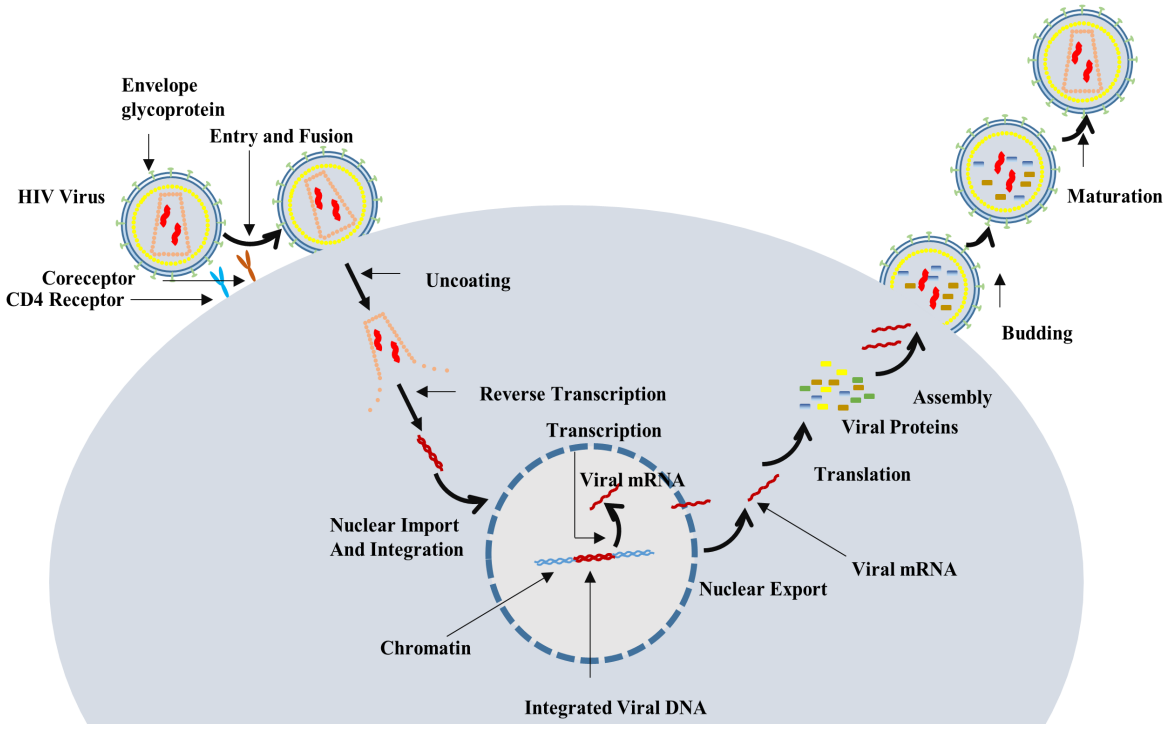



Figure 1.5 **The life cycle of HIV-1**. After HIV-1 approaches target cells, the interaction between HIV-1 envelope glycoprotein trimer and CD4 receptor/coreceptors (CCR5/CXCR4) initiates the viral entry and membrane fusion. Following that, the viral core dissembles and the uncoating occurs. Subsequently, the viral genomic RNA is released into the cytoplasm and reverse transcribed into viral DNA. The viral DNA is then imported into the nucleus and inserted into the host chromosome. Then, the following stimulation by viral protein Tat, viral mRNAs are produced from the nucleus and exported into the cytoplasm, which are subsequently translated into viral proteins. After processed by cellular enzymes, the gp160 is cleaved into gp120 and gp41 and forms a trimer on the plasma membrane. Meanwhile, assembly occurs on the inner side of plasm membrane, where viral genome and proteins assemble into immature progeny virus. HIV promotes the release of immature progeny virus by ESCRT, in which the interaction between the late assembly domain of p6 and two ESCRT components TSG101 and ALIX is required. After budding, the polyprotein Gag and Gag-pol are further cleaved and the immature progeny virus becomes mature.

**Viral entry and fusion**: When HIV-1 approaches to the host cell and binds to cellular receptor CD4 by viral glycoprotein gp120, the interaction between gp120 and CD4 receptor triggers a conformational change of gp120 as well as gp41, resulting in the rearrangements of V1/V2 of gp120, the formation of the bridging sheet, and the exposure of HR1 segment of gp41 (187, 188). Following the conformational changes in gp120, the chemokine coreceptor binding site is exposed. After gp120 binding to the chemokine receptor CCR5 or CXCR4, the interaction induces the membrane fusion, including gp41 rearrangement, exposure of gp41 fusion peptide, gp41 fusion peptide insertion into the plasma membrane, the formation of a six helix bundle of gp41 HR1 and HR2 and the formation of fusion pore (187, 189). The detailed conformational changes will be described in section 1.3.1. In addition to regulating viral entry and fusion, the interaction between HIV-1 envelope glycoprotein and host CD4 and coreceptors induces a cascade of downstream signaling, which will be described in the section 1.3.2.

**Uncoating**: After the viral entry, the viral core undergoes a disassembly process, which subsequently releases viral genomic RNA and other viral factors into the cytoplasm (190). This is an important step for HIV-1 at the early stage of infection because it's directly related to the successful formation of the pre-integration complex (191). This process is influenced by CA stability as well as several cellular factors, including Trim5α (Tripartite motif-containing protein 5), CypA (Cyclophilin A), Nup358 (Nucleoporin 358), Nup153 (Nucleoporin 153), Dynein, and Kinesin-1 heavy chain (192-197).

Previous studies have shown that the properly-scheduled uncoating is essential for the following steps, including HIV reverse transcription, nuclear import and viral integration (198). There are several models to explain when and where the uncoating occurs: (1) after viral entry, HIV uncoating immediately occurs in the cytoplasm, where is close to the plasma membrane (199). (2) HIV uncoating occurs in the cytoplasm during the transport from the plasma membrane to the nucleus and the reverse transcription occurs at the same time (200). And (3) after reverse transcription, HIV uncoating occurs at the nuclear pore (201).

**Reverse transcription**: HIV reverse transcription is a complex process, which requires the participation of viral RNA, viral proteins RT, MA, NC, CA, IN and Vpr as well as cellular factors Gemin 2 (gem-associated protein 2), INI1, SAP18 (Sin3A associated protein 18), HDAC1(histone deacetylase 1), HuR (human antigen R), AKAP1 (A-kinase anchor protein 1) and TOP1 (DNA topoisomerase 1) (154, 202). Among these cellular factors, HuR, AKAP1 and TOP1 can directly affect reverse transcription via reverse transcriptase association. HuR interacts with RT by binding to its RNase H domain and the knockdown of HuR greatly impairs the reverse transcription (203). AKAP1 also binds to the RNase H domain of RT and its downregulation inhibits reverse transcription (204, 205). TOP1 not only interacts with HIV RT to enhance RT's enzymatic activity, but also promotes viral cDNA synthesis (206, 207).

Reverse transcription is initiated by RT from the 3' end of the cellular lysyl-$tRNA^{Lys3}$ through hybridizing to the PBS (primer binding site) of viral genomic RNA

and terminated until RT reaches the 5' end of HIV-1 RNA, generating a single-stranded DNA (208). Due to its RNase H enzymatic activity, the U5 and R region of the remaining HIV-1 genomic RNA is degraded and the newly synthesized DNA hybridizes with the repeat sequence at the 3'-end of the remaining HIV-1 single-strand RNA (209). After strand transfer, RT extends the newly generated DNA and hydrolyzes the remaining RNA except for a purine-rich sequence (polypurine tract, PPT) (210). Then RT elongates the second strand DNA with PPT primer following the similar steps. Finally, it generates an integrase-competent dsDNA (208).

**Nuclear entry and Integration**: After the reverse transcription, the viral DNA and integrase recruit cellular factors to form a PIC (211). PIC is transported from the cytoplasm to the pre-nuclear area and then into the nucleus across the nuclear envelope with the assistance of viral factors MA, Vpr, IN, CA, and central DNA flap as well as cellular factors Importinα, Importinβ, Importin7 and TNPO3 (Transportin 3) (reviewed in (212, 213)).

In the nucleus, integrase binds to the target DNA with the assistance of LEDGF and strand transfer occurs, in which integrase catalyzes the interaction of HIV DNA and 3’-hydroxyl group of host DNA (214). After the strand transfer, the DNA recombination repair occurs at the breaks with the assistance of cellular enzymes, including cellular polymerase, nuclease and ligase. Finally, the viral genome is successfully inserted into the cellular chromatin (211). However, if the viral DNA enters the nucleus but does not integrate into chromatin, the circulation of viral DNA will occur with two kinds of circular viral DNA will be generated: 1-LTR and 2-LTR (215). The 2-LTR is a

byproduct of viral integration and the amount of 2-LTR is proportional to the number of imported DNA (216). 1-LTR is the product of recombination between 2-LTRs (216).

**Viral transcription**: The transcription of HIV-1 is mediated by RNAP II (RNA polymerase II) with the assistance of viral factor Tat and TAR as well as cellular factor PTEF-b complex (reviewed in (174)). The binding of Tat to TAR enhances the recruitment of cellular P-TEFb (human positive transcription elongation factor b), a complex consisting of CDK9 (Cyclin-dependent kinase-9) and CycT1 (cyclin T1), which induces the phosphorylation of RNAP II to initiate viral transcription (217, 218).

Without Tat, HIV transcription can be activated by a series of cellular factors, such as NFAT (nuclear factor of activated T cells), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and SP1 (specificity protein 1) (219). As the promotor, HIV-1 LTR is composed of a core region and an enhancer region. The core region consists of an initiator sequence, a TATA element, and three SP1 binding sites, all of which participate in the initiation of viral mRNA elongation (220-222). The enhancer region consists of two NFAT binding sites and two NF-kB binding sites. The binding of NFAT or NF-kB to the HIV LTR greatly stimulates the viral transcription (223, 224). In addition, HIV transcription also can be regulated by epigenetic modification. Previous studies have reported that the histone deacetylation of HIV LTR can efficiently suppress viral transcription and result in HIV latency, in which HDAC class I members HDAC1, 2 and 3 play an important role (225-228).

**Nuclear exportation and translation:** HIV transcription generates a large amount of nascent viral mRNAs, including unspliced mRNA, partially spliced mRNAs

and small multiply spliced mRNAs (229). Following that, HIV utilizes viral regulatory protein Rev to export these mRNAs through Rev binding to RRE (Rev response element) in mRNAs and exporting to the cytoplasm (159). After nuclear export of mRNAs, spliced mRNAs are translated into various viral proteins by cellular machinery. Unspliced mRNAs encode Gag and Gag-Pol polyprotein while partially spliced mRNAs encode Env, Vpu, Vpr, and Vif proteins and small multiply spliced mRNAs encode Nef, Tat and Rev (230). After viral protein synthesis, the monomer gp160 undergoes oligomerization and forms a trimer in the ER, the trimer is then cleaved into gp120 and gp41 by furin and undergoes oligosaccharide modification in the Golgi and finally the mature Env trimer is delivered into cell membrane by intracellular CTLA-4 containing granules (231-233).

**Assembly, budding and maturation**: HIV-1 virus assembly occurs on the inner side of plasma membrane (234). The virus particle assembly coincides with the production of all viral proteins (235). Gag polyproteins specifically bind to viral RNA with the recruitment of cellular and viral proteins, and then move to the membrane and assemble into an immature progeny viral particle (236). After that, HIV utilizes the ESCRT (endosomal sorting complexes required for transport complex) to promote membrane fusion by an assembly domain of p6 and facilitates progeny virus to release from the plasma membrane (152, 237). The late domain of $p6^{Gag}$ engages two ESCRT components TSG101 (Tumor susceptibility gene 101) and ALIX (Apoptosis-linked gene 2-interacting protein x), which is essential for HIV budding (152). Meanwhile, the polyprotein Gag will be cleaved into CA, MA, P1, P2, P6, and NC; polyprotein Gag-

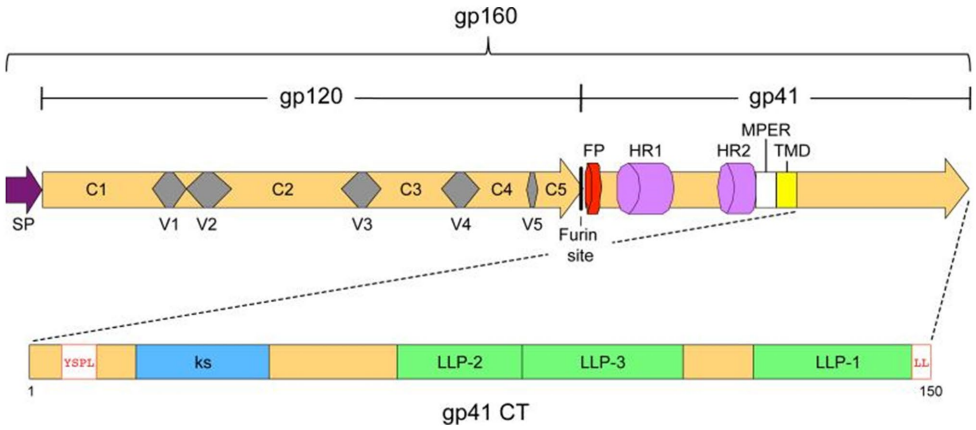
pol will be cleaved into P6, PR, RT (p51), RT (p66), and IN. These nascent viral proteins rearrange and form a fully mature and infectious progeny virus (237, 238).

## 1.3 HIV envelope glycoprotein and its induced signaling pathways

### 1.3.1 HIV envelope glycoprotein's structure

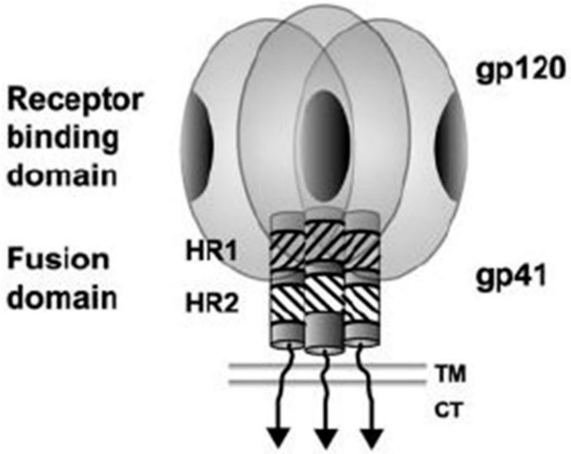
The polyprotein precursor **gp160** is initially synthesized and undergoes glycosylation and oligomerization to form a trimer on the RER (rough endoplasmic reticulum) (reviewed in (157)). The oligomerized gp160 is transported to the Golgi apparatus, where it is further cleaved into gp120 and gp41 by cellular furin and undergoes oligosaccharide modification (239, 240). The non-covalently associated gp120-gp41 complex (shown in Fig. 1.6), is carried to the plasma membrane by CTLA-4 (Cytotoxic T-lymphocyte associated protein 4) mediated secretory pathway and incorporated into progeny viral particles (reviewed in (157)).

A




(Source from MA Checkley et al, 2011, J Mol Biol)

B



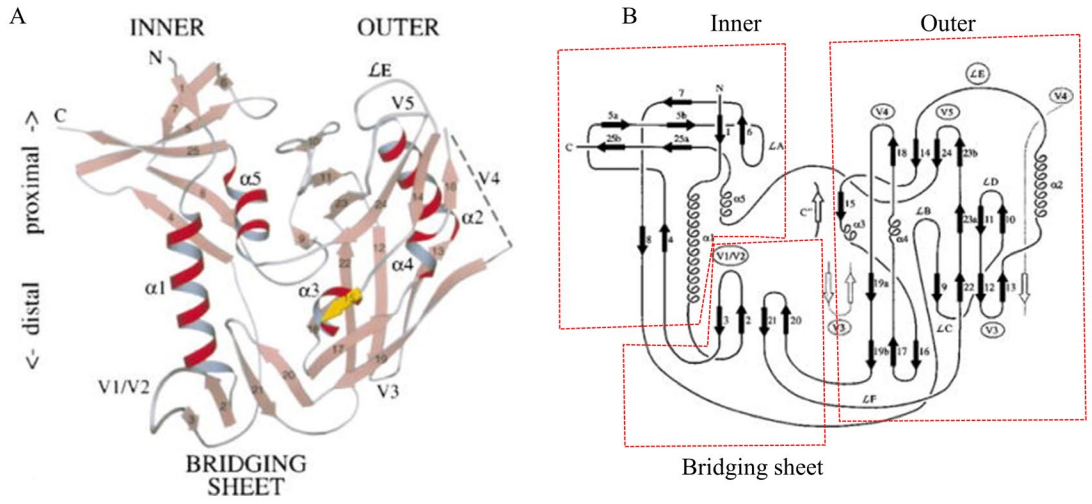

(Source from Pancera M et al, 2005, J Virol)

Figure 1.6 **The schematic representation of HIV envelope glycoprotein trimer structure**. (A) The domains of Env. Precursor gp160 is cleaved into gp120 and gp41 by cellular furin. Gp120 contains five conserved domains (C1-C5) and five variable domains (V1-V5), and gp41 contains extracellular domain (including FP (fusion peptide), HR1 (heptad repeat 1), HR2 (heptad repeat 2) and MPER (membrane-proximal external region)), transmembrane domain and cytoplasmic tail (including internalization signal YSPL and three LLPs). (B) The schematic of the structure of HIV Env trimer.

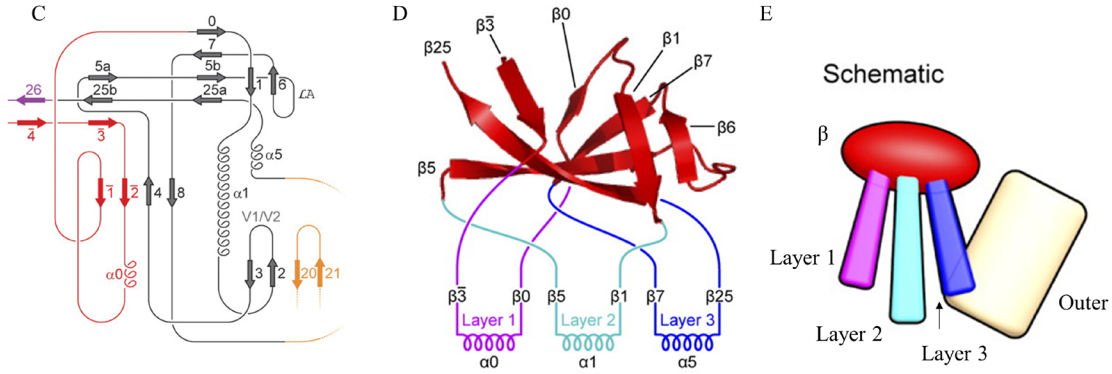
**Gp120** is the subunit of envelope glycoprotein complex, which is exposed on the outer surface of the viral particle. Gp120 contains five conserved domains and five variable domains. The relatively well-conserved elements (C1-C5) of gp120 fold into a "core", which is composed of the gp41-interactive inner domain and the highly glycosylated outer domain linked by the bridging sheet (Fig. 1.7A) (241). The inner domain contains three α-helixes (α0, α1 and α5) and a seven-stranded β sandwich (β0, β1, β3, β5-β7, and β25) (Fig. 1.7 B and C) (reviewed in (231, 242)). The loop excursions emanating from the sandwich form three separate layers, including layer 1(α0, β0 and β3), layer 2(α1, β1 and β5) and layer 3(α5, β7 and β25), which play important roles in stabilizing the gp120-CD4 complex (Fig. 1.7 D) (231, 243). This conserved inner domain contains several residues of the CD4 binding site (CD4bs) in C1, C3 and C4 region while another important CD4 binding site is located in the Phe43 cavity, which lies between the inner and outer domain (242). Also the inner domain contains the critical gp41-interactive region, which is a seven-stranded β sandwich proximal to gp41 interface (231).

The bridging sheet is exposed on the outer surface of gp120, which contains the loop V1, loop V2, β2, β3, β20 and β21 (reviewed in (242)). The region consisting of the V1/V2 is the most diverse part in length, sequence and glycosylation (244). The average length of V1/V2 is about 80 amino acids, with a possibility of large length variations derived from the two regions, one in the middle of V1 and the other near the C-terminal end of V2 (244). The V1/V2 loop shields the CD4 receptor binding site, which may participate in viral escape from immune recognition (242, 245).

The outer domain is a stacked double barrel, which is parallel to the bundle of the inner domain. The proximal end of the outer domain contains variable loop V4, V5, loop ℒ D and ℒE, and the distal end of the outer domain includes variable loop V3, loop ℒF and β20-β21 (reviewed in (242)). The V3 loop, composed of 34-35 amino acid residues, is a component of the chemokine receptor-binding region, and it is mainly responsible for co-receptor binding (246). Mutation analysis showed that the depletion of V3 region completely abolishes virus infectivity (247). The function of V4 and V5 loop has not been well defined. It is proposed that V4 loop may contribute to the neutralization escape and V5 loop may participate in the formation of CD4 binding site surface (248-250).




(Source from PD Kwong et al, 1998, Nature)



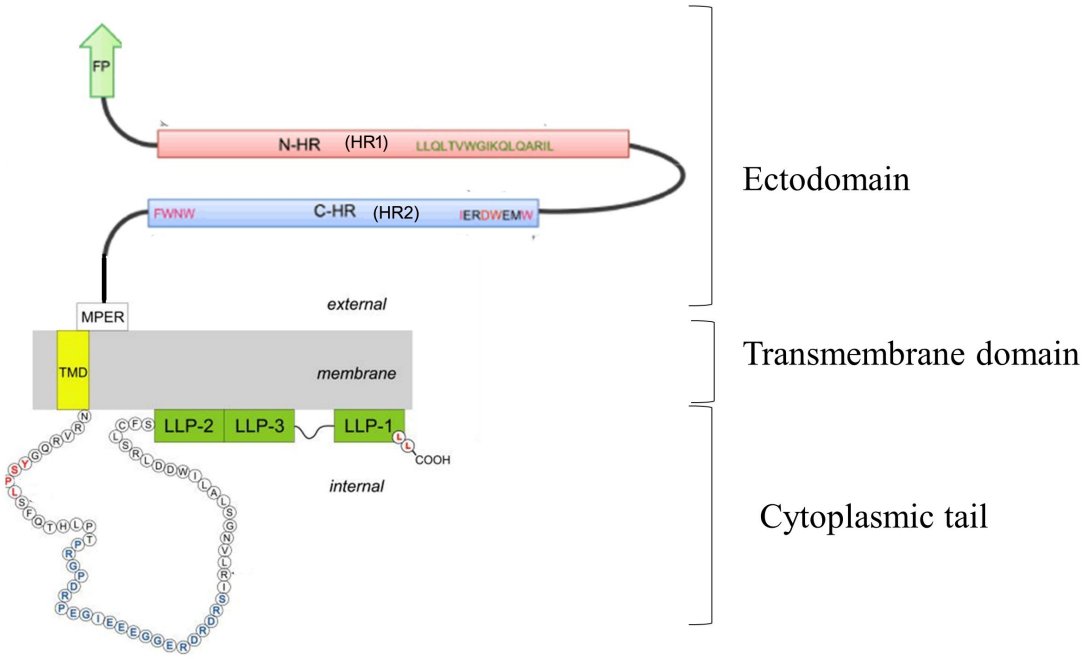

(Source from M Pancera et al, 2010, PNAS)

Figure 1.7 **The structure of gp120**. (A) Ribbon diagram of gp120 (242); (B) Topology diagram of gp120: inner domain, outer domain and bridging sheet (242); (C) Topology diagram of the gp120 inner domain with newly identified C-terminus (purple) and N-terminus (red) (231); (D) Ribbon diagram of "layered" gp120 inner domain with schematic of three layers (231); (E) The schematic of layered gp120 structure (231).

**Gp41** is a transmembrane protein and it has three prominent regions, including the ectodomain, the transmembrane domain, and the cytoplasmic tail (Fig. 1.8) (231). The ectodomain, ranging from amino acid 511 to 684, can be divided into FPPR (fusion peptide region), HR1 (N-terminal heptad repeat, N-HR), HR2 (C-terminal heptad repeat, C-HR ) and MPER (membrane-proximal external region) (251, 252). The FPPR is usually buried in the gp120-gp41 complex, which can protect the fusion peptide from interacting with other regions (253, 254). When gp120 interacts with CD4 receptor and coreceptors, this interaction results in the exposure of the fusion peptide (255). The HR1 and HR2 are linked by an immune-dominant loop and this region (HR1-HR2) is critical for efficient viral fusion. During viral fusion, three HR1 and three HR2 form a six helix bundle, which brings the viral and cellular membrane into proximity and subsequently forms a fusion pore (256). In addition, the region from Leu-550 to Leu-582 in HR1 is required for the gp160 oligomer formation and the mutations of this region makes the gp160 monomeric (257). Furthermore, the immune-dominant loop between HR1 and HR2 stabilizes the gp41 helical hairpin conformation, which is very important for the membrane fusion (258). The mutations of this loop do not have any effect on membrane lipid mixing between the viral envelope and host cells but prevent the formation of syncytia (252). The MPER mainly perform its function in membrane fusion and viral infectivity. in which the mutation of tryptophan-rich motif (amino acid 666-682 deletion mutant) severely affect the fusion activity (259, 260).

The gp41 transmembrane domain and cytoplasmic tail are buried into the viral particle. The gp41 transmembrane domain is an α-helix that consists of 25 amino acids.

This domain anchors the envelope into the lipid layer and, as reported by Liang Shang et al, the mutations of this region directly influence viral mediated membrane fusion but not affect the Env biosynthesis, cellular transport and incorporation into progeny virus (261). This cytoplasmic tail is composed of approximately 155 amino acids and it can be divided into membrane-proximal tyrosine-based sorting signal motif (YSPL) and three conserved lentiviral lytic peptides (LLP-1, LLP-2 and LLP-3) (262, 263). The sorting signal YSPL not only interacts with the AP-2 (clathrin adaptor protein complex-2) to mediate the endocytosis of Env from plasma membrane, but also influences cell-cell transmission between T cells and viral infectivity (264-266). The three lentiviral lytic peptides are involved with Env-mediated fusion, Env expression on plasma membrane, Env incorporation into progeny virus and Env-induced cell cytolysis (267-270).



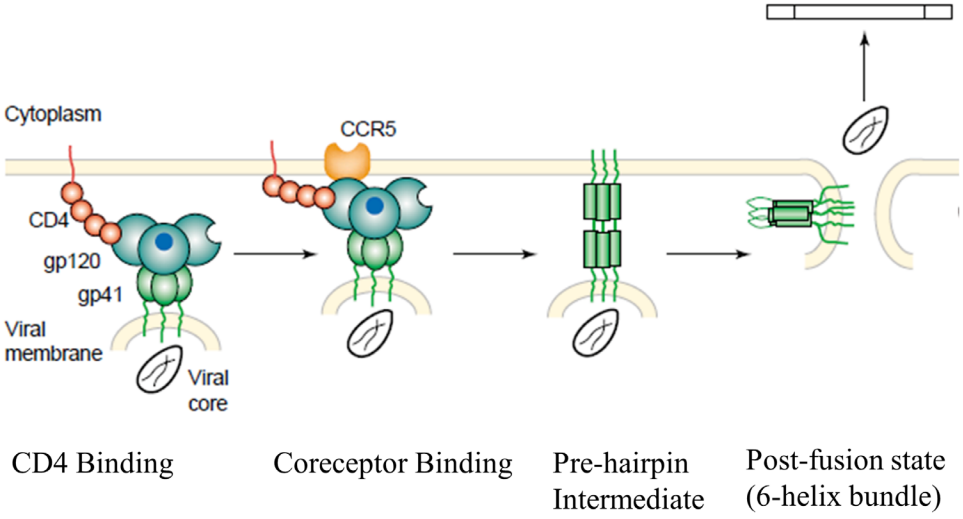

(Source from MA Checkley et al, 2011, J Mol Biol)

Figure1.8 **The structure of gp41**. Gp41 contains the ectodomain, transmembrane domain and cytoplasmic tail. The ectodomain contains FP, HR1, HR2 and MPER. The cytoplasmic tail contains tyrosine-based sorting YSPL and three lentivirus lytic peptide LLP-1, LLP-2 and LLP-3.

In nature, the envelope glycoprotein complex, composed of gp120 and gp41, is mainly present on the surface of viral particles (including infectious viral particles and defective viral particles) or infected cells. Due to the relatively weak and non-covalent interaction between gp120 and gp41, soluble CD4 and antibodies targeting to the gp120's CD4 binding site and V3 loop and the gp41's MPER region, are able to induce shedding of gp120, which is detected in the plasma of HIV positive patients (271-273). In lab, the recombinant gp120 and gp140 are used to investigate the envelope glycoprotein induced signaling pathways and the protective immune response. The recombinant gp140 is designed and generated for developing immunogens as a HIV vaccine, which is created by deleting the transmembrane domain and cytoplasmic tail of gp160 (274, 275). In addition, Env can be present on the membrane of different artificial vehicles, including liposomes, chemically inactivated virus and viral-like particles (276-279).

