Understanding the anti-HIV activity of IFITM1

by

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Abstract

Human immunodeficiency virus type 1 (HIV-1) is subject to interferon-mediated antiviral defense. Among the interferon-stimulated genes, a small protein family called IFITM (interferon-induced transmembrane) proteins have been reported to inhibit a wide range of viruses including HIV-1. Yet, little is known about how IFITM proteins inhibit the infection of HIV-1 and how HIV-1 may overcome this inhibition. My thesis project is to understand the anti-HIV activity of IFITM1 and the strategy that HIV-1 adopts to overcome this restriction. Three major findings were made and are summarized as following.

First, we have performed virus evolution assay in tissue culture and successfully selected IFITM1-resistance HIV-1 using a virus strain called HIV-1_{BH10}. Sequencing the escape viruses revealed two mutations, Vpu34 in viral Vpu protein and EnvG367E in viral envelope protein, which together enabled efficient HIV-1 replication in IFITM1-expressing SupT1 cells. These two mutations did not overcome the defects in the viral p24 expression that was caused by IFITM1, rather they enhanced HIV-1 cell-to-cell transmission. For the first time, our study demonstrates that HIV-1 is able to mutate to evade IFITM1 restriction by increasing cell-to-cell transmission.

Second, we observed that the HIV-1_{BH10} strain, but not HIV-1_{NL4-3}, was dramatically inhibited by IFITM1 in SupT1 cells. Mutagenesis analysis of the viral genome revealed that HIV-1_{NL4-3} envelope protein determines the resistance to IFITM1 at least partially resulting from its greater ability to mediate HIV-1 cell-to-cell transmission. This finding further highlights the important role of HIV-1 envelope in countering IFITM1 inhibition.

Third, we discovered that the C-terminal sequence of IFITM1 prevented IFITM1 from inhibiting HIV-1 entry. Removing the C-terminal sequence enabled IFITM1 to inhibit

HIV-1_{NL4-3} partially by diminishing virus entry. Further mutagenesis studies mapped the key amino acid residues to 117-QII-119. Importantly, HIV-1_{NL4-3} was able to escape from the inhibition by C-terminus truncated IFITM1 through mutating the Vpu and envelope proteins.

In conclusion, we have discovered a vital role of HIV-1 envelope in determining the susceptibility of HIV-1 to IFITM1 inhibition. We also, for the first time, report a role of the C-terminal sequence of IFITM1 in regulating its anti-HIV-1 function.

Résumé

Le virus de l'immunodéficience humaine de type 1 (VIH-1) doit faire face à la défense antivirale médiée par l'interféron. Parmi les gènes stimulés par l'interféron, une petite famille de protéines transmembranaires induite par l'interféron (IFITM) inhibe la réplication de plusieurs virus, y compris le VIH-1. On sait peu sur la façon dont les protéines IFITM inhibent la réplication du VIH-1 et comment le VIH-1 peut surmonter cette inhibition. Mon projet de thèse est de comprendre l'activité anti-VIH de IFITM1 et la stratégie que le VIH-1 adopte pour surmonter cette restriction. Nous avons atteint trois principales conclusions qui sont résumées ci-dessous.

Tout d'abord, nous avons atteint l'évolution du VIH-1 en culture de tissu et sélectionné avec succès des virus résistant à IFITM1 à partir d'une souche de virus VIH-1_{BH10}. Le séquençage de ces virus a révélé deux mutations : Vpu34 dans la protéine virale Vpu et EnvG367E dans la protéine d'enveloppe virale, qui ensemble permettent la réplication du VIH-1 dans des cellules SupT1 qui expriment IFITM1. Ces deux mutations ne peuvent pas surmonter le défaut d'expression de p24 causée par IFITM1, mais améliorent la transmission virale de cellule à cellule. Pour la première fois, notre étude démontre que le VIH-1 est capable de muter pour échapper à la restriction par IFITM1 grâce à l'augmentation de la transmission de cellule à cellule.

Deuxièmement, nous avons observé que la souche de VIH-1_{BH10}, mais pas VIH-1_{NL4-3}, est considérablement inhibée par IFITM1 dans les cellules SupT1. L'analyse par mutagenèse a révélé que la séquence de la protéine d'enveloppe de VIH-1_{NL4-3} détermine la résistance à IFITM1 en partie par sa capacité à permettre la transmission de cellule à cellule. Cette recherche met également en évidence le rôle important de l'enveloppe dans

l’échappement à IFITM1.

Troisièmement, nous avons découvert que la séquence C-terminale de IFITM1 empêche cette protéine d’inhiber l’entrée du VIH. La délétion de la partie C-terminale de IFITM1 permet à cette protéine d’inhiber l’entrée de VIH-1_{NL4-3}. Des études de mutagenèse ont montré que les acides aminés essentiels à cette activité sont 117-QII-119. De plus, le VIH-1_{NL4-3} peut échapper à l'inhibition par IFITM1 dont la partie C-terminale a été tronquée par le biais de mutations dans les protéines Vpu et d'enveloppe.

En conclusion, nous avons découvert un rôle vital de l’enveloppe du VIH-1 dans la sensibilité du virus à l'inhibition par IFITM1. Nous rapportons également, pour la première fois, un rôle de la partie C-terminale de IFITM1 dans la régulation de ces fonctions anti-VIH-1.

Acknowledgements

It is my great pleasure to have this opportunity to express my gratitude here to all the people supporting me in my 5 years doctoral study.

First and foremost, I would like to thank my supervisor Dr. Chen Liang, who gave me the chance to complete my Ph.D. study in his laboratory and taught me the right attitude to be a good scientist. As the director of the lab, Dr. Liang always gave us positive advices for the research even when we got unexpected results. His foresight in the project was based on his abundant readings and unique understandings, from which I learnt a lot. I would like to express my special thanks to my co-supervisor, Dr. Shan-Lu Liu. Dr. Liu was my first supervisor at McGill University. His hardworking attitude and persistence in research are always in my mind and influence all aspects of my life.

I would like to thank the members of my advisory committee Dr. Andrew J. Mouland who gave me a lot of advices from different angles and Dr. Rongtuan Lin who followed two projects of mine during the whole process of my Ph.D. study and gave me constructive advices and helpful assistance. I also want to thank the members of my comprehensive committee, Dr. Samantha Gruenheid, Dr. Anne Gatignol, and Dr. Jacques Archambault, for their advices on my project.

I would like to show my appreciation to my colleague, Dr. Marceline Côté. She taught me how to design experiments properly, how to perform experiments efficiently, and how to analyze data systematically. Her excellence in science and positive attitude are always the goals I want to achieve.

I would like to give a lot of thanks to the former and current members in Dr. Liang's lab. Special thanks to our research assistance and my collaborator, Qinghua Pan, for her

assistance in my experiments, especially during my start in the lab, and the useful advices in my life; Thanks to Rui Jia, Zhibin Liang, Zhenlong Liu and Jin Qian for their valuable help in the lab and the fun we had outside the lab. I also thank Liwei Rong, Vicky Cheng, Jianyong Zhang and Xiaoyu Li for their help when I was coming to the lab. Special thanks to Rene-Pierre Lorgeoux, Yann Le Duff and Thibault Mesplède (Dr. Mark Wainberg's lab) for the enjoyable conversations we had and their generous help in my projects.

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Preface

This thesis was written in accordance with the guidelines of "Preparation of a thesis" from "Graduate and Postdoctoral Studies" of McGill University. The candidate chose to present the study in a "Manuscript-based (Article-Based) thesis" format.

A general introduction about HIV-1 and IFITM proteins is presented in Chapter 1; Chapter 2 is based on a published manuscript; Chapter 3 and Chapter 4 are from 2 manuscripts in preparation for publication. The candidate is the first author of all manuscripts and the contributions of all authors are listed in "Contribution of Authors". Each chapter of Chapter 2, 3 and 4 is composed with its own introduction, materials and methods, results and discussion. There is preface in front of each chapter to link chapters together and introduce new chapter. The references for the whole thesis were listed in the end. The manuscripts adopted for the thesis are listed below:

- **Ding S**, Pan Q, Liu SL, Liang C, *HIV-1 mutates to evade IFITM1 restriction.* Virology, 2014. 454-455: p. 11-24. Adapted for Chapter 2;
- **Ding S**, Jia R, Pan Q, Liu SL, Liang C, *The envelope protein of HIV-1$_{NL4-3}$ confers resistance to IFITM1*, Manuscript in preparation. Adapted for Chapter 3;
- **Ding S**, Jia R, Pan Q, Liu SL, Liang C, *The C-terminal sequence of IFITM1 regulates its anti-HIV-1 activity,* Manuscript in preparation. Adapted for Chapter 4;

In addition, the candidate also contributed to other projects which are not included in this thesis but are published in the past several years, they are about the discovery of anti-HIV-1 activity of MxB, the importance of IFITM3 N-terminus to the antiviral activity, and the antiviral mechanism of IFITM proteins, as showing in the list below:

- Liu Z, Pan Q, **Ding S**, Qian J, Xu F, Zhou J, Cen S, Guo F, Liang C, The interferon-inducible MxB protein inhibits HIV-1 infection, *Cell Host Microbe.* 2013 Oct 16, 14(4):398-410.
- Li K, Markosyan RM, Zheng YM, Golfetto O, Bungart B, Li M, **Ding S**, He Y, Liang C, Lee JC, Gratton E, Cohen FS, Liu SL, IFITM proteins restrict viral membrane hemifusion, *PLoS Pathog.* 2013 Jan, 9(1):e1003124.
- Jia R, Pan Q, **Ding S**, Rong L, Liu SL, Geng Y, Qiao W, Liang C, The N-terminal region of IFITM3 modulates its antiviral activity by regulating IFITM3 cellular localization. *J Virol.,* 2012 Dec, 86(24):13697-707.
- Ndongwe TP, Adedeji AO, Michailidis E, Ong YT, Hachiya A, Marchand B, Ryan EM, Rai DK, Kirby KA, Whatley AS, Burke DH, Johnson M, **Ding S**, Zheng YM, Liu SL, Kodama E, Delviks-Frankenberry KA, Pathak VK, Mitsuya H, Parniak MA, Singh K, Sarafianos SG. Biochemical, inhibition and inhibitor resistance studies of xenotropic murine leukemia virus-related virus reverse transcriptase. *Nucleic Acids Res.* 2012 Jan, 40(1):345-59.

Contribution of Authors

Chapter 2:

HIV-1 mutates to evade IFITM1 restriction. Ding S, Pan Q, Liu SL, Liang C,

Chapter 2 is adapted from a manuscript published in the *Virology* journal and all data presented are derived from experiments performed by the candidate. Qinghua Pan performed the virus evolution experiment and extracted cellular DNA. Dr. Shan-Lu Liu was the co-supervisor of the candidate and helped with the manuscript preparation. Dr. Chen Liang and the candidate designed the experiments and wrote the manuscript.

Chapter 3:

The envelope protein of HIV-1_{NL4-3} confers resistance to IFITM1. Ding S, Jia R, Pan Q, Liu SL, Liang C,

Chapter 3 is adapted from a manuscript prepared for publication. All data presented are derived from experiments performed by the candidate, except the construction and long-term infection of HIV-1_{BH10}/HIV-1_{NL4-3} chimeras, which were performed by Rui Jia and Qinghua Pan. Dr. Shan-Lu Liu was the co-supervisor of the candidate and helped with the manuscript preparation. Dr. Chen Liang and the candidate designed the experiments and wrote the manuscript.

Chapter 4:

The C-terminal sequence of IFITM1 regulates its anti-HIV-1 activity. Ding S, Jia R, Pan Q, Liu SL, Liang C,

Chapter 4 is adapted from a manuscript prepared for publication. All data presented are derived from experiments performed by the candidate, except the construction of IFITM1

C terminus deleted mutants and virus entry assay of HIV-1_{NL4-3} in IFITM1 C terminus deleted mutants expressed SupT1 cells, which were performed by Rui Jia and Qinghua Pan. Dr. Shan-Lu Liu was the co-supervisor of the candidate and helped with the manuscript preparation. Dr. Chen Liang and the candidate designed the experiments and wrote the manuscript.

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List of abbreviations

AIDS Acquired immunodeficiency syndrome

APOBEC Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ART Antiretroviral therapy

BST-2 Bone marrow stromal cell antigen 2

CCR5 C-C chemokine receptor type 5 (β-chemokine receptor)

CDA Cytidine deaminase

CDC Centers for Disease Control

CHR N-terminal heptad repeat

CoV Coronavirus

CPZ Chlorpromazine

CRF Circulating recombinant forms

CTD C-terminal domain

CXCR4 C-X-C chemokine receptor type 4 (α-chemokine receptor)

CypA Cyclophilin A

DCs Dendritic cells

DIS Dimerization initiation site

dsDNA Double-stranded DNA

ECTO Extracellular ectodomain

ELISA Enzyme linked immunosorbent assay

ER Endoplasmic reticulum

ESCRT Endosomal sorting complex required for transport

FDA U.S. Food and Drug Administration

FeLV Feline leukemia virus

FI Fusion inhibitorg

FP Fusion peptide

Gp Glycoprotein

GPI Glycosylphosphatidylinositol

GTPase Guanosine triphosphate hydrolase

HAART Highly active antiretroviral therapy

HBV Hepatitis B virus

HFV Human foamy virus

HIF HIV-1 influencing factor

HIV-1 Human immunodeficiency virus-type 1

hnRNP Heterogenous nuclear ribonucleoprotein

HTLV-1 Human T-cell leukemia virus-type 1

IFITM Interferon-induced transmembrane protein

IFN Interferon

IN Integrase

INI Integrase inhibitor

IRES Internal ribosome entry site

IRF Interferon regulatory factor

ISE Intronic splicing enhancer

ISG Interferon stimulated gene

ISGF3 Interferon stimulated gene factor 3

ISRE Interferon stimulated response element

JSRV Jaagsiekte sheep retrovirus

Kb Kilobase

KD Kilodalton

KS Kaposi's Sarcoma

LTR Long terminal repeat

MDA-5 Melanoma differentiation associated protein-5

MLV Murine leukemia virus

MoMLV Moloney murine leukemia virus 17

mRNA messenger RNA

MSD Membrane-spanning domain

MxB Myxovirus-resistance B

NC Nucleocapsid

NER Nucleotide excision repair

NES Nuclear export sequence

NF-κB Nuclear factor-kappaB

NK Natural killer cells

NHR N-terminal heptad repeat

NLS Nuclear localization signal

nm nanometer

NMR Nuclear magnetic resonance

NNRTI Non-nucleoside reverse transcriptase inhibitor

NPC Nuclear pore complex

NRTI Nucleoside reverse transcriptase inhibitor

nt nucleotide

OA Oleic acid

OSBP Oxysterol-binding protein

PAMP Pathogen associated molecular pattern

PBS Primer binding site

pDCs Plasmacytoid dendritic cells

PI Protease inhibitor

PIC Preintegration complex

PI(4,5)P2 Plasma membrane-specific lipid phosphatidyl inositol (4,5) bisphosphate

PPT Polypurine tract

PR Protease

PRR Pattern-recognition receptor

PTEF-b Positive transcription elongation factor b

RGG Arginine- and Glycine-rich

RIG-I Retinoic acid-inducible gene I

RLRs RIG-I-like receptors

RNP Ribonucleoprotein

RRE Rev response element

rRNA ribosomal RNA

RSV Rous sarcoma virus

RT Reverse transcriptase

RTC Reverse transcription complex

SA Splice acceptor

SAMHD1 Sterile alpha motif and histidine/aspartic acid domain-containing protein 1

shRNA short hairpin RNA

siRNA small interfering RNA

SIV Simian immunodeficiency virus

SIV_{cpz} SIV from chimpanzee

SIV_{gsn} SIV from greater spot-nosed monkey

SIV_{sm} SIV from sooty mangabeys

snRNA small nuclear RNA

TAR Transactivation response region

TB Tuberculosis

TBK1 TANK-binding kinase 1

TGN Trans-Golgi network

TLPs Toll-like receptors

TNF Tumor necrosis factor alpha

TRIM Tripartite motif-containing protein

tRNA transfer RNA

U2AF U2 associated factor

UTR Untranslated region

VAPA Vesicle-membrane-protein-associated protein A

VLP Virus-like particle

VS Virological synapse

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Chapter 1 – Introduction

1.1 HIV epidemiology

1.1.1 HIV-1 isolation history

In 1981, Centers for Disease Control of the United States (CDC) reported an unusual immunodeficiency syndrome related to Kaposi's sarcoma (KS) and Pneumocystis pneumonia in homosexual men [1]. In the following several months, this syndrome was also reported in several other groups such as blood transfusion recipients, recent Haitian immigrants and the sexual partners or children of the risk groups [2-4]. In the late of 1982, the syndrome was named AIDS by CDC, standing for Acquired Immunodeficiency Syndrome. The cause of this disease was identified the following year by two groups independently. Dr. Montagnier and colleagues at the Pasteur Institute, France, published a paper in Science, reporting their finding of the cause of AIDS, which they named as lymphadenopathy virus (LAV) [5]; Dr. Gallo's group, at National Institutes of Health, the U.S., also published their finding in Science at the same time, named the etiological agent of AIDS as human T-cell leukemia virus Ⅲ (HTLV-Ⅲ) [6]. Three years late, in 1986, this virus was officially named Human Immunodeficiency Virus (HIV), which was renamed to HIV-1 after the discovery of HIV-2 [7, 8].

1.1.2 HIV-1 epidemic

More than 30 years after the discovery of HIV-1 in human, HIV/AIDS still poses a major threat to global public health, with around 35 million people living with HIV (Fig. 1.1). In 2012, around 2.3 million people are newly infected and around 1.6 million people die due to HIV/AIDS; among 6300 daily infections, around 95% are in low and middle-income countries. Sub-Saharan Africa is still the most severely affected area, which accounts for 71% of the people living with HIV worldwide (Figure 1.1) [9]. Unprotected

sexual intercourse, transfusion of contaminated blood, sharing of contaminated needles and mother to children are the main routes of HIV-1 transmission in human. Great efforts have been taken to prevent the transmission of HIV and the new global vision is “Zero new HIV infections, zero AIDS-related deaths and zero discrimination in a world where people living with HIV are able to live long, healthy lives” [10].