At present, there is a model to explain the conformational changes of HIV envelope complex during HIV entry (Fig. 1.9 (280)). The first step is the gp120 binding to the cellular receptor CD4, which induces the rearrangement of the V1/V2 region (the movement from the apical position to the peripheral site of gp120), the subsequent exposure of the V3 loop and the formation of bridging sheet (187). The exposure of the V3 loop and the bridging sheet is critical for the subsequent gp120-chemokine receptor interaction. This binding also triggers the conformational changes in the inner domain of gp120, including the apposition of layer 1 and layer 2. The rearrangement of layer1 and layer2 strengthens the gp120-CD4 interaction by decreasing the off-rate and allows

the gp41 ectodomain to undergo necessary conformal changes (243). In addition, the CD4 binding to gp120 results in the exposure of gp41 HR1, thereby allowing the subsequent interaction of HR1 and HR2 to form the six-helix bundle (254, 281). The second step is the gp120 binding to the coreceptor CCR5 or CXCR4. The chemokine receptor binding to gp120 results in the third step, the exposure of fusion peptide and the subsequent insertion of fusion peptide of gp41 into the host cell membrane as well as the release of the gp41 HR1 and HR2, which generates a pre-hairpin intermediate bridging the viral and cellular membranes (187). The fourth step is the refolding of the pre-hairpin intermediate into the 6-helix bundle of HR1 and HR2 (three HR1 in the center and three HR2 in the outer grooves) (187, 189). This step brings viral and cellular membranes together and generates a fusion pore.




(Source from SE Kuhmann et al, 2004, Trends in Pharmacological Science)

Figure 1.9 **The schematic of HIV entry**. Gp120 initially binds to CD4 receptor and then engages coreceptor (CCR5 or CXCR4), which results in the exposure of the fusion peptide and the subsequent insertion of fusion peptide into the plasma membrane. This state is called pre-hairpin intermediate. At last, the pre-hairpin intermediate refolds into 6-helix bundle and forms a fusion pore.

### 1.3.2 HIV envelope glycoprotein induced signaling pathways

As introduced in section 1.2.2, in addition to inducing viral entry and fusion, the interaction between HIV envelope glycoprotein and CD4 receptor/coreceptor also regulates cellular pathways to facilitate viral infection and replication.

**CD4 receptor signaling pathways**

CD4 receptor signaling is mainly involved in T cell activation, which is required for the efficient HIV infection (282). When CD4+ T lymphocyte encounters APCs (antigen-presenting cell), the CD4 molecule on T lymphocyte recruits a cognate peptide bound to MHC II and induces TCR (T cell receptor) signaling (283). After Ig-like domains of the CD4 interacting with MHC II, the cytoplasmic tail of the CD4 receptor recruits and activates a member of tyrosine kinases, Lck, which phosphorylates the two tyrosine residues of the ITAMs (immunoreceptor-tyrosine-based activation motifs) of CD3 and ζ chains (284, 285). Lck also phosphorylates a Syk-family tyrosine kinase, ZAP-70 (Zeta-chain-associated protein kinase 70), and cause its activation (286). The double phosphorylated ITAM recruits ZAP-70, which phosphorylates the scaffold protein SLP-76 (also named Lymphocyte cytosolic protein 2, LCP-2) and LAT (Linker of activated T cells) (286, 287). The activation of LAT and SLP-76 stimulates several downstream target proteins, eventually resulting in actin cytoskeleton rearrangements, Ras activation and calcium mobilization, which ultimately causes the activation of transcription factors and activates T lymphocytes (288, 289).

**Chemokine receptor signaling pathways**

Accumulated evidences have demonstrated that HIV utilizes chemokine receptor

pathways to enhance viral infection and replication (review in (290)). When the natural ligand (including SDF1, MIP-1α, RANTES, MIP-1β) or HIV gp120 binds to the coreceptor CXCR4 or CCR5, this interaction activates the coreceptor coupled heterotrimeric G-proteins, Gα (Gαq and Gαi) and Gβγ (291), which have been demonstrated to be involved in different signaling pathways.

Gαq protein activates PLC-c (phosphatidylinositol-specific phospholipases C-c), which subsequently hydrolyzes PIP2 (phosphatidylinositol-4,5-biphosphate) into IP3 (inositol triphosphate) and DAG (diacylglycerol) (292). This signaling can influence two downstream pathways. One is triggering the $Ca^{2+}$ flux and regulating three proteins: Calmodulin, Calcineurin and NFAT (Nuclear factor of activated T-cells) (293). C Cicala et al reported that the recombinant gp120 induces the activation of NFATs and its translocation from the cytoplasm into the nucleus in primary resting CD4+ T lymphocytes (294). The HIV LTR contain NFAT recognition sites, thereby the activation of NFAT may greatly stimulate viral transcription in HIV infected resting cells (294). Another pathway is the activation of PKC (protein kinase C), which enhances viral entry and transcription as well as activates chemotaxis and recruits neutrophils to the sites of inflammation (295, 296).

Different from Gαq, the activation of Gαi leads to the activation of lipid kinase PI3K (phosphatidylinositide 3-kinases) (297), which stimulates several downstream target regulators, including PKB (protein kinase B), NF-kB, MAP2K1 (Mitogen-Activated Protein Kinase Kinase 1), and ERK1/2 (extracellular signal-regulated kinase 1/2) (review in (298)). K Balabanian and AR Anand et al reported that recombinant

gp120 induces calcium mobilization and influences the PI3K downstream target PKB/AKT, in which the decrease of the phosphorylation of AKT induces cell apoptosis (299-301). Also, PI3K induces the phosphorylation of Pyk2 (proline-rich tyrosine kinase 2), paxillin, Crk ( also known as p38), and p130Cas (reviewed in (302)). Manuela Del Corno et al reported that both R5 tropic and X4 tropic envelope glycoproteins rapidly induce tyrosine phosphorylation of Pyk2 and subsequent inflammatory mediator (MCP-1 and MIP-1B) secretion in primary macrophages, which may contribute to the dysregulation of uninfected macrophages' function and enhance the infection in macrophages (303, 304).

In addition, the activation of Gβγ induces the GEFs (guanine nucleotide exchange factors) and the downstream target Rac (290, 305). Rac activates cellular PAK (p21-activated kinase), which results in the activation of Cofilin activity and subsequently causes the actin rearrangement (306). Balabanian et al reported that gp120 causes rapid actin cytoskeleton rearrangements and mediates membrane ruffling (301). Further, Yoder et al further confirmed that HIV gp120 utilizes CXCR4 to activate Cofilin, which induces cytoskeleton rearrangement and consequently facilitates viral infection in resting CD4+ T cells (307).

In summary, the interaction between gp120 and CD4/coreceptors induces a large number of downstream signaling pathways, facilitating different steps of viral infection. However, most of the previous studies utilized the recombinant gp120. As mentioned in section 1.3.1, virion-associated gp120 is the main form present on viral particles or infected cells, and there are conformational differences among the recombinant gp120

and virion-associated gp120. Therefore, whether the virion-associated gp120 is able to activate more signaling pathways and have more profound effect on viral replication needs more investigation.

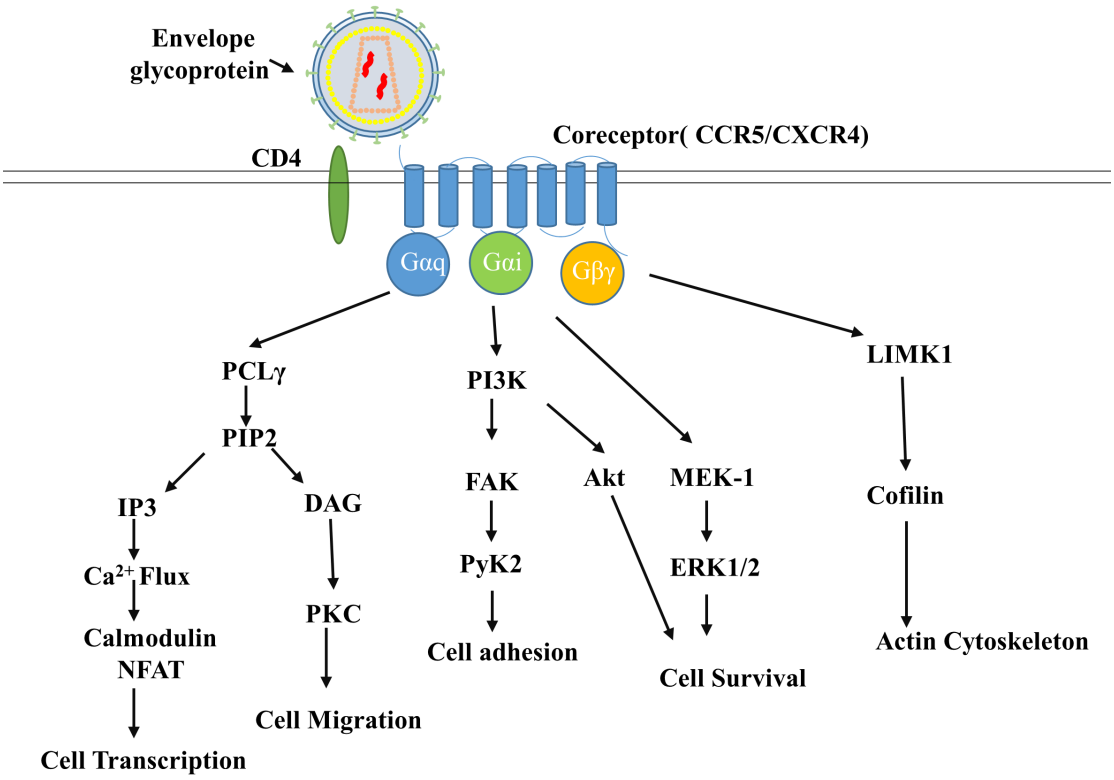



Figure 1.10 **The schematic of signaling pathways induced by the interaction between envelope glycoprotein and CD4 receptor/coreceptor (CCR5/CXCR4)**. The interaction between HIV envelope glycoprotein and cellular CD4 receptor/coreceptors can activate downstream signaling pathways, including PCLγ, PI3K, MEK-1, and LIMK1, which subsequently regulates cellular transcription, migration, adhesion, survival and actin cytoskeleton.

### 1.3.3 HIV defective particle and virion-associated envelope glycoprotein (vEnv)

It is well known that during HIV infection, infected cells produce not only infectious viruses but also large amounts of noninfectious or defective particles as a result of the highly reading-frame-error-prone reverse transcriptase (308, 309). Some studies estimate that the ratio of infectious to noninfectious particles ranges from 1 in 1,000 and 1 in 60,000 depending on the measurement methods (309-311). While the small proportion of infectious viruses continues to infect and disseminate within the body, the physiological role of the large amounts of defective particles present during infection is still not fully understood.

Previous studies have shown that these defective particles are able to induce T cell activation and cause host immune dysregulation, which plays an important role in HIV pathogenesis (308). Upon cellular activation, the HIV PIC can be transported into the nucleus and subsequently inserted into host chromatin, thereby allowing efficient viral replication (312).The exposure of primary resting CD4+ T cells to non-replicating virions (whole killed or UV-inactivated) can induce activation of these resting cells through the MHC II response (313, 314). During this process, peptides from defective particles are processed, endocytosed and degraded by APCs, presented on the surface of APC, and finally displayed to CD4+ T cells, resulting in activation of resting CD4+ T cells (315). Memory T cells are highly specific to their particular antigen (316). Therefore, If the antigens presented to CD4+ T cells are derived from the defective particles, then these cells would not recognize the infectious HIV particles and hence contribute to further viral escape (317). Thus, through MHC II response, these defective

particles not only activate resting CD4+ T cells and increase the susceptibility of resting CD4+ T cells, but also cause the dysregulation of immune system and enhance HIV immune escape. Some studies have shown that these defective particles are able to stimulate CD4+ T lymphocytes and induce their apoptosis, which contributes to the depletion of CD4+ T cells and enhances HIV pathogenesis (318-321). Herbeuval et al have shown that defective particles can specifically induce the DR5 (tumor necrosis factor-related apoptosis-inducing ligand death receptor 5) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) dependent apoptosis in CD4+ T cells (322). Ligation of TRAIL to its receptor DR5 results in multiplemerzation of the receptor and subsequent clustering of the receptor's DD (intracellular death domain), which leads to the formation of DISC (death-inducing signaling complex) (323). The formation of DISC subsequently activates caspase-8 and caspase-10, which induces caspase-3 mediated apoptosis (323). Through aforementioned mechanism, the defective viral particles drive T-cell loss by the induction of TRAIL apoptotic pathway.

At present, the understanding of the Env on defective particles is limited. Most studies utilized two forms of envelope glycoprotein, recombinant gp120 or gp140 (gp140 is created from gp160 by deleting gp41 trans-membrane and cytoplasmic tail), to investigate the functions of HIV Env. These studies have demonstrated that the interaction of recombinant gp120 with CXCR4 can activate the cellular actin-depolymerizing factor cofilin and lead to actin skeleton rearrangement, which may facilitate the process of viral integration (307). In addition to directly acting on early HIV infection, some *in vitro* studies have documented that the interaction of

recombinant gp120 and CD4/co-receptors led to the elevated expression of cytokines and chemokines (including the cytokines: IL-1β, IL-3, IL-6, IFNβ, IFNγ, TNFα, VEGF (vascular endothelial growth factor); and chemokines: CXCL-1, CXCL-3, IL-8, CCL-3, CCL-4, CCL-5 and CCL-20). IFNβ, IFNγ and TNFα have been found to activate resting CD4+ T cells and make them susceptible to HIV infection (324, 325). All these studies provide evidence as to the important role of HIV recombinant gp120 in the establishment of HIV infection and its induced pathogenesis. However, there are still several key questions remaining: what is the effect of vEnv on viral expression in HIV-infected CD4+ T cells, especially for those latently infected T cells? And can vEnv modulate the infectivity of the newly produced progeny viruses from those HIV infected T cells?

## 1.4 Cellular factors involved in HIV replication

Cellular factors play essential roles in the HIV replication, in which each step of the viral life cycle is linked to the functions of cellular proteins or RNAs. Here, I will discuss the roles of microRNAs and histone deacetylases in HIV replication.

### 1.4.1 Role of microRNA in HIV replication

Cellular microRNA (miRNA) is a small non-coding RNA molecule that is composed of 22-25 nucleotides and found in most eukaryotes (326). miRNA can interact with target mRNA through complementary base-pairing, which results in the silencing of target protein expression (327). The process of miRNA suppressing target protein expression is very complex. In most cases, the primary target mRNA engages

with the initiation factor eIF4F complex (eukaryotic initiation factor 4F, a complex composed of eIF4A, eIF4E eIF4G), and ribosome subunit to process efficient translation (328). Following miRNA synthesis, it is processed in the nucleus, exported into the cytoplasm, miRNA is then carried into a RISC (RNA-induced silencing complex). RISC directly binds to target mRNA, guides miRNA to its target mRNA and processes the downstream reaction (329). When miRNA binds to its target mRNAs, the degree of miRNA-mRNA complementarity determines the subsequent process of target mRNA. A high degree of miRNA–mRNA complementarity enhances Ago (Argonaute)-catalyzed degradation of target mRNA by mRNA cleavage whereas a low degree of miRNA-mRNA complementarity prevents mRNA cleavage but facilitates translational repression (330). At present, there are four models describing the mechanism of miRNA induced translation repression. RISC can prevent translation through one of the following mechanisms: (1) RISC competitively binds to 5' cap of the mRNA to stop efficient translational initiation; (2) RISC binds to the mRNA and inhibits the 60s ribosome recruitment; (3) RISC directly prevents the mRNA circulation; (4) RISC binds to the target mRNAs and facilitates the mRNA to detach from ribosomes (331).

During HIV replication, HIV encodes minimal genetic information, therefore it has to utilize host cellular machinery to complete replication (332). Several groups have utilized genomic, transcriptomic or proteomic methods to identify a series of miRNAs modulated by HIV and characterized their function in HIV replication (332, 333). These miRNAs can influence viral replication in both negative and positive manners (333).

Cellular miRNAs have play different role in HIV replication. First, cellular miRNAs can enhance HIV replication through various pathways. Some cellular miRNAs can enhance viral replication by indirectly regulating viral transcription. For instance, it has been demonstrated that miR-34a and -217 are able to bind the 3′UTR of mRNA of SIRT1, which repress the translational expression of the SIRT1 (silent mating type information regulation 2 homolog 1) This results in the subsequent increase of the acetylation of NF-kB and finally enhances Tat-induced transactivation of HIV LTR (334, 335). Later, Chiang Karen et al also found that HIV can specifically upregulate the expression of miR-132 and they characterized the function of miR-132 in HIV replication. They showed that miR-132 can downregulate the expression of a transcriptional factor MeCP2 (Methyl-CpG-binding protein 2), which consequently stimulates viral replication (336). Some miRNAs can influence viral replication through downregulating host antiviral proteins. The cellular miRNA Let-7c downregulates the expression of the antiviral protein p21 and the miRNA-124a and -34a-5p downregulate TASK1 (TWIK-related acid-sensitive potassium channel 1), which leads to an increase in virion release and higher copy numbers of viral genome in infected cells (337). Since TASK-1 interacts with Vpu and the overexpression of TASK-1 decreases the Vpu's mediated viral release, it is possible that miRNA-124a and -34a-5p downregulate TASK1 and increase the virion release from infected cells (337, 338).

Second, cellular miRNAs also can inhibit viral replication (333). For example, some miRNAs directly target viral mRNA and downregulate viral protein expression.

The expression of miR-29 targets nef mRNA and destabilizes it, which results in the disruption of Nef expression and consequently inhibits viral replication (339). Some miRNAs can also directly decrease the expression of cellular transcription factors required for efficient viral expression. MiR-17-5p and -20a are able to bind to the 3′-UTR of PCAF (P300/CBP-associated factor), an important histone acetyltransferase required for viral Tat's activity (340). This binding decreases the expression of PCAF, which indirectly reduces the Tat-mediated transactivation (340).

miR181A (includes miR181A1 and miR181A2) is a member of miR181 family, which consists of miR181A, 181B, 181C and 181D (341). Previous studies have revealed that miR181A is involved in multiple biological functions, including organ development, hematopoiesis, cancer formation, and autoimmune disorders (342-345). For instance, the induction of miR181A can cause apoptosis in lung cancer cells (346). In addition, miR181A was also reported to regulate HIV replication. The overexpression of miR181A is able to silence the expression of SAMHD1 (SAM Domain And HD Domain-Containing Protein 1), a restriction factor against HIV infection (347). However, whether miR181A plays an important role in HIV-1 infected cells still remains unknown.

### 1.4.2 Role of HDACs in HIV replication

Histone deacetylase (HDAC) are a class of enzymes that can remove acetyl groups from a ε-N-acetyl lysine amino acid on histone or non-histone proteins (348). In humans, there are 18 members in this family and they can be divided into four groups: HDAC

class I includes HDAC1, 2, 3 and 8; HDAC class II includes HDAC4, 5, 6, 7, 9 and 10; HDAC10 class III includes SIRT1-7; HDAC10 class IV includes HDAC11 (348, 349). By their enzymatic activity, HDACs mediate multiple biological processes, including transcription, cell cycle and autophagy (348). For example, HDAC1, 2 and 3 were reported to inhibit cellular transcription through their enzymatic activity (350). They target histone H3 and H4 and induce the deacetylation of lysine on these histones, which enhances the binding of the histone to DNA, making the chromosome more condensed and inhibiting cellular transcription (350).

To date, the roles of HDACs in HIV replication have received extensive investigation. Accumulated evidence shows that HDACs is important for HIV latency formation (105). Class I HDAC members, including HDAC1, 2 and 3, are essential for HIV transcription through epigenetic regulation (105). These HDAC members can be particularly recruited to the HIV LTR and induce the deacetylation of histone H3 and H4 to mediate the degree of chromatin compaction, which inhibits viral transcription and subsequently contributes to the formation of HIV latency (105). Based on this mechanism, a series of HDACs inhibitors were developed to reactivate HIV latent reservoirs and their clinical potency is under investigation (105). Also, HDACs can mediate viral transcription through regulating cellular transcriptional factors. SIRT1 can deacetylate NF-kB subunit p65 and block T cell activation, while SIRT1 can be suppressed by Tat to induce NF-kB pathway, which results in the T cell hyperactivation and subsequent increase of viral transcription (351). In addition to the regulation in viral transcription, HDAC6 was shown to inhibit HIV entry and cell fusion by preventing

the acetylation status of cortical tubulin and preventing Vif-mediated A3G degradation to reduce progeny virus infectivity (352, 353). However, the roles of other HDAC family members in HIV replication, such as HDAC10, are still not clear.

HDAC10 is a member of HDAC class IIB (354). It contains an Hda1p-related putative deacetylase domain in N-terminal and a leucine-rich domain in C-terminal (354). HDAC10 was reported to play multiple functions in the biological process, including cellular transcription, autophagy and cell cycle (355-357). However, whether HDAC10 plays an important role in HIV replication and what is the function of HDAC10 in HIV replication remains unknown.

## 1.5 Hypothesis and Objectives

During my thesis, I investigated the effect of vEnv on HIV transcription. Based on previous studies, I hypothesized that ***vEnv can stimulate viral transcription in HIV infected cells through modulating multiple cellular pathways***.

To demonstrate the hypothesis, I set up four objectives:

(1) Demonstrate the vEnv can specifically stimulate viral transcription in HIV infected CD4+ cells. The effect of vEnv on viral transcription would be examined in TZMb1 cells and HIV infected CD4+ T cell, including J-Lat 6.3 cell line, HIV infected primary resting CD4+ T cell and PBMCs isolated from aviremic HIV-infected patients.

(2) Identify the cellular genes involved within this interaction of vEnv and CD4/coreceptors by whole transcriptome sequencing. The cellular genes modulated by vEnv would be screened by transcriptome sequencing and chosen genes (miR181A2

and HDAC10) would be further identified by quantitative RT-PCR and western blots

(3) Identify the genes involved in viral transcriptional activation and investigate how these genes stimulate viral transcription in HIV infected cells. Based on the RNA-seq results, I would identify the genes acting in viral transcription and characterize their roles by knockdown or overexpression methods.

(4) Identify the genes associated with different steps of viral replication (including integration and transcription). The interaction between vEnv and CD4/coreceptors plays an important role in the HIV life cycle. It not only mediates viral entry and fusion, but also regulates the other steps of viral replication. Based on the RNA-seq result, I would identify cellular inhibitor factors that act in viral replication and investigate how they perform their inhibitory function in HIV replication.

# Chapter 2

# Material and methods

## 2.1 General reagents

### 2.1.1 Plasmids

HIV-1 plasmid carrying a secreted Gaussia luciferase (Gluc) gene (ΔRT/ΔEnv/Gluciferase), HIV-packaging plasmids pCMVΔ8.2, CMVinGag-Pol, HIV-1 envelope glycoprotein plasmids pLET-Lai (X4-tropic) and pLET-JRFL (R5-tropic), CMV-VSV-G, CMV-HA, CMV-ZGP plasmids (pCAGGS-ZEBOV-GP) and HIV-1 plasmid NL4.3 (PNL4.3) were described previously (358-361). Lentiviral-vector encoding hs-miR-181a-2-3p miRNA, hsa-miR-181a-2 and miR control plasmids were purchased from Applied Biological Materials Inc (Richmond, BC). HDAC10 shRNA-1 (target: ATCCCATCTAAGAGGTACAGG) and HDAC10 shRNA-2 (target: TGCGGTGTCATTTCTGCGGTG) lentiviral plasmid and Scramble control plasmid (target:CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG) were purchased from Open Biosystem. HDAC6-FLAG expression plasmid was purchased from Addgene (362). HDAC10-Myc expression plasmid was purchased from Sino Biological Company. pFLAG-HDAC10 H135A was kindly provided by Dr Li Y (357). GFP-IN and GFP-IN mutants were described in (363) and IN-YFP was described in (364).

### 2.1.2 Chemicals

Bicyclam JM-2987, TAK 779, vorinostat (VOR), recombinant HIV-1 gp120 (CM) and HIV-1 gp120 (Bal) were obtained from the NIH AIDS Research and Reagent Program. Valproic acid (VPA) and phytohemagglutinin (PHA) were purchased from Sigma and Roche. Protein A beads was purchased from Sigma. SYBR GREEN kit was purchased from Roche. EasySep Human CD4+ T Cell Isolation Kit was purchased from Stemcell Technologies. Protease Inhibitor Cocktail Set III, puromycin (puro) and NP-40 were purchased from Calbiochem. ECL kit was purchased from PerkinElmer Life Sciences. WST-1 kit was purchased from Roche.

### 2.1.3 Antibodies

The antibodies used for Western blotting (WB) were as follows: rabbit anti-green fluorescent protein (anti-GFP) polyclonal antibody (Molecular Probes), rabbit polyclonal antibodies against avian influenza NA protein (Cedarlane Lab, Ontario), mouse anti-PCAF (Santa Cruz), mouse anti-HDAC10 (Santa Cruz), rabbit anti-HDAC6 (Santa Cruz), anti-T7-HRP conjugated antibody (Novagen), mouse anti-Tubulin antibody (Sigma) and rabbit monoclonal antibodies against acetyl- group in lysine protein (Abcam). HIV-1 gp120 monoclonal antibody (ID6), human HIV antibody NIH45-46G54W and HIV-1 p24 monoclonal antibody were obtained from the NIH AIDS Research and Reagent Program. Mouse monoclonal antibody (2G4) against EBOV glycoprotein was described previously (365). HRP-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were purchased from Amersham Biosciences

(Mississauga, Ontario).

## 2.2 Cells and Virus

### 2.2.1 Primary cell isolation and cell culture

Human embryonic kidney HEK 293T and TZM-b1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin. HIV latently infected cell J-Lat 6.3, CD4+ T cell line Jurkat cell and C8166 T cell were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy adult volunteers or HIV aviremic patients by sedimentation in Ficoll-Hypaque (Sigma-Aldrich St Louis, MO). CD4+ T lymphocytes were isolated from peripheral blood mononuclear cells by negative selection with an EasySep Human CD4+ T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). Purified resting CD4+ T cells and PBMCs were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin and 10μg/ml IL-2. All HIV-related analyses in isolated PBMCs from HIV aviremic patients were conducted in accordance with our internal ethics committee and were approved by the Ethics Committee from the University of Manitoba. Written informed consent was obtained from all subjects.

### 2.2.2 Virus and virus-like particle (VLP) preparation

Nevirapine-resistant X4 tropic HIV-1 (N119, Cat1392) was obtained from the NIH AIDS Research and Reference Reagent Program. HIV-1 vEnv (Env-VLP) was generated by co-transfecting HEK 293T cells with Gag-pol expresser and pLET-Lai (X4-tropic) or pLET-JRFL (R5-tropic). H5N1-VLP, EBOV-VLP and VSV(G)-VLP were produced by co-transfecting HEK 293T cells with pCMVΔ8.2 and CMV-VSV-G, CMV-HA or CMV-ZGP, respectively. Lentiviral particles expressing MiR-181A2, MiR-181A2-3p were produced by co-transfecting HEK 293T cells with VSV-G expression plasmid, pCMVΔ8.2 and lentiviral vector HSA-MiR-181A2-3p or HSA-MiR-181A2 or miR Control, respectively. One cycle of replicating virus (Gluc+) was generated by cotransfecting HEK 293T cells with HIV ΔRT/ΔEnv/Gluciferase plasmid, CMVinGag-Pol, and VSV-G plasmid. Lentiviral particles expressing Scramble shRNA, HDAC10 shRNA-1 and HDAC10 shRNA-2 were produced by co-transfecting HEK 293T cells with VSV-G expression plasmid, pCMVΔ8.2 and lentiviral vector TRC-Scramble shRNA or TRC HDAC10 shRNA-1 or TRC HDAC10 shRNA-2, respectively. Progeny virus (PNL4.3) was generated by co-transfecting HEK 293T cells with PNL4.3 /GFP or PNL4.3/HDAC10 or PNL4.3/HDAC10 H135A plasmid. After 48 hours of transfection, all supernatants were collected and spun at 3000 rpm for 30 minutes to clear debris, and the viruses or VLPs were concentrated by ultracentrifugation at 35000 rpm for 90 minutes. Concentrated virus or VLP stocks were quantified for Gag p24 levels using an anti-p24 ELISA kit.

Virus inactivation: HIV virus was treated with indicated concentrations of aldrithiol-2 (AT-2) for 1 hr at 37℃ and filtered with centrifuge filters (Amicon Ultra-4) to remove AT-2 for further analysis (278).

### 2.2.3 Cell infection

For PHA-stimulated PBMCs, isolated PBMCs were first cultured in RPMI 1640 containing IL-2 (10μg/mL) and PHA (3 μg/ml) for three days. Then, $1x10^6$ stimulated PMBCs were infected with 10 ng (p24 gag) of VSV-G pseudotyped one cycle Gluc+ HIV-1 for two hours, washed and cultured in RPMI medium without PHA. Forty-eight hours later, the infected cells were prepared for further experiments. For CD3/CD28-stimulated PBMCs, isolated cells were cultured with anti CD3/CD28 (25μl/1 million cells) for two days followed by infection with HIV-1 (N119) MOI=0.1 for two hours. After additional culturing for 48 hours, the infected cells were ready for further experiments. Resting primary CD4+ T lymphocytes isolated from peripheral blood mononuclear cells of healthy donors were cultured in RPMI supplemented with 10% FBS and IL-2 (10 μ/ml) for 2 days, and $1x10^6$ cells were then infected with HIV-1 (N119) MOI=0.5 by spinoculation at 1400 rpm for 2 hours at room temperature. After overnight incubation, cells were washed and cultured in RPMI containing IL-2 for three days to establish infection.

### 2.2.4 Env-VLP, gp120, and shedding gp120 treatment of cells

Various VLPs were generated by transfecting 293T cells with plasmids encoding

gag-pol expression plasmid and various viral envelope glycoprotein (HIV, VSVG, H5N1 or Ebola virus) respectively.TZM-b1 cells in each well of a 24-well plate were treated with varying concentrations of HIV-1 Env-VLP (1-10 ng Gag p24), VSV, H5N1 (A/Hanoi/30408/2005) or Ebola virus envelope pseudotype-VLPs (4 ng Gag p24) or recombinant HIV-1 gp120 (0.5-2 μg), HIV-1 Bal gp120 (40-80 ng) for the indicated time points, ranging from 4 to 48 hours. To enhance the binding of envelope to cell receptors, TZM-b1 cells were spinoculated at 1200 rpm for 30 min after adding VLPs. To assess the specific effect of gp120-CD4/coreceptors interactions on the action of Env-VLP, TZM-b1 cells were first incubated with varying concentrations of HIV-1 neutralizing antibody NIH45-46G54W (targeted to the CD4/gp120 interaction region), TAK-779 or Bicyclam JM-2978 for two hours following 24 hours of HIV Env(X4/R5)-VLP treatment. The treated cells were collected and subjected to quantitative RT-PCR or luciferase activity assays as described in the section 2.3.2 and 2.3.4.

For the assessment of the effect of the HIV shedding gp120 on HIV LTR-transcription, shedding gp120 was produced from HIV-1-transfected HEK 293T cells or infected C8166 cells. Briefly, HEK 293T cells were transfected with PNL4.3 plasmid for 48 hours, while C8166 T cells were infected with HIV PNL 4.3 for 48 to 72 hours. The supernatants from transfected HEK 293T cells or infected C8166 cells were collected following ultracentrifugation to separate the shedding gp120 from pelleted viruses. The shedding gp120 in the supernatants was further concentrated using a Vivaspin 6 centrifugal concentrator (100MWCO PES). Various volumes of shedding gp120 (detail in figure legends) were added to TZM-b1 cells for 24 hours, and the

luciferase activity of cells was measured by luciferase assay (Promega).

J-Lat 6.3, infected stimulated or resting PBMC cells and PBMCs isolated from patients were treated with Env-VLP at a concentration of 6 μg of p24 per $1\times10^6$ cells, spinoculated at 1200 rpm for 30 minutes and cultured for 24 or 48 hours for further analysis.

PBMCs were isolated from various healthy individuals, and CD4+ T cells were isolated from PBMCs using a Human CD4+ T cell enrichment kit (Stem Cells). The freshly purified CD4+ T cells were treated with Env-VLP for 24 hours and washed with PBS twice for further analysis.

### 2.2.5 Cell transfection and transduction

**Cell transfection:** The day before transfection, $2.5\times10^6$ HEK 293T cells were plated into 10 cm plate and incubated with complemented DMEM medium. On the second day, the old medium was replaced with fresh DMEM without FBS 2 hours prior to transfection. The plasmid was diluted with PBS to 0.1 μg/μl and PEI was diluted with PBS to 0.1 μg/μl. Both of them were mixed and incubated for 20 minutes at 37 ℃. The mixer was added into cells and after 2 hours, the DMEM medium was replaced with fresh complemented DMEM medium and incubated for 48 hours for further analysis.

**Cell transduction:** To overexpress miR-181A2 or miR-181A2-3p, the miR-181A2-VLPs or miR-181A2-3p–VLPs were used to transduce J-Lat 6.3 T cells, and the cells were washed after 24 hours of transduction. After 96 hours, the cells were collected and used for further analysis. To generate HDAC10 knockdown cells, the

Jurkat T cells were transduced with the TCR-HDAC10 shRNA-1-VLP, TCR-HDAC10 shRNA-2-VLP or TCR-scramble shRNA -VLP, respectively. After 96 hours, the cells were selected with 2 μg/ml puromycin for 2 days and collected for further analysis.

## 2.3 General methods

### 2.3.1 Bacteria transformation and maxi preparation

**Bacteria transformation:** The competent bacteria AG1 was taken out of -80℃ and thawed on ice. 100ng of DNA was added to 50 μL of competent AG1 bacteria in a 1.5mL tube and gently mix by flicking for a few times. Then the mixture of the competent cell and DNA were incubated on ice for 20-30 minutes, followed by heat shock. Each transformation tube was placed into a 42°C water bath for 90 seconds and then on ice for 2 minutes. 1 ml liquid LB medium (Lysogeny broth medium) was added into the mixture and the tube was kept shaking at 37℃ for 30 minutes. At last, the heat shocked bacteria were plated on LB solid plate with specific antibiotics and incubated at 37℃ overnight.

**Maxi preparation**: the process of maxi preparation was described in the protocol of GenElute™ HP Plasmid Maxiprep Kit on the Sigma website.

### 2.3.2 Quantitative RT-PCR and data analysis

**Measurement of transcription by quantitative RT-PCR**:

Different Env-VLP-treated cells were collected and washed with PBS twice. Total RNA was isolated from cells using a High Pure RNA Isolation Kit (Roche) and reverse-

transcribed into cDNA with M-MLV reverse transcriptase (Promega). The relative expression levels (comparative transcription) of target mRNA was detected by quantitative RT-PCR analysis and normalized using the household gene *gapdh*. Quantitative RT-PCR reactions were performed by a two-step reaction in a total volumn of 20 μl using a LightCycler® 480 SYBR Green I Master kit（Roche), and in a thermocycler (Stratagene MX3000) with the following protocol: one cycle at 95 ℃ for 10 minutes; and 40 cycles at 95 ℃ for 10 seconds; 60℃ for 30 seconds; and 72℃ for 30 seconds. The data were organized using the Prism program, and the p value was calculated using unpaired t tests. The primer sequences are as follows:

5'-Gag: ATCAAGCAGCCATGCAAATG;

3'-Gag: CTGAAGGGTACTAGTAGTTCC;

5’-Gfp: GGTGGTGCAGATGAACTTCA;

3’-Gfp: AACCACTACCTGAGCAC;

5’-*Gapdh*: TGGGTGTGAACCATGAGAAG;

3’-*Gapdh*: ATGGACTGTGGTCATGAGTC;

5’-PCAF: TGCTGTCAGTATTTTAACACCC;

3’-PCAF: GCACTAAACTGGAATCCCAAG;

5’- miR-181A2: TCAGAGGACTCCAAGGAACATT;

3’-miR-181A2: GCTAACGGTCAG TGGTTTTTTC;

5’-HDAC10: ATGTGGCTGTTCGGAGAGGC;

3’-HDAC10: CTGCACTCCTGGCTGCAATG.

**Measurement of total viral DNA, integrated DNA and 2-LTR viral DNA**:

After infection and incubation, cells were collected and washed twice with PBS. Total DNA was isolated from cells by QIAmp blood DNA mini kit from Qiagen Company. The levels of HIV-1 total DNA, integrated DNA, and 2-LTR viral DNA were quantified by Mx3000P real-time PCR system (Stratagene, CA) following steps described in (366). The HIV total DNA was quantified using the primers (TD- Gag Fr and TD-Gag Rv) targeting the Gag region. The integrated DNA was first amplified by an Alu-LTR-nested PCR and then quantified by quantitative RT-PCR with the TD-GAG Fr and Rv primers. The 2-LTR viral DNA was quantified by primers MH535 and MH536 and the 2LTR probe, targeting LTR-LTR junctions.

The primers sequences are given below:

TD-Gag Fr: 5′-ATCAAGCAGCCATGCAAATG-3′

TD-Gag-Rv: 5′-CTGAAGGGTACTAGTAGTTCC-3′

Alu-Fr: 5′-TCCCAGCTACTCGGGAGGCTGAGG-3′

Int-Gag: 5′-GTCCAGAATGCTGGTAGGGCTATACA-3′

MH535: 5′-AACTAGGGAACCCACTGCTTAAG-3′

MH536: 5′-TCCACAGATCAAGGATATCTTGTC-3′

2LTR probe: 3′-6-carboxyfluorescein-[FAM]-ACACTACTTGAAGCAC

TCAAGGCAAGCTTT-6-carboxytetramethylrhodamine [TAMRA]-5′

### 2.3.3 Co-IP assay in HEK 293T cells

**Co-IP**: For the interaction of HDAC10 and HIV integrase, $2.5 \times 10^6$ HEK 293T cells were plated into 10 cm plate before the transfection. On the second day, these cells

were transfected with the indicated amount of HIV integrase expression plasmid (GFP-IN), HDAC10 expression plasmid (HDAC10-Myc) and GFP plasmid. After 48 hours, cells were collected and washed with PBS three times. Cells were lysed by 1 ml RIPA buffer supplemented with PI (protease inhibitor) for 30 minutes on the ice. The supernatant was collected by centrifuge at 14,000 rpm for 30 minutes. Following that, the cell lysis supernatant was added with anti-GFP or anti-Myc antibody and incubated overnight followed by adding protein A beads for 2 hours. After the incubation period, the beads were washed three times with RIPA buffer and boiled with 2× loading buffer for 10 minutes for western blots analysis.

For the interaction of HIV integrase and LEDGF, after transfection, cells were collected and washed three times with PBS. Cells were lyzed by CSK buffer (0.5% NP-40, 10% (w/v) sucrose, 10mMPipes pH6.8, 1mM $MgCl_2$, 1mM DTT, 400mM NaCl and PI) for 30 minutes on the ice. The supernatant was collected by centrifuge at 14,000 rpm for 15 minutes. Afterwards, the supernatant was added with anti-GFP antibody and incubated overnight followed by adding protein A beads for 2 hours. After the incubation, these beads were washed with CSK wash buffer (0.5% NP-40, 10 mM Pipes pH 6.8, 10% (w/v) sucrose, 1 mM DTT, 1 mM $MgCl_2$, 100 mM NaCl and protease inhibitor) three times and boiled with 2× loading buffer for 10 minutes for western blots analysis.

### 2.3.4 Luciferase assay and Western blotting

**Luciferase assay：** Following treatment with Env-VLPs or infected with HIV,

TZMb1 cells were collected and washed three times with PBS. The cell pellet was lysed with lysis buffer (Promega) and the luciferase activity in the supernatant was detected using a GLOMAX Luminometer (Promega) and normalized using the total protein concentration.

**Western blots:** To detect target protein expression, samples were lysed in RIPA buffer and directly loaded onto an 8% or 10% SDS-PAGE gel. Then the protein was transferred onto the nitrocellulose membrane overnight. Following being blocked by 5% milk in PBS, the membrane was washed three times with PBS and incubated with the relevant primary antibody (dilution 1:1000) and secondary antibody (dilution 1:5000). Following three washes in PBS, the target protein expression was visualized by the reaction of secondary antibody and ECL substrate.

### 2.3.5 WST-1 assay and Trypan blue staining

**Cell proliferation measurement**: $5\times10^4$ cells were plated into 96 well plate and cultured in 100μl/well culture medium in the cell incubator. The reagent WST-1 10μl was added to each well at the indicated time points and incubated at 37℃ for 4 hours. After incubation, the cells and reagents in the 96 well plate were mixed thoroughly for 1 minute and the absorbance of samples was detected at the wavelengths of 450nm and 650nm (reference wavelength). The proliferation ratio was calculated by A450-A650.

**Cell viability measurement**: Cells were suspended in 1 ml culture medium and 10μl of the cell suspension was added with 10 ul Trypan blue. The cell viability was measured by countess II FL automated cell counter.

### 2.3.6 ChIP (chromatin immunoprecipitation)

According to the steps described previously (367) , these treated cells were collected, and cross-linked with formaldehyde to a final concentration of 1% for 10 min at 37℃ followed by lysing on ice for 30 minutes. The cell debris was cleared by centrifugation at 13,000 rpm for 10 minutes. The supernatant was transferred to a new tube and sonicated 15 times of 10-second bursts. Next, the histone H3 antibody or IgG (Millipore) was added to the lysis mixture, and the liquid was incubated with Protein A beads (Sigma) at 4℃ overnight. The beads were then washed and mixed with 10% Chelex*100 slurry (Sigma) and boiled for 10 minutes. The samples underwent proteinase K treatment, and the proteinase K was inactivated at 55℃ for 30 minutes. Finally, the DNA was purified and prepared for further analysis. The copy of the LTR -109-+82 region was detected by quantitative RT-PCR with the primer pair:

LTR -109-+82 (F: TACAAGGGACTTTCCGCTGG);

LTR -109-+82 (R: AGCTTTATTGAGGCTTAAGC),

and the fold enrichment was calculated using $2^{-(CtIP-CtIgG)}$ (367).

### 2.3.7 RNA-Seq: Total RNA-seq library preparation and sequencing

Whole-genome sequencing was carried out at the next-generation sequencing (NGS) facility of the Manitoba Institute of Child Health (MICH) using the Illumina MiSeq platform. Briefly, J-Lat 6.3 T cells were either treated with Env-VLP or left untreated for 24 hours. Total RNA was extracted from J-Lat 6.3 T cells using a Nucleospin kit (Macherey-Nagel, Germany) according to the protocol provided by the

manufacturer. Total RNA was depleted of ribosomal RNA using the Epicentre Ribo-Zero Magnetic Kit, fragmented using RNaseIII and assessed for yield and size by the Agilent Bioanalyzer 2100. The fragmented RNA was hybridized and ligated to SOLiD adaptors, and reverse transcription was performed. The cDNA was size-selected to remove fragments smaller than 100 bp using Agencourt AMPure (Beckman Coulter) magnetic beads and subjected to limited PCR amplification. After purification, the resulting libraries were quantitated using Qubit and assessed again for size and quality using a Bioanalyzer. The libraries were pooled to be equimolar and were amplified on SOLiD sequencing beads using the EZ bead system (ABI). The beads were loaded into one lane of a SOLiD flowchip and sequenced on the SOLiD5500xl platform (ABI) using paired-end (50X35bp) tags.

### 2.3.8 Statistical analysis

Statistical analysis of Env-VLP functional assays, including the results of luciferase assays, comparative quantitative RT-PCR and p24 ELISA, were performed using the unpaired t test (considered significant at $P \leq 0.05$) or multiple t test (with correction using the Holm-sidak method) using GraphPad Prism 6.01 software.

RNA-seq bioinformatics data analysis: A total of 201,127,972 pairs of SOLiD sequence reads were generated from the samples. After quality checking, 158,718,406 of these reads were mapped onto the human reference genome (hg19) using Lifescope v2.5.1 software (Life Technologies) with the 2-mismatch setting. The mapped reads were quantified against the gene features of the seq database. The gene expression

values were normalized as RPKM (reads per kilobase per million reads). The bioconductor package edgeR, based on a Negative binomial model, was used to infer the differential expression gene (DEG) expression (368).

# Chapter 3

## vEnv stimulates viral transcription in HIV infected cells

### 3.1 Introduction

During HIV infection, infected cells produce not only infectious viruses but also large amounts of noninfectious or defective particles as a result of highly reading-frame-error-prone reverse transcriptase (308, 309). Some studies estimated that the ratio of infectious to noninfectious particles is between 1 in 1,000 and 1 in 60,000 depending on the measurement methods (309-311). Compared with infectious virus, most defective particles still keep an intact structure, on which the envelope glycoprotein retains its function. Although they still can enter target cells, the reverse transcription, integration or subsequent replication is blocked due to the mutation in viral proteins. Some studies have shown that these defective particles are able to stimulate CD4+ T lymphocytes and induce their apoptosis, which contribute to HIV infection and pathogenesis (318-321). However, the physiological role of the large amounts of defective particles present during infection is still not fully understood.

During natural infection, HIV envelope glycoproteins gp120 and gp41 can be present in several forms, including trimer on virion, trimer on infected cell surface and

shedding form. The binding of envelope glycoproteins to cell receptor CD4 and coreceptors (CCR5 or CXCR4) is essential for viral entry into CD4+ T lymphocytes, monocytes and macrophages (369, 370). Meanwhile, the interaction of gp120 with CXCR4 has been shown to activate downstream signaling to facilitate viral infection and replication. Whether the envelope glycoproteins expressed on defective particle have important functions in HIV pathogenesis remains unknown.

Based on the above information, I proposed that vEnv stimulates viral LTR controlled transcription in HIV infected cells. Therefore I designed four objectives to test this hypothesis:

(1) Demonstrate that vEnv stimulates LTR controlled transcription in TZMb1 cells.

(2) Demonstrate that the interaction between envelope glycoprotein and CD4/coreceptor (CCR5 or CXCR4) is required for viral transcription activation

(3) Compare the ability of activating viral transcription among shedding gp120, recombinant gp120 and virion-associated gp120.

(4) Confirm that vEnv stimulates viral transcription in HIV-1 infected CD4+ T cells, including J-Lat 6.3 cell line, HIV-1 infected resting PBMCs and resting PBMCs isolated from aviremic HIV-infected patients

## 3.2 Results

### 3.2.1 HIV non-infectious virus-associated envelope glycoprotein (vEnv) stimulates HIV LTR-driven gene expression

Although these defective viruses are not able to infect hosts, they are far from innocuous (308). To investigate whether these noninfectious viral particles play any role in HIV-infected cells, I treated the HIV viruses (N119) with aldrithiol-2 (AT-2), which can inactivate viruses by preferential covalent modification of internal viral proteins (NC) while preserving the structural and functional properties of the viral envelope protein (371), and used them to infect C8166 T cells. The results showed that AT-2-treated virus lost its infectivity (Fig.3.1 A). Concurrently, the AT-2 viral particles were used to treat TZMb1 cells, which express CD4, CCR5, and CXCR4 and contain a reporter gene firefly luciferase (Luc) driven by HIV LTR (372). Interestingly, the inactivated viruses were able to activate HIV LTR-controlled Luc expression (Fig. 3.1B), suggesting a stimulating effect on HIV-LTR driven transcription in TZMb1 cells.

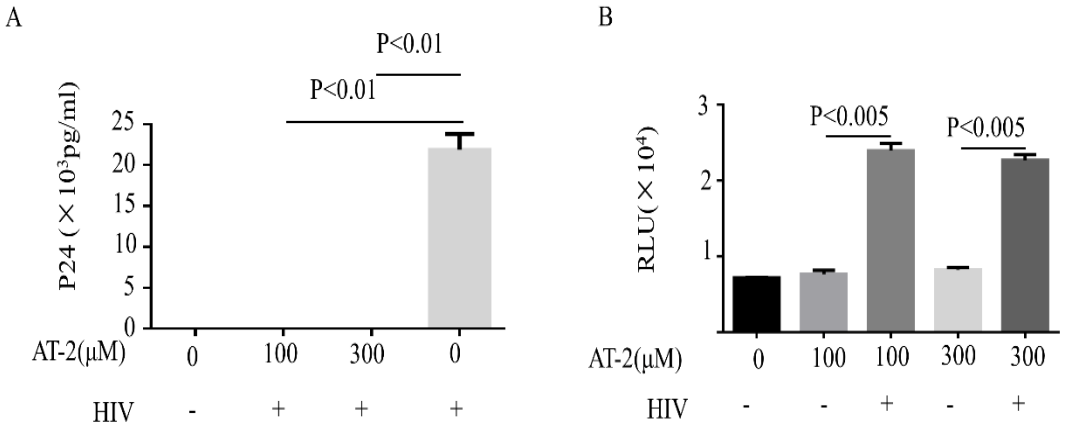



Figure 3.1 **Inactivated virus can stimulate HIV-1 LTR controlled transcription.** (A) Detection of infectivity of AT-2-treated HIV virus. Wild-type HIV virus was treated with the indicated concentrations of AT-2 for 1 hour at 37℃ and then used to infect C8166 cells for three days. The p24 level in the supernatant was detected by p24 ELISA (n=2). (B) Luciferase expression in TZMb1 cells infected with AT-2-treated virus for 24 hours (n=2). HIV-1 virus N119 was treated with aldrithiol-2 (AT-2) for 1 hour at 37℃ and used to treat TZMb1 cells. After 24 hours treatment, the cells were collected and the endogenous firefly luciferase was detected, indicating the activation of HIV-1 LTR controlled transcription. (two-tailed unpaired t-test)

To identify the main determinant in viral particles for the activation of HIV-1 LTR, I produced envelope glycoprotein-incorporated HIV virus-like particles (Env-VLP) by co-transfecting HEK 293T cells with HIV X4- or R5-tropic envelope glycoprotein-expressing plasmid and HIV gag-pol expression plasmid (Delta 8.2), as described previously (358) (Fig. 3.2 A). The presence of X4- or R5-tropic vEnv on purified VLPs was confirmed by western blot analysis using anti-gp120 and anti-p24 antibodies respectively (Fig. 3.2 B). To test whether X4- or R5-tropic Env-VLP are able to stimulate HIV LTR-derived transcription, TZMb1 cells were treated with equal amounts of X4- or R5- Env-VLP, and the HIV LTR-driven Luc activity was measured after 24 hours. The results showed that both X4- or R5-Env-VLP induced 7-fold- or 4-fold-increased Luc activity respectively. In contrast, VLP lacking Env had no effect on HIV LTR-driven Luc expression (Fig. 3.2 C). I also showed that the Env-VLP-mediated stimulating effect was dose- and time-dependent (Fig. 3.2 D and E). Briefly, I treated the same amount of TZMb1 cells with the different amount of Env(X4)-VLP and detected the relative firefly luciferase activity after 24 hours. Concurrently, I treated the same amount of TZMb1 cells with the same amount of Env(X4)-VLP for different time points and detected relative firefly luciferase activity. The results indicated that both X4- and R5-envelope glycoproteins may be the main determinants in HIV VLP responsible for HIV LTR activation.

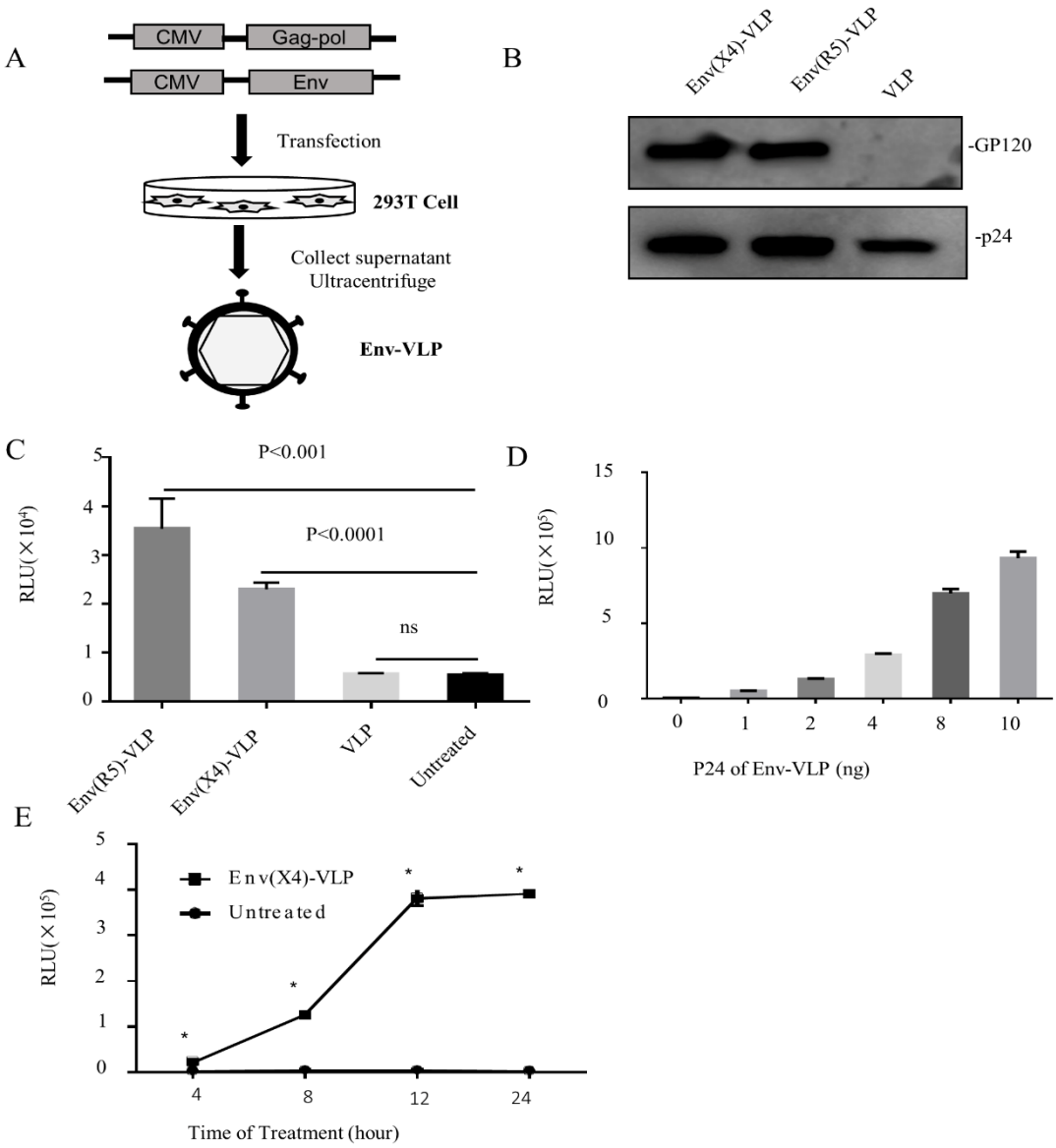



Figure 3.2 **HIV-1 vEnv can efficiently stimulate HIV-1 LTR controlled transcription.** (A) Schematic for producing HIV Env-VLP. HIV Gag-pol (Δ8.2) and Env (X4/R5-tropic) plasmids were co-transfected into HEK 293T cells; after 48 hours, Env-VLP in the supernatant was collected and concentrated by ultracentrifugation. Purified Env-VLPs were used to treat the various cells. (B) Western blot confirming the presence of gp120 and p24 of Env-VLP. (C) Luciferase expression in TZMb1 cells treated with Env(X4)-VLP, Env(R5)-VLP or VLP or untreated for 24 hours (n=3). TZMb1 cells were treated with vEnv (R5/X4) for 24 hours and the endogenous firefly luciferase was detected and normalized by total protein concentration. (D) Does-dependent assay. Luciferase expression in TZMb1 cells treated with varying amounts (0-10 ng) of Env(X4)-VLP (n=3). (E) Time-dependent assay. Luciferase expression in TZMb1 cells treated with Env(X4)-VLP for different periods (n=3). (two-tailed unpaired t-test; multiple-t test; correction for multiple comparison using the Holm-Sidak method)

### 3.2.2 HIV vEnv (X4) can specifically induce LTR controlled transcriptional increase.

Following the demonstrating that HIV vEnv (X4) can stimulate HIV LTR controlled transcription, I next tested whether other viral envelope proteins could also exhibit similar effects on HIV LTR-derived gene expression. The virus entry-competent VSV-G, H5N1 (A/Hanoi/30408/2005 strain) or Ebola (Mayinga strain) envelope glycoprotein-pseudotyped HIV VLPs (viral-like particle) were produced as described previously (373, 374), and the VLP-incorporated glycoproteins were detected by western blots (Fig. 3.3 A). Equal amounts of each Env pseudotyped VLP were used to treat the TZMb1 cells. Interestingly, I observed that only HIV Env-VLP induced significantly high Luc activity (Fig. 3.3B), while no other viral Env-VLP had any stimulating effect on HIV LTR, indicating a unique and specific effect of HIV-1 envelope glycoprotein on LTR-driven expression.

Additionally, I tested whether Env-VLP acts on HIV LTR transcription. To do so, TZMb1 cells were treated with HIV Env-VLP, and after 24 hours treatment, Luc gene mRNA was measured using quantitative RT-PCR. Treatment with HIV Env-VLP, but not VSV Env-VLP, induced a 4-fold increase in Luc mRNA (Fig. 3.3 C) in TZMb1 cells, which is correlated with elevated levels of Luc activity (Fig. 3.3 D). Altogether, the data indicated that HIV envelope glycoprotein is the main determinant that stimulates HIV LTR-driven gene transcription and expression.