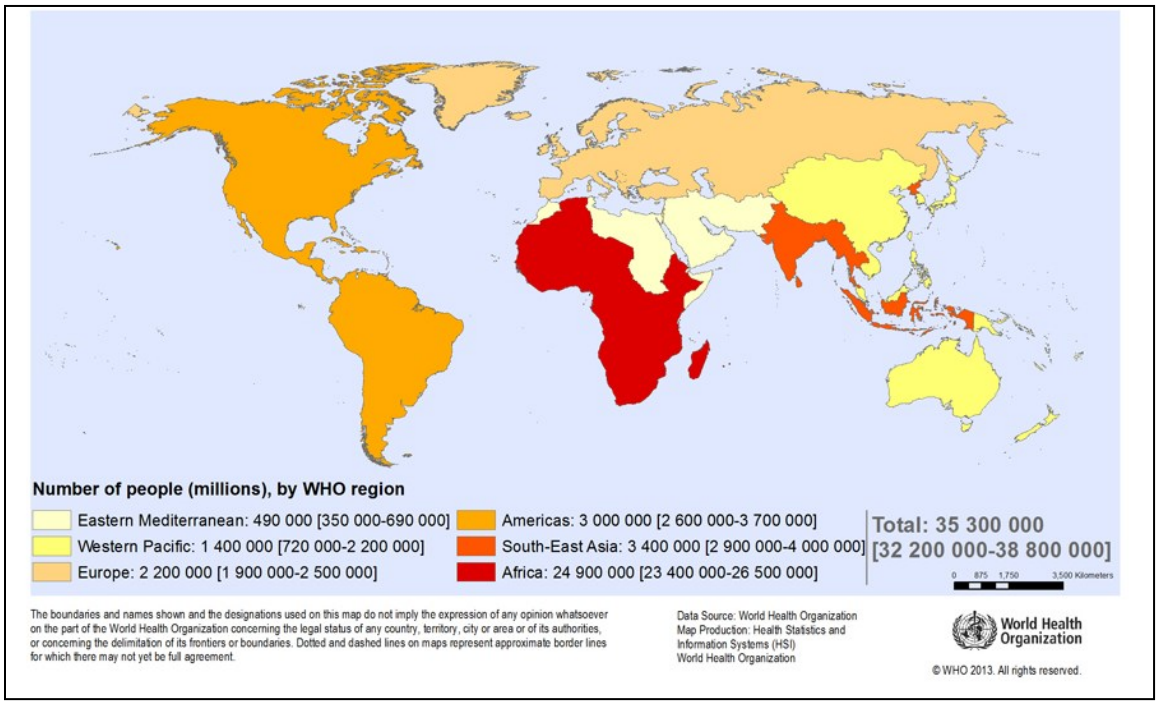


Figure 1.1: Adults and children estimated to be living with HIV, 2012, by WHO region [9].

The typical course of HIV-1 infection in humans is divided into three stages including acute HIV syndrome, clinical latency and clinically apparent disease (AIDS). As shown in Figure 1.2, after primary infection, around 50% to 70% patients develop high levels of viremia accompanied with flu-like syndromes and CD4 T cells declining from 1 week to 3 months. At the end of this period, most of the virus is cleaned in plasma by HIV-specific immunity and peripheral-blood CD4 T cells is recovered, although HIV-1 is still

replicating in lymph nodes. During the following 2 to 10 years, HIV-1 multiplicates slowly with low or non-detectable plasma viral particles. However, the peripheral CD4 T cells continue to decline concomitant with the deterioration of the immune system. As the number of CD4 T cells drops below to a certain level, the plasma viral load increases dramatically, the patients shows acquired immunodeficiency syndromes (AIDS) including opportunistic infection diseases and cancer [11].

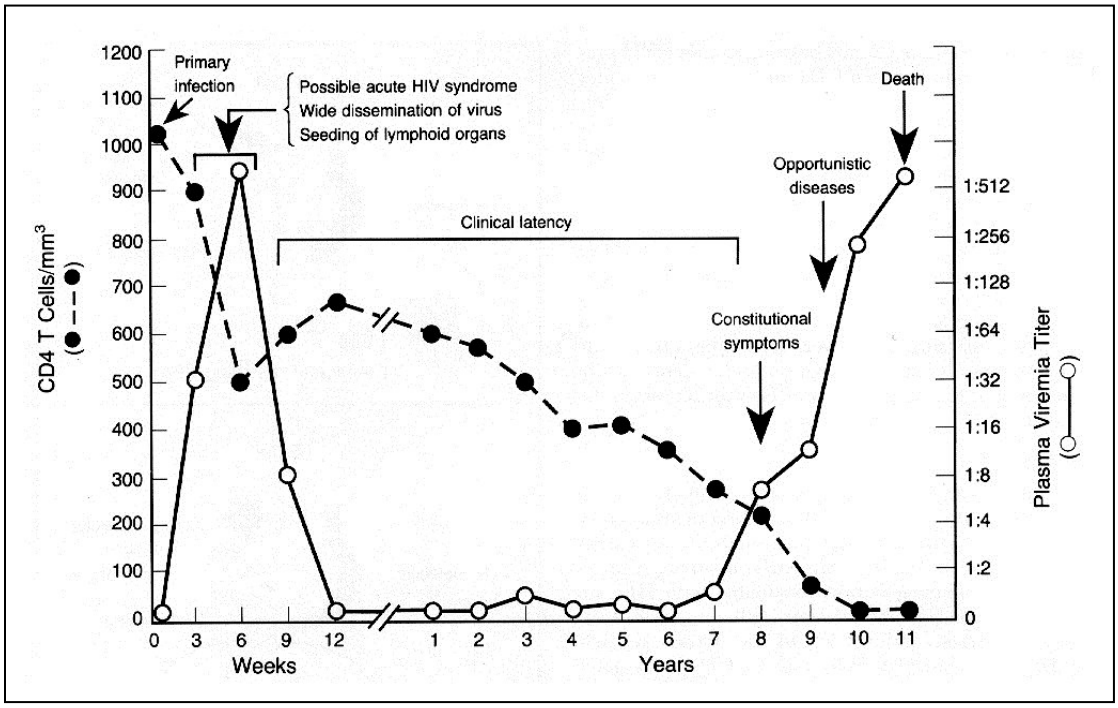


Figure 1.2: Typical course of HIV infection.
After primary infection of HIV-1, most patients experience a sharp increase of plasma viral load and a drop of peripheral-blood CD4 T cells in the first 3 months' acute infection period; followed by 2 to 10 years period called clinical latency when very low viral load in plasma is detected and continuously declining CD4 T cells; at last, patients develop AIDS illness when CD4 T cell counts drop to <200 cells/μl [11].

1.1.3 HIV origins

Both HIV-1 and HIV-2 are zoonotic primate lentiviruses originated from simian immunodeficiency viruses (SIV) found in non-human primates [12] [13]. HIV-2 was derived from SIV_{sm} (sooty mangabey). HIV-2 and SIV_{sm} are common in genomic and

have closely related phylogenetic. Sooty mangabeys have close contact with human because they are usually the targets of hunting and pet in Africa [13] [14] [15]. HIV-1 has a unique Vpu-coding sequence, which is not common among most of the SIVs except SIV_{cpz} (chimpanzee) and SIV_{gsn} (greater spot-nosed monkey) [16]. SIV_{cpz}, more specifically, the SIV_{cpz} from *Pan troglodytes troglodytes* (*P. t. troglodytes*) subspecies became the origin of HIV-1 because of the close relationship between chimpanzees and humans in West-central Africa [12].

1.2 HIV-1 virology

1.2.1 HIV classification

HIV belongs to the retrovirus family, the *Retroviridae*. The hallmark of retrovirus infection is reverse transcription from RNA to DNA and integration of viral DNA into host cell chromosomal DNA during the life cycle. Based on the characteristics of virus morphology and biochemical properties, retrovirus genera are classified into two major classes: *simple* retroviruses and *complex* retroviruses. The genome of *simple* retroviruses mainly encodes Gag, Pro, Pol, and Env proteins, while that of *complex* retroviruses encodes regulatory and accessory proteins. As shown in Table 1, *simple* retroviruses include alpharetroviruses, betaretroviruses, and gammaretroviruses; *complex* retroviruses include deltaretroviruses, epsilonretroviruses, lentiviruses, and spumaviruses.

Retrovirus genera	Genus	Examples
Simple retroviruses	Alpharetrovirus	Rous sarcoma virus
	Betaretrovirus	Jaagsiekte sheep retrovirus (JSRV)
	Gammaretrovirus	Murine leukemia virus (MuLV)
Complex retroviruses	Deltaretrovirus	HumanT-lymphotropic virus (HTLV)
	Epsilonretrovirus	Walleye dermal sarcoma virus
	Lentivirus	Human immunodeficiency virus (HIV) type 1 and type 2 (HIV-1, HIV-2)
	Spumavirus	Human foamy virus

Table 1.1: Retrovirus genera

HIV, SIV and other immunodeficiency viruses belong to the lentivirus family. HIV has two types, HIV-1 and HIV-2. They differ in their origins and also exhibit different clinical outcomes. HIV-2 has much lower mortality rates than HIV-1 in humans [17]. HIV-1 is further divided into three groups: M (main), O (outlier) and N (non-M, non-O). More than 95% of the HIV-1 virus isolates are M group. M group has 9 subgroups (clade A, B, C, D, E, F, G, H, J and K) and 15 circulating recombinant forms (CRF) that are the recombinants between different M group subgroups [18] [19]. The O group and N group isolates are only found in Africa [20]. All subgroups of M group can be found in Africa, but their distributions worldwide are different. Clade C viruses now are the majority subtype and cause half of the infection worldwide. Clade B viruses cause most of infection in Europe and North America. Clade A viruses are responsible for infection in

eastern and central African countries [21] [22].

1.2.2 Structure of HIV

1.2.2.1 HIV genome

As a member of the complex retrovirus, HIV encodes not only Env, Gag, Pol, but also several small regulatory and accessory proteins including Tat, Rev, Vpu (Vpx in HIV-2), Vpr, Vif, and Nef (Figure 1.3). At the 5' and 3' ends of HIV genome, there are two long terminal repeats (LTR) composed by U3, R and U5. LTRs are involved in viral reverse transcription and integration of viral DNA. The Gag gene encodes Gag precursor protein which is processed into mature structure proteins Matrix (MA), Capsid (CA), Nucleocapsid (NC) and P6. Gag/Pol is the polymerase precursor for protease (PR), reverse transcriptase (RT) and integrase (IN). Env is a glycoprotein precursor – gp160, which is processed into surface unit (SU, gp120) and transmembrane domain (TM, gp41). Two single strands of viral RNA form a RNA dimer through noncovalent link initiating from the 5' end of each RNA molecule (reviewed in [23]).

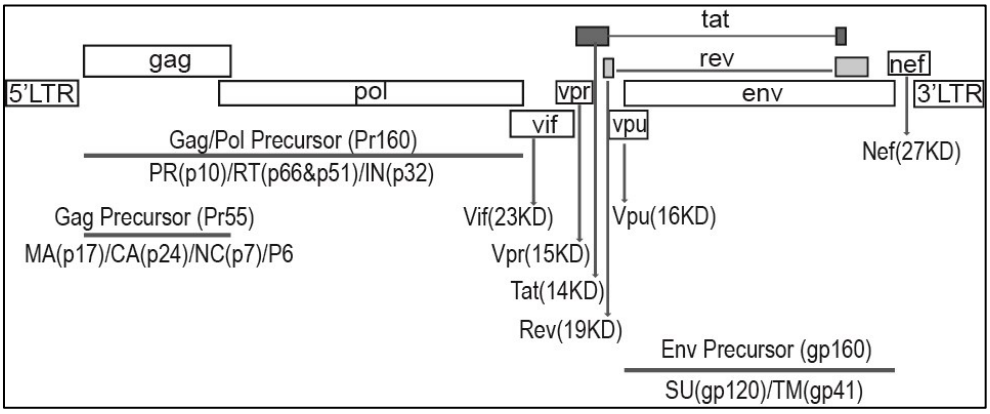


Figure 1.3: HIV-1 genome.

1.2.2.2 HIV morphologic structure

Like most of the other retroviruses, the diameter of mature HIV viral particle is around 100 nm (Figure 1.4). The mature HIV particle is featured by a cone-shaped core surrounded by a lipid membrane derived from cells. Tens of trimetric envelopes are located on viral membrane, with gp120 (SU) on the surface and gp41 (TM) anchored in the membrane. Between the membrane and viral core, matrix (MA) forms a thin layer. Capsid (CA) forms a cone-shaped core encaging the viral RNA dimer. The nucleocapsid (NC) binds to viral RNA and helps the encapsidation of RNA during viral assembly. Viral protease (PR), integrase (IN), reverse transcriptase (RT) and viral protein R (vpr) are found inside the core and play essential roles in the new infection.

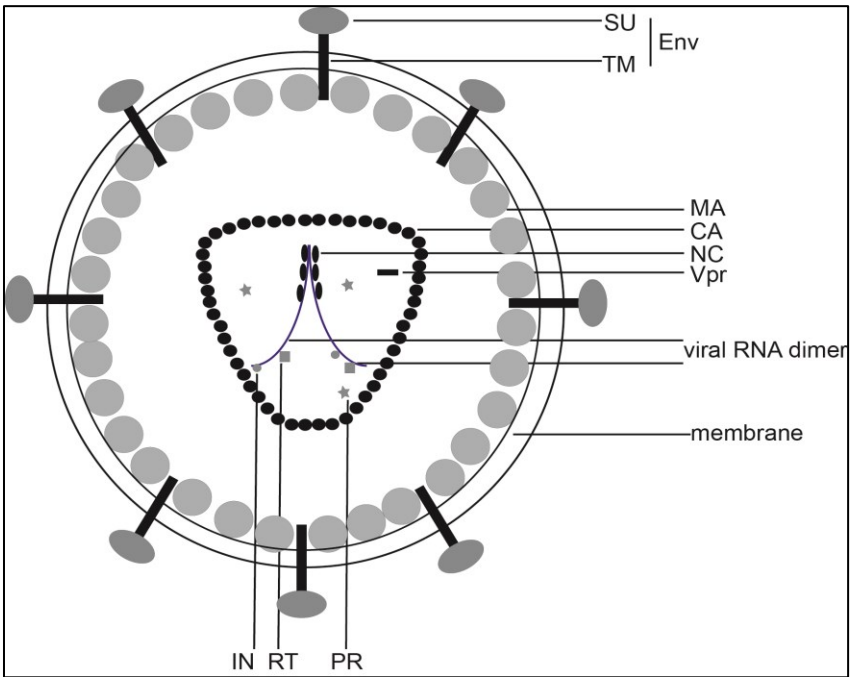


Figure 1.4: Schematic representation of mature HIV-1 particle.

1.2.3 HIV-1 replication

The replication cycle of HIV-1 is usually divided into two phases [23]. The early phase starts with virus attachment to host cell membrane, followed by membrane fusion between virus and cell, viral RNA reverse transcription and viral core uncoating, viral DNA nuclear import and integration. The late phase includes viral gene transcription, mRNA translation, viral RNA encapsidation, viron assembly, budding and maturation (Figure 1.5). The viral DNA integration into host chromosome is the fundamental cause of HIV latency [24].

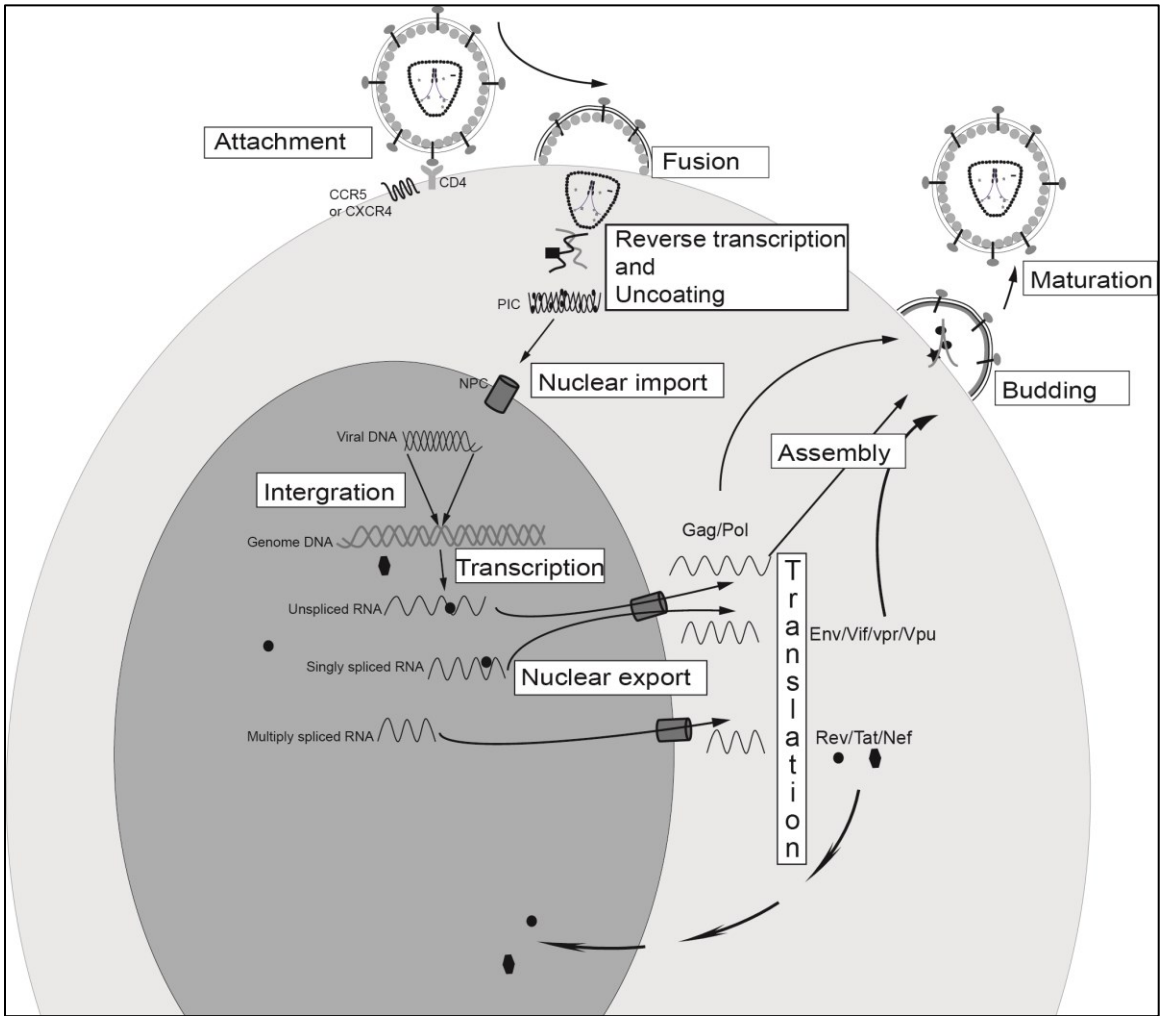


Figure 1.5: HIV-1 life cycles.
All the events are labeled in the texts. All the components during the life cycle are labeled with black words only.

1.2.3.1 Entry

HIV receptor and co-receptors

To start a new infection, HIV needs to recognize their target cells by interacting with specific cell surface molecules, which are called receptors. The types of receptors are different from virus to virus and some viruses utilize more than one receptor, as shown in Table 2. Five years after the discovery of HIV-1, glycoprotein CD4 was identified as the receptor required by the entry of HIV/SIV, the first retrovirus receptor being discovered [25] [26] [27]. It was then observed that HIV-1 isolates showed different infection tropisms with some viruses only infecting macrophage (M-tropic) while the others only replicating in T cells (T-tropic) [28] [29]. This suggested the existence of new receptors for HIV-1. After nearly ten years, in 1996, several groups independently reported that two seven-transmembrane domain proteins - α-chemokine receptor CXCR4 (Fusin) and β-chemokine receptor CCR5, which belonged to G protein-coupled receptor superfamily, were essential for HIV-1 infection and act as co-receptors of HIV/SIV ([30] [31] [32] and reviewed in [33]). HIV using CCR5 as its co-receptor are usually M-tropic and are designated as R5 viruses. They dominate through the HIV-l infection in most patients [34]. HIV viruses only utilizing CXCR4 are designated as X4 viruses and are usually T-tropic. The X4 HIV often emerges at the later symptomatic stage of infection in about half of infected individual [35]. Dual tropic R5/X4 viruses use either CXCR4 or CCR5 as co-receptors.