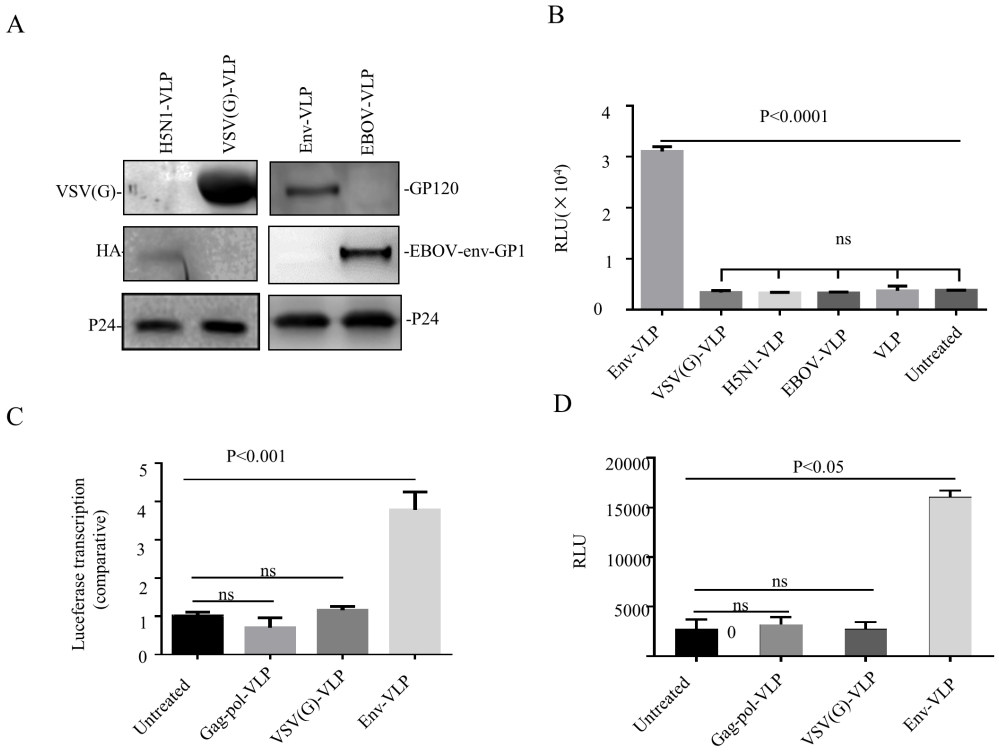



Figure 3.3 **vEnv (X4) can specifically induce HIV-1 LTR controlled transcriptional increase, compared with other viral glycoproteins**. (A) Detection of the presence of different viral glycoproteins in HIV VLP by western blot. Each VLP stock was lysed, and glycoproteins were detected using corresponding antibodies (data in the figures A and B are from two experiments). (B) Luciferase expression was detected in TZMb1 cells treated with Env-VLP, VSVG-VLP, H5N1-VLP, EBOV-VLP or VLP (without Env) or untreated for 24 hours, and luciferase activity was measured (n=3). (C) Luciferase comparative transcription (luciferase/*gapdh*, n=3) in TZMb1 cells treated with Env-VLP, VSVG-VLP, VLP (without Env) or untreated. (D) Luciferase activity was measured in TZMb1 cells treated with Env-VLP, VSVG-VLP or VLP or untreated cells (n=2). Data presented as mean and sd. Ns, not significant $p>0.05$; *, $p<0.05$. (two-tailed unpaired t-test; multiple-t test; correction for multiple comparison using the Holm-Sidak method)

### 3.2.3 The interaction between vEnv and CD4/coreceptors (CCR5 and CXCR4) is essential for HIV transcription activation

It is well accepted that in addition to mediating membrane fusion, the interaction of HIV-1 gp120 with its chemokine coreceptor (CCR5 /CXCR4) triggers cellular signaling transduction (290). I sought to determine whether the interaction of vEnv with CD4/coreceptors is required for the activation of HIV transcription. Briefly, Env(X4)-VLPs were incubated with TZM-b1 cells in the presence of a highly potent neutralizing HIV-1 antibody NIH45-46$^{G54W}$, which specifically targets the CD4 binding site of the HIV-1 envelope glycoprotein (375). Luc activity was measured after 24 hours. NIH45-46$^{G54W}$, in concentrations ranging from 0.5-2μg/ml, completely inhibited Env(X4)-VLP-induced Luc activities (Fig. 3.4 A), indicating that the gp120/CD4 interaction is essential for the action of vEnv on HIV LTR activation. Concurrently, I treated TZM-b1 cells with CCR5 chemokine receptor antagonist TAK-779 (376) or CXCR4 inhibitor Bicyclam JM-2987 (377) for 2 hours prior to incubating the cells with X4- or R5-tropic Env-VLP. Bicyclam JM-2987 is a CXCR4 inhibitor, which inhibits HIV-1 entry by blocking the chemokine receptor CXCR4 (378). TAK779 is a chemokine receptor CCR5 inhibitor which inhibits viral entry by blocking the chemokine receptor CCR5 (379). The results revealed that TAK-779 almost completely inhibited the activating effect of Env(R5)-VLP (Fig. 3.4 B) but had a moderate effect on the action of Env(X4)-VLP (Fig. 3.4 C). In contrast, Bicyclam JM-2987 had no effect on Env(R5)-VLP (Fig. 3.4 B) but significantly decreased the Luc activity induced by Env(X4)-VLP (Fig. 3.4 C). The mechanism by which TAK-779 could partially block Env(X4)-VLP-mediated

activation is still unclear. Nevertheless, my aforementioned results clearly indicated that the interactions of gp120 with CD4/coreceptors (CCR5 and CXCR4) are necessary for vEnv-induced HIV transcription.

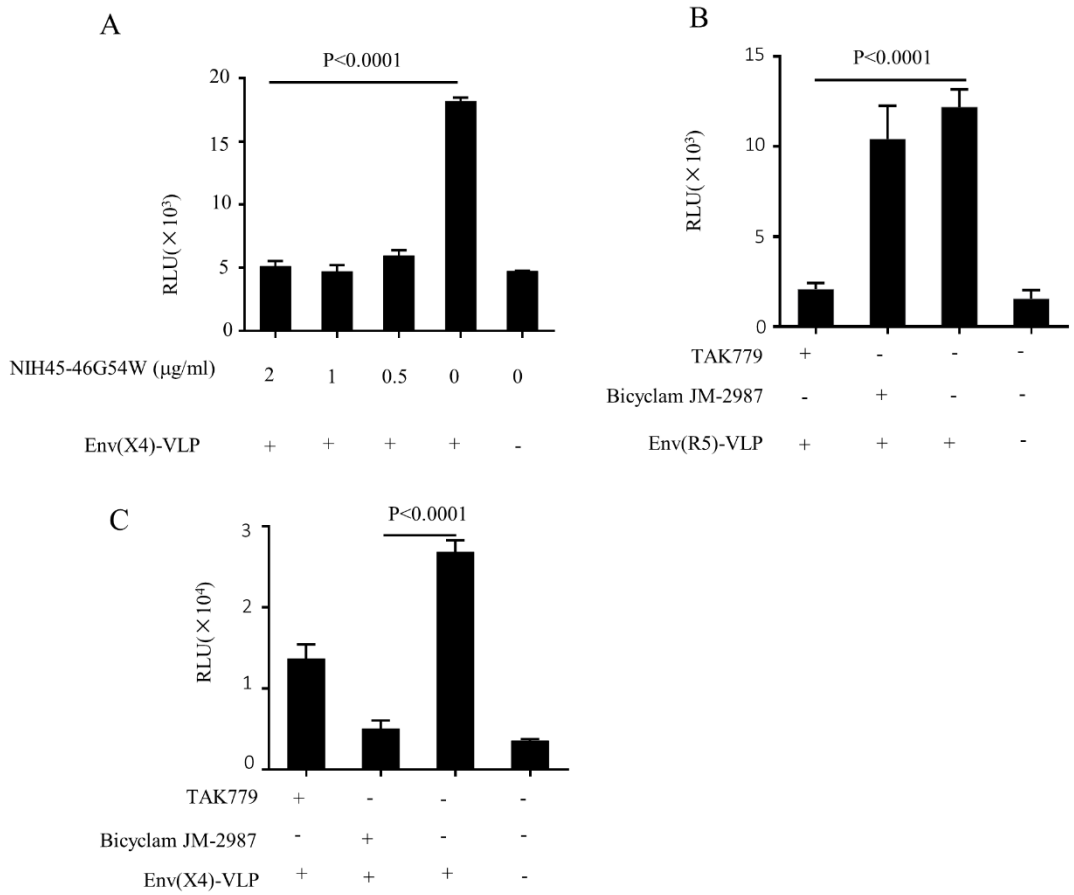



Figure 3.4 **Interaction between HIV virus-associated envelope glycoprotein (vEnv) and CD4/coreceptors is essential for HIV transcription activation.** (A) Expression of luciferase in TZMb1 cells treated with Env-VLP in the presence of different concentrations of an anti-gp120 neutralizing antibody NIH45-46G54W, which targets the interaction region between CD4 and gp120 (HXB2 strain). After 24 hours, luciferase activity in the TZMb1 cells was detected (n=3). TZMb1 cells were first cultured in the presence of CCR5 inhibitor TAK779 or CXCR4 inhibitor Bicyclam JM-2987 for 2 hr. Then cells were treated with Env(R5)-VLP (B) or Env(X4)-VLP (C) in the presence of CCR5 inhibitor TAK779 or CXCR4 inhibitor Bicyclam JM-2987. After 24 hours, cells were collected, lysed and used to measure luciferase activity (n=4). Data are presented as mean and sd. Ns, not significant $p>0.05$ (two-tailed unpaired t-test).

### 3.2.4 Virus-associated envelope glycoprotein is a significantly more effective activator of HIV transcription than shedding or recombinant HIV gp120 proteins.

It is known that shedding gp120 is present in patients' serum *in vivo* (380). As introduced in the section 1.3.1, shedding gp120 is generated by the disassociation of the gp120 and the gp41 on viral particles. Recombinant gp120 is generated from the supernatant of the cells expressing gp120. Given the ability of HIV-1 vEnv to activate HIV transcription, I next asked whether HIV shedding gp120 or recombinant gp120 could also act on HIV transcription. First, I compared vEnv and shedding gp120 for their effects on viral transcription. Briefly, the shedding gp120 from transfected HEK 293T cells or HIV (pNL4.3)-infected C8166T cells were obtained as described in the Materials and Methods section and detected by western blot (Fig. 3.5 A, lower panel). Next, shedding gp120 was added to TZMb1 cells for 24 hours. In parallel, an equal volume of Env(X4)-VLP was used as a control. The results showed that while Env(X4)-VLP induced a significantly increased Luc activity ($p<0.001$), the Luc activity produced from the cells treated with sgp120 was only moderately increased ($p<0.05$), (Fig. 3.5 A, upper panel). These results suggest that shedding gp120 may have only a limited effect on HIV transcription.

We also tested the effect of recombinant HIV-1 gp120 (CM) or HIV-1 gp120 (Bal) on HIV transcription, as these recombinant gp120s have been reported to be capable of inducing apoptosis (381, 382). Briefly, TZMb1 cells were treated with different concentrations of recombinant gp120 (CM) (Fig. 3.5 B) or recombinant gp120 (Bal) (Fig. 3.5 C) for 24 hours, and the Env(X4)-VLP or Env(R5)-VLP were used as

positive controls. The results showed that neither recombinant gp120 could activate HIV LTR gene expression (Fig. 3.5B/C, upper panels), even at much higher input amounts of recombinant gp120 compared to vEnv (Fig. 3.5 B/C, lower panels). Notably, the molecular weight of both recombinant gp120 proteins was less than 120 kDa, probably due to varied glycosylation in different production systems. Nevertheless, the data indicated that vEnv, but not the shedding gp120 or recombinant gp120 tested in this study, has a significant effect on HIV LTR transcription in TZMb1 cells.

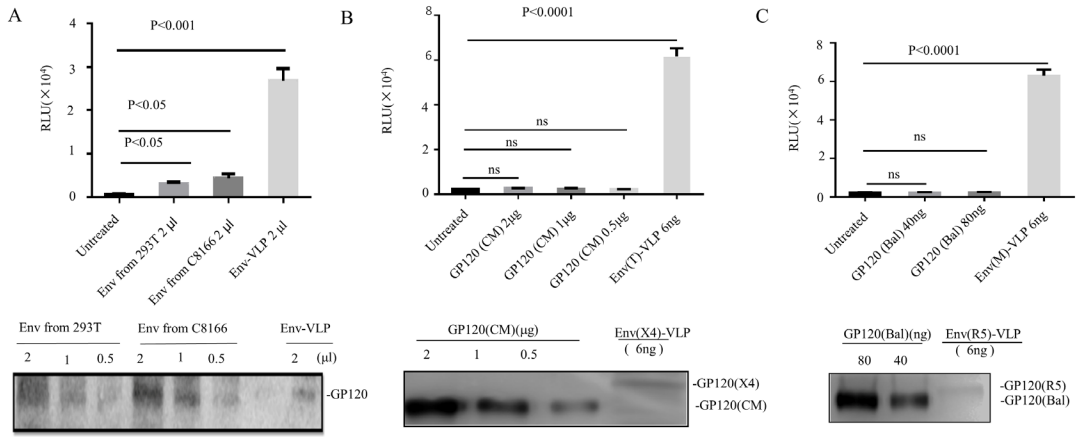



Figure 3.5 **vEnv can more efficiently stimulate HIV-1 LTR controlled transcription than shedding gp120 and recombinant gp120.** Various HIV envelope glycoproteins were compared, including vEnv, shedding Env (Env from transfected HEK 293T cells or HIV (pNL4.3)-infected C8166T cells) and recombinant gp120 (from CM strain or Bal strain). TZMb1 cells treated with Env(X4 or R5)-VLP, shedding Env proteins (A), recombinant gp120 (CM) (B) or the recombinant gp120 (Bal) (C). After 24 hours, cells were collected, lysed and used to measure luciferase activity (n=3). Data are presented as mean and sd. Ns, not significant $p>0.05$ (two-tailed unpaired t-test).

### 3.2.5 HIV vEnv stimulates viral transcription in HIV-latently-infected CD4+ T cells (J-Lat 6.3), HIV-infected non-stimulated PBMCs and PBMCs from ART-treated patients

Upon indicating that vEnv(X4) can stimulate LTR controlled transcription in TZMb1 cells, my next step was to demonstrate whether vEnv(X4) can activate viral transcription in HIV-1 infected cells. To specifically test whether vEnv induces HIV transcription in HIV-infected cells, I first tested the action of vEnv in J-Lat 6.3 T cells that harbor an Env-defective HIV provirus containing the green fluorescent protein (GFP) in the *nef* region, which has been widely used as an HIV latently infected cell model (383). Upon the treatment of HIV Env(T)-VLP, HIV transcription was monitored by the viral mRNA or GFP expression. J-Lat 6.3 T cells were treated with Env(X4)-VLP for 24 hours, the total mRNA was isolated and the gag mRNA and GFP mRNA levels were measured using quantitative RT-PCR. The gag mRNA expression increased 14- to 16-fold in Env(X4)-VLP-treated cells compared to mock-treated cells (Fig. 3.6 A). As expected, six hours of the treatment with a lower dose of Env(X4)-VLP resulted in a significantly increased mRNA level (Fig. 3.6 B). A higher mRNA and protein level in HIV LTR-driven GFP was detected in Env(X4)-VLP-treated cells by quantitative RT-PCR and western blot (Fig. 3.6 C). The data suggested that vEnv is able to stimulate HIV transcription in HIV latently infected J-Lat 6.3 T cells.

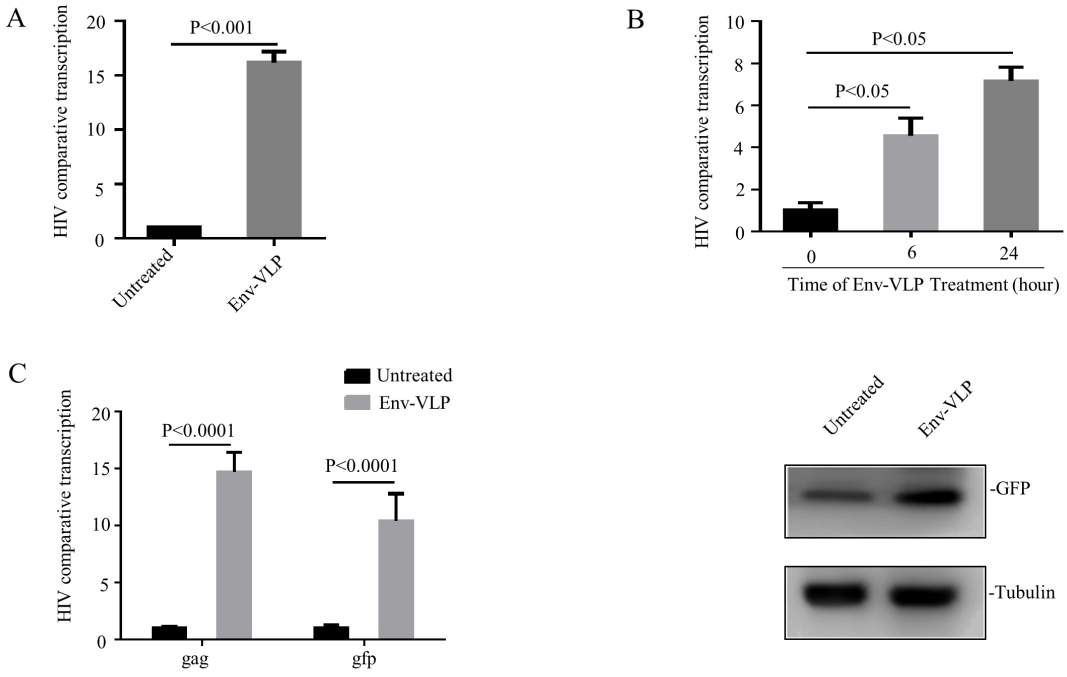



Figure 3.6 **vEnv induces viral transcription in HIV-infected CD4+ T cell line J-Lat 6.3.** (A) Increased transcription of HIV gag in J-Lat 6.3 T cells treated with Env(X4)-VLP. J-Lat 6.3 T cells were either treated or non-treated with Env(X4)-VLP for 24 hours. HIV gag mRNA was detected by quantitative RT-PCR, normalized by the housekeeping gene *gapdh* (n=3), and expressed as comparative transcription level. (B) J-Lat 6.3 T cells were treated with Env(X4)-VLP for 0, 6, and 24 hours (as indicated) and after 24 hours, cells were lysed and HIV comparative transcription levels (gag/*gapdh*) were detected. (C) Left panel: increased comparative transcription levels (GFP/*gapdh*) of the reporter gene GFP were detected by quantitative RT-PCR. Right panel: increased expression of the reporter gene GFP was detected by western blots. In addition, the Env(X4)-VLP treated or untreated J-Lat 6.3 T cells were lysed and the expression of GFP was detected by western blot with corresponding antibody (right panel). Data are presented as mean and sd. Ns, not significant p>0.05. (two-tailed unpaired t-test)

Next, I explored whether vEnv could induce HIV transcription in HIV-infected primary PBMCs. To address this question, I mimicked HIV infection in two different ways. First, PBMCs were isolated from five healthy donors and infected with HIV-1 N119 strain (MOI 0.5) overnight (384, 385). Three days post infection, infected PBMCs were treated with Env(X4)-VLP, and 24 hours later, cells were collected and HIV mRNA in the cells was measured using quantitative RT-PCR. The results showed that compared with the infected and mock-treated cells, HIV transcription was increased at various levels in the infected PBMCs treated with Env(X4)-VLP (Fig. 3.7 A). I also detected significantly greater infectious virus production from the vEnv-treated HIV infected resting PBMCs by co-culturing with C8166 T cells (Fig. 3.7 B). These results indicated that vEnv is able to induce viral transcription in infected primary resting PBMCs. Concurrently, I tested whether vEnv could effect on HIV transcription in PHA- or anti-CD3/CD28-stimulated HIV-infected PBMCs. The results showed no significant difference between the vEnv-treated and untreated groups (Fig. 3.7 C/D), suggesting that vEnv was unable to further stimulate viral transcription in PHA or anti-CD3/CD28 pre-stimulated HIV-infected PBMCs.

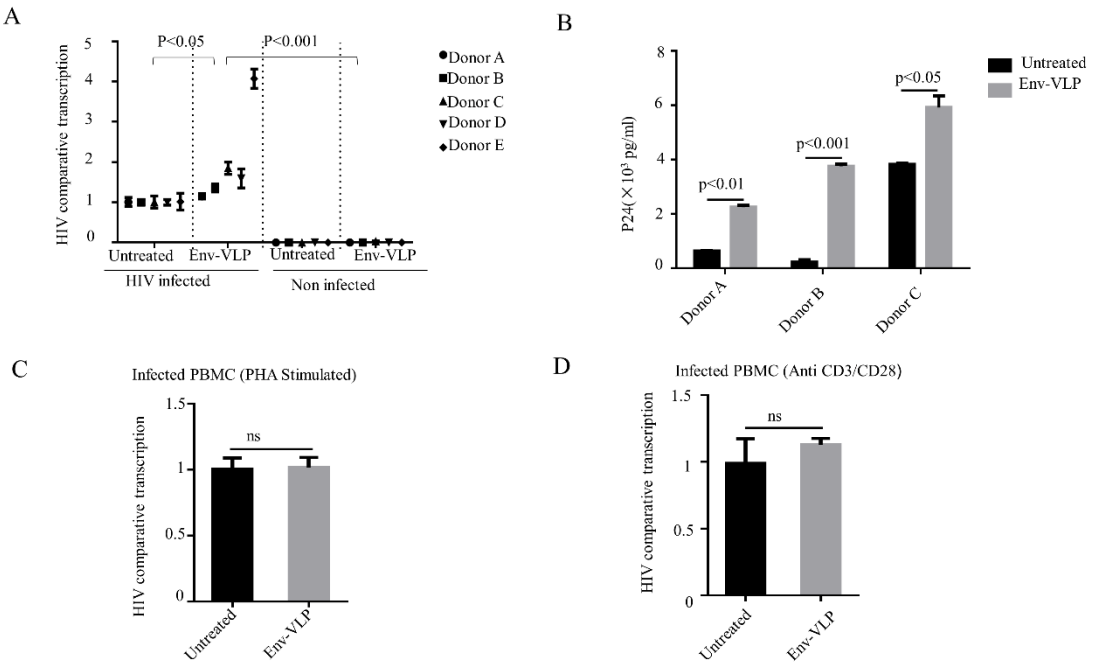



Figure 3.7 **vEnv induces viral transcription in HIV-infected non-stimulated PBMCs.** (A) HIV comparative transcription levels in HIV-infected resting PBMCs treated with Env(X4)-VLP or untreated (n=3). PBMCs were isolated from five donors and infected with HIV virus (MOI=0.5) for 24 hours without stimulation and the infected PBMCs were washed and kept in culture medium for 2 days. Then, cells were treated with Env(X4)-VLP or untreated. Meanwhile, the untreated HIV-infected PBMCs, untreated and uninfected PBMCs or Env(X4)-VLP-treated uninfected PBMCs were used as controls. After 24 hours of treatment, HIV comparative transcription levels (gag/*gapdh*) were detected by quantitative RT-PCR. (B) Concurrently, the infected resting PBMCs treated or untreated with Env-VLP, were co-cultured with C8166 T cells for three days. Then, the HIV p24 levels in the supernatant of co-cultures were measured by anti-HIV p24 ELISA (right panel). (C and D) HIV comparative transcription levels were detected in PHA or anti-CD3/CD28 stimulated HIV-infected PBMCs followed by the treatment with Env-VLP or not (n=3). Data are presented as mean and sd. Ns, not significant p>0.05. (two-tailed unpaired t-test)

The above observations indicated that vEnv is able to induce HIV transcription in the HIV-infected J-Lat 6.3 T cell line and unstimulated human PBMCs. I then interrogated whether vEnv activates HIV transcription in resting PBMCs isolated from combination antiretroviral therapy (cART)-treated aviremic HIV-infected patients. Seven patients were selected based on the following criteria: HIV-1 infected; currently treated with cART; a plasma HIV-1 RNA <50 copies per ml for at least six months; and a CD4 count >300 per ml. Following the isolation of PBMCs without stimulation, cells from each donor were either treated with Env(X4)-VLP or mock-treated. After 48 hours of treatment, cells were collected and HIV transcription was analyzed by detecting the gag mRNA level via quantitative RT-PCR. Interestingly, the results revealed the vEnv treatment led to an increased HIV transcription in PBMCs from 5 of 7 patients, with a maximum 2-fold higher transcription than in the mock-treated group (Fig. 3.8).

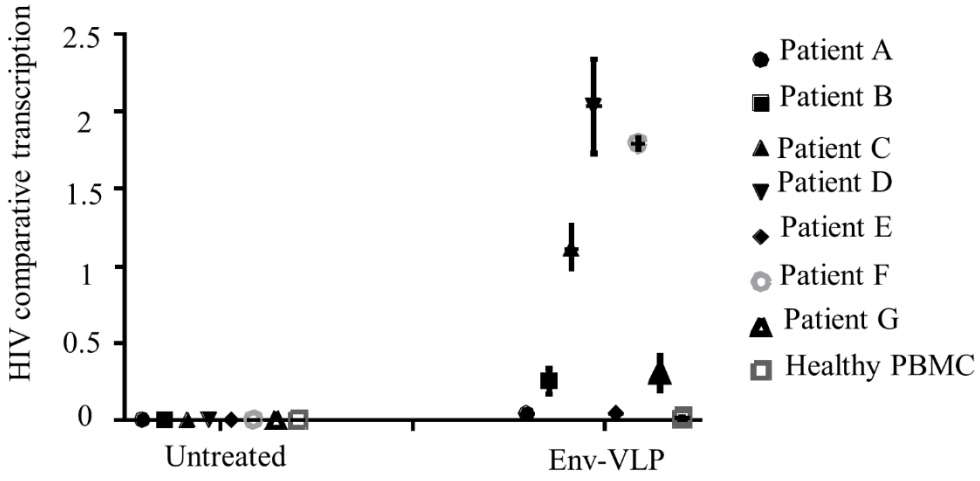



Figure 3.8 **HIV vEnv induced viral transcription in latent-infected PBMCs in ART-treated patients.** HIV comparative transcription levels in non-stimulated PBMCs isolated from seven HIV aviremic patients, that were either treated or non-treated with Env(X4)-VLP for 24 hours, and then the HIV gag mRNA was detected by quantitative RT-PCR (n=3). Data are presented as mean and sd. Ns, not significant $p>0.05$. (two-tailed unpaired t-test)

## 3.3 Discussion

During HIV infection, it is known that a large proportion of produced progeny viral particles are defective, as a result of error-prone viral reverse transcription (308, 309, 386). Although numerous studies have shown that these defective particles, especially the envelope glycoproteins present on these viral particles, significantly contribute to HIV pathogenesis *in vivo* (308, 387), their roles in the late-stage of viral replication remain elusive. In this study, I investigated the effect of the envelope glycoprotein (X4) present on HIV noninfectious viral particles on HIV-LTR-driven transcription. My study showed that noninfectious viral particles are able to stimulate HIV transcription in virus-infected cells, on which the vEnv is essential for this transcription activation. Here, this phenotype was observed in various HIV-infected cells, including the HIV-infected J-Lat 6.3 T cells, HIV-infected resting PBMCs and resting PBMCs isolated from ART-treated HIV patients.

The binding of gp120 with T cell receptor CD4 and coreceptors (CCR5 or CXCR4) is a necessary step for HIV entry into CD4+ T lymphocytes, monocytes and macrophages (369, 370, 388). This study provides new evidence that vEnv is able to activate HIV LTR-driven transcription in HIV LTR-integrated cells through the interaction of HIV vEnv and CD4/coreceptors. Moreover, it demonstrated that vEnv was able to activate viral transcription in HIV latently-infected cells. These observations suggest that the presence of HIV-defective viral particles *in vivo* may be able to stimulate HIV-infected resting T cells and that at least some of the cells become productive HIV infected cells that contribute to a higher viral load and facilitate HIV

dissemination.

Accumulated evidence suggests that HIV replication may occur in resting CD4+ T cells residing in the lymphoid tissue from HIV-1 infected individuals, either early in the disease or after long-term ART (389, 390). A recent study showed that HIV-infected resting CD4+ T cells can produce Gag protein without spreading infection (391). Thus, the observation of low-level productive infection in the presence of ART and a rapid rebound of viral replication after ART interruption suggest that undefined factors, including viral factors, are able to stimulate viral expression and replication *in vivo*, especially in human lymphoid tissue. These factors result in more infected CD4+ T cells and the establishment and /or maintenance of latent viral reservoirs. Combined with previous studies revealing that vEnv may exist in the lymphoid tissue of HARRT-treated patients (389), my observation may imply that HIV vEnv in lymphoid tissue may be the main factor that co-stimulates viral transcription and causes viral rebound. However, once HIV-infected resting PBMCs were stimulated by PHA or anti-CD3/CD28, no further effect was observed in the presence of vEnv. This is expected, since PHA or anti-CD3/CD28 treatment induces a strong activation status for T cells, enhancing viral transcription and therefore masking the effect of vEnv.

In patients' serum, gp120 can be present in several forms. One is associated with viral envelope glycoprotein (gp41), which is present on the surface of the virus and/or on the infected cells as a trimer (112, 392, 393). Another is the shedding gp120, which is present in serum in a soluble form (272). Whether they share the same stimulating activity on HIV transcription merits additional investigation. Although

studies by Claudia in 2006 indicated that recombinant gp120 also activates NFAT1 and induces NFAT1 translocation from the cytosol into the nucleus in resting CD4+ T cells isolated from HIV-infected individuals (294), my study showed that vEnv, but not shedding gp120, had a profound effect on HIV transcription. It is known that the mature Env complex is composed of three gp120 exterior subunits and three gp41 transmembrane subunits. In addition, MA trimerization is required to form a lattice capable of accommodating the long cytoplasmic tail of HIV-1 envelope trimer (136). Thus, it is reasonable that Env glycoprotein associated with virus-like particles forms the biologically active complex that may play a major role in HIV-1 pathogenesis. The reason for the minimal activity of shedding gp120 in my study may be that it is not in a functional conformation. However, I did not further test whether the shedding gp120 could induce other biological activities.

Overall, for the first time, this study provided evidence for the important role of vEnv in modulating viral transcription in the HIV infected cells.

## Chapter 4

# Identification of genes that are modulated by HIV envelope glycoprotein and investigation of microRNA 181A2's (miR-181A2) function in HIV transcription activation in latently infected cells

## 4.1 Introduction

The act of HIV envelope glycoprotein (Env) binding with cell receptor CD4 and coreceptors (CCR5 or CXCR4) leads to two major events: viral entry and signaling transduction. Presently, the role of envelope glycoproteins in viral entry has been well studied. However, the relevant signal pathways induced by Env are not fully understood. The HIV Env-mediate signaling events have been suggested to facilitate viral replication in non-proliferating target cells (301, 325, 394). In 2002, Claudia et al reported that over 600 genes are modulated by envelope glycoprotein gp120 in PBMCs and MDMs. These genes are involved in cytokines, growth factors, chemokines, cell motility, cell migration, cell transcription, viral replication and cell proliferation (325). In my preliminary study, I demonstrated that HIV vEnv could stimulate HIV transcription and expression by inducing signaling processes, especially in HIV-1 infected resting CD4+ T cells. However, the underlying mechanism of this phenotype still remains undetermined.

To understand the underlying mechanism, I investigated how vEnv (X4) stimulate viral transcription and what genes are involved in this activation. I proposed that the

binding of vEnv to CD4 receptor/coreceptor regulates some specific genes and subsequently activates viral transcription. In order to test my hypothesis, I utilized whole transcriptome sequencing to identify genes modulated by vEnv in HIV-1 infected CD4+ T lymphocytes. Whole RNA transcriptome sequencing (RNA-Seq) is an NGS (next-generation sequencing) which reveals the quantity of RNA in a sample at an indicated time point (395). RNA-Seq can be used to show alternative gene spliced transcripts, changes of gene expression, transcriptional modification, or different gene expression following different treatments (396). In addition to mRNA, RNA-Seq also can detect other type of RNA, including non-coding RNA (ncRNA), microRNA, tRNA and ribosomal RNA (397). Therefore, whole transcriptome sequencing is an excellent tool to identify cellular gene expression changes in HIV vEnv modulation.

MicroRNAs (miRNAs) are an important class of small, non-coding RNAs (398). Mature miRNAs bind primarily to the 3′UTR of target mRNAs by base-paired complementarity, resulting in reduced protein synthesis via repressed translation or mRNA degradation (398). A large number of studies have shown that miRNAs are important in multiple cellular processes including metabolism, transcription, differentiation and cell death (review in (399-401)). Since the virus has evolved to utilize cellular machinery to facilitate replication, HIV manipulates a series of host cellular factors to complete its life cycle, including miRNAs. Previous researches have revealed that miRNAs play dual-roles in HIV replication. For instance, miR-28, -125b, -150, -223 and -382 can bind to the 3′ UTR of viral mRNAs to inhibit HIV replication

(402), while miR-132 is able to enhance viral replication by downregulating cellular transcriptional cofactor MeCP2 (Methyl-CpG binding protein 2 ) (336). MiR-181A2 (5′-AACAUUCAACGCUGUCGGUGAGU-3′) is a member of miR181 family, which include miR-181A, B, C and D. Among them, miR-181A and miR-181B are well studies. MiR181A has been found to participate in cell differentiation, T cell sensitivity, vascular development, cerebellar neurodegeneration, diabetes mellitus and breast cancer (403-409). Recently, several studies also have shown that miR181A is important in HIV pathogenesis. For instance, Jin C et al reported that miR181A specifically regulates the post-transcriptional level of SAMHD1 (SAM domain and HD domain-containing protein 1) by directly binding to the 3' UTR of *samhd1* mRNA, which is considered as an HIV-1 host restriction factor (410, 411). Thus it is believed that the level of miR181A is involved in the susceptibility of primary cells to HIV infection. However, whether miR-181A2 functions in HIV transcription in HIV infected cells remains unknown.

Based on the above information, I proposed that HIV vEnv induced activation may be involved in multiple pathways. Here, I designed two objectives:

(1) Identify these genes modulated by vEnv in HIV infected cells J-Lat 6.3.

(2) If these genes altered by vEnv include miR-181A2, I would investigate whether miR-181A2 plays an important role in viral transcription and how it regulates viral transcription.

## 4.2 Results

### 4.2.1 vEnv induced HIV transcription activation is associated with the alteration of multiple cellular pathways

Since HIV vEnv is able to induce viral transcription in both HIV-infected unstimulated PBMCs and latently infected PBMCs from ART-treated patients, it becomes interesting to explore its underlying mechanism. Therefore, I utilized whole-RNA transcriptome sequencing to analyze the overall gene expression in J-Lat 6.3 T cells upon treatment of vEnv. Briefly, J-Lat 6.3 T cells were either treated with Env(X4)-VLP or untreated for 24 hours and then the total RNA was isolated, reverse transcribed into cDNA and processed using total RNA profile analysis.

The results of RNA-Seq analysis showed that among 28,802 transcripts, there were 1,349 differentially expressed genes whose expression levels had significant changes ($p<0.05$) in the Env(X4)-VLP treated group. Among these significantly altered genes, there were 49 upregulated genes with fold changes greater than 2, and 82 downregulated genes with fold changes less than 0.5. The heat map outlined the distinct genome-wide expression profiles from the hierarchical clustering of RNA-seq data from J-Lat 6.3 T cells which were either treated with Env(X4)-VLP or mock-treated (Fig. 4.1 A).

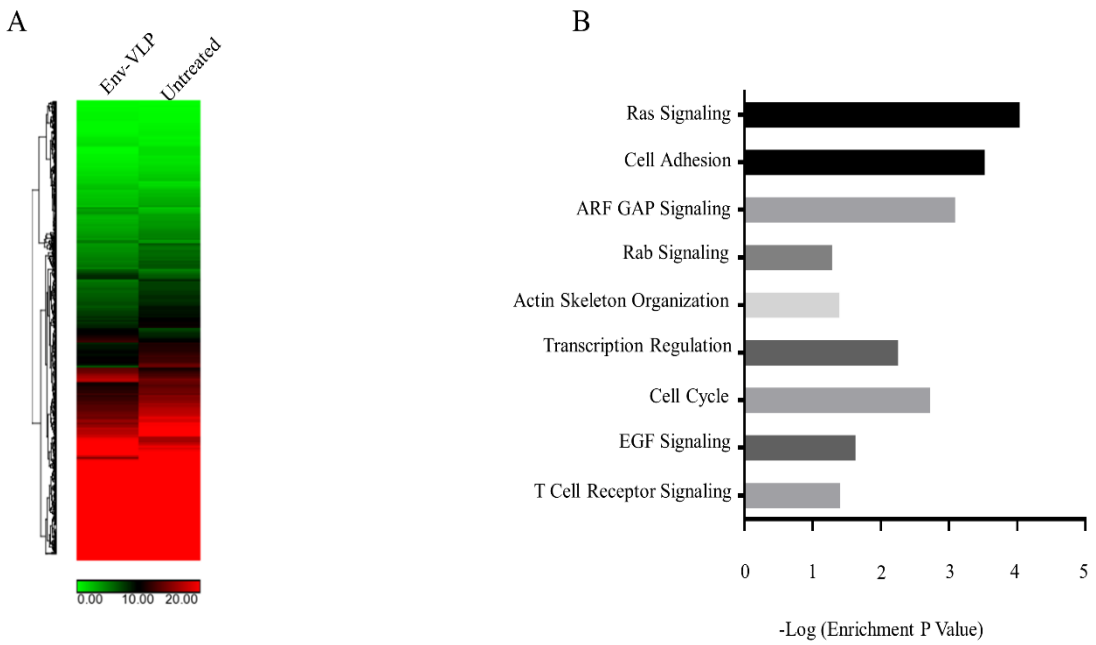



Figure 4.1 **Treatment of vEnv(X4) of J-Lat 6.3 T cells induces the transcriptional changes of multiple cellular genes involved in different signaling pathways.** (A) Heatmap of genes ($p<0.05$) in J-Lat 6.3 T cells untreated (n=2) or treated with Env(X4)-VLP (n=3) in technical replicates showing hierarchical clustering. (Lifescope v2.5.1 software from Life Technologies with the 2-mismatch setting). (B) Enrichment of genes modulated by vEnv. The fold enrichment was calculated based on the frequency of genes annotated to the term compared to their frequency in the genome in the DAVID Bioinformatics database.

I then investigated the 1,349 genes functions using KEGG and DAVID bioinformatics resources (385, 412) and divided them into groups according to their known functions and pathways (Fig. 4.1 B). The gene analysis revealed that vEnv treatment modulates the expression of genes involved in multiple cellular signaling pathways, including transcription regulation, actin skeleton organization, and T cell receptor signaling, as well as various microRNAs. Among them, many genes encoding proteins that have been reported to be either directly or indirectly regulated by HIV viral proteins (Table 1). Some of the upregulated gene encoding proteins were shown to be modulated by HIV glycoprotein gp120 (CD2, CD40L, CCR5, FN1, MANEA, SDC2, TJP1, and UBE2D1), gp41 (AP1S2, AP3M1, FN1), Tat (TAF9, LAMC1, MED21, SDC2, CCR5, CDK8, FN1), Vpr (NUP54), Protease (PLS1) and Nef (CD40L, AP1S2, AP3M1, CCR5, FN1). These genes are usually associated with cell surface receptors, adhesion molecules and RNA polymerase II positive regulators. Among the downregulated genes, several genes encoded proteins that were previously shown to be regulated by viral protein gp120 (FCAR, ESR2, GAA, NFKBIB, TNFRSF10D), gp41 (ARHGEF1, GAA), Vpr (MKNK2), and Tat (EEF1D, NFKBIB). These cellular genes are usually negative regulators or host immune receptors (as summarized in Table 3).

Table 3 Genes encoding host proteins associated with HIV proteins

| Gene Name | Regulation | Functional Category | Proposed Role in HIV Replication |
|---|---|---|---|
| CD2 | + | Cell adhesion molecules | HIV-1 latency |
| CD40L | + | TNF superfamily member | Immunological control of viral replication |
| TAF9,CDK8,MED21 GTF2A2,GTF2H1 | + | Facilitate RNAP Ⅱ complex function | Regulate Tat mediated HIV-1 transactivation; Regulate HIV transcription |
| AP1S2 | + | mediates the recruitment of clatherin | Interact with HIV-1 gp41 or Nef protein |
| AP3M1 | + | Facilitates the budding of vesicles | Viral Budding |
| CCR5 | + | Chemokine receptor | Viral entry, Chemokine signaling pathway |
| FN1 | + | Bind to integrin | Binds to HIV-1 gp120/160 and increase infection |
| LAMC1 | + | extracellular matrix glycoproteins | Upregulated by HIV infection or Tat protein |
| MANEA | + | Endomannosidase | Process of HIV-1 gp160 formation |
| NUP54 | + | Nuclear pore protein | Nuclear import of PIC |
| PLS1 | + | Actin-binding protein | Interact with HIVgp41 |
| SDC2 | + | Syndecan proteoglycan | Interact with HIV gp120, Tat and Matrix |
| TJP1 | + | Tight junction proteins | gp120-mediated tight junction disruption |
| UBE2D1 | + | Ubiquitin enzyme | Ubiquitination of HIV-1 Tat |
| FCAR | - | Immunoglobulin | The phenotype and function of monocytes |
| MKNK2 | - | Protein kinases (CAMK) | MEK2-ERK pathway |
| ARHGEF1 | - | Rho GTPases | Binds to HIV gp41 |
| ESR2 | - | Estrogen receptor | HIV gp120-induced cell death |
| EEF1D | - | Translation elongation | Enhance the translation of viral protein |
| GAA | - | Glucosidase | Processing of HIV-1 gp120 |
| NFKBIB | - | NF-kappa-B inhibitor | NF-kappa-B pathway |
| TNFRSF10D | - | TNF-receptor | TRAIL mediated cell death |

+, upregulated; -, downregulated.

In addition to the previously reported genes, RNA-seq analysis also identified many genes not previously reported to be functionally associated with HIV envelope glycoproteins. Here, I listed the fold change and p value of the genes and their involved cellular pathways (Table 4). This table illustrated that genes encoding positive transcriptional regulators are upregulated, including TAF9, TAF9B, ZNF143, NPAT, CDK8, RNF2 and GTF2A2. In contrast, genes encoding negative transcriptional regulators were downregulated, including HDAC10, HDAC6, HDAC7, HSPBP1, DMAP1, RCOR2, and SMYD1. In addition, I noticed that the expression levels of several microRNAs were altered following the interaction between HIV vEnv and CD4/CXCR4 coreceptor. As shown in Table 2, the expression of several microRNAs was downregulated, including that of miR-181A2/B2, miR25, miR320A, miR342, miR423 and miR638. The RNA-seq data provided the evidence that vEnv-regulated cellular gene expression may have a great impact on HIV gene expression and/or viral replication, which deserves further investigation.

Table 4 List of genes modulated by Env-VLP

| Category | Gene ID | FC | P-Value | Category | Gene ID | FC | P-Value | Category | Gene ID | FC | P-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Positive Regulation of Transcription** | TAF9 | 1.36 | 0.006262 | **Chemokine Signaling** | RAP1B | 1.34 | 0.016442 | **Microtubule Modification Signaling** | MAP1B | 1.85 | 0.028223 |
| | ZNF143 | 1.25 | 0.03816 | | CCR5 | 2.04 | 0.011009 | | MID1 | 1.40 | 0.047273 |
| | TAF9B | 1.39 | 0.002465 | | CCR9 | 1.53 | 0.01863 | | PLK4 | 1.28 | 0.025818 |
| | NPAT | 1.26 | 0.031909 | | GNB3 | -1.45 | 0.039175 | | SKA3 | 1.26 | 0.030978 |
| | CDK8 | 1.26 | 0.032946 | | GNG5 | 1.46 | 0.010546 | | TACC3 | -1.23 | 0.042907 |
| | RNF2 | 1.43 | 0.002864 | | IKBKG | -1.46 | 0.009372 | | IQGAP3 | -1.26 | 0.043145 |
| | GTF2A2 | 1.28 | 0.019587 | | NFKBIB | -1.36 | 0.047192 | | ARHGEF1 | -1.43 | 0.00163 |
| | HSBP1 | -1.36 | 0.018719 | | BCAR1 | 1.92 | 0.02482 | | ARPC2 | 1.30 | 0.029475 |
| **Negative Regulation of Transcription** | HDAC10 | -1.52 | 0.004067 | **Cytokine/ Cytokine Receptor Signaling** | IL11RA | -1.52 | 0.034023 | | FN1 | 1.65 | 0.006954 |
| | HDAC6 | -1.25 | 0.031393 | | IL7R | 1.29 | 0.022709 | | INS-IGF2 | -1.81 | 0.048713 |
| | HDAC7 | -1.43 | 0.000938 | | IFNGR1 | 1.27 | 0.021442 | | ITGB6 | 2.31 | 0.006102 |
| | HSPBP1 | -1.36 | 0.018719 | | CSF1 | -1.57 | 0.030169 | **Micro RNA** | MIR-181A2 | -2.15 | 0.017717 |
| | DMAP1 | -1.35 | 0.020367 | | RELT | -1.28 | 0.036626 | | MIR181B2 | -1.39 | 0.036086 |
| | RCOR2 | -1.38 | 0.015575 | **Ras Signaling** | ARFGAP1 | -1.44 | 0.002863 | | MIR25 | -2.74 | 0.004789 |
| | SMYD1 | -1.95 | 0.01964 | | ARFGAP2 | -1.32 | 0.009121 | | MIR320A | -1.97 | 0.03999 |
| **Cell Cycle Regulation** | E2F2 | -1.27 | 0.022243 | | ARHGAP27 | -1.36 | 0.003443 | | MIR342 | -2.68 | 0.001442 |
| | E2F4 | -1.26 | 0.031867 | | ARHGEF2 | -1.26 | 0.033349 | | MIR423 | -1.79 | 0.012911 |
| | ANAPC2 | -1.29 | 0.039934 | | SGSM2 | -1.32 | 0.007924 | | MIR638 | -2.50 | 0.021011 |
| | CDC25B | -1.25 | 0.033301 | | SGSM3 | -1.26 | 0.028747 | **JAK-STAT Signaling** | IFNGR1 | 1.27 | 0.021442 |
| | CCNE2 | 1.27 | 0.029139 | | PAK4 | -1.42 | 0.043744 | | PIAS4 | -1.39 | 0.005369 |
| **Cell Adhesion Regulation** | CD2 | 1.24 | 0.043637 | | SH3GL2 | 1.99 | 0.008399 | | SPRED1 | 1.27 | 0.047374 |
| | CD40L | 1.65 | 0.00482 | | SH3GL3 | 1.49 | 0.024412 | | SOCS2 | 1.35 | 0.034987 |
| | CD58 | 1.45 | 0.006601 | | | | | | TYK2 | -1.30 | 0.014046 |
| | ITGAL | -1.32 | 0.007246 | | | | | | | | |
| | CD226 | 1.31 | 0.014234 | | | | | | | | |
| | HLA-C | -1.27 | 0.030582 | | | | | | | | |

### 4.2.2 vEnv downregulates miR-181A2 and upregulates PCAF level in HIV infected CD4+ T lymphocytes.

The RNA-seq analysis from section 4.2.1 revealed that the expression of miR-181A2/B2 was downregulated by vEnv. Interestingly, a recent study reported that miR181A could downregulate PCAF expression by destabilizing PCAF mRNA (413). PCAF is an acetyltransferase that uses the cofactor acetyl coenzyme A (AcCoA) to acetylate lysine 14 of histone 3, and the overexpression of PCAF enhances histone H3 acetylation (414, 415). Therefore, I speculated that HIV vEnv-mediated downregulation of miR-181A2 leads to the increased expression of PCAF, which facilitates histone H3 acetylation of HIV LTR, and subsequently activates viral transcription. First, I validated the miR-181A2 and PCAF expression in vEnv-treated J-Lat 6.3 T cells using individual quantitative RT-PCR assays. Briefly, I treated the J-lat 6.3 cells with vEnv(X4) for 24 hours and then the cellular mRNA miR-181A2 was measured and normalized by host house-keeping gene *gapdh*. The results showed that the expression of miR-181A2 was reduced by 60-70% in vEnv-treated cells compared to mock-treated cells (Fig. 4.2 A). Furthermore, this result was confirmed in primary CD4+ T lymphocytes. Here, PBMCs were isolated from three different individuals and primary CD4+ T cells were purified by negative selection kit. The primary CD4+ T cells were treated with vEnv(X4) for 24 hours, following which, the cellular mRNA miR-181A2 was measured and normalized by host house-keeping gene *gapdh*. Consistent with the results in J-Lat 6.3 cells, the miR-181A2 was also downregulated in primary CD4+ T cells (Fig. 4.2 B).

As mentioned above, PCAF is downregulated by miR-181A2. PCAF is an acetyltransferase, which can modify histone acetylation and enhance transcription (415). Thus, upon indicating vEnv(X4) downregulates miR-181A2, I investigated the PCAF expression changes. Briefly, the J-lat 6.3 cells were treated with vEnv(X4) for 24 hours, following which, the cellular *pcaf* mRNA was measured and normalized by host house-keeping gene *gapdh* and the PCAF protein level was detected by western blots and normalized by cellular protein tubulin. The results showed that the expression of PCAF was upregulated without affecting the level of PCAF mRNA (Fig. 4.2 C and D).

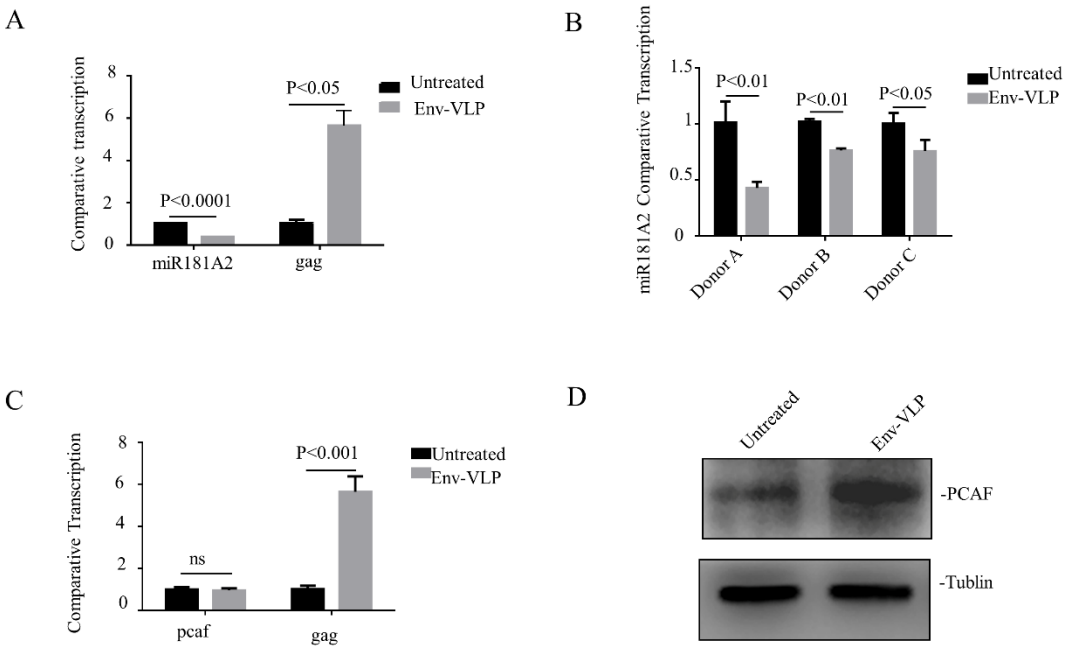



Figure 4.2 **vEnv downregulates miR-181A2 and upregulates PCAF expression**. (A) The miR-181A2 mRNA level was reduced in J-Lat 6.3 T cells treated with Env(X4)-VLP for 24 hours, as compared to the untreated cells (n=3). (B) The detection of miR-181A2 expression upon vEnv treatment by quantitative RT-PCR in primary CD4+ T cells from three different individuals. (C) The detection of pcaf mRNA upon vEnv treatment by quantitative RT-PCR in J-Lat 6.3 cells. (D) The detection of PCAF protein expression upon vEnv treatment by western blots in J-Lat 6.3 cells. Data are presented as mean and sd. Ns, not significant $p>0.05$. (two-tailed unpaired t-test)

### 4.2.3 The downregulation of miR-181A2 increases PCAF expression and subsequent HIV transcription

Upon demonstrating vEnv(X4) downregulates miR-181A2 expression in HIV-1 infected CD4+ T cells, I also characterized the role of miR-181A2 in HIV transcription. I evaluated the effect of miR-181A2 on HIV transcription by overexpressing either miR-181A2 or a miR-181A2 inhibitor (miR-181A2-3p (413)) in J-Lat 6.3 T cells using a lentiviral vector technique. After four days of introducing miR-181A2 or miR-181A2 inhibitors, HIV *gag* mRNA expression was measured using quantitative RT-PCR and normalized by host house-keeping gene *gapdh*. The results showed that the overexpression of miR-181A2 led to reduced HIV transcription, while the overexpression of miR-181A2 inhibitor significantly increased HIV transcription (Fig. 4.3 A).

After characterizing the role of miR-181A2 in viral transcription, I proposed that PCAF expression changes are involved with miR-181A2 mediated transcriptional activation. To further address the relationship between miR-181A2 and the expression of PCAF, I investigated the levels of PCAF in miR-181A2 or miR-181A2 inhibitor-overexpressed J-Lat 6.3 T cells by quantitative RT-PCR and western blot. Here, J-Lat 6.3 cells were transduced with miR-181A2, miR-181A2 inhibitor or negative control. After 4 days, I characterized their effect on PCAF expression through quantitative RT-PCR and western blot. Interestingly, the overexpression of miR-181A2 significantly downregulated PCAF mRNA and protein levels (Fig. 4.3 C), while miR-181A2 inhibitor efficiently increased the synthesis of PCAF protein but did not significantly

change the PCAF mRNA level (Fig. 4.3 B). The data suggested that the miR-181A2/PCAF pathway is involved in the action of vEnv on viral transcription.

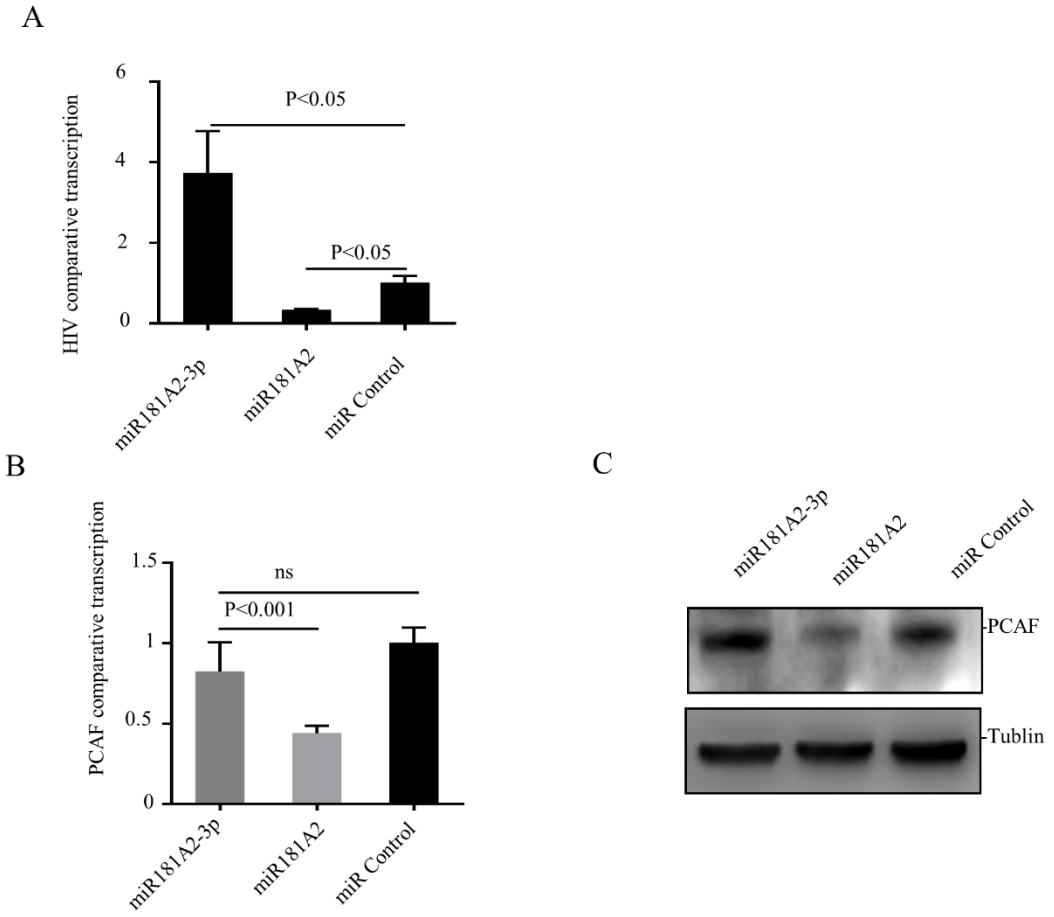



Figure 4.3. **MiR-181A2 can significantly inhibit HIV-1 viral transcription by upregulating PCAF protein expression.** (A) The HIV gag transcription levels in J-Lat 6.3 T cells overexpressed with either miR-181A2 or miR-181A2-3p (inhibitor), as compared to the cells transduced with empty lentiviral vectors (n=3). J-Lat 6.3 T cells were transduced with lentiviral vectors encoding miR-181A2, miR-181A2-3p or transduced with empty vector for 24 hours and kept on culture for another 72 hours. Then, cells were collected and the HIV comparative transcription (*gag*/*gapdh*) was measured by quantitative RT-PCR. (B) The detection of *pcaf* mRNA upon miR-181A2 overexpression or inhibition. (C) The detection of PCAF protein expression upon miR-181A2 overexpression or inhibition. Data are presented as mean and sd. Ns, not significant $p>0.05$. (two-tailed unpaired t-test)

### 4.2.4 vEnv activates viral transcription through increasing histone acetylation in NUC-1.

In HIV-1 infected cells, viral DNA is inserted into host chromatin and tightly packed in a highly organized chromatin structure, which is directly associated with LTR controlled viral transcriptional activity (416, 417). Previous studies have shown that LTR activity is linked to the post-translational modifications of the histones in nucleosome NUC-1 (416, 418). In particular, the histone acetylation of HIV-1 LTR, mediated by HAT (histone acetyltransferases), is required for transcriptional activation (418). As PCAF plays a direct role in transcriptional regulation by enhancing histone H3 acetylation, it is necessary to investigate whether HIV LTR-associated H3 acetylation level changes upon stimulation with HIV vEnv. The J-Lat 6.3 T cells were treated with Env-VLP for 24 hours, followed by CHIP with anti-acetylated-histone H3 antibody and quantitative RT-PCR with primers located within the nucleosome-1 (nuc-1) of the HIV-1 LTR promoter (Fig. 4.4 A). In parallel, cells treated with either histone deacetylase inhibitor vorinostat (VOR) (419) or valproic acid (VPA) (420, 421) were included as positive controls. VOR and VPA are histone deacetylase inhibitors, which can increase histone acetylation in NUC-1(422, 423). The results showed that Env(X4)-VLP treatment was able to facilitate histone H3 acetylation in the 5' LTR region (5-fold) compared to the untreated control, while VPA and VOR induced 11-fold and 8-fold acetylation respectively (Fig. 4.4 B). I then explored whether this enhanced acetylation is HIV LTR-specific. The level of histone H3 acetylation in J-Lat 6.3 T cells treated with Env-VLP, VOR or VPA was measured by western blot using an anti-acetylated-

histone H3 antibody (Fig. 4.4 C). The scanning analysis revealed that the global H3 acetylation in Env-VLP treated cells was approximately 1.79-fold higher than in mock-treated cells, which was not as high as that in cells treated with VOR (2.59-fold) or VPA (2.38-fold). All the above observations provide evidence that miR-181A2/PCAF/H3 acetylation is one cellular pathway manipulated by vEnv to activate HIV transcription.