Retrovirus receptors		
Virus	**Receptor Name**	**Function**
MuLV Ecotropic	CAT-1	Basic amino acid transporter
MuLV Amphotropic	Ram-1/GLVR2/PiT-2	Phosphate transporter
MuLV 10A1; FeLV-B	GLVR1/PiT-1	Phosphate transporter
M813 Ecotropic	SMIT-1	Na/Inositol transporter
FeLV-C	Flvcr	Organic anion transporter
MMTV	TfR1	Transferrin receptor
ASLV-A	tv-a	LDLR-like
ALV-B,D,E	tv-b, -e	Fas receptorlike
ALV-C	tv-c	Butyrophilinlike
RD114, BaEV, MPMV, HERV-W	RDR, RDR2/ASCT1,2	Neutral amino acid transporter
BLV	Blvr	AP-3 delta subunitlike
JSRV	HYAL2	Hyaluronidase receptor
HTLV-1	GLUT-1	Glucose transporter
HIV-1, HIV-2, SIV	CD4 plus CCR5,CXCR4	T-cell differentiation markers

Table 1.2: Retrovirus receptors (modified from TABLE 55.4 of *Fields virology*. 5th [36])

(**MuLV,** murine leukemia virus; **FeLV,** feline leukemia virus; **MMTV,** mouse mammary tumor virus; **ASLV, ALV,** avian leukosis viruses; **Perv-A,** porcine endogenous virus-A; **RD114,** virus isolated from RD114 cells; **BaEV,** baboon endogenous virus; **M-PMV,** Mason-Pfizer monkey virus; **HERV,** human endogenous retroviruses; **BLV,** bovine leukemia virus; **JSRV,** Jaagsiekte sheep retrovirus; **HTLV,** human T-cell lymphoma or leukemia virus; **HIV,** human immunodeficiency virus; **SIV,** simian immunodeficiency virus.)

Initial binding

The interaction between HIV virus and host cell initiates with the interaction of HIV envelope and host cell CD4 receptor. HIV envelope protein is composed of gp120 (SU) and gp41 (TM). Three copies of non-covalent heterodimers of gp120 and gp41 form a trimeric spike on the virus surface. Around ten envelope protein spikes spread on the surface of a virus particle [37]. Gp120 is mainly responsible for interacting with CD4 on cell surface. Gp120 possesses variable domains (V1 to V5) and conserved domains (C1 to C5) according to the conservation of the amino acids. C3 and C4 are the main regions on gp120 that interact with the extracellular domain of CD4, although the other discontinuous regions of gp120 also contribute to this interaction [38]. Gp120 is divided into two domains: outer domain which initially binds with CD4 and inner domain which interact with gp41 [39]. Upon binding with CD4, a bridging sheet composed with β-sheet forms connects the outer and inner domains [39]. Three flexible layers (layers 1, 2, and 3) in inner domain were also reported to facilitate gp120-CD4 interaction though they do not directly bind with CD4 [40] [41]. The association between gp120 and CD4 then consolidates the interaction between HIV and the cell surface. More important, this interaction triggers conformational changes of gp120, prepares gp120 for binding to co-receptors – CXCR4 or CCR5.

Co-receptor binding and membrane fusion

Conformational change of gp120 exposes the binding sites for co-receptors. Rearrangement of V1, V2 and V3, especially the V3 region on gp120, allows interaction with co-receptors [42]. Gp41 is subdivided into three domains: extracellular ectodomain (ECTO), a membrane-spanning domain (MSD) and a long cytoplasmic tail. ECTO has

the fusion peptide (FP), the N-terminal heptad repeat (NHR, or HR1), and the CHR (or HR2), which are critical for membrane fusion (Figure 1.6). FP is hydrophobic and normally is buried inside the trimeric envelope protein. Following formation of CD4-gp120-CXCR4 (CCR5) complex, gp120 changes its conformation and FP is exposed. Insertion of FP into host cell membrane links viral and host cell membranes together. In the meantime, three NHRs and CHRs of gp41 form a six-helix bundle (6HB) [43]. Formation of the 6HB further brings the viral and cellular membranes together, which leads to formation of a fusion pore. HIV viral core is then released into host cell through the fusion pore (Figure 1.6).

It is generally believed that unlike influenza virus and vesicular stomatitis virus which enter cell by receptor-dependent endocytosis, HIV can fuse with membrane on the cell surface, although in some cell types (such as macrophages) HIV may undergo endocytosis before entering cells [44] [45]. A recent research using time-resolved imaging of single viruses revealed that HIV virus may complete fusion in endosomes and is dynamin-dependent [46].

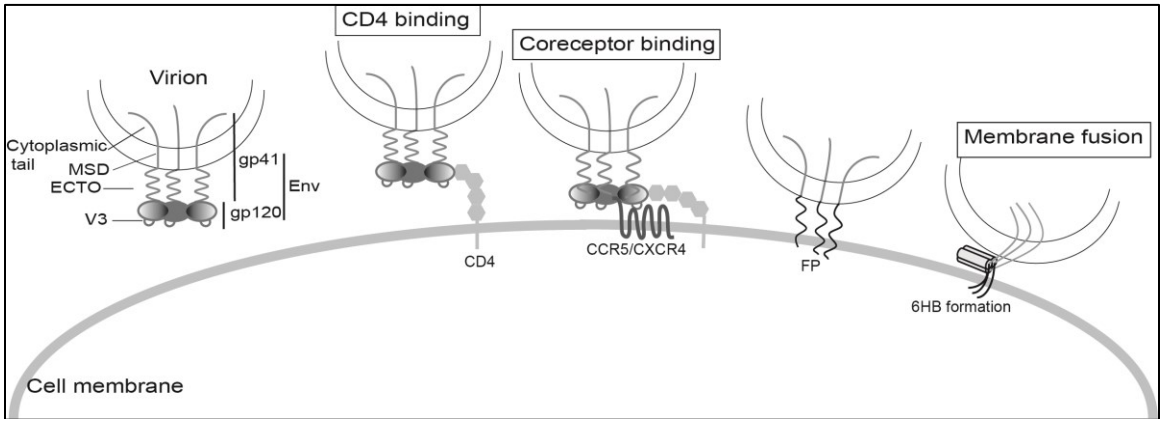


Figure 1.6: HIV entry.
HIV entry is divided into 3 steps including CD4 binding, co-receptor binding and membrane fusion.

1.2.3.2 Reverse transcription

After entry, the viral core, containing two copies of viral ssRNA, RT, IN, Vpr, Vif, Nef and host cellular factors (e.g. tRNALys3), develops to be reverse transcription complex (RTC) where viral RNA is reverse transcripted into double stranded viral DNA. The reverse transcriptase (RT) is a heterodimer of two subunits – p51 and p66. P66 is a 560 amino acids enzyme with polymerase and RNase H activities. P55 is derived from p66 by the removal of the C-terminal 15-kd fragment, it mainly serves as a structural role [47]. Reverse transcription is divided into 3 steps as shown in Figure 1.7.

1. RT utilizes host $tRNA^{Lys3}$ as primer to initiate the synthesis of minus strand ssDNA (minus strong – stop DNA). $tRNA^{Lys3}$ binds to primer binding sequence (PBS) region of viral DNA. RT initiates the synthesis of DNA from PBS until the 5' end of RNA is reached. The RNA component of the RNA/DNA hybrid is digested by the RNase H of RT (Figure 1.7 – steps A and B);

2. There are two repeating sequences - R at both 5' and 3' ends of HIV genome. They bridge the newly generated minus strong-stop DNA to the 3' end of viral RNA. After being synthesized, minus strand strong-stop DNA is transferred to the 3' end through the binding of the 5'R of viral DNA to the 3' R of viral RNA. RT then continues viral DNA synthesis. The RNase H of RT degrades the template RNA, only retains two purine-rich sequences called polypurine tract (PPT) (Figure 1.7– steps C and D). One PPT close to the 3' end serves as primer to initiate the plus strand DNA synthesis, while the other one in the middle of genome (central PPT) may contribute to viral DNA import into the nucleus [48];

3. Plus-stand DNA synthesis leads to copying the first 18 nucleotides of the $tRNA^{Lys3}$ primer that is complementary to viral PBS. With the help of this 18-nt $tRNA^{Lys.3}$

sequence, the plus strand DNA is transferred to the 3' of minus-strand DNA, which is called "the second strand transfer". RT then completes both minus and plus strand DNA synthesis to form a double-stranded linear viral DNA (dsDNA) (Figure 1.7 – steps E, F and G).

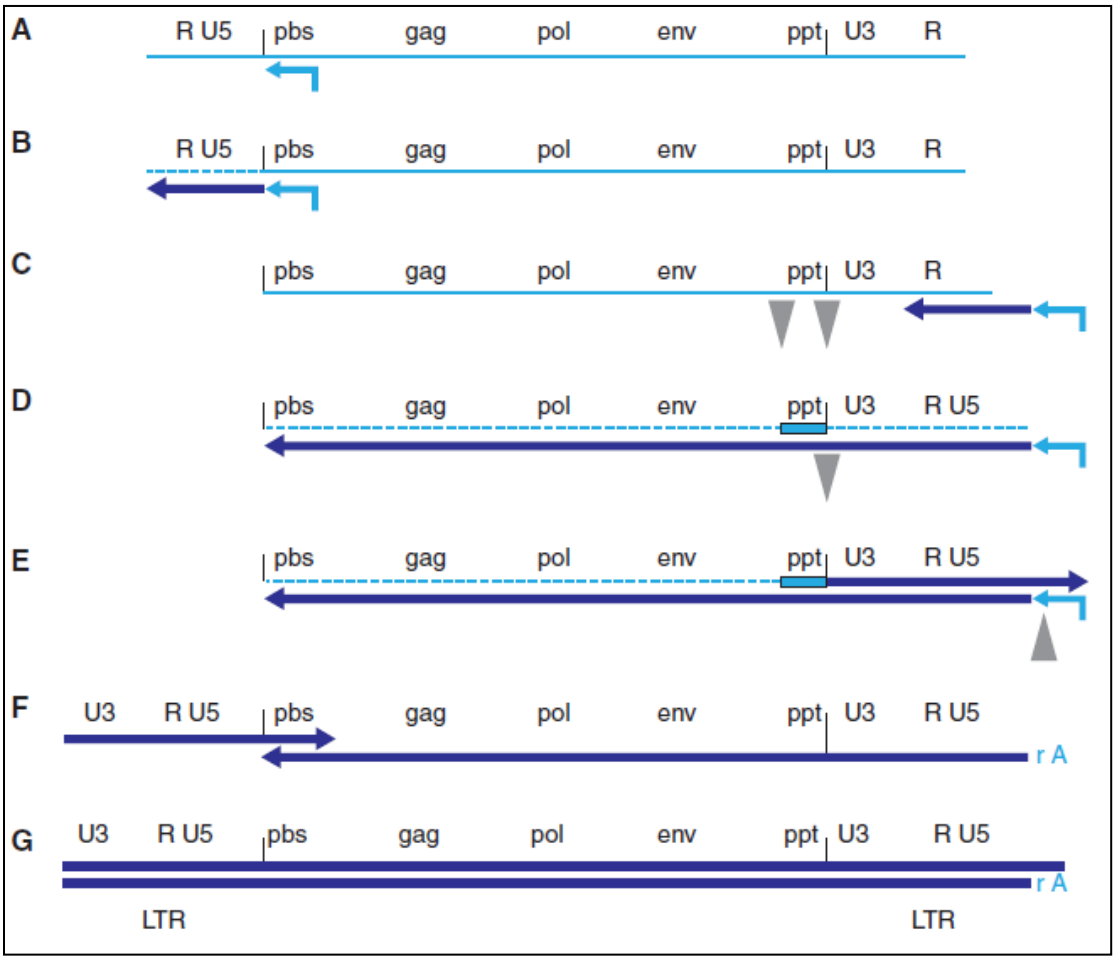


Figure 1.7: Reverse transcription of HIV.
The whole process of reverse transcription is divided into 3 steps illustrated in A to G. A and B are the synthesis of minus strong-stop DNA, C and D are the synthesis of minus-strand DNA, E to G are the synthesis of plus-strand DNA (Adapted from [49]).

1.2.3.3 Nuclear import and integration

During or after the formation of double-stranded viral DNA, RTC follows the microtubule network to approach nucleus [50]. RTC becomes the pre-integration

complex (PIC) containing viral double-strand DNA, viral proteins (NC, MA, Vpr, IN) and cellular proteins (transportin 3, Nup358) ([51] [52] and reviewed in [53]). Unlike the other retroviruses (e.g. MuLV) that only infect dividing cells, lentiviruses (e.g. HIV) are able to infect non-dividing cells by delivering PIC into nucleus through the nuclear pore. PIC may directly interact with nuclear pore protein when crossing the nuclear pore. The detailed mechanism is actively investigated (reviewed in [54]).

Integration of viral DNA into host chromosomal DNA is the second special feature of retrovirus. This step is accomplished by viral IN. IN is a 32-kd protein having three domains: N-terminal zinc binding domain, a catalytic core domain and a relatively nonconserved C-terminal domain. N-terminal domain and catalytic core domain perform the catalytic functions with C-terminal domain binding to viral genome RNA [55] [56]. Integration occurs through two major steps. First, IN removes 2 nucleotides from the 3' end of viral DNA and generates 3’-recessed sticky ends; Then the viral DNA attacks the target host DNA and undergoes a process called strand transfer. The integration sites of HIV are located in active transcription units, which are favorable to the HIV gene transcription [57] [58]. Both 3’ ends of viral DNA attack the phosphodiester bonds in the target DNA and joint to the 5’ end of target DNA. Five nucleosides are between the two sites that viral DNA integrates referring to HIV. Two nucleosides of 5’ ends of viral DNA are removed. The single strand gap between viral DNA and host DNA is filled by the cellular enzymes (reviewed in [54]). The integrated HIV DNA is also called provirus, which is the template for transcription of viral RNAs.

The following steps of HIV replication are named as the late stage which includes the viral gene transcription, translation, virus assembly and budding and virus maturation. At

some of these steps, the accessory proteins of HIV play essential roles, which will be described in the following sections.

1.2.3.4 Provirus transcription and mRNA nuclear export

The LTR contains U3 (unique 3' end), R (repeated) and U5 (unique 5' end) sequences that are important for transcription [59]. The transcription start site divides U3 and R regions. TATA box and three SP1 (Transcription factor Sp1)-binding sites comprise the core promoter that recruit transcription factor IID (TFIID) and SP1 followed by the binding of TFIIB. The cellular RNA pol II is then recruited to initiate transcription. Upstream of the SP1-binding sites, two nuclear factor-kappaB (NF-κB) binding motifs and two activator protein 1 (AP1) elements together form the enhancer region that profoundly stimulates the transcription efficiency of HIV [60].

The transcription efficiency of HIV promoter is very low without the stimulation by viral accessory protein Tat (transactivating factor) [61]. Tat protein contains a highly basic RNA binding domain that recognizes a sequence at the 5' end of newly synthesized viral mRNA - the transactivation response region (TAR). TAR is a conserved structure of HIV viral mRNA containing a base-paired stem, a non-base-paired bulge, and a G-rich loop. Upon binding to the bulge sequence, Tat recruits two cellular factors, Cyclin T1 (Cyc T1) and CDK9, that form heterodimer and are the member of positive-transcriptional elongation factor b (P-TEFb). CDK9 then hyper-phosphorylates the C-terminal domain (CTD) of RNA Pol II and thus promotes the elongation of viral mRNA synthesis (reviewed in [62]).

After transcription in nucleus, the full-length viral RNA is spliced into more than 40 different species by cellular spliceosome [63]. These viral RNAs are separated into three groups: unspliced viral RNA that encodes gag/pol protein and also serves as viral

genome; the singly spliced viral RNAs encoding env/vpu, vpr, vif and the first exon of nef; the completely spliced mRNAs which are approximately 1.8 kb and encode rev, nef and both exons of tat. The complexity of viral RNA splicing results from the existence of multiple splicing sites (ss) on both 5' (4 sites) and 3' (7 sites) of mRNA as well as the enhancers (exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs)) and silencers (intronic and exonic splicing silencers) [64].

The completely spliced viral RNAs leave the nucleus into cytoplasm with the help of the cellular RNA export machinery that exports the majority of cellular mRNAs. Yet, the unspliced and incompletely spliced viral RNAs adopt a special mechanism for nuclear export. This mechanism involves a viral protein called Rev (regulator of expression of viral proteins) and a viral RNA sequence RRE (Rev responsive element). Rev contains two functional domains. One is responsible for nucleus localization and binding to RRE, the other has the nuclear export sequence (NES). RRE is a stem-loop structure of 351nt located in envelope protein gene and exists in unspliced viral RNA and singly spliced viral RNAs but not in completely spliced viral mRNAs [61]. Eight copies of Rev synergistically bind to RRE. The NES of Rev binds to cellular karyopherin family member Crm1 (exportin 1). Viral mRNA is then exported out of nucleus through NPC with the help of Ran/GTPase-GTP complex that is recruited by Crm1. Once in the cytoplasm, Rev is recycled back to the nucleus through binding to importinβ (nuclear import factor) [65]. Cellular translation machinery is then engaged to produce viral proteins.

1.2.3.5 Virus assembly, budding and maturation

Assembly

An infectious HIV particle needs two copies of the positive sense genomic viral RNA,

cellular $tRNA^{Lys3}$, Env, the Gag polyprotein, and three viral enzymes: PR, RT, and IN. Gag polyprotein is the major structural component that drives the assembly process [66] (Figure 1.8). MA mediates Gag-plasma membrane interaction. The binding between MA and cellular PI(4,5)P2 (plasma membrane-specific lipid phosphatidyl inositol (4,5) bisphosphate) exposes myristoyl group at the amino-terminal of MA. The myristoyl group anchors Gag into membrane inner leaflet [67]. The Env precursor gp160 is translated and is glycosylated in the endoplasmic reticulum (ER). The transport of gp160 to the plasma membrane is independent of Gag, and MA was reported to help the incorporation of gp160 during assembly through binding with the cytoplasmic domain of gp41 [68] [69]. Two copies of viral RNA are non-covalently linked by forming "kissing loop" at their dimer initiation site (DIS) at the 5' untranslated region (UTR). Dimerization is necessary for RNA packaging [70]. NC of Gag contains two zinc-finger motifs, which are called "CCHC" and are responsible for recognizing and binding to viral RNA. NC specifically interacts with the viral RNA packaging signal (Ψ - site) at 5' UTR of viral RNAs. In the meantime, binding to viral RNA also promotes Gag multimerization and assembly [71].