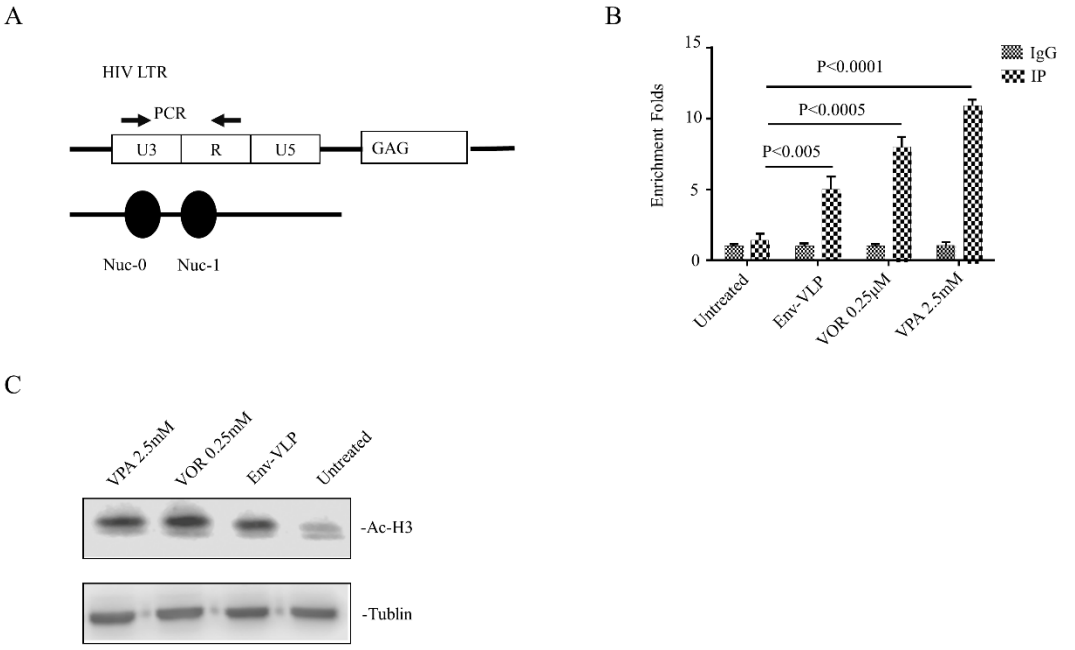



Figure 4.4 **vEnv induces the increase of histone H3 acetylation in NUC-1 in HIV LTR.** (A) The schematic diagram of the positions of the nucleosomes bound to the HIV-1 LTR U3 and R5 region and the relevant primers used for the real-time PCR in the ChIP assay. (B) The detection of histone H3 acetylation ratio in HIV LTR -109-+82 by CHIP (n=3). J-Lat 6.3 T cells were treated with Env(X4)-VLPs for 24 hours. In parallel, cells treated with histone deacetylase inhibitor VOR or VPA were included as positive controls and the untreated cells act as a negative control. After treatment, cells were lysed and analyzed by CHIP assay (C) The detection of the global histone H3 acetylation in J-Lat 6.3 T cells treated with Env(X4)-VLP, VOR, VPA or untreated were detected by western blot with anti-histone H3 acetylated antibody. Data are presented as mean and sd. Ns, not significant $p>0.05$. (Two-tailed unpaired t-test).

## 4.3 Discussion

During HIV infection, HIV envelope glycoprotein performs two functions, mediating viral entry, and regulating downstream signaling to facilitate viral infection and replication. As mentioned in chapter 3, my study demonstrated that vEnv can interact with CD4 receptor/coreceptor (CCR5/CXCR4) and this interaction can efficiently stimulate viral transcription in HIV-1 infected CD4+ T lymphocytes. Upon finding this phenomenon, it is necessary to question: what is the underlying mechanism of vEnv function? And which cellular genes are involved with this activation? In order to reveal the underlying mechanism, I utilized whole RNA transcriptome sequencing and identified 1,349 genes that are involved with the vEnv induced modulation. The results revealed that the host genes modulated by vEnv are associated with Ras signaling, cell adhesion, cell cycle regulation, actin skeleton organization, and transcription regulation. Among these genes, I identified that miR-181A2 is responsible for regulating viral transcription in HIV infected cells. The downregulation of miR-181A2 induced by vEnv can upregulate the expression of PCAF, which increases the acetylation of histone H3, and consequently stimulates viral transcription.

Whole transcriptome sequencing is a powerful tool to study the cellular or viral RNA changes during viral replication. My RNA-seq results revealed that the host genes modulated by vEnv are associated with multiple cellular pathways. In addition to the genes reported previously, my RNA-seq results showed a number of genes, including several micro RNAs, which are responsive to vEnv stimulation (listed in Table 2). Among these genes, the microRNA miR-181A2 was significantly downregulated by

vEnv. Moreover, in HIV latently infected J-Lat 6.3 T cells, I found that the downregulation of miR-181A2 enhanced the expression of PCAF and activated HIV LTR-associated histone H3 acetylation, potentially contributing to the activation of HIV transcription. Therefore, it can be speculated that HIV can use its envelope glycoprotein to stimulate its transcription by down-regulating miR-181A2 and enhancing LTR histone acetylation, thus providing one possible mechanism for the rapid viral rebound after HARRT treatment ceases. In HIV latently infected cells, HIV transcription is absent or rare due to the low expression of Tat and high deacetylation in the HIV LTR region. Once HARRT treatment ceases, the envelope glycoprotein present on small amounts of viruses can interact with the receptor/coreceptors on HIV-infected resting T cells and modulate multiple cellular signaling factors, including the down-regulation of cellular miR-181A2 and facilitation of HIV LTR acetylation. These in turn may contribute to the viremia rebound. Therefore, the down-regulation of host gene miR-181A2 may be an attractive strategy that can be used to induce HIV reactivation in chronically infected individuals to eliminate HIV reservoirs.

In addition to the genes associated with viral transcription, I also found a number of genes, which were reported to contribute to the other aspects of HIV pathogenesis. For example, I found that vEnv can decrease the expression of HDAC6, which may lead to the changes of viral fusion and progeny virus infectivity (352, 353). Additionally, I found that there are several genes that may be involved in HIV induced immune dysregulation. For instance, vEnv can increase the expression of CD40L (CD40 ligand). CD40L is a protein that primarily expressed on the activated T cells, which can be

incorporated into progeny virus (424). The host derived CD40L acquired by HIV progeny virus can interact with the CD40 on B cell surface, which induces CD40 signaling and activates B cells (425). The B cell activation may lead to elevated serum levels of immunoglobulins and autoantibodies, and eventually cause the B cell dysregulation (426). Thus, it is possible that the increase of CD40L on cell surface induced by vEnv may facilitate the dysfunction of humoral immunity by CD40 signaling. However, the detailed mechanism requires further investigation.

In summary, my results illustrated the genes modulated by HIV envelope glycoproteins and provided a potential mechanism for the transcriptional activation induced by HIV envelope glycoproteins.

## Chapter 5

## Characterization and role of HDAC10 in HIV integration and progeny virus infectivity

### 5.1 Introduction

To date, a series of histone deacetylase (HDAC) family members have been reported to act as inhibitory factors against HIV infection (427). For instance, HDAC1 and HDAC2 induce the deacetylation of histone H3 and H4 to increase the degree of chromatin compaction, which inhibits viral transcription and subsequently contributes to the formation of HIV latency (96). Moreover, HDAC6 has been reported to have an inhibitory effect on HIV entry and cell fusion by preventing the acetylation status of cortical tubulin (428). HDAC6 has also been shown to inhibit Vif-mediated A3G degradation to reduce progeny virus infectivity (352). Previously, my studies revealed that another member of HDAC family, HDAC10 was downregulated by HIV vEnv in HIV infected cell line J-Lat 6.3 (429). HDAC10 is a member of the HDAC class II B family (354). In 2002, Tong and Guardiola et al identified for the first time that HDAC10 consists of 669 amino acids with a Hda1p-related putative deacetylase domain in the N-terminus and a leucine-rich domain in the C-terminus (354, 430). HDAC10 is located in the cytoplasm due to its leucine-rich domain though it can enter into the nucleus by passive diffusion or through the assistance of nuclear proteins (354). HDAC10 has been reported to play multiple functions in biological processes,

such as the mediation of homologous recombination, autophagy-mediated cell survival and cell cycle, which are dependent on its deacetylase activity (356, 357, 431). While it also regulates cellular transcription and cell proliferation independent of its deacetylase activity (355, 432). In addition to mediating cellular processes, HDAC10 has reportedly been involved in viral replication (433). For instance, HDAC10 was demonstrated to interact with HCV-IE1 (Human Cytomegalovirus Immediate-Early Protein 1), a viral factor which is important for viral gene expression at the early stage of viral replication (433). However, whether HDAC10 plays an important role in HIV replication and what this role may be remains unknown.

HIV integrase (IN) is a 32 kDa viral protein, which consists of 288 amino acids (211). It consists of three functional domains, including an N-terminal core domain (residues 1-49), a catalytic core domain (residues 50-212 ) and a C-terminal core domain (residues 213-288) (434). HIV integrase is a key viral enzymatic protein that acts in several viral replication steps, including viral integration and progeny virus assembly. (211). During the process of integration, HIV integrase recruits a series of cellular factors to form a large pre-integration complex (PIC) and catalyzes the insertion of viral DNA into the host genome (155). Presently, several cellular factors have been identified that interact with HIV integrase and act as a component of PIC, including BAF, HMGA1, INI1/hSNF5, LEDGF, EED, HRP2, HSP60 and p300 acetyltransferase (435, 436). The interaction of HIV integrase and these cellular factors is essential for the stability and catalytic activity of HIV integrase, ensuring efficient integration (437). For instance, HSP60 interacts with HIV integrase and stimulates integrase's processing

and joining activities, protecting this enzyme from denaturation (438). In addition to catalyzing the insertion of viral DNA into host chromatin, HIV integrase is also involved in the morphology and the infectivity of progeny virus (439). Previous mutagenesis analysis has shown that some mutations of HIV integrase not only cause aberrant morphology of viral particles, but also lead to defective progeny virus by impairing subsequent reverse transcription and integration in incoming target cells (440-443). The disruption of the interaction between HIV integrase and cellular factors also results in an infectivity loss of progeny virus due to HIV integrase abnormal multimerization (444). Collectively, evidence has indicated that HIV integrase is important for the early and late stages of viral replication. However, whether HDAC10 can interact with HIV integrase and influence viral integration or progeny virus's infectivity is still not clear.

Our previous studies have shown that HDAC10 was downregulated by HIV vEnv in HIV infected cell line J-Lat 6.3. However, how HIV downregulates HDAC10 expression and whether the downregulation of HDAC10 affects viral replication remain unknown. Here I proposed that the downregulation of HDAC10 may benefit HIV replication. Thus I designed four objectives to testify my hypothesis:

(1) Investigate the HDAC10 expression during HIV infection

(2) Investigate the impact of HDAC10 downregulation on HIV replication

(3) Identify which viral step is regulated by HDAC10 downregulation

(4) Explore the underlying mechanism of the HDAC10 regulatory function in HIV replication

## 5.2 Results

### 5.2.1 Endogenous HDAC10 is downregulated during HIV-1 replication

Prior to investigating the role of HDAC10 in HIV replication, I first detected the endogenous HDAC10 expression changes during HIV replication by infecting $CD4^+$ T lymphocytes Jurkat cells with HIV-1 (N119) (MOI=0.5) overnight. The cells were then collected at 0, 48 and 96 hours for further mRNA or protein expression analysis. The *hdac10* mRNA was detected by quantitative RT-PCR and normalized by host gene *gapdh*. After detecting the *hdac10* mRNA, HDAC10 protein synthesis was detected by western blot analysis with anti-HDAC10 specific antibody and normalized by host house-keeping protein Tubulin. Results showed that the *hdac10* comparative transcription was downregulated to 50% at 48 hours and was further downregulated to 30% at 96 hours (Fig. 5.1 A left panel). Consistent with the changes of *hdac10* mRNA, the HDAC10 protein level was downregulated to 70% at 48 hours and 50% at 96 hours (Fig. 5.1 A middle and right panel). This result was further confirmed by detecting HDAC10 expression changes in another $CD4^+$ T lymphocytes C8166 cells. Similarly, I found that during HIV infection, HDAC10 mRNA and protein level were also rapidly downregulated post-infection in C8166 cells. Consistent with the results in Jurkat cells, the HDAC10 level was downregulated into almost 40% at 48 hours and 20% at 96 hours (Fig. 5.1B).

In addition, I confirmed these results by detecting the HDAC10 expression changes following HIV infection in primary resting $CD4^+$ T cells. Primary resting $CD4^+$T cells were isolated from three healthy individuals and subjected to HIV

infection (MOI=0.5) overnight followed by washing twice with PBS. After 96 hours' incubation, HIV infection was confirmed by detecting HIV gag mRNA expression and the HDAC10 expression changes were measured by *hdac10* mRNA and normalized by house-keeping gene GAPDH. HIV gag mRNA was detected in these primary infected $CD4^+$ T cells in all individuals (Fig. 5.1 C right panel) while HDAC10 was downregulated to 50%, 25%, and 40% respectively in each individuals (Fig. 5.1 C left panel). In a word, these results indicate that HDAC10 was downregulated by HIV through transcription inhibition, which is consistent with my preliminary results (429).

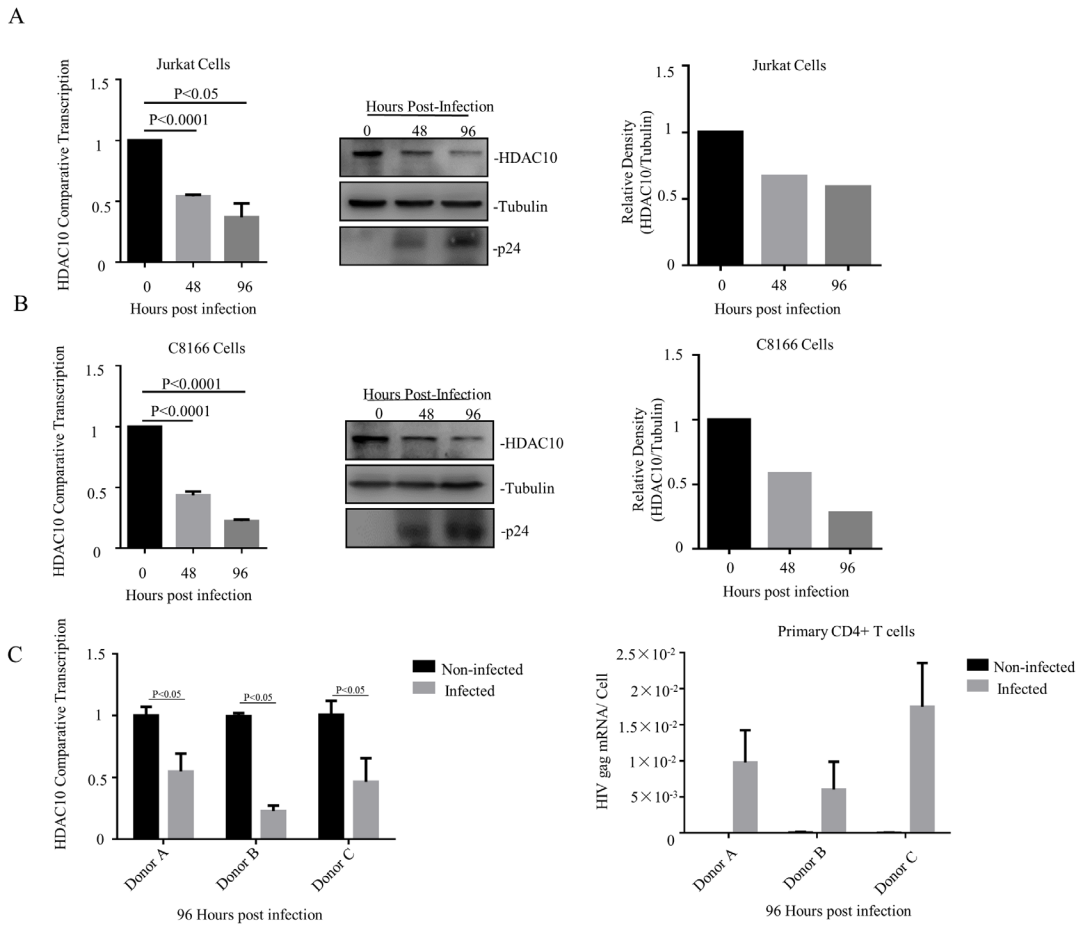



Figure 5.1 **Endogenous HDAC10 is downregulated in CD4+ T cells during HIV replication.** (A, B) The *hdac10* mRNA (left panel) and protein (middle and right panel) expression changes during HIV replication in $CD4^+$ T cell line Jurkat cells or C8166 cells. Jurkat cells or C8166 cells were infected with HIV (for Jurkat cell MOI=0.5; for C8166 cell MOI=0.1) overnight and collected at indicated time points 0, 48, and 96 hours. The endogenous HDAC10 mRNA was isolated, reverse transcribed into cDNA and detected by quantitative RT-PCR and normalized by house-keeping gene *gapdh* while HDAC10 protein production was detected by western blot and normalized by host protein Tubulin. (C) Left panel: the *hdac10* mRNA expression changes during HIV infection in primary $CD4^+$ T cells. Right panel: the expression of HIV gag mRNA expression in HIV-1 infected primary resting $CD4^+$ T cells. Primary resting $CD4^+$T cells were isolated from healthy individuals and subjected to HIV infection (MOI=0.5) overnight. After 96 hours post-infection, endogenous HDAC10 mRNA and HIV gag mRNA were monitored by quantitative RT-PCR. Data are mean and sd. (Two-tailed unpaired t-test)

### 5.2.2 HDAC10 downregulation benefits viral replication

After demonstrating the downregulation of HDAC10 during HIV infection, it is necessary to investigate the effect of this downregulation on viral replication. In order to study the influence of HDAC10 downregulation on HIV infection and replication, HDAC10 knockdown cells were generated by RNA interference. Briefly, lentiviral particles encoding scramble shRNA or HDAC10 shRNAs were produced by transfecting HEK 293T cells with Δ8.2 plasmid, CMV-VSVG plasmid, and TCR-Scramble shRNA or TCR HDAC10 shRNA-1 or TCR HDAC10 shRNA-2 plasmids, respectively. After 48 hours' transfection, these particles were collected and purified by ultracentrifuge. Jurkat cells were transduced with scramble shRNA or HDAC10 shRNA lentiviral particles respectively and selected by puromycin (2 μg/ml) for 48 hours. The selected cells were collected and the HDAC10 protein level was measured by western blot analysis with anti-HDAC10 specific antibody and normalized by host house-keeping protein Tubulin. The western blot results showed that HDAC10 protein could be detected in scramble shRNA transduced cells while it could not be detected in HDAC10 shRNA-1 or shRNA-2 transduced cells (Fig. 5.2A). This result suggests that HADC10 expression was efficiently knocked down in Jurkat cells by HDAC10 shRNA-1 and shRNA-2.

After identifying that both HDAC10 shRNAs were able to knock down endogenous HDAC10 expression, I investigated the biological impact of this knockdown on cell viability and proliferation. First, I demonstrated that HDAC10 knockdown did not affect cell viability in the 6 days following transduction. After

transduction and a 48 hours' selection, the cell viability of transduced positive cells was detected by Trypan blue stain and counted by countess II FL automated cell counter. The results showed that there was no significant difference in the viability between scramble shRNA and HDAC10 shRNA transduced cells, meaning that the HDAC10 knockdown did not have an effect on cell viability in 6 days after transduction and selection (Fig. 5.2B). I also demonstrated that HDAC10 knockdown did not influence cell proliferation in the 6 days after transduction. Briefly, I transduced these cells with HDAC10 shRNA or scramble shRNA overnight and plated $5\times10^4$ transduced cells per well in 96 well plates. The cell proliferation was detected with WST-1 assay at 0, 2, 4 and 6 days. The results showed no significant differences between the scramble shRNA and HDAC10 shRNA transduced cells' proliferation on aforementioned dates, indicating that HDAC10 knockdown did not influence cell proliferation in the 6 days following transduction and selection (Fig. 5.2C).

While investigating the effect of HDAC10 KD on cell viability and proliferation, I accessed the influence of HDAC10 downregulation on viral replication. Briefly, the positively selected HDAC10 KD cells or control cells were infected with HIV multiple cycle virus N119 overnight. The supernatant was collected at indicated time points and the viral protein p24 production in the supernatant was detected by anti-p24 ELISA. The results showed that compared to the scramble transduced cells, there was increased production of p24 in the supernatant from HDAC10 knockdown cells, which suggested that HADC10 knockdown cells produced more progeny virus (Fig. 5.2D). Therefore it can be concluded that HDAC10 downregulation enhances viral replication.

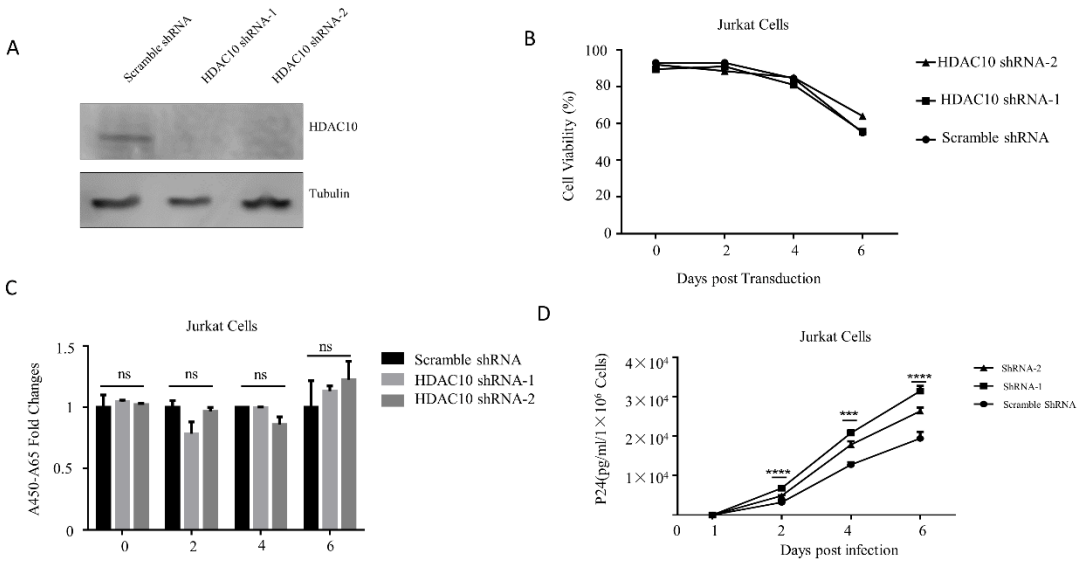



Figure 5.2 **HDAC10 downregulation benefits HIV replication.** (A) The HDAC10 protein expression in HDAC10 shRNA transduced cells or scramble shRNA transduced cells. Jurkat cells were transduced with HDAC10 shRNAs or scramble shRNA and selected with 2 μg/ml puromycin. After 48 hours, cells were harvested and the endogenous HDAC10 protein expression was detected by western blots and normalized by host protein Tubulin. (B) The cell viability of HDAC10 shRNA transduced cells or scramble shRNA transduced cells on days 2, 4 and 6 following transduction and selection. After transduction and selection, the same amount of positive cells were collected and the viability of transduced cells was measured by trypan blued staining on 2nd, 4th and 6th day after transduction and selection. (C) The cell proliferation of HDAC10 shRNA transduced cells or scramble shRNA transduced cells on day 2, 4 and 6 following transduction and selection. After transduction and selection, the same amount of positive cells were collected and the proliferation of transduced cells was detected by WST-1 assay at on day 2, 4 and 6 after transduction and selection. (D) The detection of viral protein p24 in the supernatant of HDAC10 shRNA transduced cells or scramble shRNA transduced cells at the indicated time points. After transduction and selection, the same amount of HDAC10 shRNA or scramble shRNA transduced cells were infected with HIV overnight. The supernatant was collected on days 2, 4 and 6 following infection and the viral protein p24 in supernatant was detected by anti-p24 ELISA. Data are mean and sd. Ns, no significance, $p>0.05$; *, $<0.05$;***, $<0.005$;****, $<0.001$. (Two-tailed unpaired t-test)

### 5.2.3 HDAC10 downregulation enhances viral integration

After demonstrating that HDAC10 knockdown benefits viral replication, I investigated which step of replication was facilitated by HDAC10 downregulation. At first, I examined the impact of HDAC10 downregulation at the early stage of viral replication. Here, I infected the HDAC10 KD cells and control cells (scramble transduced cells) with HIV and detected the total viral DNA, integrated viral DNA and 2-LTR viral DNA in these cells. The DNA analysis showed that there were no significant differences of total viral DNA between HDAC10 knockdown cells and scramble shRNA transduced cells (Fig. 5.3A). However, compared with scramble shRNA transduced cells, the integrated viral DNA increased to 2-3 fold while the 2-LTR DNA decreased to 50%-70% in HDAC10 KD cells (Fig. 5.3B and 5.3C). These results indicate that HDAC10 downregulation enhanced HIV replication through promoting viral integration.

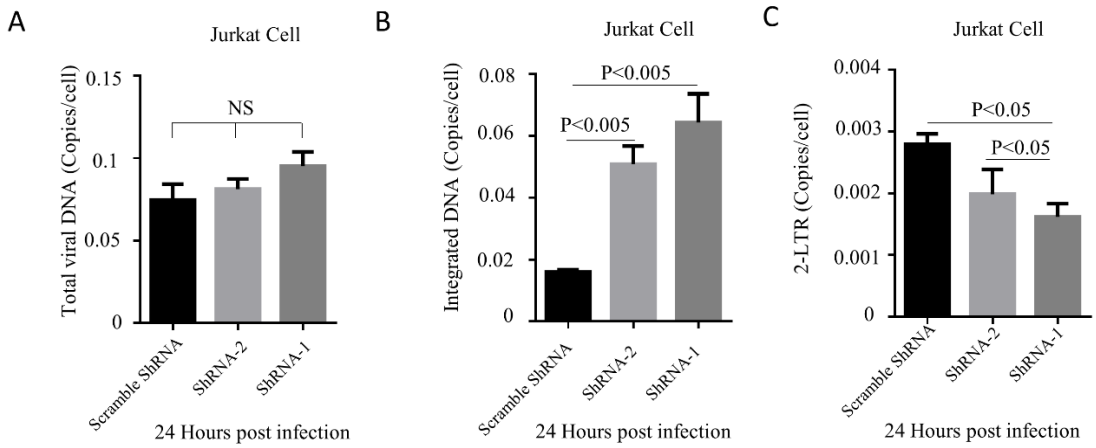



Figure 5.3 **HDAC10 downregulation enhances viral integration**. The detection of total viral DNA (the left panel), viral integrated DNA (the middle panel) and 2-LTR viral DNA (the right panel) in HDAC10 shRNA or scramble shRNA transduced cells at 24 hours after infection. After transduction and selection, positively selected Jurkat cells were subjected to HIV infection for 2 hours and then washed with PBS twice. After 12 hours, cells were treated with 5'-AZT for a further12 hours and then collected. The total viral DNA, viral integrated DNA and 2-LTR DNA were analyzed by quantitative RT-PCR. Data are mean and sd. Ns, no significance. (two-tailed unpaired t-test)

### 5.2.4 HDAC10 can interact with HIV integrase through binding to IN 55-165

During the process of viral integration, HIV integrase is a key enzyme that catalyzes the integration of viral genome into host chromatin (445). HIV integrase recruits several cellular cofactors to generate a pre-integration complex and process efficient integration (171). However, there is no publication reporting the interaction between HDAC10 and HIV integrase. In an attempt to investigate the underlying mechanisms by which HDAC10 downregulation enhances viral integration, I investigated the interaction between HIV integrase and HADC10. Here, I examined the interaction of HDAC10 and HIV integrase (IN) using a co-immunoprecipitation assay. Briefly, HEK 293T cells were co-transfected with HDAC10-Myc/GFP-IN, HDAC10-Myc/GFP (negative control), or GFP-IN (negative control). After 48 hours, cells were subjected to lysis buffer and the supernatant was collected for anti-Myc immunoprecipitation. The results showed that by pulling down HDAC10, GFP-IN was detected in the pellet of the HDAC10/GFP-IN group, while the GFP was not detected in the pellet of the HDAC10-Myc/GFP group, indicating an interaction between HDAC10 and HIV integrase (Fig. 5.4 A).