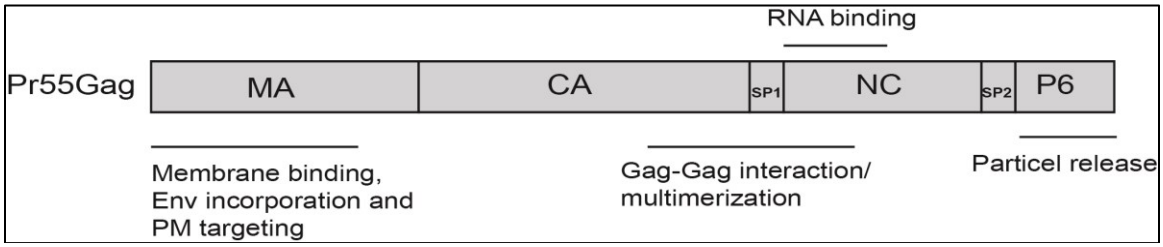


Figure 1.8: Schematic organization of Gag and its functional domains (Adapted from [72])

Budding and maturation

After all components of HIV particle being encapsulated, HIV hijacks the cellular

endosomal sorting complexes required for transport (ESCRT) for the release of virus particle [73] [74]. ESCRT contains more than 30 proteins and catalyze cell membrane fission [75]. P6 of HIV Gag contains two motifs: "PTAP" and "YPXL" that are called late domains. "PTAP" binds UEA domain of TSG101 of ESCRT-1 complex, whereas "YPXL" binds to the V domain of ALIX in ESCRT. These two interactions lead to the recruitment of ESCRT-III proteins (CHMP1, CHMP2, and CHMP4), which then promote the closure of the membrane "neck" between cell and virus particle [69] [75].

During the budding process, virus particle is immature, with amino-terminal of MA binding to viral membrane and carboxy-terminal of p6 facing inside. The viral PR catalyzes the maturation of viral particle. The formation of Gag multimers after budding concentrates PR monomers to form a PR dimer, which activates PR [66]. PR cleaves at five sites in $Pr55^{Gag}$ to generate MA, CA, NC, SP1, SP2 and p6, and at five sites in $Pr160^{Gag\text{-}Pro\text{-}Pol}$ to generate RT and IN [76]. This maturation process is essential to produce infectious HIV particles.

1.3 HIV-1 treatment

1.3.1 Challenges of HIV-1 treatment

1.3.1.1 HIV mutation

High mutation rate allows HIV to escape from the inhibition by human immune responses and drug treatment. "No two HIV-1 isolates were identical" [36] indicates the high degree of variations of HIV genome. High turnover rate of HIV and large population of infected individuals also amplify the mutation rate [77]. High mutation rate is mainly a result of the lack of proofreading of HIV RT during reverse transcription. The mutation rate is approximately 3×10^{-5} mutations/nucleotide/replication cycle in *vivo* [78]. During reverse transcription, two copies of viral RNA templates switch frequently and generate

HIV recombinants. The intra- and inter- molecular jump further increase mutation rate of HIV [23] [79]. As a result, multiple-drug-resistant HIV viruses have been seen in patients and high mutation rate is one of the major obstacles for achieving effective and durable treatments.

1.3.1.2 HIV latency

After integration of HIV DNA into host chromosome, not all of the proviruses proceed to transcription and produce viruses. A portion of these viruses remain transcriptionally silent and go into latency [24]. It is estimated to take approximately 70 years for human body to clear HIV reservoir under the treatment with HAART (Highly Active Antiretroviral Therapy) [80]. Latency makes HIV infection a chronic disease, which needs life-long treatment of HAART.

1.3.2 HIV-1 treatment

1.3.2.1 Antiretroviral (ARV) Drugs

From the discovery of HIV-1 in 1980s until 1996, only a few antiretroviral (ARV) drugs were approved by FDA (U.S. Food and Drug Administration) to treat HIV-1 infected patients. As shown in Table 1.4, these drugs are mainly nucleoside-analog reverse transcriptase inhibitors (NRTI) and one protease inhibitor (PI). Before 1996, HIV patients received monotherapy of the single ARV [81], which was not efficient with quick development of drug resistant virus in patients [82]. In addition to find new drugs, combination of two NRTIs was tried for the treatment of patients [83] [84].

In June 1996 at the 11th World AIDS Conference (Vancouver, Canada), Dr. David Ho presented their breakthrough research on the combination therapy of HIV – HAART, which was quickly practiced worldwide. Typically, HAART adopts the combination of 3 ARVs targeting at least 2 distinct HIV molecular sites in order to inhibit the evolution of

drug resistance virus [85]. Application of HAART dramatically inhibits the replication of virus and AIDS-related mortality decreased steadily since 1996.

Now, more HIV drugs have been discovered and approved for HIV care. By 2013, 37 (3 are not in the market) HIV ARVs have been approved by FDA, which are divided into six classes (Table 1.3):

1. Nucleoside Reverse Transcriptase Inhibitor (NRTI) was the first FDA approved ARV class. It is also the largest class so far. By being incorporated into the newly synthesized viral DNA, NRTIs prevent the incorporation of incoming 5'-nucleoside triphosphates and thus terminate the elongation of viral DNA [86]. HIV virus adopts two mechanisms to escape the inhibition of NRTIs: one is to remove NRTIs from the viral DNA chain by ATP-dependent pyrophosphorolysis [87], and the other involves mutations such as M184V/I and K65R to reduce the binding of NRTIs [88] [89];

2. Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs) target the same step of viral life cycle as NRTIs but with different mechanism. They directly bind to HIV-1 RT and change the conformation of substrate binding site [47]. HIV mutates the sites surrounding the NNRTIs' binding pocket to escape from this inhibition [90] [91];

3. IN inhibitors (INIs) target viral DNA strand transfer reaction. They have two active components, one is a metal-binding pharmacophor which blocks integrase active site and the other is a hydrophobic group that binds to viral DNA. Two INIs (RAL and DTG) have been approved by FDA to treat HIV infection and the drug resistance mutations for one INI often show cross-resistance to the other one [92];

4. Protease Inhibitor (PI) targets viral protease and prevent the cleavage of gag, gag-pol proteins. More than 10 PIs have been approved by FDA for the treatment of HIV

infection and they have similar chemical structures. PIs resistance mutants emerge by accumulating primary and secondary mutations stepwisely [93]. Primary resistance mutations are in the protease gene to restore the enzymatic function and secondary mutations are located in the major protease cleavage sites to make these sites to be better substrates for the mutated protease[94] [95];

5. Only one fusion inhibitor (FI) – enfuvirtide (T20) has been approved by FDA so far. It is an analogue of CHR at gp41. It competes for binding with NHR to prevent formation of 6-helix bundle between gp41 CHR and NHR during virus entry [96]. Mutations are mainly in the sequence of NHR, which reduce the binding between NHR and T20. However, these mutations also affect the replication of the virus because of the less efficient formation of 6-helix bundle.

6. As an entry inhibitor, Maraviroc (MVC) is the only FDA approved drug that targets cellular factor. MVC is a small molecule that specially binds to the hydrophobic transmembrane cavity of CCR5. This action blocks the interaction between viral gp120-V3 loop and CCR5. Drug resistance mutations do not specifically target the interaction between MVC and CCR5, but utilize other mechanism to escape the inhibition such as tropism exchange (CXCR4) and increasing coreceptor affinity (reviewed in [93]).

Class	Brand Name	Generic Name	Manufacturer Name	Approval Date
Multi-class Combination Products	Atripla	efavirenz, emtricitabine and tenofovir disoproxil fumarate	Bristol-Myers Squibb and Gilead Sciences	12-Jul-06
	Complera	emtricitabine, rilpivirine, and tenofovir disoproxil fumarate	Gilead Sciences	10-Aug-11
	Stribild	elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate	Gilead Sciences	27-Aug-12
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Combivir	Lamivudine and zidovudine	GlaxoSmithKline	27-Sep-97
	Emtriva	Emtricitabine, FTC	Gilead Sciences	02-Jul-03
	Epivir	Lamivudine, 3TC	GlaxoSmithKline	17-Nov-95
	Epzicom	Abacavir and Lamivudine	GlaxoSmithKline	02-Aug-04
	Hivid	zalcitabine, dideoxycytidine, ddC **(no longer marketed)**	Hoffmann-La Roche	19-Jun-92
	Retrovir	zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline	19-Mar-87
	Trizivir	abacavir, zidovudine, and lamivudine	GlaxoSmithKline	14-Nov-00
	Truvada	tenofovir disoproxil fumarate and emtricitabine	Gilead Sciences, Inc.	02-Aug-04
	Videx EC	Enteric coated didanosine, ddI EC	Bristol Myers-Squibb	31-Oct-00
	Videx	Didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9-Oct-91
	Viread	Tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01
	Zerit	Stavudine, d4T	Bristol Myers-Squibb	24-Jun-94
	Ziagen	Abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Edurant	Rilpivirine,	Tibotec Therapeutics	20-May-11
	Intelence	Etravirine, ETR	Tibotec Therapeutics	18-Jan-08
	Rescriptor	Delavirdine, DLV	Pfizer	4-Apr-97
	Sustiva	Efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98
	Viramune	Nevirapine, NVP	Boehringer Ingelheim	21-Jun-96
	Viramune XR	Nevirapine, NVP	Boehringer Ingelheim	25-Mar-11
	Agenerase	Amprenavir, APV **(no longer marketed)**	GlaxoSmithKline	15-Apr-99

Protease Inhibitors (PIs)	Aptivus	Tipranavir, TPV	Boehringer Ingelheim	22-Jun-05
	Crixivan	Indinavir, IDV,	Merck	13-Mar-96
	Fortovase	saquinavir **(no longer marketed)**	Hoffmann-La Roche	7-Nov-97
	Invirase	Saquinavir mesylate, SQV	Hoffmann-La Roche	6-Dec-95
	Kaletra	lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15-Sep-00
	Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03
	Norvir	Ritonavir, RTV	Abbott Laboratories	1-Mar-96
	Prezista	Darunavir,	Tibotec, Inc.	23-Jun-06
	Reyataz	Atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03
	Viracept	Nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-Mar-97
Fusion Inhibitors	Fuzeon	Enfuvirtide, T-20	Hoffmann-La Roche & Trimeris	13-Mar-03
Entry Inhibitors: CCR5 co-receptor antagonist	Selzentry	Maraviroc, MVC	Pfizer	06-Aug-07
HIV integrase strand transfer inhibitors	Isentress	Raltegravir, RAL	Merck & Co., Inc.	12--Oct-07
	Tivicay	Dolutegravir, DTG	GlaxoSmithKline	13-Aug-13

Table 1.3: Antiretroviral drugs used in the treatment of HIV infection as of October, 2013 (modified from FDA data)

1.3.2.2 HIV Vaccines

HAART is the main and the most effective treatment of HIV infection, but it also has some disadvantages, including the multiple-drug resistance and adverse effects. Adverse effects are important to determine the adherence of HAART and adherence is the most significant determinant of the regimen [97]. The adverse effects of current HAART contain short term and long term toxicities. Taking HAART might cause gastrointestinal

toxicities [98], rash [99], hypersensitivity reaction [100], etc. in short term, and cardiovascular events [101], renal adverse events [102], lipodystrophy [103], ect. in long term period. Most importantly, the treatment of HAART can not eradicate HIV reservoirs and is not a cure of HIV infection. HIV vaccine may work in multiple pathways: generating antibodies to neutralize the existence virus and prevent the establishment of new infection or generating T-cell response ("T-cell vaccination") through antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cell-mediated virus inhibition (ADCVI) ([104] [105] [106] and reviewed in [107]). The main challenge for developing HIV vaccine is the high viral diversity as a result of high mutation rate [108]. Other challenges include that immune response generated by HIV infection does not clear viruses, has limited effect on disease progression, and does not prevent the establishment of HIV latent state in memory T cells (reviewed in [109]). The current design of HIV vaccines focuses on utilizing synthesized HIV protein(s). Dozens of trials have been carried or are ongoing. Most did not generate protection effect [110]. The 2009 Thai "Phase Ⅲ" trial showed that the RV144 vaccine produced an efficacy of 31.2% against HIV infection after 42 months of follow up using prime-boost strategy [111]. This gives hope for the HIV vaccine development and also for the HIV treatment in the future.

1.3.2.3 Other treatments

Besides the treatments of ART drugs and vaccine, several other ways were proved to have effect in preventing the transmission of HIV. Circumcision is a WHO recommended method which reduces the risk of HIV infection in men who have sex with infected female partner but not in men who have sex with infected men [112] [113]. Pre-exposure prophylaxis (PrEP) is a way used to treat healthy person who is at the risk of having a special disease, which is common used for people traveling to an area where malaria is a

risk. For the prevention of HIV infection, HIV ART Truvada was proved by the FDA for PrEP in 2012 and Truvada based PrEP was also proved to prevent the HIV transmission from mother to child [114]. To prevent HIV transmission from the early stage of HIV positive person, treatment as prevention (Tasp) is applied to HIV positive person by treating them with ARTs regardless of whether their immune system is damaged. Tasp is effective to prevent the HIV transmission, though the controversies exist in the drug side effects and drug resistance [115].

1.4 Innate Immunity related to HIV-1 infection

1.4.1 Pathogen-associated molecular patterns for HIV

To protect from pathogens infection such as bacteria and viruses, host develop immune responses to eliminate the pathogens. The immune responses include innate immune response and adaptive immune response. Activation of innate immune response induces special genes expression such as interferon (IFN) in response to invasion of pathogens.

Upon HIV infection, pattern-recognition receptors (PRRs) in cell host, either on endosomes or in the cytosol, recognize HIV components such as viral RNA, DNA or proteins as pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) are two types of PRRs that sense HIV viral RNA on endosome or plasma membranes before virus entry and viral RNA in the cytosol, respectively [116] [117]. In plasmacytoid dendritic cells (pDCs), TLR7 binds to HIV viral ssRNA in endosome, whereas in dendritic cells (DCs), TLR8 responds by binding to viral RNA [118]. During HIV replication, different RLRs in the cytosol may recognize viral RNA: RIG-I prefers binding to shorter dsRNA and 5’ppp-ssRNA while MDA5 binds to dsRNA [119] [120]. Tetherin (BST-2) and TRIM5 are cellular factors that restrict HIV infection. They have also been reported to induce the innate immune response to HIV

infection [121] [122] [123].

After sensing HIV components, PRRs recruits interferon regulator factor 3 or 7 (IRF3 or IRF7). IRF3 or IRF7 is then phosphorylated by TANK-binding kinase 1 (TBK1) and forms homodimer or heterodimer, which is transported into the cell nucleus. Binding of IRF3 or IRF7 dimer to IFN-stimulated response element (ISRE) on cellular genome triggers the expression of IFN (reviewed in [124]).

1.4.2 IFN and IFN induction

As a key cytokine induced by virus infection, IFN represents the first line of antiviral defense. Based on their receptors, IFN is separated into three classes: types Ⅰ, Ⅱ and Ⅲ IFN. Type Ⅰ IFNs have seven members, which are IFNα, IFNβ, IFNκ, IFNω, IFNδ, IFNo and IFNε, they utilize heterodimer of IFNAR1 and IFNAR2 as the receptor (Figure 1.9). IFNα has 13 subtypes. Together with IFNβ，they are the two main type Ⅰ IFN produced by leukocytes and fibroblasts, respectively [125]. Type ⅡIFN recognize its own receptor composed by IFNGR1 and IFNGR2. Type ⅢIFN has three types of IFNλ and is considered to be the ancestral type I IFNs [126]. The receptor of type ⅢIFN is composed by IFNLR1 and a special IL-10R2 (Figure 1.9).

Upon binding of IFNs to their receptors, tyrosine kinases (JAK1 and TYK2 (tyrosine kinase 2), JAK1 and JAK2 in type Ⅱ IFN), which associate with the dimer of receptors, phosphorylate the receptors and also recruit signal transducers and activators of transcription (STATs – STAT1 and STAT2). Phosphorylated STATs form heterodimer and associate with IFN-regulatory factor 9 (IRF9). Together the complex is called IFN-stimulated gene factor 3 (ISGF3). Type Ⅱ IFN causes phosphorylation of STAT1. The

homodimer of STAT1 is the IFNγ activation factor (GAF). ISFG3 and GAF then transport into the nucleus and bind to IFN-stimulated response elements (ISREs) or IFN-γ-activated site (GAS) promoter elements, respectively. Hundreds of IFN stimulated genes (ISGs) are induced, they perform different functions such as antiviral activity (Figure 1.9) [127].

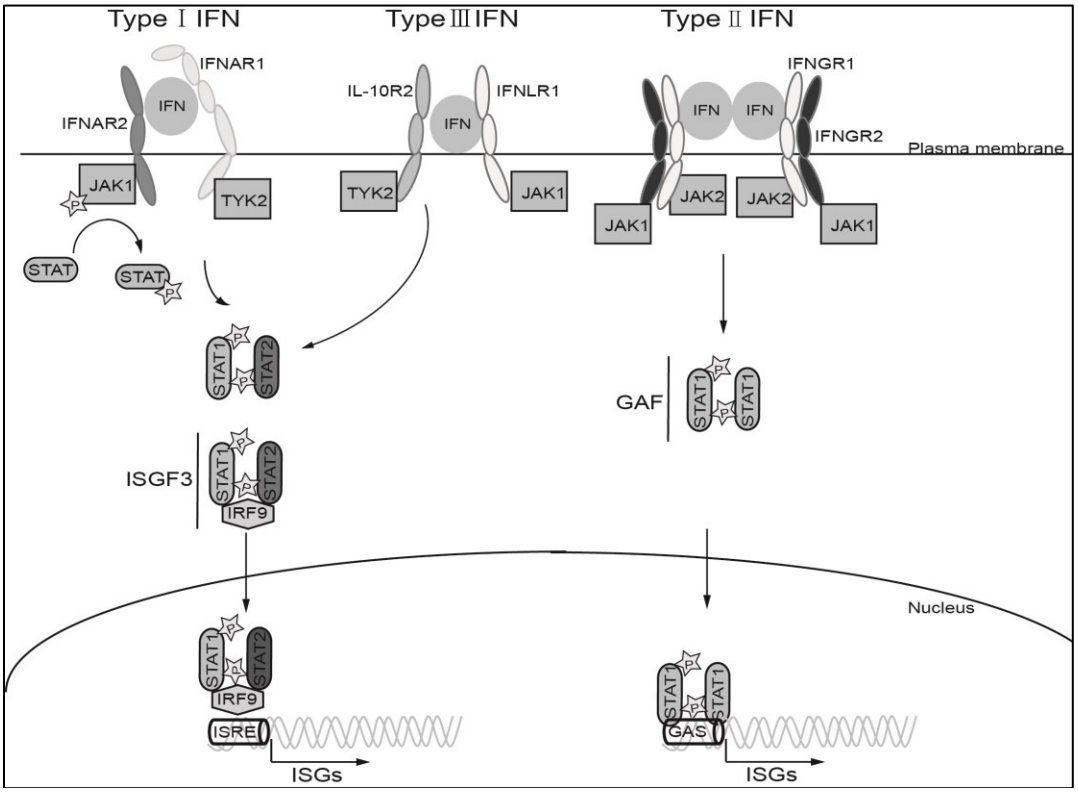


Figure 1.9: IFN signaling.
Three types of IFN adopt different pathways to activate the expression of ISGs. Type Ⅰ and Ⅲ IFNs binds with their own receptors and then utilize the same pathway containing the formation of ISGF3 and the binding with ISRE. Type Ⅱ IFN binds to its own receptor IFNGR2 and then activates the formation of GAF, which binds to GAS to activate the expression of ISGs.

1.4.3 IFN stimulated genes (ISGs) and HIV

Some ISGs have been reported to affect the replication of HIV at different steps of virus life cycle. Tripartite motif-containing protein (TRIM5α) is reported to block HIV

uncoating. Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC3) and sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1 (SAMHD 1) impedes HIV viral reverse transcription. Human myxovirus-resistance B (MxB) protein interferes with a step after reverse transcription. Bone marrow stromal antigen 2 (Bst-2 or Tetherin) and T-cell immunoglobulin and mucin domain (TIM) proteins block the release of HIV viral particles. The interferon-induced transmembrane proteins (IFITMs) act by interfering with virus entry.

1.4.3.1 TRIM5a

It has been long known that HIV-1 replicates in human and chimpanzee cells but not in Old World monkey cells. It was suspected that an Old World Monkey-specific factor acts as the species barrier of HIV infection [128]. This host factor was subsequently discovered as TRIM5α in rhesus macaques and also as TRIM-Cyp in a New World Monkey - owl monkey [129] [130] [131] [132]. Tripartite motif-containing protein (TRIM) family proteins have three domains: RING, B-Box2 and Coiled-Coil domains. N terminal RING exists in all TRIM proteins and has E3 ubiquitin ligase activity [133] [134]. B-Box and Coiled-coil domains are responsible for protein multimerization. C-terminal domains of TRIM5 proteins such as B30.2/PRYSPRY (SPRY) domain of TRIM5α or cyclophillin A (CypA) domain in TRIM-Cyp, recognizes viral capsid core [135] [136]. Binding of TRIM5 proteins to viral core leads to premature uncoating in a proteasome-dependent manner ([137] [138] [139] and reviewed in [140]). Besides C-terminal, Coiled-Coil domain of TRIM5 also contributes to specific recognition of viral capsid [141].

1.4.3.2 APOBEC3G

Vif is essential for HIV to replicate in some cell types such as primary CD4 T cells and

CEM cell but not in some other cell lines such as SupT1 and CEM-SS [142] [143]. Through comparing the genome variation of CEM and CEM-SS cell lines using cDNA subtraction method, APOBEC3G was identified as the inhibitor of HIV replication [144]. Like the other members of APOBEC family, APOBEC3G contains cytidine deaminase (CDA) domain that catalyzes the deamination of cytidine (C), leading to the generation of uridine (U) [145]. By deaminating C to U in the minus strand viral DNA , APOBEC3G causes G to A mutations in HIV genome [146] [147] [148]. APOBEC3G is packaged into virion in the absence of Vif through the interactions with viral NC region of viral Gag and also with the viral RNA [149] [150] [151]. Wild type HIV/SIV use Vif protein to counteract APOBEC3G. Vif acts by binding to APOBEC3G and cullin5-elongin B/C-Rbx ubiquitin ligase at the same time, which induces polyubiquitylation and degradation of APOBEC3G, thus avoiding APOBEC3G incorporation into HIV particles [152] [153].

1.4.3.3 SAMHD1

The main target cells of HIV-1 are activated CD4 positive T lymphocytes. But the non-dividing CD4+ T-lymphocytes and myeloid cells such as monocytes, dendritic cells and macrophages are also potential target cells of HIV-1 infection, except that HIV-1 is much less infectious in these non-dividing cells [154] [155]. It has been known that a SIV protein called Vpx dramatically increases HIV-1 infection in monocyte-derived dendritic cells (MDDCs) under the the existence of SIV Vpx protein [156], whereas Vpr from HIV-1 does not have the same function despite that Vpx may have been derived from Vpr. Mass spectrometry analysis of Vpx-binding proteins in differentiated human monocytic THP-1 cells leads to the discovery of SAMHD1 protein that is responsible for inhibition of HIV-1 infection and is counteracted by Vpx [157]. SAMHD1 has the N terminal sterile alpha motif (SAM) and C terminal HD domain containing conserved

histidine and aspartate residues. The dNTPase activity of SAMHD1 diminishes the level of dNTP in cells, which hampers the synthesis of HIV DNA by viral RT [158] [159]. Vpx from SIV or HIV-2 counteracts the function of SAMHD1 by recruiting E3 ubiquitin ligase complex to degrade SAMHD1 via polyubiquitilation [160]. However, HIV-1 does not have a strategy to counteract SAMHD1, which may have helped the virus not activate the innate immune responses in myeloid cells [161].

1.4.3.4 BST-2

BST-2 (Tetherin) was independently reported by two groups to inhibit the release of Vpu deficient HIV-1 particles in 2008 [162] [163]. Subsequently, a wide range of enveloped viruses or their viral like particles (VLP) (arenaviruses, herpesviruses, filoviruses, orthomyxoviruses etc.) were also reported to be subject to BST-2 restriction (reviewed in [164]). BST-2 comprises N-terminal cytoplasmic tail (NT), transmembrane region (TM), an ectodomain (ED), and a C-terminal glycosylphosphatidylinositol (GPI) anchor. All three domains are necessary for tethering viral particles on cell surface. The three conserved cysteines in ED help the formation of BST-2 homodimer through the formation of disulfide bonds between BST-2 molecules [165]. One possible mechanism by which BST-2 inhibits the release of viral particle is that both ends of the dimmer – TM and GPI anchor insert into either cell membrane or the viral membrane during viral budding, allowing the ED dimer as a bridge connecting host and viral membrane [166]. The other possible mechanism is that BST-2 may cluster around the neck of budding virus and prevent the release of mature virions (viewed in [164]). Trapped virus particles are then internalized and degraded in the host cells [167]. To counteract the inhibition of BST-2, viruses evolve countering strategies. HIV-1 has an accessory protein Vpu, whose transmembrane domain interacts with BST-2 and degrades BST-2 by recruiting ubiquitin

ligase [168]. Kaposi-Sarcoma Herpesvirus (KSHV) also uses its K5 protein to bind with NT of BST2 and induces BST-2 degradation [169]. HIV-2 and Ebola viruses utilize their envelope proteins to counteract BST-2. Binding of HIV-2 Env redistribute BST-2 to Golgi but not on cell membrane, whereas Ebola Env interaction does not reduce the BST-2 level on cell surface [170] [171] [172]; Interaction between SIV Nef protein and BST-2 also relocates BST-2 and removes it from the cell surface [173] [174].

1.4.3.5 MxB

Myxovirus-resistance (Mx) proteins are large GTPase and belong to dynamin superfamily. Humans have MxA and MxB. MxA has long been known to inhibit a wide range of viruses including bunyaviruses , coxsackie virus, hepatitis B virus (HBV) and influenza virus [175] [176] [177] [178]. MxA recognizes viral nucleocapsids or other viral components after viral infection and degrades them to prevent virus replication at early stage [179] [180]. In 2013, three groups, including our group, independently reported that human MxB efficiently inhibits HIV-1 [181] [182] [183]. MxB likely acts by recognizing HIV capsid and blocks the nuclear import of viral DNA ([182] [183]) and viral DNA integration ([181]). Capsid mutations A88, P90 and N74 render HIV-1 resistant to MxB [181] [182] [183].

1.4.3.6 TIM family proteins

The T-cell immunoglobulin and mucin domain (TIM) protein family consists of three members in human: TIM-1, TIM-3 and TIM-4 [184]. Their roles in cells are related with immune response, as TIM-1 plays important role in the development of autoimmune disease and TIM-3, TIM-4 function in immune tolerance [185] [186] [187] [188]. Several research groups also found the connections between TIM proteins and viral infection such as hepatitis A virus [189] [190]. Recently, Li. et al. discovered that all human TIM

proteins efficiently inhibited HIV-1 virus release and replication [191]. Moreover, they found that TIM-1 holds the HIV-1 virion on cell surface through its phosphatidylserine (PS)-binding capability, indicating the possible antiviral activities of PS-binding proteins.

1.5 Interferon-induced transmembrane proteins (IFITMs)

IFITMs were first reported in 1984 in cDNA library screening of Type Ⅰ IFN treated neuroblastoma cells [192]. They are ubiquitously expressed in different tissues, and conserved from Zebrafish to human [193]. IFITM proteins are involved in cell development, cell adhesion, germ cell homing and oncogenesis ([194] [195] [196] and reviewed in [193]). In 2009, IFITM proteins were reported to potently inhibit influenza A virus, West Nile virus and Dengue virus [197].

1.5.1 IFITMs genes and structure

Humans have 5 IFITM genes. IFITM 1, 2, 3 and 5 are located on chromosome 11. IFITM 10 is in the other locus of chromosome 11 and is conserved in all vertebrates but its function is unknown [198] (reviewed in [199]). There are two copies of ISRE upstream the 5' UTR of IFITM 1, 2, 3 but not IFITM 5, indicating the involvement of IFITM1, 2 and 3 in interferon induced innate immunity [193]. IFITM proteins have two hydrophobic transmembrane (TM) domains and a conserved hydrophilic intracellular loop. The N-terminal TM domain together with the intracellular loop of IFITMs are called CD225, which is shared by members of the CD225/pfam04505 family, albeit that function of the other members of CD225 family is not much known [200] [199]. IFITM1, 2 and 3 are activated by Type I and Type II IFNs *in vitro*. IFITM1 is seen at plasma membrane and early endosomes [201] [202] [203]. IFITM2 and IFITM3 are mainly located in late endosomes and/or lysosomes [203] [204] [205].

The membrane topologies of IFITM proteins have been largely elucidated, although

controversies still exist. IFITM proteins were originally called transmembrane proteins, indicating that both the N- and C- termini of IFITM1, 2 and 3 are luminal or extracellular (Figure 1.10 I). This topology was supported by studies that detected the N- or C-terminal tags of IFITMs by flow cytometry [206] [207]. However, Yount *et al.* [208] later reported that the N-terminal region of IFITM3, although contains glycosylation sites, was not modified, which suggests the cytosolic localization of the N terminus. Similar studies also support the cytosolic localization of the C-terminal region of IFITM3. This membrane topology is supported by the phosphorylation of the N-terminal residue Tyr-20 as well as the palmitoylation of the C-terminal cysteine residues of mouse IFITM1 [209] [210] (Figure 1.10 II). A recent study by Bailey *et al.* [211] showed that the C-terminus of mouse IFITM3 was readily detected on the cell surface. By adding the ER retention motif KDEL to C-terminus of IFITM3, they observed ER sequestration of this modified IFITM3, further supporting the luminal localization of the C-terminus. Moreover, they found that the tag at the C-terminus, but not at the N-terminus, was removed in lysosomes. On the basis of these results, they propose that IFITM3 is a type Ⅱ transmembrane protein (Figure 1.10 Ⅲ) with cytosolic N-terminus and extracellular/luminal C-terminus [212]. The topologies of IFITM1 and IFITM2 are under investigation, there is some unpublished data showing that IFITM1 also adopts the topology similar to IFITM3 [213].

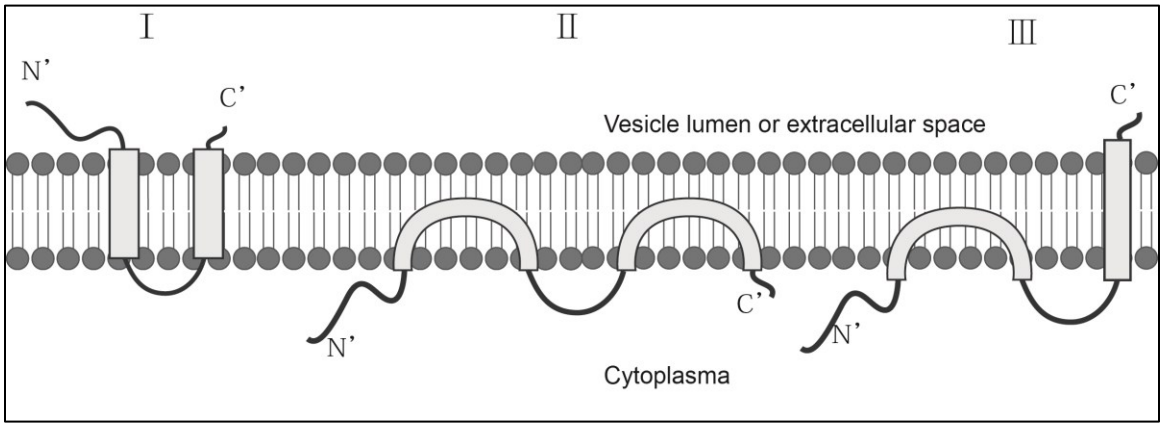


Figure 1.10: Topologies of IFITMs on membrane.
Three topologies differ in the locations of N and C termini: both termini of model Ⅰ are extracellular; both terminus of model Ⅱ are intracellular; and N terminus of model Ⅲ is intracellular with C terminus is extracellular.

1.5.2 Function of IFITM proteins in cell biology

It was reported that IFITMs were involved in cell adhesion and growth control, cancer development and germ cell differentiation. The interaction between IFITM1 and CD81 is involved in the cell adhesion to extracellular matrix proteins. Murine IFITMs were reported to interact with OPN-osteopontin, which contributes to the establishment of germ cell niche [214] [215]. The IFITMs may have diverse roles in cancer development. For example, gastric cancer cells have higher IFITM1 expression, breast carcinoma has increased IFITM3 expression at the invasive stage [216] [217], the level of IFITM1 decreases in the brain samples of astrocytoma patients [218]. The role of murine IFITM1&3 in embryo cell (primordial germ cell) development is unsettled. Tanaka et al. reported that IFITM1 was required for PGC translocation from the mesoderm into the endoderm; however, Ulrike et al. showed that knockout of IFITM locus had no effect on the mouse development [219] [220].

1.5.3 Antiviral spectrum of IFITMs

The antiviral spectrum of IFITMs has been greatly expanded since the discovery of inhibition of influenza A virus by IFITMs. Viruses of 13 different families, including both enveloped and non-enveloped, RNA and DNA viruses, have been tested for their sensitivity to IFITMs (summarized in Table 1.4). In 2009, using functional genomic screen, Brass et al. [197] discovered that human IFITM1, 2, 3 inhibit influenza A virus at the early stage of infection. Knockdown of IFITM3 in both IFN treated U2OS and A549 cells dramatically increased the infection of wild type and pseudotyped influenza A viruses. Importantly, the infection of influenza A virus greatly increased in MEF cells when the ifitm loci was deleted. IFITMs also inhibited the early infection of flaviviruses (dengue virus, West Nile virus, yellow fever virus and Omsk hemorrhagic fever virus). In contrast, infection of pseudotyped arenaviruses (lymphocytic choriomeningitis virus, Lassa virus, and Machupo virus) and the MLV retrovirus were not affected. Studies by Huang et al. [221] extended IFITMs inhibition to Filoviridae (Marburg virus and Ebola virus) and Coronaviridae (SARS-CoV). They also found that the infection of different viruses is affected by IFITM1, 2, 3 proteins to different extents. For example, influenza virus is restricted more by IFITM3 than by IFITM1, as opposed to a greater inhibition of Marburg virus, Ebola virus and SARS-CoV by IFITM1 than by IFITM3. This virus-specific inhibition by IFITMs was also observed for bunyaviruses. Mudhasani et al. [222] found that IFITM1, 2, 3 all inhibited La Crosse virus, Hantaan virus, Andes virus, but only IFITM2 and IFITM3 inhibit Yellow fever virus, and none of the three IFITM proteins inhibited Crimean-Congo hemorrhagic fever virus. The difference of inhibition may result from the different localization of IFITMs in cells. In addition to all enveloped

viruses, IFITM3 also inhibit the entry of reovirus, a non-enveloped virus of Reoviridae that utilize endocytic pathway during entry [223]. Reovirus is the only non-enveloped virus found so far being inhibited by IFITM3. Not all enveloped viruses are inhibited, human papillomaviruses, cytomegalovirus and adenovirus type 5 are not affected by IFITMs [224]. Intriguingly, Zhao et al. [225] recently found that one member of the *Coronaviridae* – HCoV-OC43 could utilize IFN-induced IFITM2 and IFITM3 but not IFITM1 to promote its infection rather than being inhibited. However, the N terminus of IFITM3, which is important for the inhibition of influenza A virus and SARS-CoV, is not critical for increasing the infection of HCoV-OC43. Moreover, deletion of C terminus of IFITM1 increases the infectivity of HCoV-OC43 but had no effect to the inhibition of influenza A virus and SARS-CoV. Taken together, each member of IFITM proteins exhibits distinct inhibition of viruses partially as a result of their different subcellular localizations. Different viruses are differently affected, some are highly inhibited, and some are resistant.

IFITMs have important roles in the *in vivo* antiviral defense. Everitt et al. [226] challenged the IFITM3 knockout mice model with two influenza virus strains: low-pathogenicity murine-adapted H_3N_2 influenza A virus (A/X-31) and pandemic H1N1/09 Eng/195. The IFITM3 knockout mice showed higher mortality and morbidity, and cannot clear the viruses in lung. The same study also showed that hospitalized patients suffering influenza A virus infection have a much higher percentage of a SNP that results in deletion of the N-terminal 21 amino acids of IFITM3 and the loss anti-influenza activity of IFITM3 [209] [212]. Studies by Wakim et al. [227] further confirmed the importance of IFITM3 *in vivo*. They observed the persistence expression of IFITM3 in lung anti-

influenza CD8+ resident memory T cells (T_{RM} cells) after first influenza infection, which helped mice to survive and also protect from the next round of influenza virus infection.

Summary of viruses tested by IFITM proteins					
Family	**Virus**	**pH dependence (Y-yes, N-no)**	**Restricts infectivity**	**Pseudotyped virions (P) or live virus (L)**	**IFITM specificity**
Enveloped					
Orthomyxoviridae	Influenza A virus	Y	Y	P & L	IFITM3 > IFITM2 > IFITM1
	Influenza B virus	Y	Y	L	IFITM3 > IFITM2 > IFITM1
Flaviviridae	West Nile virus	Y	Y	P	IFITM3 > IFITM1 > IFITM2
	Dengue virus	Y	Y	P	IFITM3 > IFITM1 > IFITM2
	Yellow fever virus	Y	Y	P	IFITM3 > IFITM1 > IFITM2
	Omsk hemorrhagic fever virus	Y	Y	P	IFITM3 > IFITM1 > IFITM2
	Hepatitis C virus	Y	Y or N	P & L	IFITM1, but not IFITM3
Rhabdoviridae	VSV	Y	Y	P & L	IFITM3 > IFITM1 > IFITM2
	Rabies virus	Y	Y	P	IFITM1
	Lagos Bat virus	Y	Y	P	IFITM1
Filoviridae	Marburg virus	Need Cathespin L	Y	P & L	**A549:**IFITM3 > IFITM1 > IFITM2 **Vero E6**:IFITM1 > IFITM2/IFITM3 **HUVEC**:IFITM3 > IFITM1 > IFITM2 **293T:**IFITM3 > IFITM2 > IFITM1
	Ebola virus	Need Cathespin L	Y	P & L	**A549:**IFITM3 /IFITM1 > IFITM2 **Vero E6**:IFITM1 > IFITM2/IFITM3 **HUVEC**:IFITM3/IFITM1 > IFITM2 **293T:**IFITM3 > IFITM2/IFITM1
Coronaviridae	SARS coronavirus	Need Cathespin L	Y	P & L	**A549**: IFITM3/IFITM2 ≥ IFITM1 **Vero E6**:IFITM1 > IFITM2 > IFITM3
	HCoV-OC43	Need Cathespin L	Increase	L	Utilizes IFITM2 or 3, not IFITM1
Retroviridae	HIV-1	N	Y or N	P & L	**SupT1**:IFITM1 > IFITM2/IFITM3, No inhibition of IFITM3 in TZM-bl-Hela
	MLV	N	N	P & L	N
	Jaagsiekte sheep retrovirus	Y	Y	P	**HTX**: IFITM1 > IFITM2 > IFITM3 **293**: IFITM1 > IFITM3 > IFITM2

					Cos7: IFITM1 > IFITM3
Arenaviridae	Lassa virus	Y	N	P	N
	Machupo virus	Y	N	P	N
	Lymphocytic choriomeningitis virus	Y	N	P	N
Alphaviridae	Semliki Forest virus	Y	Y	L	IFITM2/IFITM3 > IFITM1
Bunyaviridae	La Crosse virus	Y	Y	L	IFITM1/IFITM2/IFITM3
	Hantaan virus	Y	Y	L	IFITM1/IFITM2/IFITM3
	Andes virus	Y	Y	L	IFITM1/IFITM2/IFITM3
	Rift Valley fever virus	Y	Y	L	IFITM2/IFITM3
	Crimean–Congo haemorrhagic fever virus	Y	N	L	N
Non-enveloped					
Reoviridae	Reovirus	Y	Y	L	IFITM3
Papillomaviridae	Human papillomaviruses	Y	Increase	P & L	Increase when IFITM1 & 3 overexpressed, not IFITM2
Herpesviridae	Human cytomegalo virus	Y	N	P	N
Adenoviridae	Adenovirus type 5	Y	N	P	N

Table 1.4: Summary of viruses tested by IFITM proteins (Modified from [199] [213]).