I also demonstrated the interaction between HDAC10 and HIV integrase by pulling down GFP-IN. Briefly, HEK 293T cells were co-transfected with HDAC10/GFP (negative control) or HDAC10/GFP-IN. Then I pulled down GFP-IN or GFP by anti-GFP antibody and detected HDAC10 in the pellet. The results showed the presence of HDAC10 in the pellet of the HDAC10/GFP-IN group while HADC10 was not detected in the pellet of HDAC10/GFP group (Fig. 5.4B). All these results

demonstrated that HDAC10 was able to interact with HIV integrase.

I further confirmed this interaction of HDAC10 and HIV integrase by co-transfecting HIV genome plasmid and HDAC10. Briefly, I transfected HEK 293T cells with Bru-IN-HA/HDAC10, Bru-IN-HA/GFP (negative control), or PNL4.3/HDAC10 (negative control) for 48 hours. Cells were then subjected to lysis buffer and IN-HA was pulled down by the anti-HA antibody. The results revealed that HDAC10 could only be detected in the pellet of Bru-IN-HA/HDAC10 group while no bands could be detected in the Bru-IN-HA/GFP or PNL4.3/HDAC10, suggesting that HDAC10 could interact with HIV integrase (Fig. 5.4C).

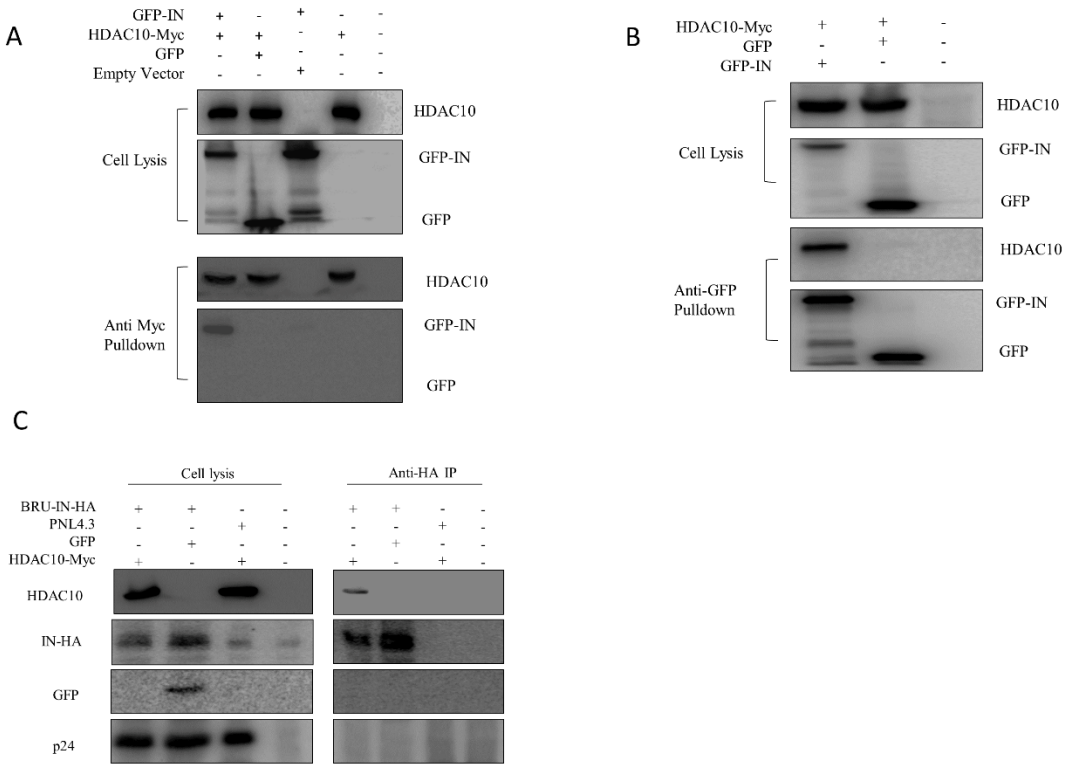



Figure 5.4 **HDAC10 interacts with HIV integrase.** (A) The interaction of HDAC10 and HIV integrase in HEK 293T cells. GFP-IN (or GFP) was co-expressed with HDAC10-Myc in HEK 293T cells. After 48 hours, cells were subjected to co-IP analysis. HDAC10 was immuno-precipitated by using anti-Myc antibody and HDAC10-bound integrase was detected by anti-GFP antibody. (B) The interaction of HDAC10 and HIV integrase in HEK 293T cells. GFP-IN (or GFP) was co-expressed with HDAC10-Myc in HEK 293T cells for 48 hours and cells were subjected to co-IP analysis. GFP-IN (or GFP) was immuno-precipitated by using rabbit anti-GFP antibody and IN-bound HDAC10 was detected by anti-HDAC10 antibody. (C) The interaction of HDAC10 and HIV integrase in HEK 293T cells. HIV expression plasmid Bru-IN-HA or PNL4.3 was co-expressed with HDAC10 or GFP in HEK 293T cells. After 48 hours, cells were collected and subjected to co-IP analysis. Co-IP was performed by using a rabbit anti-HA antibody to pull down IN-HA and an anti-HDAC10 antibody to detect IN-bound HDAC10.

Following the identification of the interaction between HDAC10 and HIV integrase, I further investigated the region of integrase that is essential for HDAC10 binding. HIV integrase is composed of three independent functional domains, including the N terminal domain (amino acid residues 1-49), the catalytic core domain ( amino acid residues 50-212) and the C terminal domain (amino acid residues 213-288) (446). To investigate which region of integrase is essential for HDAC10 binding, I constructed a series of integrase deletion mutants tagged with GFP, including IN 55-288, IN 165-288, IN 1-212, IN 1-230 IN 1-250 and IN 1-270 (Fig. 5.5A) (447). I co-transfected HEK 293T cells with the deletion mutant expression plasmids and HDAC10 respectively. After 48 hours, I collected the cells and detected the expression of these plasmids in the cell lysis. The cell lysis was then subjected to anti-GFP pulldown and the presence of HDAC10 in the pellet was detected by western blot analysis. The results showed that the co-expression of HDAC10 and HIV integrase (or integrase mutants) were detected in the cell lysis. When the integrase or integrase mutant was pulled down by anti-GFP antibody, GFP and GFP-IN 165-288 did not bind to HDAC10 whereas GFP-IN and other truncated integrase mutants (including GFP-IN 55-288, 1-212, 1-230, 1-250 and 1-270) still retained their HDAC10 binding ability, which suggests that IN 55-165 is essential for HDAC10 binding (Fig. 5.5B).

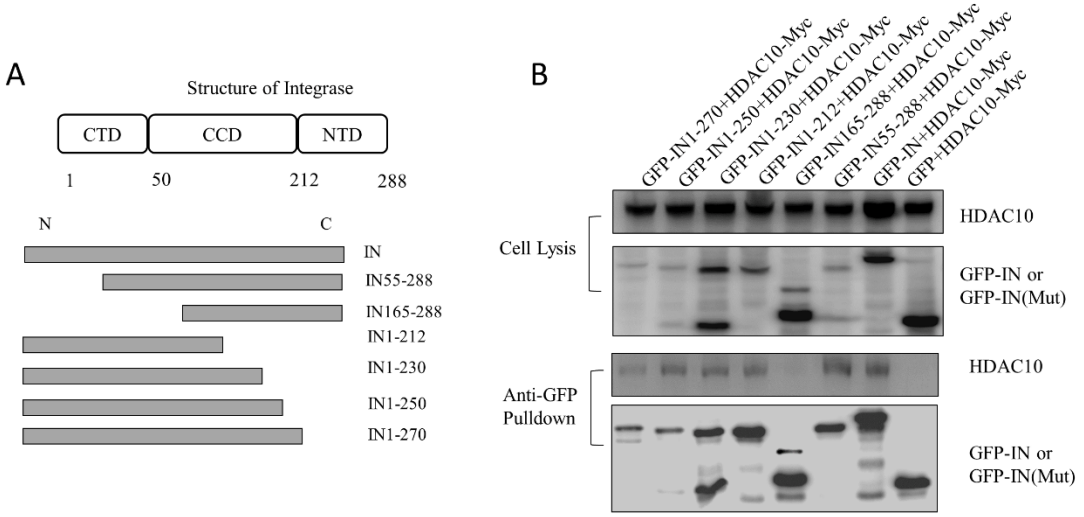



Figure 5.5 **HDAC10 binds to the domain of IN55-165**. (A) The Schematic of HIV integrase deletion mutants tagged with GFP. (B) The interaction of HDAC10 and HIV integrase deletion mutants. GFP tagged HIV integrase or its deletion mutant was co-expressed with HDAC10-Myc in HEK 293T cells. Cells were treated with 20μM/ml of MG-132 for 24 hours and then subjected to Co-IP analysis. GFP-IN (or GFP and GFP-IN mutants) was immuno-precipitated by using anti-GFP antibody, and HIV integrase (or integrase mutants)-bound HDAC10 was detected by anti-HDAC10 antibody.

### 5.2.5 Presence of HDAC10 does not regulate HIV integrase's lysine acetylation but enhances the interaction of HIV integrase and LEDGF

Following the finding that HDAC10 can interact with HIV integrase, it is necessary to investigate whether this interaction is specific for HDAC10. As mentioned above, HDAC6 and HDAC10 belong to the HDAC class II B family and contain a unique, putative second catalytic domain not found in other HDACs (355). Although HDAC6 and HDAC10 share some unique features, they have some structural differences (355). For example, HDAC6 has two putative enzymatic domains while HDAC10 has only one functional enzymatic domain in N terminus while the other enzymatic domain in C terminus is non-functional (355). Although HDAC6 and HDAC10 shares structural homology, whether HDAC6 can act similarly to HDAC10 and interact with HIV integrase remains unknown. Here it is proposed that the interaction between HDAC10 and HIV integrase is HDAC10 specific. Briefly, HEK 293T cells were transfected with GFP-IN/ HDAC10, GFP/HDAC10 (negative control), GFP-IN/HDAC6, or GFP/HDAC6 (negative control). After 48 hours' transfection, cells were subjected to lysis and the GFP-IN or GFP was pulled down by anti-GFP antibody. The western blot results showed that the presence of HDAC10 was detected in the anti-GFP pull down pellet of GFP-IN/ HDAC10 group. However, HDAC6 could not be detected in the anti-GFP pull-down pellet of GFP-IN/HDAC6 group (Fig. 5.6A). This result indicated that HIV integrase was able to interact with HDAC10 but not HDAC6, which suggested that the interaction of HDAC10 and HIV integrase is specific.

Although the results above showed HDAC10 can specifically interact with HIV

integrase by binding to IN 55-165, how this interaction influences viral integration is still unclear. As an essential viral protein, HIV-1 integrase interacts with a large number of cellular cofactors and shows multifunctional properties that are impacted by different post-translational modifications(448, 449). Previous publications have shown that there are four kinds of post-translational modifications in HIV integrase, including ubiquitination, SUMOylation, acetylation, and phosphorylation (130, 450-453). The increase of acetylation of HIV integrase can increase the binding of HIV integrase to DNA and enhance the activity of DNA strand transfer, which subsequently enhances viral integration (450, 454). Here I propose that HDAC10 might interact with HIV integrase to post-translationally modify the lysine acetylation state of HIV integrase. Briefly, HEK 293T cells were co-transfected with HDAC10/GFP-IN or GFP/GFP-IN plasmids for 48 hours. Cells were then subjected to lysis buffer and the HIV integrase was pulled down by the anti-GFP antibody. The expression of both HIV integrase and the acetylated lysine in HIV integrase were detected by anti-GFP or anti-acetylated lysine antibody respectively. The results showed that regardless of HDAC10 expression, the lysine acetylation state of HIV integrase was not significantly different, indicating that HDAC10 did not influence on integration through regulating the lysine acetylation of integrase (Fig. 5.6B).

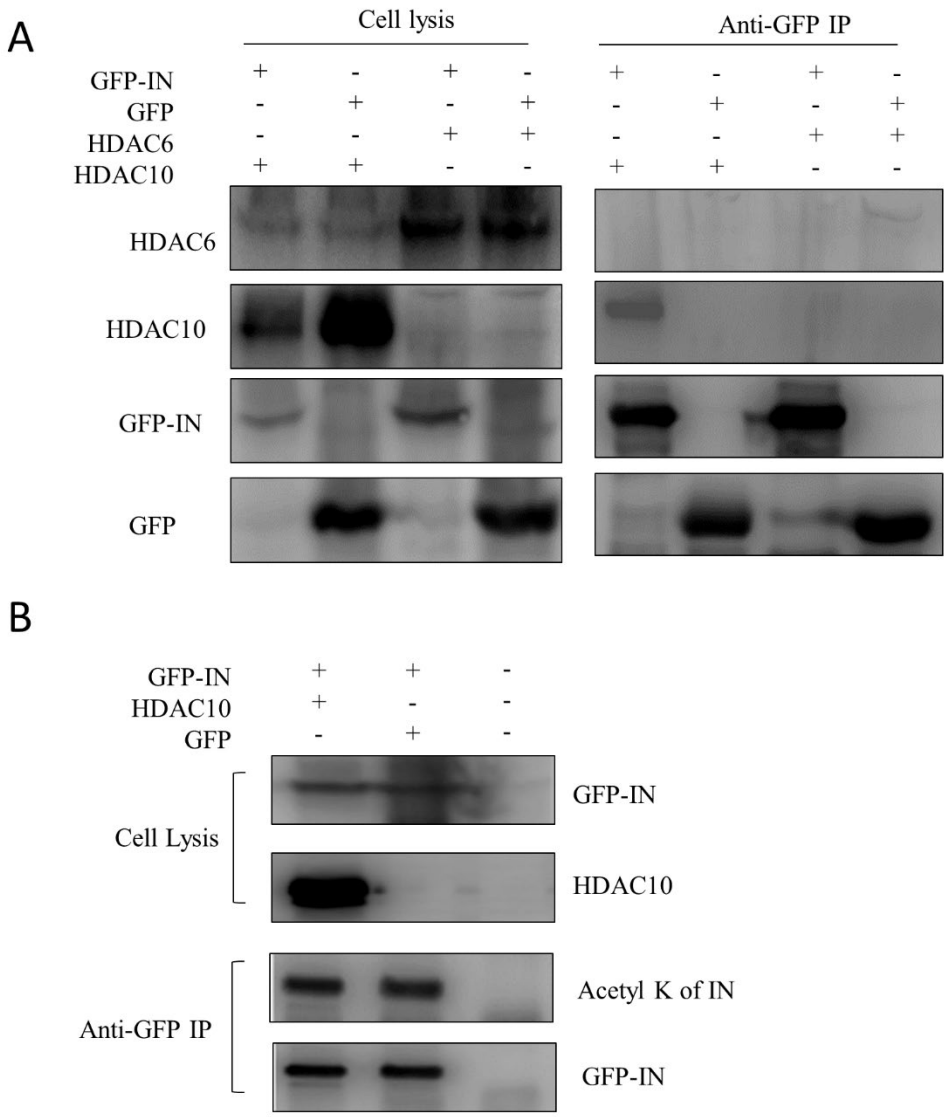



Figure 5.6 **HDAC10 does not mediate the lysine acetylation of HIV integrase.** (A) The interaction of HDAC10 and HIV integrase is specific. GFP or GFP tagged HIV integrase was co-transfected with HDAC10 or HDAC6 in HEK 293T cells. After 48 hours transfection, cells were collected and subjected to co-IP analysis by pulling down GFP-IN or GFP. GFP and GFP-IN were detected by anti-GFP antibody while IN-bound HDAC10 or HDAC6 were detected by anti-HDAC10 or anti-HDAC6 antibody. (B) The interaction of HDAC10 and HIV integrase did not change the lysine acetylation state of HIV integrase. GFP-IN was co-expressed with HDAC10 or GFP in HEK 293T cells for 48 hours. Cells were subjected to co-IP analysis by pulling down HIV integrase. The expression of GFP-IN was detected by anti-GFP antibody while the lysine acetylation state of integrase was detected by anti-acetyl K antibody.

In addition to the post-translational modifications, HIV integrase can also interact with cellular proteins to form a pre-integration complex, an essential required for viral integration (437). For instance, Ku70-a DNA repair protein part of the NHEJ (non-homologous end-joining pathway)-binds to HIV integrase and protects integrase from proteasomal degradation (130, 455-457). Similarly, LEDGF, a co-transcription factor, has been reported to tether HIV integrase into the host chromatin and induce subsequent viral integration (458, 459). Here I speculate that HDAC10 could regulate viral integration through mediating the interaction of HIV integrase and Ku70. Briefly, HEK 293T cells were co-transfected with IN-YFP alone (negative control), T7-Ku70 alone (negative control), IN-YFP/T7-Ku70/Empty-vector (negative control) or IN-YFP/T7-Ku70/HDAC10. After 48 hours' transfection, cells were collected and subjected to the lysis buffer. The HIV integrase was pulled down by anti-GFP antibody and HDAC10 or Ku70 were detected by anti-HDAC10 or T7 antibody respectively. The western blot results showed that both of HDAC10 and Ku70 were detected in the anti-GFP co-IP pellet and there was no significant difference between the protein bands of Ku70 between the two groups (IN-YFP/T7-Ku70/Empty-vector or IN-YFP/T7-Ku70/HDAC10), indicating that HDAC10 can not mediate the interaction between Ku70 and HIV integrase (Fig. 5.7A).

Similarly, it's investigated whether the presence of HDAC10 could influence the interaction between HIV integrase and LEDGF. Briefly, HEK 293T cells were co-transfected with T7-LEDGF alone (negative control), IN-YFP/T7-LEDGF/Empty-vector (negative control), or IN-YFP/T7-LEDGF/HDAC10 for 48 hours. IN-YFP was

pulled down by anti-GFP antibody and IN-bound HDAC10 or LEDGF were detected by anti-LEDGF antibody. Interestingly, the western blots results showed that compared to the negative control (IN-YFP/LEDGF/Empty vector), the presence of HDAC10 significantly decreased the interaction of HIV integrase and LEDGF, implying that HDAC10 might regulate viral integration by mediating the interaction of HIV integrase and LEDGF (Fig. 5.7B). Moreover, compared with wildtype HDAC10 -although the substitution of His (H) with Ala (A) at the residue 135 decreased the interaction of HDAC10 and HIV integrase -this HDAC10 mutant (HDAC10 H135A) was able to weaken the interaction of HIV integrase and LEDGF (Fig. 5.7C). Thus, it could be concluded that the presence of HDAC10 weakens the interaction of HIV integrase and LEDGF.

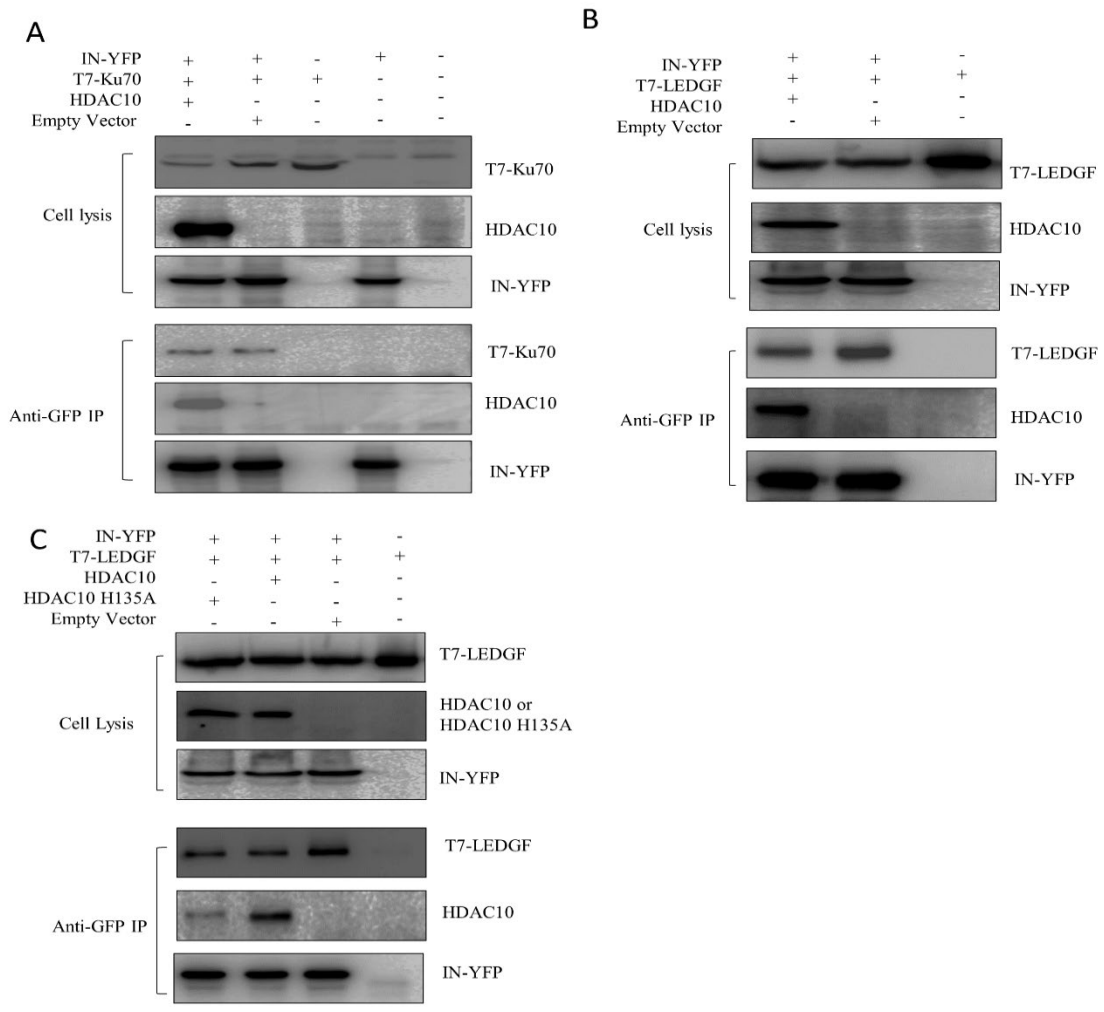



Figure 5.7 **HDAC10 specifically weakens the interaction of HIV integrase and LEDGF.** (A) HDAC10 does not mediate the interaction of Ku70 and HIV integrase. T7-Ku70 was co-transfected with IN-YFP/HDAC10 or IN-YFP/Empty vector in HEK 293T cells. After 48 hours transfection, cells were subjected to co-IP analysis by pulling down HIV integrase. IN-YFP was detected by anti-GFP antibody while IN-bound Ku70 was detected by anti-T7 antibody and IN-bound HDAC10 was detected by anti-HDAC10 antibody. (B) HDAC10 weakens the interaction of LEDGF and HIV integrase. LEDGF was co-expressed with IN-YFP/empty vector or IN-YFP/HDAC10 in HEK 293T cells. After 48 hours transfection, cells were subjected to co-IP analysis by pulling down HIV integrase. IN-YFP was detected by anti-GFP antibody while IN-bound LEDGF was detected by anti-LEDGF antibody and IN-bound HDAC10 was detected by anti-HDAC10 antibody. (C) The HDAC10 enzymatically inactive mutant can weaken the interaction of HIV integrase and LEDGF. LEDGF was co-expressed with IN-YFP/Empty vector, IN-YFP/HDAC10, or IN-YFP/HDAC10 H135A in HEK 293T cells. After 48 hours' transfection, cells were subjected to co-IP analysis by pulling down HIV integrase. IN-YFP was detected by anti-GFP antibody while IN-bound LEDGF was detected by anti-LEDGF antibody and IN- bound HDAC10 or HDAC10 H135A was detected by anti-HDAC10 antibody.

### 5.2.6 HDAC10 downregulation facilitates progeny virus infectivity

In order to study how HDAC10 downregulation benefits HIV replication, I studied the impact of HDAC10 downregulation on the late stage of HIV replication. Here I evaluated the infectivity of the progeny virus isolated from HDAC10 shRNA transduced cells or scramble shRNA transduced cells (control). Briefly, the progeny virus was isolated from the supernatant of HIV infected HDAC10 shRNA transduced cells or scramble shRNA transduced cells. Then the concentration of progeny virus was detected by anti-p24 ELISA. Following that, the same amount of progeny virus from HDAC10 shRNA transduced cells or scramble shRNA transduced cells was used to infect TZMb1 cells, in which the cellular relative firefly luciferase activity was detected after 48 hours infection. Results showed that the progeny virus from HDAC10 shRNA transduced cells induced higher relative firefly luciferase activity (increase to 2-3 folds) compared with controls, which suggest that virus from HDAC10 knockdown cells was more infectious than that from control cells (Fig. 5.8A). I also confirmed this result in $CD4^+$ T lymphocytes C8166 cells. The same amount of progeny virus from HDAC10 shRNA transduced cells or scramble shRNA transduced cells was used to infect CD4+ T cell line C8166 overnight. After 72 hours, I detected the presence of p24 in the supernatant by ELISA. Results showed that the progeny virus from HDAC10 knockdown cells could more effectively infect C8166 cells and produce more virus in the supernatant (increased to 2-2.2 folds). This suggests the progeny virus from HDAC10 knockdown cells was more infectious than that from control cells (Fig. 5.8B).

Next, I tested whether HDAC10 overexpression influences the infectivity of

progeny virus. Briefly, HEK 293T cells were co-transfected with HIV plasmid PNL4.3 /HDAC10 or HIV plasmid PNL4.3/GFP. Then the progeny virus was purified from the supernatant by ultracentrifugation. The concentration of progeny virus was detected through anti-p24 ELISA. As mentioned above, the same amount of progeny viruses from HDAC10 overexpression cells or control cells were used to infect TZMb1 or C8166 cells and the firefly luciferase activity or viral protein p24 in the supernatant was detected on the days 3 and 7 post-infection. Results showed that the progeny virus from HDAC10 overexpression cells induced less firefly luciferase activity in TZMb1 cells and produced less p24 in C8166 cells, which suggest that HDAC10 overexpression inhibits the progeny virus's infectivity (Fig. 5.8C and 5.8D). In summary, the above results demonstrate that progeny virus infectivity is inversely correlated with cellular HDAC10 level.

After demonstrating that the downregulation of HDAC10 enhances the infectivity of progeny virus, I investigated the impacts of the enzymatic domain of HDAC10 on the infectivity of progeny virus. In doing this, HEK 293T cells were co-transfected with HIV plasmid PNL4.3/GFP (negative control), HIV plasmid PNL4.3/ HDAC10 or HIV plasmid PNL4.3/HDAC10 H135A (catalytically inactive mutant) (357). After that, the progeny virus was purified from the supernatant by ultracentrifugation. The concentration of progeny virus was detected by anti-p24 ELISA. Same amounts of progeny virus from HDAC10 overexpressed cells, HDAC10 H135 overexpressed cells or control cells, were used to infect TZMb1 or C8166 cells and the firefly luciferase activity or viral protein p24 in the supernatant was detected on days 3 post-infection.

The results showed that the virus from HDAC10 H135A overexpressed cells induced higher firefly luciferase activity in TZMb1 cells and produced more p24 in the supernatant from C8166 cells compared with HDAC10 overexpressed group. However, when compared with the negative control group (HIV plasmid PNL4.3/GFP), the virus from HDAC10 H135A overexpressed cells produced less firefly luciferase activity in TZMb1 cells and produce less p24 in supernatant from C8166 cells (Fig. 5.8E and 5.8F). These results suggest that the enzymatic domain of HDAC10 may be partially responsible for the ability of HDAC10 inhibiting progeny virus infectivity.