1.5.4 IFITMs and HIV

The anti-HIV-1 activity of IFITMs is now considered to be cell type dependent. At the beginning when Brass et al. found the antiviral activity of IFITMs, they tested the anti-HIV-1 activity of IFITM3 by knocking down TZM-bl Hela cells using HIV-1(IIIB) virus and did not see an increase of the infectivity of HIV-1 [197]. But then in SupT1 cell, our group [203] first reported the increase of HIV-1 infectivity in IFN-treated cell when IFITM1 was knocked down. Furthermore, overexpression of either IFITM1, 2 or 3 profoundly suppressed the replication of HIV-1 in SupT1. IFITM2 and IFITM3, but not IFITM1, diminished the entry of HIV-1 [209] [228]. Results of our study showed that all IFITM1, 2 and 3 need to be depleted in TZM-bl cells in order to observe an increase of HIV-1 infection. In support of the inhibitory role of IFITMs in HIV-1 infection, co-

transfection of IFITMs with HIV-1 DNA into 293 cells dramatically decreased the expression of HIV-1 Gag, Vif and Nef [229]. Expressing of IFITMs also reduced HIV-1 production in a dose-dependent manner. More studies are underway to delineate the anti-HIV mechanisms of IFITMs.

1.5.5 Antiviral mechanisms of IFITMs

A series of studies have demonstrated that IFITMs inhibit virus entry [197] [203] [206] [221] [222], although IFITMs may also inhibit other steps of virus infection [203] [229]. Through monitoring cell-cell fusion that was induced by JSRV ENV and HA of influenza virus in cells overexpressing IFITMs, Li et al. [206] discovered that IFITMs efficiently prevented membrane fusion. Oleic acid (OA), but not chlorpromazine (CPZ), was shown to rescue IFITM inhibition of JSRV Env-mediated membrane fusion. Since CPZ promotes membrane fusion from hemifusion to full fusion and OA promotes hemifusion, it was proposed that IFITMs interfere with membrane hemifusion triggered by JSRV Env. Importantly, the fluidity of cell membranes was reduced by IFITMs, which explains the impaired membrane hemifusion caused by IFITMs.

How do IFITMs affect membrane fluidity? One possible mechanism involves accumulation of cholesterol multivesicular bodies as a result of the disruption of the binding between vesicle-membrane-protein-associated protein A (VAPA) and oxysterol-binding protein (OSBP) by IFITM3 and the consequent disruption of intracellular cholesterol homeostasis [230].

A recent study proposed a different model. Desai et al. [231] monitored the movement of single virus in A549 cells and found that overexpression of IFITM3 did not prevent the hemifusion between virus membrane and membrane of endosomes. Next, they generated pseudovirus with influenza NA and HA and labeled the viruses with YFP-Vpr

(encapsidated into the pseudovirus by binding with HIV gag protein) and Gag-iCherry. The release of capsid core was dramatically inhibited. They concluded that IFITM3 inhibited the fusion pore formation. They also observed moderate increase of cholesterol in endosomes as a result of IFITM3 overexpression. However, treatment with 40 μM U18666A or knocking down of NPC1, both leading to cholesterol accumulation, did not inhibit influenza virus membrane fusion. This latter data suggest that cholesterol accumulation itself does not solely account for IFITM3 inhibition.

The antiviral activity of IFITMs is modulated by their posttranslational modifications. Three major types of modifications have been reported, they are S-Palmitoylation, phosphorylation and ubiquitination. Ubiquitination of IFITM3 occurs mostly on Lys 24, Lys-83, Lys-88, and Lys-104. Mutating these lysines leads to the loss of ubiquitination of IFITM, greater localization to the late endosomes, and increase in their antiviral function [208] [200]. Yount et al. [204] reported that the antiviral activity of IFITM3 depends on S-Palmitoylation of cysteines at positions 71, 72 and 105. Mutating these cysteines cause a dispersed distribution of IFITMs within the cytoplasm. The tyrosine reside at position 20 in IFITM3 is phosphorylated [200] [209] [212]. Mutating Y20 severely attenuates the ability of IFITM3 to inhibit influenza virus, but the same mutants retain the function to inhibit HIV-1 [209] [212]. This tyrosine is a key residue of the YEML protein sorting motif that binds to μ2 subunit of the AP-2 complex and therefore controls the intracellular trafficking of IFITM3.

1.6 Research objectives

Viruses often evolve measures to counter host restrictions. A number of viruses from *arenaviridae,* adenoviruses and papilomaviruses are resistant to IFITM inhibition, although the underlying mechanisms remain unexplored. On the basis of our results that IFITM1 profoundly inhibits HIV-1 replication in a CD4+ cell line SupT1, we asked whether and how HIV-1 escapes from IFITM1 inhibition. Three projects were pursued:

1. We hypothesized that HIV-1 is able to evolve in tissue culture to escape from IFITM1 inhibition. To test this, we monitored HIV-1 replication in IFITM1-expressing SupT1 cells until escape mutants emerged. Sequencing the entire genome of the escape viruses revealed the resistant mutations. The resistance mechanisms were further investigated.

2. We observed that the HIV-1 strain BH10 was profoundly inhibited by IFITM1, but another HIV-1 strain called NL4-3 was resistant. We hypothesized that protein(s) of HIV-1_{NL4-3} were able to overcome IFITM1 inhibition. To identify the underlying viral protein, we generated a series of chimeras between HIV-1_{BH10} and HIV-1_{NL4-3} and identified the involvement in resistance to IFITM1 inhibition.

3. IFITM1 has a relatively longer C-terminal region than IFITM2 and IFITM3. To investigate the role of the C-terminal sequence in the antiviral activity of IFITM1, we deleted this sequence and tested the mutants on the infection HIV-1_{NL4-3}. Although HIV-1_{NL4-3} is resistant to the wild type IFITM1, it is drastically inhibited by these IFITM1 mutants. Again, HIV-1_{NL4-3} was able to escape from these IFITM1 mutants by mutating Vpu and Env.

Results of these studies, for the first time, demonstrated virus escape from IFITM inhibition and revealed a role of the viral envelope glycoprotein in countering IFITM proteins.

Chapter 2 – HIV-1 mutates to evade IFITM1 restriction

Preface

Interferon-induced transmembrane (IFITM) proteins inhibit the infection of a wide range of viruses including human immunodeficiency virus type1 (HIV-1). At present, little is known about how viruses overcome IFITM restriction. In this study, we have utilized HIV-1 as a model and selected IFITM1-resistant viruses after multiple passages of HIV-1 in IFITM1-expressing SupT1 cells. Sequencing the entire viral genome revealed several mutations in the vpu and envelope genes, among which mutations Vpu34 and EnvG367E together enable efficient HIV-1 replication in IFITM1-expressing cells. Vpu34 introduces a stop codon at amino acid position 35 of Vpu, whereas EnvG367E changes the G367 residue at the CD4-binding site of gp120. These two mutations do not appear to overcome the downregulation of viral p24 expression caused by IFITM1, but rather enhance HIV-1 replication by promoting cell-to-cell virus transmission. Altogether, our data demonstrate that HIV-1 can mutate to evade IFITM1 restriction by increasing cell-to-cell transmission.

2.1 Introduction

Interferon (IFN) inhibits virus infection through inducing the expression of hundreds of cellular genes collectively known as interferon-stimulated genes (ISGs) [127]. The antiviral functions of some of these ISGs have been well characterized. Examples include the RNase L and OAS proteins, protein kinase R (PKR), myxovirus-resistance (Mx) proteins, ISG15, APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G), TRIM5α (Tripartite motif-containing protein 5α), tetherin, SAMHD1 (SAM domain and HD domain-containing protein 1), Schlafen 11, and MxB [127, 129, 162, 181, 232-237]. A recent addition to this list is the interferon-induced transmembrane (IFITM) proteins that inhibit a number of highly pathogenic human viruses [197, 203, 207, 221, 238, 239]. IFITM proteins are distinct from all other ISGs in that they inhibit viral entry, particularly the step of membrane fusion [197, 203, 205, 221] (reviewed in [240, 241]).

Humans have IFITM1, 2, 3, 5 and 10, all of which are located on the chromosome 11 [242]. IFITM1, 2, 3 and 5 are clustered in a 26.5 kb region, whereas IFITM10 is located 1.4 Mb apart. IFITM5 is also called bone-restricted IFITM-like (Bril) protein due to its strict expression in osteoblasts and its role in bone mineralization and maturation [243, 244]. The function of IFITM10 is unknown, but it is highly conserved in different species [242]. IFITM1, 2 and 3 have been shown to promote anti-proliferation, homotypic cell adhesion, and apoptosis [194, 245-247]. They are also overexpressed in some tumor cells, such as human colorectal tumors, suggesting a possible role in oncogenesis [193, 248]. In addition to their ubiquitous expression in different tissues [249, 250], IFITM1, 2 and 3 also respond to the stimulation by interferon [251], supporting their roles in host antiviral defense. Indeed, IFITM1, 2 and 3 have been reported to inhibit the infections by

influenza A virus (IAV), flavivirus (West Nile virus, Dengue virus, Yellow Fever virus), filovirus (Ebola virus, Marburg virus), SARS coronavirus, vesicular stomatitis virus (VSV), hepatitis C virus (HCV), reovirus, Rift Valley fever virus, as well as human immunodeficiency virus type 1 (HIV-1) [197, 203, 205, 207, 222, 238, 239, 252, 253]. In addition to this broad antiviral activity, knockout of *ifitm3* in mice or IFITM3 deficiency in humans renders the hosts highly vulnerable to IAV infection [227, 254-256], highlighting the importance of IFITM proteins in host antiviral defense *in vivo*.

Human IFITM1, 2 and 3 are of 125, 132 and 133 amino acids in length, respectively. They are predicted to have two transmembrane domains [193]. Results of cell-surface immunostaining and flow cytometry experiments suggest that their amino- and carboxy-termini project toward the extracellular space or luminal compartments [197, 207]. However, recent evidence also supports the cytoplasmic localization of the N-terminus [257, 258]. In addition to the plasma membrane, IFITM proteins are also observed in the endoplasmic reticulum (ER) and endosomes [197, 203-205, 209, 259-261]. The localization of IFITM3 in late endosomes is important for inhibiting IAV infection, because ectopic expression of IFITM3, or its induced expression by interferon, causes expansion of late endosomes and lysosomes and results in the sequestration of endocytosed IAV particles in these acidic membrane compartments [205, 221]. By taking advantage of lipid analogs and fluorescence labeling, we recently showed that oleic acid (OA), but not chlorpromazine (CPZ), rescues the inhibitory effect of IFITMs on cell-to-cell fusion induced by Jaagsiekte sheep retrovirus (JSRV) Env and IAV hemagglutinin (HA), indicating that IFITM proteins interfere with the hemifusion stage of virus entry, possibly by changing membrane fluidity and curvature [206]. This conclusion is further

strengthened by the fact that IFITM proteins increase lipid order of membranes [206]. This latter property of IFITM proteins is at least partially attributed to their interaction with VAPA (vesicle-membrane-protein-associated protein A) and consequent disturbance of cholesterol homeostasis [230].

Viruses often evolve mechanisms to evade or antagonize host restrictions [262], and this strategy should also be operative for the IFITM proteins. Indeed, HCV infection increases the expression of miR-130a that targets the 3' untranslated region of IFITM1 mRNA and thus diminishes IFITM1 expression [263]. Additionally, arenaviruses, which require low pH for entry, are refractory to IFITM restriction [197], although the underlying mechanism still remains unclear. In order to better understand the viral evasion of IFITM restriction, we investigated whether HIV-1 can develop resistance to IFITM1 in CD4+ SupT1 cells. The results showed that long-term culture led to the emergence of IFITM1-resistant HIV-1 mutants, and we further mapped the escape mutations to the viral Vpu and Env proteins.

2.2 Materials and methods

2.2.1 Plasmids, cell lines and antibodies

The tetracycline-inducible IFITM1 SupT1 cell line was generated as previously described [203]. The HIV-1_{BH10} proviral DNA clone was obtained from the NIH AIDS Reference and Reagent Program. The mutations Vpu34, EnvR311K, EnvG367E and g7178a were engineered using the site-directed mutagenesis kit (Stratagene). The anti-Flag and anti-β actin antibodies were purchased from Sigma, anti-tubulin antibody from Santa Cruz biotechnology, anti-HIV-1 p24 antibody from ID Lab Inc., phycoerythrin (PE)-conjugated anti-human CD4 antibody from BD Biosciences, Dylight-649-

conjugated anti-Flag antibody from Rockland, FITC-conjugated anti-HIV-p24 antibody from Beckman. G418 was purchased from Invitrogen, puromycin and doxycycline from Sigma.

2.2.2 Virus infection

HIV-1 stocks were produced by transfection of the human embryonic kidney cell line (HEK293T) with HIV-1 proviral DNA. The culture supernatants were clarified by passing through the 0.2 μm filter (VWR) to remove the cell debris. Amounts of viruses were determined by measuring viral p24 (CA) levels using the HIV-1 p24 Antigen Capture Assay kit (Cat. 5447, ABL Inc.)

Virus infection was measured by four assays. First, infection of the TZM-bl indicator cells that express CD4/CXCR4/CCR5 and contain the HIV-1 LTR-Luc reporter [264]: TZM-bl cells were first seeded into 24-well plates (4 $X10^4$cells/well) one day before virus infection. Forty hours after infection, cells were lysed with 1 X passive lysis buffer (Promega) and the levels of luciferase activity were measured using the luciferase assay kit (Promega). Second, short-term infection of the SupT1 cells: SupT1 cells were first exposed to virus equivalent to 200 ng viral p24 antigen. Forty hours after infection, the infected cells were washed with cold 1 X phosphate-buffered saline and fixed with 1% paraformaldehyde. The infected cells were stained with anti-HIV-1 p24 antibody and scored by flow cytometry. Third, long-term infection of SupT1 cells: SupT1 cells were infected with virus equivalent to 10 ng viral p24. Viral replication was monitored by measuring the levels of viral reverse transcriptase activity in supernatants over various time intervals. Lastly, we used the CEM-Rev-Luc cells to examine the sensitivity of HIV-1 to inhibition by different agents. The CEM-Rev-Luc cells express luciferase in a HIV-1 Rev-dependent manner [265]. These cells are a gift of Dr. Yuntao Wu (George Mason

University). Cells were first pretreated for one hour with different doses of each of the five agents, including soluble CD4 (sCD4), broadly neutralizing antibody VRC03, anti-CD4 antibody (SIM4), CXCR4 antagonist (AMD3100) and CCR5 antagonist maraviroc (all agents were obtained from the NIH AIDS Reference and Reagent Program). The cells were then infected with the same amounts of different HIV-1 stocks. Forty hours after infection, cells were lysed and luciferase activity was measured to determine virus infection.

2.2.3 Identification and cloning of the escape viruses

We started by infecting IFITM1-expressing SupT1 cells with HIV-1 to monitor the development of resistant viruses. When marked cytopathogenic effect and high levels of viral RT activity in the supernatant were detected 3 weeks after infection, we used these newly produced viruses to infect fresh IFITM1-expressing SupT1 cells. After five such passages, HIV-1 was able to reach peak level of its replication at 6 to 8 days instead of 20 days as observed in the initial round of infection. We harvested SupT1 cells that were infected by the highly replicable HIV-1 and extracted the total cellular DNA using the DNeasy Blood & Tissue kit (Qiagen). Viral genomic DNA was amplified with three primer pairs to cover the entire genome. The PCR products were cloned into the PCR-Blunt II-TOPO vector with Zero Blunt TOPO PCR Cloning Kit (Invitrogen). An average of 7 positive DNA clones for each PCR reaction was sent to McGill University and Quebec Innovation Center for sequencing. We also grew HIV-1 in the control SupT1 cells that do not express IFITM1 for the same period of time, and viral genomes were similarly amplified by PCR, cloned, and sequenced.

2.2.4 shRNA knockdown of CD4 in SupT1 cells

We purchased four shRNA clones that target human CD4 mRNA (Sigma-Aldrich,

catalogue number SHCLNG-NM_000616). The sequences of these four shRNA are:

shRNA1-

CCGGCCAGATAAAGATTCTGGGAAACTCGAGTTTCCCAGAATCTTTATCTGGTT

TTTG,

shRNA2-

CCGGCCTTCTTAACTAAAGGTCCATCTCGAGATGGACCTTTAGTTAAGAAGGT

TTTTG,

shRNA3-

CCGGCCTGATCATCAAGAATCTTAACTCGAGTTAAGATTCTTGATGATCAGGTT

TTTG,

shRNA4-

CCGGAGAGCGGATGTCTCAGATCAACTCGAGTTGATCTGAGACATCCGCTCTT

TTTTG.

These shRNA clones were transfected into HEK293T cells together with plasmid DNA pLP1 (encoding HIV-1 Gag and Gag-Pol), pLP2 (encoding HIV-1 rev) and pVSV-G (encoding VSV G protein) to produce VSV-G pseudotyped lentiviral particles that package the shRNA. SupT1 cells were then infected with these virus particles and subject to selection with 2 μg/ml puromycin for stably transduced cells. The empty shRNA vector was used to generate the control cell line. The total amounts of CD4 in these SupT1 cell lines were determined by Western blotting. To determine the cell surface level of CD4, cells were first incubated with the PE-conjugated anti-human CD4 antibody on ice for 30 min, then fixed with 1% paraformaldehyde. The positively stained cells were scored by flow cytometry. Only shRNA3 significantly decreased CD4 level.

2.2.5 HIV-1 virion fusion assay

The experiment was performed as described previously [203, 266]. Briefly, 3 μg of HIV-1 DNA was co-transfected with 1 μg pCMV-BlaM-Vpr DNA into 293T cells. Supernatants containing virions were filtered with 0.22 μm filter and then concentrated by ultracentrifugation at 100,000xg for 1 hour at 4°C. Pelleted viruses were suspended with DMEM, quantified for viral RT activity, aliquoted and stored at -80°C. For virion fusion assay, SupT1 cells were infected with same amounts of wild type HIV-1 and mutant viruses by spinoculation for 45 minutes at room temperature, followed by incubation for 2 hours at 37°C. Cells were then washed with CO2-independent medium (Invitrogen), mixed with 100 μl loading solution (CCF2/AM substrate, Invitrogen) for 1 hour at room temperature in the dark. After washing off the loading solution, cells were incubated in 200 μl of development medium in dark for 16 hours at room temperature. Cells were washed and fixed with 3.7% formaldehyde. The cleavage of CCF2/AM was measured by flow cytometry.

2.2.6 Western blotting

Cell lysates were separated in 1% sodium dodecyl sulfate-12% polyacrylamide gels (SDS-PAGE) by electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche). The membranes were blocked with 4% skim milk (in 1 X phosphate-buffered saline) and further probed with anti-p24 (1:5000), anti-β actin (1:5000) or anti-tubulin (1:5,000) antibodies. After a further incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare), the protein bands were visualized by exposure to X-ray films following a brief treatment of the membranes with the ECL (enhanced chemiluminescence) reagents.

2.2.7 Cell-to-cell transmission assay

SupT1 cells were first infected with HIV-1 particles bearing VSV-G protein. VSV-G protein was used to effectively increase the infection rate of donor cells; however, the subsequent virus transmission from donor cells to target cells was mediated by HIV-1 Env. Forty hours after, the infected cells (designated as donor cells) were washed with complete medium prior to mixing with un-infected SupT1 cells (designated as target cells) that had been labeled with cell tracker BMQC (Invitrogen). After 8 hours, the cell mixtures were fixed with 1% paraformaldehyde and permealized with 0.1% Triton X-100. After staining with FITC-conjugated anti-HIV-1 p24 antibody, the p24-positive donor and target cells were scored by flow cytometry. In order to control for the infection by free HIV-1 particles, infections were also set up with trans-wells that separated the donor from target cells but allowed the free movement of HIV-1 particles. In addition, we performed control experiments by adding the HIV-1 fusion inhibitor T-20 (2 mM, obtained from the NIH AIDS Reference and Reagent Program) to the mixed donor and target cells in order to block HIV-1 envelope-dependent cell-to-cell fusion.

HIV-1 transmission between cells was also determined by measuring the production of viral late cDNA as described in [267]. Briefly, the donor and target SupT1 cells were collected at time 0 (before mixing) and 8 hours after mixing. Total cellular DNA was prepared using the DNeasy Blood & Tissue kit (Qiagen). The same amounts of DNA from different infection samples were subjected to real-time PCR to quantify HIV-1 late DNA and cellular GAPDH DNA as described in [203]. The amounts of GAPDH DNA serve as internal controls.

2.3 Results

2.3.1 HIV-1 mutates to escape from the inhibition by IFITM1 in SupT1 cells

We previously reported that IFITM1, 2 and 3 suppressed HIV-1 replication in SupT1 cells, with IFITM1 exhibiting the greatest inhibition [203]. In order to investigate whether HIV-1 is able to develop resistance to IFITM restriction, we grew HIV-1 in IFITM1-expressing SupT1 cells and observed that the virus gradually became refractory to IFITM1 inhibition and replicated to high levels (Fig. 2.3.1 A). As a control, we also grew HIV-1 in SupT1 cells without ectopic expression of IFITM1 for the same time interval. We then sequenced the entire genomes of these two virus populations. Five mutations were identified only in IFITM1-resistant viruses, not in those that had replicated in the control SupT1 cells (Fig. 2.3.1 B). Two mutations are located in Vpu, namely Vpu28 and Vpu34. Vpu28 was seen in 2 out of the 7 sequenced viral DNA clones, Vpu34 in 5 clones, indicating that the virus either carried the Vpu28 or the Vpu34 mutation. Both mutations created a stop-codon, resulting in the premature termination of Vpu translation (Fig. 2.3.1 C). The other three mutations were found in the Env coding region (Fig. 2.3.1 B). EnvR311K resides in the variable loop 3 (V3), which is critical for co-receptor-binding (Fig. 2.3.1 D). EnvG367E is in the CD4-binding site in constant region 3 (C3) (Fig. 2.3.1 D). The g7178a mutation does not cause any amino acid change in envelope, nor in the Rev or Tat proteins. This mutation was found in 5 out of the 7 sequenced DNA clones. These data suggest that HIV-1 is able to escape from IFITM1 inhibition by mutating its Vpu and Env proteins.

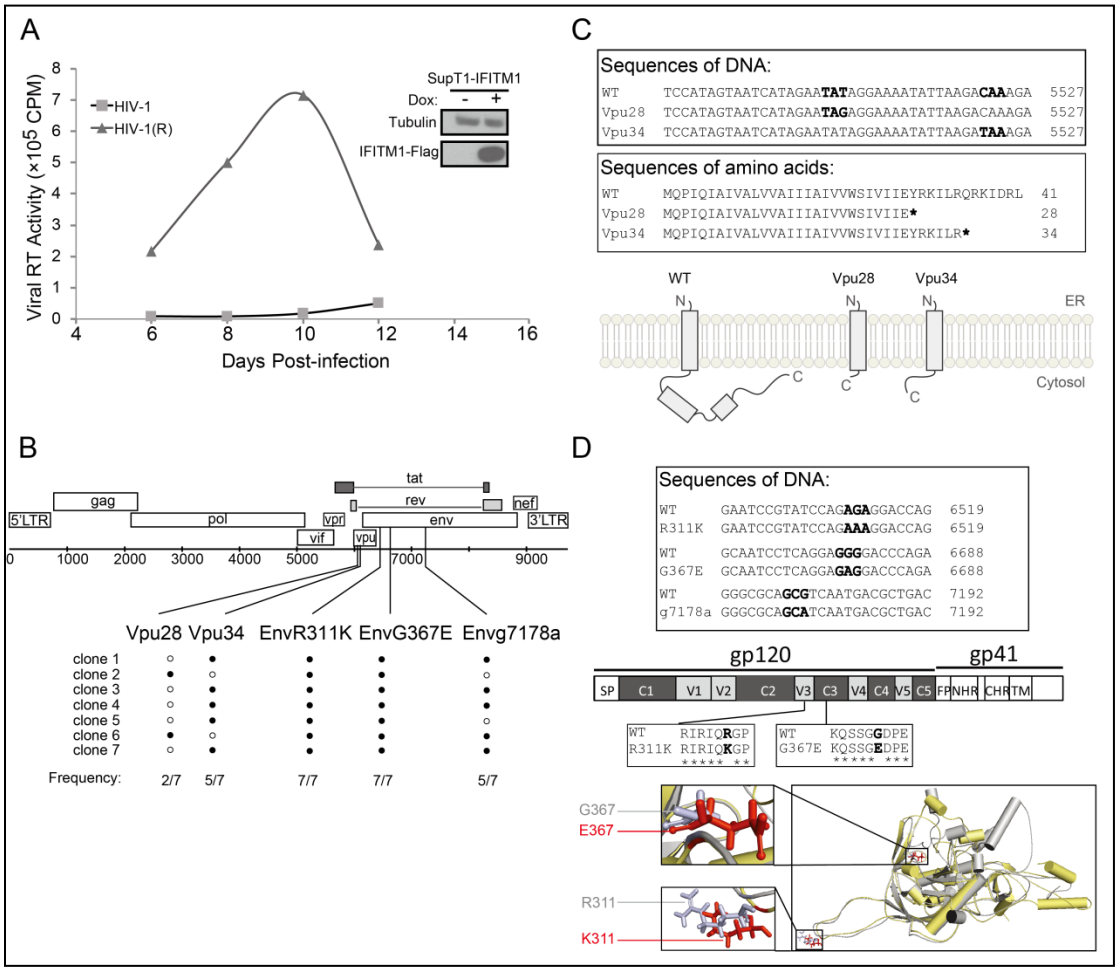


Figure 2.3.1: Identification of escape mutations.

(A) Replication of wild type HIV-1 and the escape viruses named HIV-1(R) in IFITM1-expressing SupT1 cells. Same amounts of viruses were used to infect SupT1 cells expressing IFITM1 with induction by doxycycline. Amounts of viruses in the supernatants were determined by measuring the viral reverse transcriptase activity. The expression of IFITM1-Flag in SupT1 cells was examined by Western blotting with doxycycline (Doxycycline) induction. (B) Illustration of the escape mutations in HIV-1 genome. Seven viral DNA clones were sequenced. The frequency of each mutation in seven clones is shown. (C) Vpu28 and Vpu34 mutations cause premature termination of Vpu translation. Part of the DNA sequences and amino acid sequences of the wild type and mutated Vpu are shown. The Vpu fragments of the Vpu28 and Vpu34 mutants are illustrated. (D) Mutations EnvR311K and EnvG367E are located in the V3 and C3 regions of envelope protein, respectively. The g7178a mutation does not cause amino acid change. Part of the DNA sequences and amino acid sequences of the EnvR311K and EnvG367E mutations are presented. Locations of these two mutations in the gp120 structural model are shown. The structural models were generated using the I-TASSER server for protein 3D structure prediction [268].

2.3.2 The Vpu34 and EnvG367E mutations rescue HIV-1 replication in IFITM1-expressing SupT1 cells

We next asked which of these five mutations are sufficient to rescue the IFITM1 inhibition of HIV-1. Since Vpu28 and Vpu34 mutations both resulted in truncation of Vpu, and Vpu34 was seen in 5 out of 7 viral DNA clones, we chose to further characterize Vpu34 by performing the following experiments. We inserted Vpu34, EnvR311K, EnvG367E and g7178a into the HIV-1 proviral DNA either individually or in different combinations, and generated 11 mutated HIV-1 DNA clones (Table 2.3.1). We then transfected these viral DNA clones into HEK293T cells to produce viruses, and used viruses of the same viral p24 amounts to infect SupT1 cells that were induced with doxycycline to express IFITM1. Infection experiments were also performed without doxycycline as the control for the IFITM1-negative condition. In the absence of IFITM1 expression, HIV-1(Mut4) (containing Vpu34, EnvR311K, EnvG367E and g7178a mutations), Vpu34/EnvG367E, and Vpu34/EnvR311K/G367E grew slightly faster than the wild type virus and other viruses (Fig. 2.3.2). With IFITM1 expression, viruses HIV-1(Mut4), Vpu34/EnvR311K/G367E, and Vpu34/EnvG367E exhibited much robust replication, in strong comparison to the wild type and other mutants which were inhibited by IFITM1 (Fig. 2.3.2). We thus conclude that the Vpu34 and EnvG367E mutations together are sufficient to overcome IFITM1 inhibition, and that EnvR311K and g7178a do not play an important role in this regard.

The Vpu34 mutation allows the expression of the first 34 amino acids of Vpu (Fig. 2.3.1 C). In order to test whether this truncated form of Vpu contributes to the rescue of HIV-1, we mutated the translation start codon AUG of Vpu to ACG in order to

completely eliminate the Vpu expression. We inserted this Vpu(-) mutation into the wild type and the mutated HIV-1 DNA, and generated mutants HIV-1/Vpu(-), HIV-1(Mut4)/Vpu(-), Vpu34/EnvG367E/Vpu(-), and Vpu34/EnvR311K/G367E/Vpu(-). We observed that, in contrast to the complete inhibition of HIV-1/Vpu(-) by IFITM1, the other three viruses replicated efficiently (Fig.2.3.3 A). We further showed that the Vpu34 mutation was sufficient to block the downregulation of cell surface CD4 by HIV-1 (Fig. 2.3.3 B). Therefore, the loss of Vpu expression, but not the expression of the first 34 amino acids of Vpu, enables the virus to escape IFITM1 inhibition.

		Mutations			
		Vpu34	R311K	G367E	g7178a
HIV-1 DNA clones	HIV-1(Mut4)	√	√	√	√
	Vpu34	√			
	EnvR311K		√		
	EnvG367E			√	
	Envg7178a				√
	EnvR311KG367E		√	√	
	Vpu34/EnvR311K	√	√		
	Vpu34/EnvG367E	√		√	
	Vpu34/Env/g7178a	√			√
	EnvR311K/G367E/g7178a		√	√	√
	Vpu34/EnvR311K/G367E	√	√	√	

Table 2.3.1: The mutated HIV-1 clones

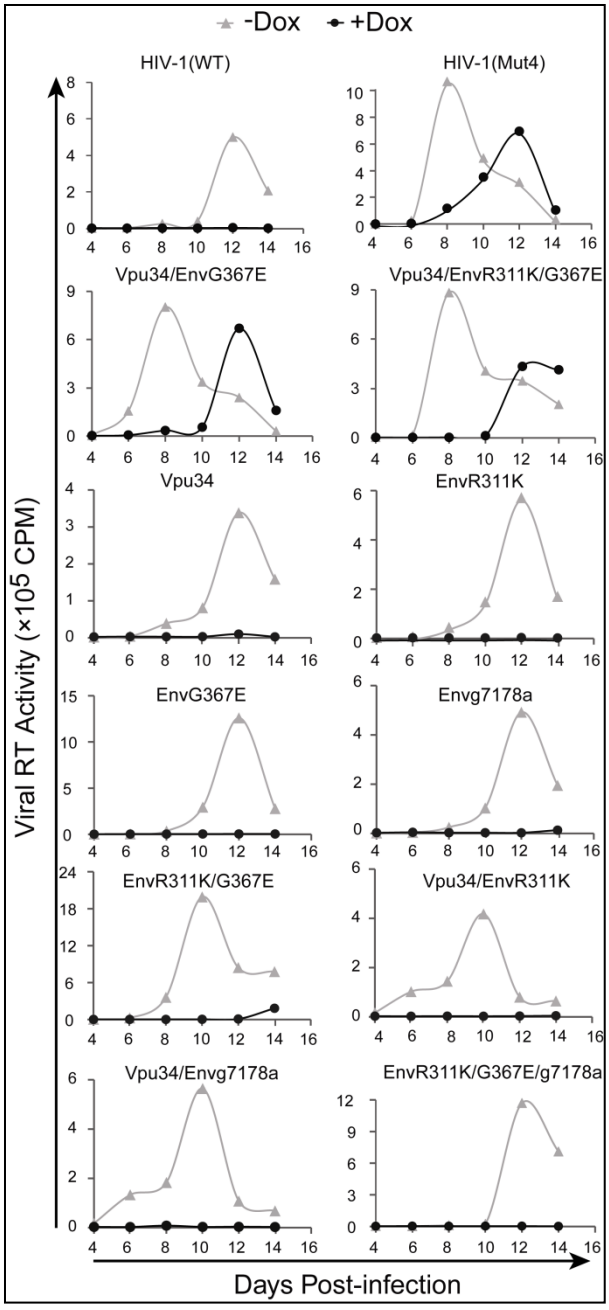


Figure 2.3.2: Mutations of Vpu34 and EnvG367E rescue HIV-1 replication in IFITM1-expressing SupT1 cells.

The mutations Vpu34, EnvR311K, EnvG367E and g7178a were inserted into HIV-1 DNA either individually or in different combinations. The HIV-1(Mut4) virus contains all four mutations. These viruses were used to infect SupT1 cells with or without IFITM1 induction by doxycycline. Viral replication was determined by measuring levels of viral reverse transcriptase activity in culture supernatants. Results shown represent three independent infection experiments

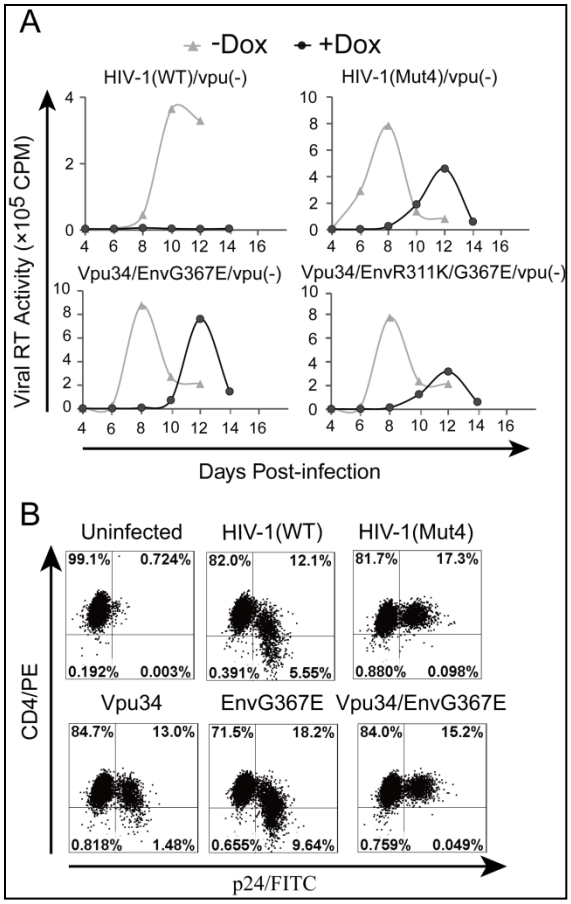
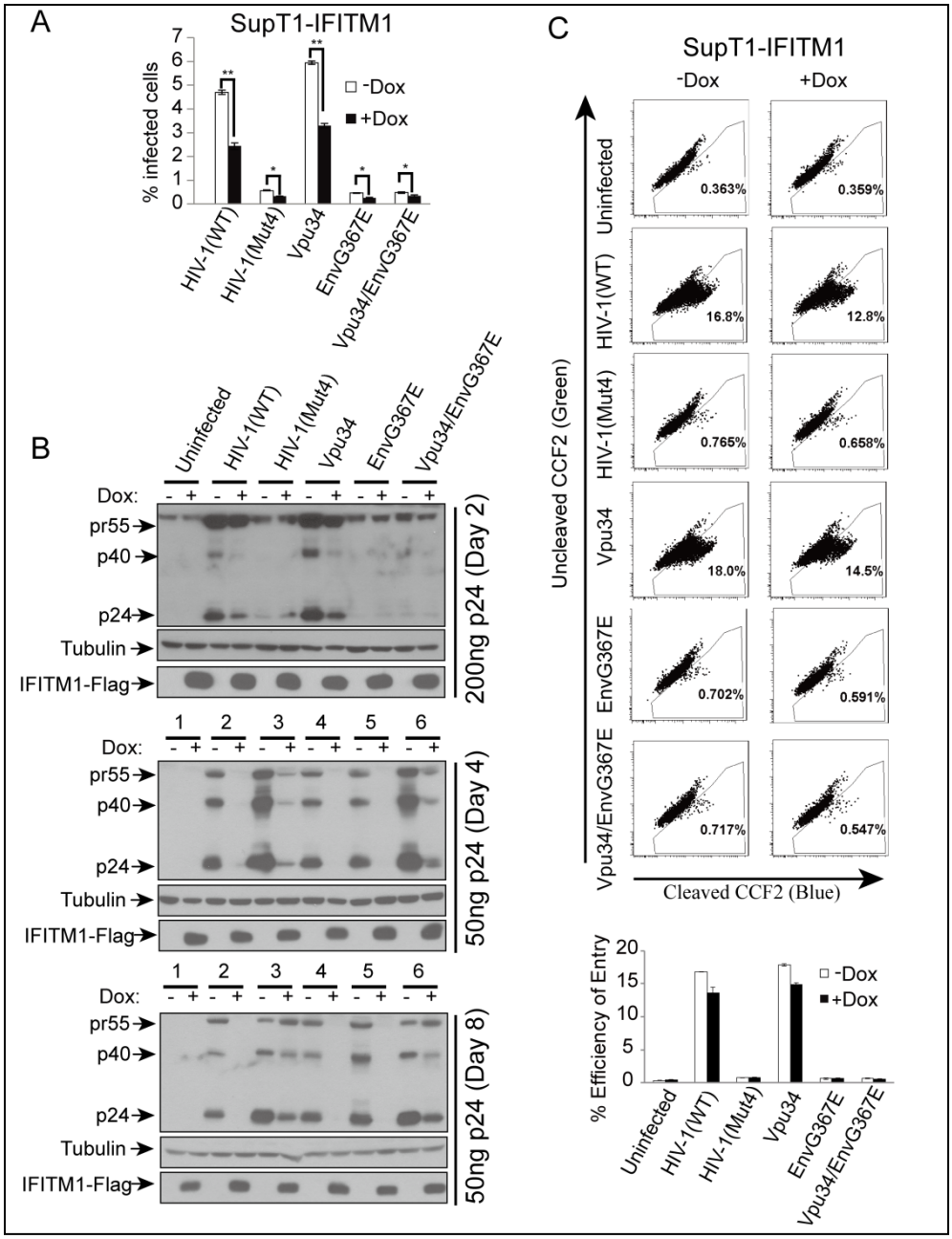