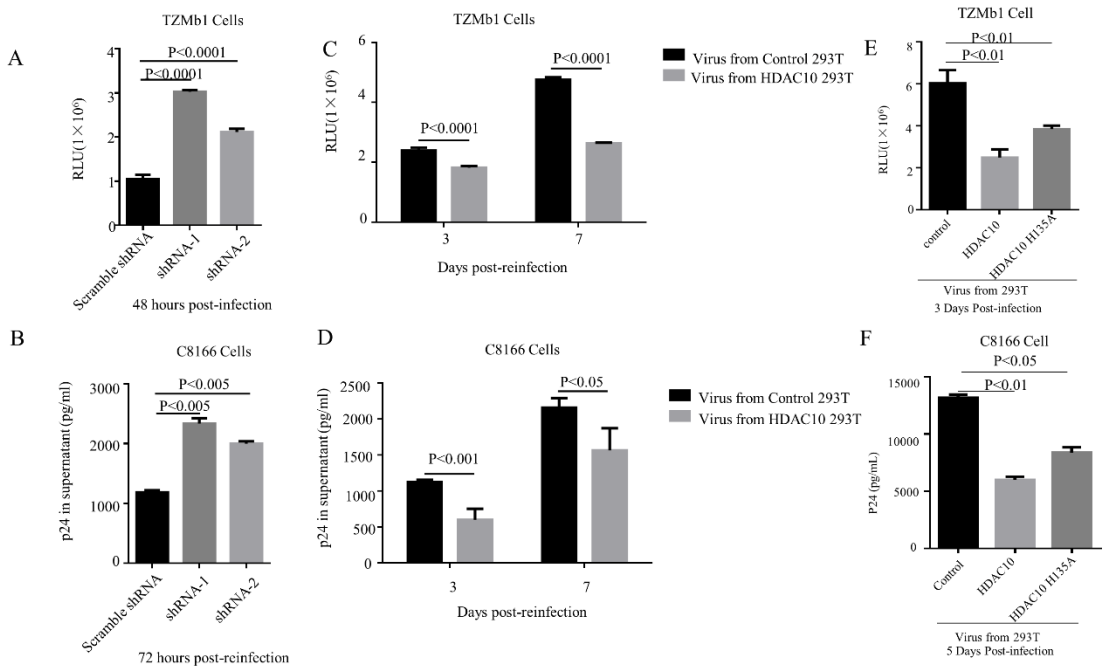



Figure 5.8 **HDAC10 downregulation enhances progeny virus infectivity.** (A, B) The detection of progeny virus infectivity from HDAC10 shRNA transduced cells or scramble shRNA transduced cells. Progeny virus was purified from either transduced cell lines. The same amount of progeny virus was then used to infect TZMb1 or C8166 cells. The infectivity of progeny virus was detected by measuring the luciferase activity in TZMb1 cells or p24 production in the supernatant of C8166 cells. (C, D) The comparison of progeny virus infectivity from HDAC10 overexpressed cells and control cells. The progeny virus was purified from PNL4.3/HDAC10 overexpressed or PNL4.3/GFP (control) HEK 293T cells. The same amount of progeny virus was used to infect TZMb1 cells or C8166 cells and the infectivity of progeny virus was detected by measuring the luciferase activity in TZMb1 cells or p24 production in the supernatant from C8166 cells. (E, F) The detection of progeny virus infectivity from PNL4.3/HDAC10, PNL4.3/HDAC10 H135A overexpressed cells, or PNL4.3/GFP control cells. The progeny virus was purified from HDAC10 overexpressed, HDAC10 H135A overexpressed, or control HEK 293T cells. The same amount of progeny virus was used to infect TZMb1 cells or C8166 cells and the infectivity of progeny virus was detected as mentioned above. Data are mean and sd. (Two-tailed unpaired t-test)

## 5.3 Discussion

In this study, I identified HDAC10 as an HIV inhibitory factor and studied its inhibitory function during HIV replication. By quantitative RT-PCR and western blot, the results revealed that HDAC10 is transcriptionally downregulated by HIV in CD4+ T cells. It also showed that HDAC10 downregulation promotes viral replication through enhancing viral integration. To investigate the underlying mechanism, I demonstrated that HDAC10 can interact with HIV integrase by using cell-based co-immunoprecipitation assay. Deletion analysis on HIV integrase revealed that HDAC10 interacts with HIV integrase by binding to IN55-165 region. Furthermore, my results showed that the interaction of HDAC10 with HIV integrase does not alter the lysine acetylation of HIV integrase but weakens the interaction of HIV integrase and LEDGF, which contributes to the inhibition of viral integration. In addition to mediating viral integration, my results showed that the infectivity of progeny virus is inversely correlated with cellular HDAC10 level and the HDAC10 downregulation increased the infectivity of progeny virus. In summary, my study suggests that HDAC10 acts as an inhibitory factor against HIV and its downregulation is beneficial for the early and late stages of HIV replication.

Previous studies have identified several restriction factors and investigated the relevant HIV evading mechanism (162). For instance, A3G catalyzes the deamination of cytidine to uridine and causes mutations of the viral genome but it can be degraded by viral protein Vif (460). BST-2 prevents the release of HIV-1 virions on the cell surface but it can be degraded by viral protein Vpu (461). Here I report that the cellular

protein HDAC10 is downregulated by HIV during HIV replication. Similar to A3G and BST-2, I found that HDAC10 level decreased rapidly and it can be, achieving up to a 50% reduction in less than 48 hours. However, the downregulation of HDAC10 is not by proteasomal degradation as A3G or BST-2, but by decreasing its mRNA expression. In my preliminary studies, the RNA-seq results showed that HIV vEnv caused the downregulation of endogenous HDAC10. However, with the exception of envelope glycoprotein, whether other viral proteins also directly contribute to the downregulation remains unknown (429).

HIV integration is an essential step during the early stage of HIV replication (462). HIV integrase is a key enzyme for this process and it interacts with many cellular factors to form a pre-integration complex to mediate viral integration (449, 463). Some of these cellular factors can enhance HIV integration, such as Ku70, while others can inhibit this process, such as KAP1 (130, 464). This study provided another example of a host cellular factor of HIV integrase, HDAC10, acting as an inhibitory factor in viral integration. These results showed that HDAC10 downregulation enhances viral integration and HDAC10 can interact with HIV integrase through binding to the IN55-165 region. The interaction of HIV integrase and cellular cofactors can influence the HIV integrase activity in several ways, such as post-translational modifying HIV integrase (449). Previous publications reported that the acetylation state of HIV integrase is associated with the activity of HIV integrase (450, 454). Acetylation increases the binding of integrase to DNA and facilitates DNA strand transfer activity, which consequentially enhances viral integration (450). However, my results showed

that HDAC10 does not influence the lysine acetylation of HIV integrase. It is possible that HDAC10 is a poor lysine deacetylase but a robust polyamine deacetylase, which implies HDAC10 may not perform its normal enzymatic function, but rather, have other regulatory functions during viral integration (465). It is of note that although HADC10 does not alter the acetylation of HIV integrase, interestingly, I found that the presence of HDAC10 weakens the interaction of HIV integrase and LEDGF. The interaction of HIV integrase and LEDGF is essential for viral integration. Previously, several groups have reported that the impaired interaction of HIV integrase and LEDGF can directly destroy viral integration (466). For instance, in 2005, Emiliani et al demonstrated that the HIV integrase mutants defective for LEDGF binding can impair viral integration and later in 2007, Busschotes et al also observed similar phenomenon (467, 468). In 2013, Desimmie et al revealed that the disruption of the interaction between HIV integrase and LEDGF by specific inhibitors can also destroy viral integration (469). Based on the information, it is possible that HDAC10 weakens the interaction of HIV integrase and LEDGF, consequently inhibiting viral integration.

Finally, this study also provided evidence of HDAC10 mediating the late stage of viral replication. During the late stage of viral replication, many cellular factors can influence progeny virus assembly and maturation, and consequently regulate progeny virus infectivity (470). For instance, in addition to preventing the release of viral particles, BST-2 also impairs HIV progeny virus infectivity through the accumulation of Gag pr55 as well as Gag p40 intermediates, which results in the loss of a mature core in of HIV-1 viral particles (471). In my study, I found that the level of cellular HDAC10

is inversely correlated with the infectivity of progeny virus. The downregulation of HDAC10 can enhance progeny virus infectivity while the upregulation of HDAC10 can inhibit progeny virus infectivity. This suggests that endogenous HDAC10 performs an inhibitory function in progeny virus infectivity. Previous studies have also reported that progeny virus infectivity is associated with the interaction of endogenous LEDGF and HIV integrase in producer cells. In 2002, Frauke Christ et al reported that the disruption of HIV integrase binding to endogenous LEDGF can significantly decrease the progeny virus infectivity (444). Later, another independent group, Rouzic et al, reported similar results, finding that the inhibition of the interaction of integrase and endogenous LEDGF can greatly decrease the progeny viral infectivity (472). For the underlying mechanism, Desimmie et al found that the disruption of the interaction of newly synthesized HIV integrase and endogenous LEDGF mediates integrase multimerization and about 70% of the progeny virus do not form a mature core due to a mislocalized electron dense ribonucleoprotein (469). Therefore, it is possible that the presence of HDAC10 decreases the interaction of HIV integrase and endogenous LEDGF, which causes aberrant core formation and consequentially contributes to the loss of progeny viral infectivity. However, the role of HDAC10 in mediating progeny virus infectivity still needs more investigation.

In summary, this study showed that HDAC10 is a potent cellular factor that influences viral integration and progeny virus infectivity. The investigation of HADC10 in HIV replication can provide a new insight into the interaction between cellular factors and virus, which offers us a potential target for HIV therapy.

# Chapter 6

# General discussion and future direction

## 6.1 Major findings

In addition to mediating viral entry, HIV envelope glycoprotein has been found to induce cellular signal pathways to benefit viral infection and replication. Previous studies have shown that the interaction between the envelope glycoprotein, CD4 receptor, and coreceptor (CCR5/CXCR4) are essential for the early stage of HIV infection. These interactions can induce actin cytoskeleton rearrangement, stimulate cellular transcription and modulate cytokine and chemokine expression to facilitate HIV infection. However, how vEnv regulates the late stage of HIV infection is poorly understood. The work presented in this thesis describes the role of vEnv in inducing HIV viral transcription in HIV infected CD4+ T cells and its underlying mechanism, which can be used for developing new strategies for HIV therapy.

As first described in chapter 3, this thesis revealed that AT-2 inactivated HIV could induce HIV LTR controlled transcription in TZMb1 cells. The inactivated virus lost its infectivity due to AT-2 covalently modifying NC and inhibiting viral reverse transcription. Since the viral genome of the inactivated virus cannot be integrated into host chromatin, viral regulatory protein Tat cannot be produced and activate the subsequent viral transcription. Therefore I proposed that there was another viral protein activating HIV transcription. Based on the above information, I mimicked the inactivated virus and generated the HIV virus like particles. Luciferase assay results

indicated that the HIV envelope glycoprotein incorporated into viral particles could specifically induce viral transcription in TZMb1 cells. Furthermore, I demonstrated that this transcriptional activation required the interaction between envelope glycoprotein and CD4 receptor/coreceptor (CCR5/CXCR4) and if the receptor was inhibited by a specific antibody or reagent, the viral transcription could not be activated. In addition, the efficiency of inducing transcriptional activation was compared between the shedding gp120, recombinant gp120 and vEnv. The results showed that vEnv could activate viral transcription more efficiently than the other two forms of envelope glycoproteins. Finally, this phenotype was also observed in HIV-1 infected CD4+ T lymphocytes, including J-Lat 6.3 cell line, HIV-1 infected resting PBMCs, and resting PBMCs isolated from ART-treated patients. As stated above, these results suggested that vEnv could significantly activate viral transcription in HIV-1 infected cells.

As shown in chapter 4, to investigate the underlying mechanism of vEnv activating viral transcription, I utilized whole RNA transcriptome sequencing, to efficiently and accurately describe the presence of RNA and their expression changes. The RNA-seq results showed that there were 1,349 differentially expressed genes whose expression levels had significant changes ($p<0.05$) in the vEnv (X4) treated group. In the heat map, the hierarchical clustering of RNA-seq data from Env(X4)-VLP or mock-treated cells demonstrated the different genome-wide expression profiles. The gene analysis revealed that vEnv treatment modulated genes involved in multiple cellular signaling pathways, including transcription regulation, actin skeleton organization, and T cell receptor signaling. Several pathways are consistent with previously published results.

For example, a previous study reported that HIV envelope glycoprotein can stimulate cellular cytoskeleton reorganization and my RNA-seq results also showed the cellular cytoskeleton associated genes were significantly modified (307). In addition to previously reported genes, my RNA-seq results revealed genes not previously reported to participate in HIV replication. Among these genes, I identified that microRNA181A2 was associated with viral transcription. Initially, the downregulation of miR-181A2 was confirmed in J-Lat 6.3 cells as well as primary CD4+ T cells upon vEnv (X4) treatment. The results showed the inverse correlation of miR-181A2 and PCAF expression after vEnv treatment. To characterize the role of miR-181A2 in viral transcription, I used a lentiviral system to overexpress miR-181A2 or its inhibitor. The inhibition of miR-181A2 could increase PCAF expression and subsequently enhance viral transcription, whereas the overexpression of miR-181A2 could downregulate PCAF expression and consequently inhibit viral transcription. Subsequently, using a chromatin immunoprecipitation assay, I demonstrated that vEnv downregulated miR-181A2 and increased histone H3 acetylation in HIV LTR, contributing to the activation of viral transcription.

Among these genes modulated by envelope glycoprotein, several genes participate in other steps of viral replication. As outlined in chapter 5, I investigated the role of HDAC10 in viral replication. Consistent with the RNA-seq results, HDAC10 was downregulated in CD4+T cell lines as well as primary CD4+ T cells during HIV infection. To investigate the influence of HDAC10 downregulation on viral replication, I knocked down the endogenous HDAC10 in CD4+ T cells, monitored viral replication

and analyzed viral DNA in these cells. The results showed that HDAC10 downregulation enhanced viral replication by promoting viral integration. While investigating the underlying mechanism by which HDAC10 downregulation enhances viral integration, it was observed that HDAC10 could interact with viral integrase in HEK 293T expression system. Furthermore, deletion analysis suggested that a region in the catalytic domain of HIV integrase, amino acid 55-165, was required for HDAC10 binding. Interestingly, it was found that the interaction of HDAC10 and HIV integrase did not alter the lysine acetylation state of integrase, but rather, specifically decreased the interaction of LEDGF and HIV integrase, consequently leading to the inhibition of viral integration. In addition, I investigated the role of HDAC10 in the late stage of viral replication through detecting the infectivity of progeny virus produced from HDAC10 knockdown cells or HDAC10 overexpressed cells. My results revealed that progeny viral infectivity was inversely correlated with cellular HDAC10 level. The downregulation of HDAC10 led to the increase of progeny virus infectivity while the overexpression of HDAC10 decreased it. Overall, these findings provided evidence that HDAC10 acts as an important inhibitory factor in early and late stages of HIV replication.

In summary, the HIV vEnv interacts with CD4/coreceptors to stimulate viral transcription and increase the infectivity of progeny virus in HIV infected cells, which enhances viral pathogenesis.

## 6.2 General discussion and future directions

HIV envelope glycoprotein is an essential viral protein required for viral entry and fusion. The mechanism of viral entry and fusion has been well studied. However, HIV envelope glycoprotein induced chemokine-signaling is poorly understood and the impact of these signaling pathways is not well-characterized. In this study, I revealed the role of HIV envelope glycoprotein in HIV transcription and investigated the mechanism of its action.

### 6.2.1 HIV envelope glycoprotein has a profound effect on HIV replication.

HIV envelope glycoprotein is the initial protein that interacts with host cells. Its function is primarily characterized as an essential viral protein for viral entry and membrane fusion. However, recent studies have revealed that HIV envelope glycoprotein not only mediates viral entry and membrane fusion, but also affects processes, such as viral reverse transcription, cytoplasmic transportation, integration, and transcription (290, 307). For instance, HIV envelope glycoprotein induced signaling can activate cellular factor Cofilin and stimulate cytoskeleton rearrangement, which directly affects subsequent reverse transcription, cytoplasmic transportation and nuclear import (307). As shown in the chapter 3 and 4, HIV-1 vEnv downregulates miR-181A2 and increases PCAF expression, which results in the increases of histone H3 acetylation at NUC-1 and consequently activates viral transcription. The above evidences suggest that HIV envelope glycoprotein has a profound effect on the HIV replication, not only limited to viral entry and membrane fusion.

My study results showed that the interaction of HIV envelope glycoprotein and CD4/coreceptors is required for the initiation of envelope glycoprotein induced chemokine signaling pathways. If CD4 or coreceptors (CCR5 or CXCR4) were blocked by specific antibody or reagents, the viral transcriptional activation would not occur. In addition, my study demonstrated that the conformation of HIV envelope glycoprotein is important for the successful activation of chemokine signaling pathways. My results showed that the HIV envelope glycoprotein trimer on viral particles can more efficiently stimulate viral transcription than shedding or recombinant gp120, which suggests that the conformation of the HIV envelope glycoprotein determines the activation of chemokine pathways. It is possible that the naïve envelope glycoprotein spikes present on viral particles contain the cytoplasmic tail and membrane proximal external region, which may affect the trimer structure to interact with CD4/ coreceptors. Therefore, both the conformation of Env and the presence of host receptors are essential for viral transcriptional activation.

What's more important, the findings revealed a potential mechanism of the reactivation of HIV latency. At present, HIV latency is a state of nonproductive infection in a small number of infected cells. These HIV latently infected cells harbor viral genome but produce little to no viral proteins (89). Therefore, latently HIV infected cells can evade immune recognition and persist in the host body for a long time even under HAART treatment (473). Once HAART treatment is ceased, the viral load will rapidly rebound as a result of the reactivation of HIV latency (473). However, how these latently infected cells are reactivated so rapidly is still poorly understood. As

shown in chapter 3, HIV vEnv can efficiently stimulate viral transcription in HIV latently infected cells, including HIV-infected J-Lat 6.3 T cells, HIV-infected resting PBMCs and resting PBMCs isolated from ART-treated HIV patients. In addition, previous studies revealed that HIV vEnv exists in the lymphoid tissue of HARRT-treated patients (389). Based on these observations, it is reasonable to believe that the small amount of vEnv can stimulate viral transcription in these latently infected cells in lymphoid, which results in the rapid rebound of viral load in HIV patients once HAART is ceased.

### 6.2.2 MicroRNA181A2 is a potential target for the reactivation of HIV latency

Based on the finding that HIV envelope glycoprotein can induce viral transcription in HIV latently infected cells, I utilized whole transcriptome sequencing to identify cellular genes modulated by envelope glycoprotein. Among these genes, I found that miR-181A2 participated in viral transcriptional activation.

MicroRNAs are a class of small, non-coding RNA molecules that participate in multiple biological processes (474). Previous researches have demonstrated that miRNAs play important roles in HIV pathogenesis. For instance, miR-28, 150, 223 and 382 were reported to be associated with cellular resistance against HIV infection (475). The high level of these miRNAs makes cells resistant to HIV infection while the low level of these miRNAs increases susceptibility (475). Recent studies have also revealed that microRNAs can mediate the formation of HIV latency. For instance, miR-198 was reported to suppress cellular cofactor Cyclin T1, an important component of P-TEFb

which is necessary for the activation of viral transcription (476, 477). As shown in chapter 4, miR-181A2 participates in the formation of latency through regulating viral transcription. Previous studies reported that MiR-181A2 can target to 3'UTR of PCAF and repress its expression. PCAF is an important histone lysine acetyltransferase, which strongly acetylates histone H3 but weakly modifies histone H4 (478). In normal conditions, HIV recruits PCAF to the HIV LTR promotor and increases the acetylation of HIV LTR to stimulate viral transcription (479). Instead, the decrease of PCAF caused by miR-181A2 can directly reduce the lysine acetylation of histone H3 and subsequently suppress viral transcription. To facilitate viral transcription, HIV utilizes envelope glycoprotein to downregulate miR-181A2 and promote PCAF expression, which increases histone H3 acetylation of NUC-1 at HIV LTR promotor, thereby stimulating viral transcription.

My findings suggest an explanation of how HIV envelope glycoprotein causes the rapid viral rebound after HAART treatment ceases. In HIV latently infected cells, HIV transcription is absent or rare due to the high deacetylation state of histone H3 and H4 in the HIV LTR region (89). Once HAART is stopped, HIV envelope glycoprotein present on a small amount of virus interact with host receptors and initiates chemokine signaling pathways to regulate downstream genes' expression, including downregulating miR-181A2. These activated cellular pathways contribute to the increase of histone H3 acetylation on HIV LTR and subsequent activation of viral transcription. Therefore, miR-181A2 is a good candidate for the reactivation of HIV latency to eliminate HIV latent reservoir.

To date, the utilization of miRNAs in viral therapy has received extensive concerns. For instance, Miravirsen is a small RNA composed of 15 RNA molecules which are complementary to human miR-122 (480). miR-122 can ferry an Argonaute protein which provides protection for Hepatitis C viral RNA while Miravirsen can complementarily bind to miR-122 and remove this protection, which suppresses viral production (480, 481). Recent data from phase II clinical trials has shown that administration of Miravirsen to HCV patients resulted in viral load reduction to undetectable level (482). Similarly, downregulating miR-181A2 or inhibiting miR-181A2's function can be a good strategy for reactivating HIV latency. However, the putative use of miR-181A2 inhibitor in HIV latency reactivation needs further investigation.

### 6.2.3 HDAC10 is an inhibitory factor involved in multiple steps of HIV replication

From the results of whole transcriptome sequencing, I identified another cellular factor HDAC10 which is downregulated by HIV envelope glycoprotein in J-Lat 6.3 cells. As mentioned previously, the interaction between HIV envelope glycoprotein and CD4/coreceptors has a profound effect on HIV replication, which implies that HDAC10 may mediate other steps of viral replication not limited to viral transcription.

HDAC10 is a member of HDAC class IIB. Different from HDAC class I members, HDAC10 is mainly located in the cytoplasm because of its leucine-rich domain though it can enter the nucleus with the assistance of other proteins (355). Although it was reported that HDAC10 participates in multiple biological processes, such as

homologous recombination, autophagy-mediated cell survival, and cell cycle, the role of HDAC10 in HIV replication is poorly understood (356, 357, 431). For the first time, my study revealed that HDAC10 acts as an inhibitory factor against HIV replication. As described in chapter 5, HDAC10 inhibits the early stage of viral replication by impairing viral integration. This impairment occurs by HDAC10 interacting with HIV integrase and weakening the interaction between HIV integrase and LEDGF. The interaction of HIV integrase and LEDGF is essential for tethering the PIC into the host chromatin and the disruption of this interaction can prevent viral integration (483, 484). Additionally, the interaction of HDAC10 and HIV integrase affects the late stage of viral replication. My results found that the progeny virus infectivity is inversely correlated with the cellular HDAC10 expression. Previous studies have shown that the interaction of LEDGF and HIV integrase is important for viral assembly. Christ and Rouzic et al, reported that the disruption of HIV integrase binding to endogenous LEDGF can significantly decrease the progeny virus infectivity (444, 472). Because the disruption of the interaction of newly synthesized HIV integrase and endogenous LEDGF mediates integrase multimerization during viral assembly and about 70% of the progeny virus do not form a mature core due to a mislocalized electron dense ribonucleoprotein (469). In my study, I showed that HDAC10 can impair the interaction of HIV integrase and LEDGF, by which the presence of HDAC10 decreases the infectivity of progeny virus. In summary, HDAC10 negatively modulates HIV replication.

### 6.2.4 Future directions

How to effectively reactivate viral expression in latently infected cells is an important component of "shock and kill" strategy, which is developed to eliminate latent reservoirs in ART treated patients. Based on the RNA-seq results, I found there are over 1,000 genes modulated by HIV envelope glycoprotein. Among these genes, I proposed that some of their encoding products may participate in the reactivation of viral expression in latently infected cells, and I found two gene products, E2F2 and miR423, which may mediate viral expression by different mechanisms. E2F2 is a member of E2F family, which is a group of transcriptional factors that play an important role in G1/S transition in mammalian cells. Based on the homology, E2F1 and E2F2 belong to the activating group of E2F family as both can induce S phase entry in quiescent cells (485, 486). Previous studies have shown that E2F1 binds NF-κB p50 to form a complex targeting HIV LTR enhancer region, which suppresses HIV transcription regulated by NF-κB p50/p65 complex (487). The RNA-seq results showed downregulation of E2F2 but not E2F1 upon vEnv treatment. Whether E2F2 can act like E2F1 and interact with NF-κB p50 to inhibit viral transcription is not clear. Therefore we propose that HIV vEnv downregulates E2F2 to activate NF-κB p50/p65 mediated viral transcription in HIV latently infected cells and are carrying out the relevant research to testify this hypothesis. To test this hypothesis, I plan to demonstrate the downregulation of E2F2 in HIV latently infected cell lines and PBMCs from aviremic patients. Following that, I will characterize the function of E2F2 in HIV transcription by knocking down the E2F2 level in HIV infected cell lines and detect viral mRNA

expression. Next I plan to examine the impact of downregulation of E2F2 on NF-κB p50/p65 by detecting the translocation of NF-κB p50/p65. If the result showed that the downregulation of E2F2 induces the translocation of NF-κB p50/p65, it is suggested that the downregulation of E2F2 by vEnv may effect viral transcription through activating NF-κB p50/p65.

There is another cellular gene miR423, which may be associated with the reactivation of HIV latency induced by HIV vEnv. miR423 (also named miR423-3p) is a microRNA, which participates in several biological processes, including promoting cell growth, regulating cell cycle, stimulating cancer progression and activating autophagy (488, 489). Recently, Adam W. Whisnant et al used the PAR-CLIP (photoactivatable ribonucleoside-enhanced cross linking and immunoprecipitation) and found that miR423 inhibits viral expression by binding to the viral transcripts (490). Thus it is reasonable to speculate that the downregulation of miR423 may activate viral expression through a post-transcriptional regulation in HIV latently infected cells. Whether this hypothesis is correct still needs investigation. To test my hypothesis, I plan to demonstrate that miR423 participates in viral expression by transducing miR423 inhibitor in HIV latently infected cells. Besides, I will identify the sequence of viral transcripts bound to miR423 by RNA sequencing, and access the impact of miR423 on the expression of these viral transcripts by detecting their mRNA and protein level.

## 6.3 Conclusion

HIV envelope glycoprotein has multiple functions in HIV replication. In addition

to regulating viral entry and membrane fusion, my results demonstrated that HIV-1 envelope glycoprotein can efficiently activate viral transcription in HIV-1 infected CD4+ T cells, including HIV infected cell line, HIV-1 infected resting PBMCs and resting PBMCs isolated from aviremic HIV-infected patients. The transcriptional activation requires the interaction of HIV envelope glycoprotein and CD4/coreceptors. Additionally, the virion-associated HIV envelope glycoprotein can more efficiently induce the activation of viral transcription than shedding or recombinant gp120. This activation is linked to multiple cellular pathways and more than 1,000 genes have been identified that can be modulated by HIV envelope glycoprotein, including miR-181A2 and HDAC10. The downregulation of miR-181A2 by envelope glycoprotein can activate viral transcription through increasing PCAF expression, which enhances the histone H3 acetylation of nuc-1 in HIV LTR and subsequently activates viral transcription. While the downregulation of HDAC10 by HIV envelope glycoprotein can enhance viral integration and increase progeny virus infectivity through preventing the impairment of the interaction of HIV integrase and LEDGF (shown in Fig. 6.1). In summary, this work revealed a potential new role of HIV envelope glycoprotein in HIV pathogenesis, which helps us to better understand the action of envelope glycoprotein and develop new HIV therapies.

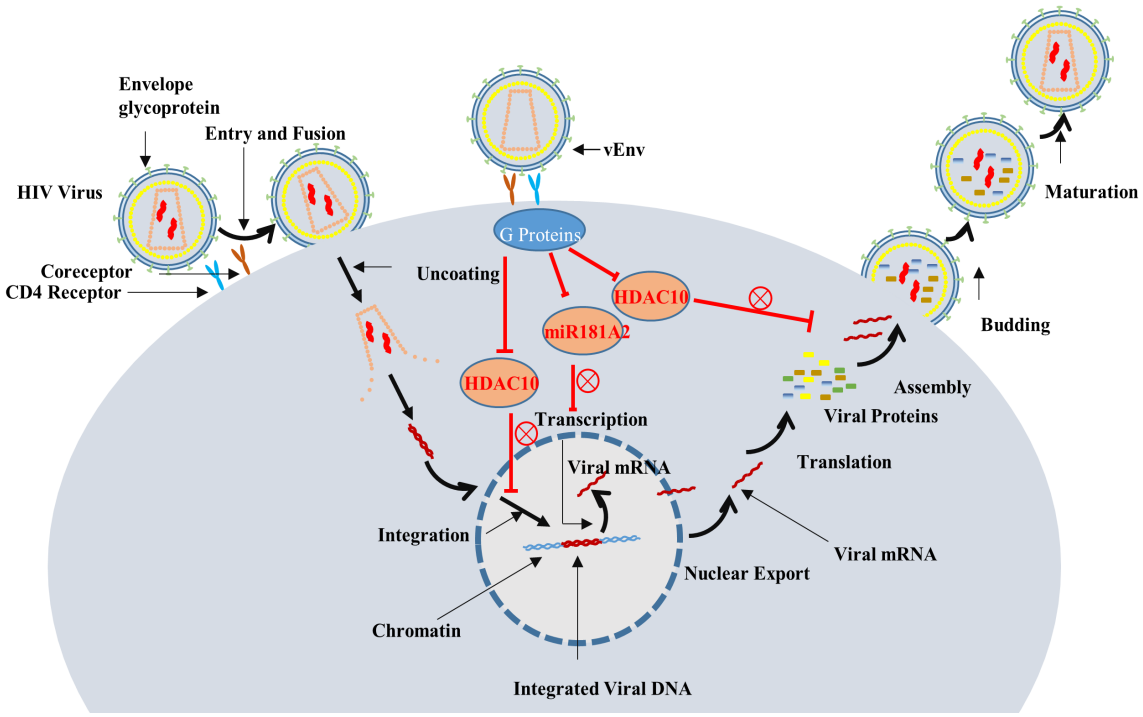



Figure 6.1. **The schematic of the vEnv (X4) induced pathways in CD4+ T cells during HIV replication.** HIV-1 vEnv (X4) can efficiently interact with CD4 receptor and CXCR4 coreceptor and activate cellular pathways in HIV-1 infected CD4+ T cells. This activation involves the regulation of several genes, including miR-181A2 and HDAC10. The downregulation of miR-181A2 can activate viral transcription by enhancing histone H3 acetylation of nuc-1 in HIV LTR. While the downregulation of HDAC10 can enhance viral integration and increase progeny virus infectivity.

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