Figure 2.3.3: Deleting Vpu assists HIV-1 to escape IFITM1 inhibition.
(A) Mutating the translation initiation codon ATG of Vpu to ACG does not affect the ability of Vpu34 mutation to rescue HIV-1 replication in IFITM1-expressing SupT1 cells. The first ATG of Vpu was changed to ACG, which is named Vpu(-). This Vpu(-) mutation was inserted into viral DNA clones HIV-1(Mut4), Vpu34/EnvG367E, and Vpu34/R311K/EnvG367E such that the expression of the first 34 amino acids of Vpu in these viruses are eliminated. Virus replication in SupT1 cells, with or without IFITM1 induction, was monitored by measuring levels of viral reverse transcriptase activity in culture supernatants. One representative result of three independent infections is shown**.** (B) The Vpu34 mutation abolishes the ability of HIV-1 to downregulate cell surface CD4. The wild type HIV-1 and its mutants were used to infect SupT1 cells. Forty hours after infection, cells were stained with PE-conjugated anti-CD4 antibody and FITC-conjugated anti-p24 antibody followed by flow cytometry analysis. Results shown represent three independent experiments.

2.3.3 The Vpu34 and EnvG367E mutations do not correct the defect in HIV-1 p24 expression caused by IFITM1.

We next performed short-term infection to investigate the rescue mechanism conferred by Vpu34 and EnvG367E. We have previously shown that IFITM1 inhibits the expression of HIV-1 p24 [203], an observation that is further supported by the study of Chutiwitoonchai et al [229]. To test whether the Vpu34 and EnvG367E mutations can overcome this defect, SupT1 cells were infected with the same amounts of wild type and mutated HIV-1 particles, and viral Gag/p24 expression was measured by staining cells with FITC-conjugated anti-p24 antibody. In the absence of IFITM1 expression, viruses HIV-1(Mut4), Vpu34/EnvG367E and EnvG367E generated 10-fold fewer p24-positive SupT1 cells than the wild type virus and the Vpu34 mutant (Fig. 2.3.4 A). This observation was confirmed by the results of Western blots measuring cell-associated HIV-1 Gag and p24 (Fig. 4B, 200 ng p24 virus infection). This deficiency is likely a result of the impaired virus entry, since results of HIV-1 virion fusion experiments showed that the HIV-1(Mut4), Vpu34/EnvG367E and EnvG367E mutants were more than 10 fold less efficient in entering cells than the wild type and the Vp34 mutant (Fig. 2.3.4 C). Given that HIV-1(Mut4), Vpu34/EnvG367E and EnvG367E all bear the EnvG367E mutation, we suspected that this envelope protein mutation likely have dramatically diminished HIV-1 infectivity. Supporting this speculation, we found that this defect in infection was fully corrected when the wild type and mutated viruses carried the VSV G protein that is not affected by IFITM1 [207] (Fig. 2.3.4 D). Interestingly, when IFITM1 was induced to express, both the wild type and the mutated viruses either bearing HIV-1 envelope or VSV G protein, still gave 50% fewer p24-positive SupT1 cells (Fig.

2.3.4 A, D). This decrease is more pronounced when levels of cell-associated HIV-1 p24 were measured by Western blotting, and this decrease persisted until later time points of infection such as day 4 and day 8 (Fig. 2.3.4 B). In addition, the results of Western blots showed decreased ratio of p24 to pr55 in IFITM1-expressing cells (Fig. 2.3.4 E), which indicates a defect in Gag processing. These defects were further reflected by a similar reduction in virus particles that were released into the supernatants (Fig. 2.3.4 F). Therefore, the Vpu34 and EnvG367E mutations are unable to repair the defect in viral p24 expression caused by IFITM1, suggesting that they must have rescued HIV-1 replication in IFITM1-expressing SupT1 cells by a new mechanism.


A
SupT1-IFITM1
% infected cells
-Dox
+Dox
HIV-1(WT)
HIV-1(Mut4)
Vpu34
EnvG367E
Vpu34/EnvG367E
B
Uninfected
HIV-1(WT)
HIV-1(Mut4)
Vpu34
EnvG367E
Vpu34/EnvG367E
Dox:
pr55
p40
p24
Tubulin
IFITM1-Flag
200ng p24 (Day 2)
50ng p24 (Day 4)
50ng p24 (Day 8)
C
SupT1-IFITM1
-Dox
+Dox
Uninfected
HIV-1(WT)
HIV-1(Mut4)
Vpu34
EnvG367E
Vpu34/EnvG367E
0.363%
0.359%
16.8%
12.8%
0.765%
0.658%
18.0%
14.5%
0.702%
0.591%
0.717%
0.547%
Uncleaved CCF2 (Green)
Cleaved CCF2 (Blue)
% Efficiency of Entry

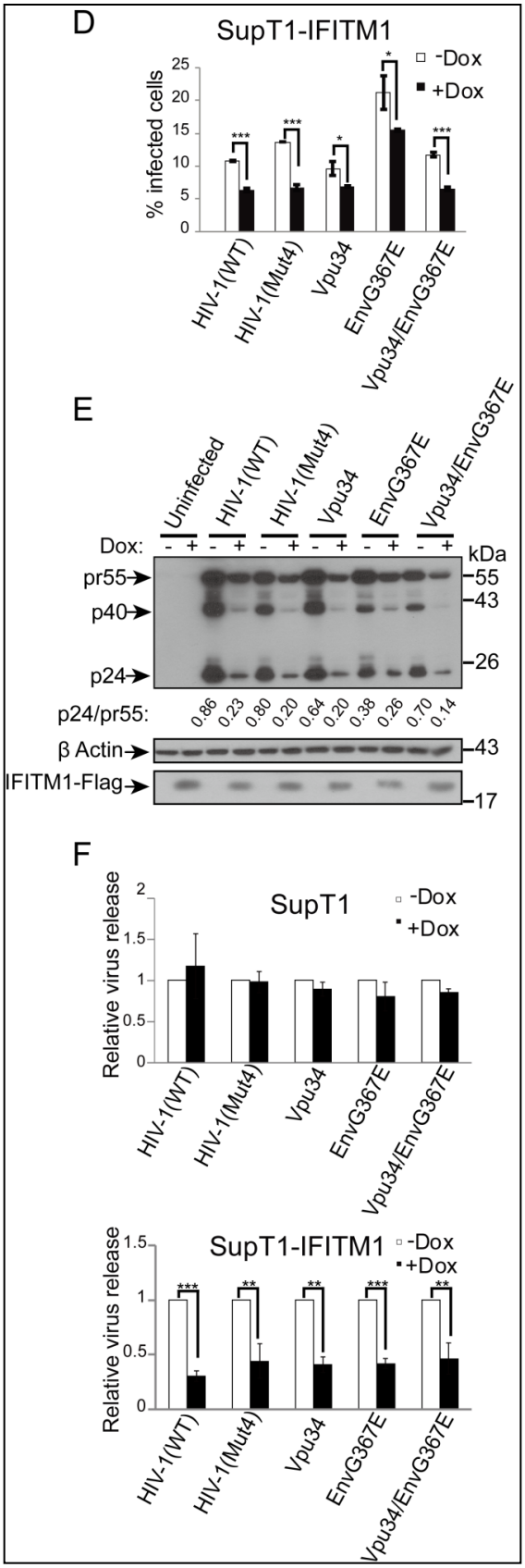

D
SupT1-IFITM1
% infected cells
-Dox
+Dox
HIV-1(WT)
HIV-1(Mut4)
Vpu34
EnvG367E
Vpu34/EnvG367E
E
Uninfected
Dox:
kDa
pr55
p40
p24
55
43
26
p24/pr55:
0.86 0.23 0.80 0.20 0.64 0.20 0.38 0.26 0.70 0.14
β Actin
IFITM1-Flag
17
F
SupT1
Relative virus release
SupT1-IFITM1

Figure 2.3.4: The Vpu34 and EnvG367E mutations do not correct the defect caused by IFITM1 in the short-term infection of HIV-1.
(A) The wild type and mutant viruses were used to infect SupT1 cells with or without IFITM1 induction. Forty hours after infection, the infected cells were stained with FITC-conjugated anti-p24 antibody and scored by flow cytometry. Results of three independent infections are summarized in the bar graph. The p values were calculated and the significance is indicated by * (<0.05) and ** (<0.01). (B) The infected cells were collected 2, 4 and 8 days after infection. Levels of HIV-1 Gag/p24 were examined in Western blots. (C) The cell entry efficiency of wild type and mutant viruses were examined by Blam-Vpr virion fusion assay. The cleavage of CCF2/AM by Blam-Vpr was measured by flow cytometry. Results of three independent infections are summarized in the bar graph. (D) The wild type and mutant HIV-1 were pseudotyped with VSV G protein and used to infect SupT1 cells with or without IFITM1 induction. Forty hours after infection, the infected cells were stained with FITC-conjugated anti-p24 antibody and scored by flow cytometry. Results of three independent infections are summarized in the bar graph. The p values were calculated and the significance is indicated by * (<0.05) and *** (<0/001). (E) Levels of viral Gag/p24 expression in the infected cells were determined by Western blotting. The intensities of pr55 and p24 protein bands were determined with the Image J software (NIH). The ratios of p24 to pr55 were calculated and shown below the Western blot. (F) Amounts of viruses in the culture supernatants were determined by measuring viral reverse transcriptase activity. Virus amount that was produced by the wild type HIV-1 in the absence of doxycycline induction is arbitrarily set as 1. Results shown are the averages of three independent infections. The p values were calculated and the significance is indicated by ** (<0.01) and *** (0.001).

2.3.4 The EnvG367E mutation impairs the usage of CD4 receptor

It is not surprising that the EnvG367E mutant is poorly infectious because the EnvG367E mutation alters the conserved G367 amino acid at the CD4-binding site (Fig. 2.3.1D). Such a mutation is expected to diminish the affinity of envelope for CD4. Indeed, we observed that as little as 0.1 μg/ml of soluble CD4 (sCD4(D1/D4) was able to reduce the infection of wild type HIV-1 and the Vpu34 virus by 10-fold, as opposed to less than 30% decrease for the EnvG367E, Vpu34/EnvG367E and HIV-1(Mut4) viruses (Fig. 2.3.5 A). We further tested the usage of CD4 receptor using an antibody named VRC03 that recognizes the CD4-binding site on gp120 [269, 270]. Again, viruses HIV-1(Mut4), EnvG367E and Vpu34/EnvG367E, which all carry the EnvG367E mutation, exhibited greater resistance to VRC03 inhibition than the wild type virus (Fig. 2.3.5 B).

We speculated that the diminished usage of CD4 by the EnvG367E mutant may render the virus more sensitive to the cell surface CD4 level. To test this possibility, we first used the CD4 antibody SIM4 to block cell surface CD4 and then measured its effect on HIV-1 infection. The results showed a much greater inhibition of the Vpu34/EnvG367E and EnvG367E viruses as compared to the wild-type HIV-1 and the Vpu34 mutant (Fig. 2.3.5 C). Next, we used shRNA to knock down CD4 expression in SupT1 cells. Out of the four shRNA clones tested, one clone diminished CD4 level by approximately 40% (Fig. 2.3.5 D), the other three did not significantly affect CD4 expression (data not shown). This CD4-knockdown SupT1 cell line was then infected by either the wild type HIV-1 or the HIV-1(Mut4) virus. Compared to their replication in the control SupT1 cells, the growth of HIV-1(Mut4), but not the wild type HIV-1, was delayed in the CD4-knockdown cells (Fig. 2.3.5 D). Results of Figure 5E and 5F further showed that the

Vpu34 and EnvG367E mutations did not affect the usage of CXCR4 as the co-receptor, since all mutated viruses were as sensitive as the wild type virus to the inhibition by the CXCR4 antagonist AMD3100 (Fig. 2.3.5 E), and none was inhibited by the CCR5 antagonist maraviroc (Fig. 2.3.5 F). These results suggest a deficient engagement of CD4 by the mutated viral envelope EnvG367E.

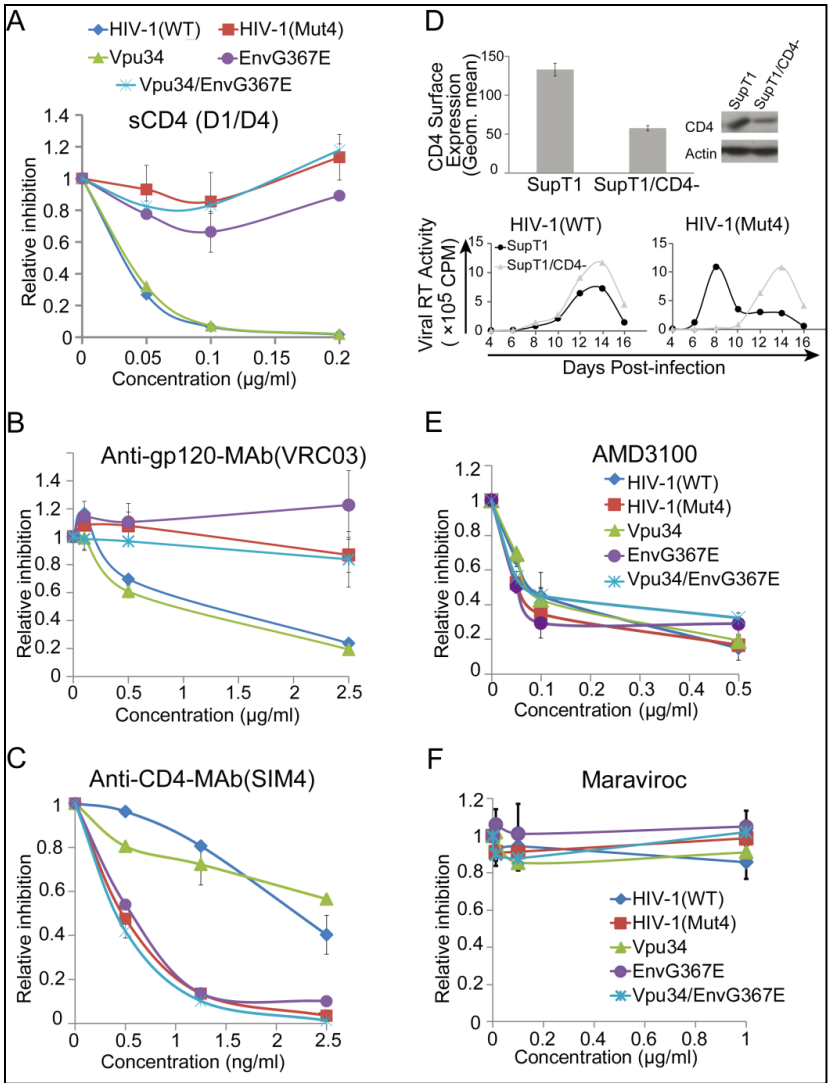


Figure 2.3.5: The EnvG367E mutation diminishes the usage of CD4 receptor.
(A) Same amounts of wild type or mutated HIV-1 were used to infect the CEM-Rev-Luc indicator cells in the presence of increasing amounts of soluble CD4 (sCD4). Virus infection was determined by measuring levels of luciferase activity in the infected CEM cells. Infection by each virus without sCD4 is arbitrarily set as 1. Results are the averages of three independent infections. (B) Sensitivity of the wild type and HIV-1 mutants to the inhibition by the VRC03 antibody that recognizes the CD4-binding site on gp120. (C) Inhibition of the wild type and mutated viruses by the anti-CD4 antibody SIM4. (D) Knockdown of CD4 delays the replication of HIV-1(Mut4). The shRNA targeting CD4 mRNA was used to create a stable SupT1 cell line. The cell surface level of CD4 was determined by staining with anti-CD4 antibody followed by flow cytometry, the result is presented in the bar graph. The total amount of CD4 was assessed by Western blotting. Replication of the wild type and the HIV-1(Mut4) viruses was examined in the CD4-knockdown SupT1 cells and the control SupT1 cells by measuring levels of viral reverse transcriptase. (E) Inhibition of the wild type and mutated viruses by the CXCR4 antagonist AMD3100. (F) Sensitivity of the wild type and mutated viruses to the CCR5 antagonist maraviroc.

2.3.5 The Vpu34 and EnvG367E mutations enhance HIV-1 cell-to-cell transmission.

It is intriguing that the Vpu34/EnvG367E virus particles were 10-fold less infectious than the wild type HIV-1 (Fig. 2.3.4), yet this mutant replicates as efficiently as does the wild type virus in SupT1 cells (Fig. 2.3.2). We also noted that, although infection of the mutated viruses was initially impaired as shown by the results of Western blotting at day 2 after infection, the HIV-1(Mut4) and Vpu34/EnvG367E mutant produced similar levels of viral p24 as compared to wild type HIV-1 at day 4 and day 8 after infection (Fig. 2.3.4 B). Given that cell-to-cell transmission of HIV-1 is known to dominate virus spread over free-virus infection [271], it is possible that the Vpu34/EnvG367E mutant compensates for its reduced infectivity by enhancing cell-to-cell transmission. To test this, we first infected the donor SupT1 cells with the wild type or mutated HIV-1 particles bearing the VSV-G protein, ensuring that both wild type and mutated HIV-1 would generate similar numbers of infected donor cells. Forty hours after infection, donor cells were mixed with target SupT1 cells that were labeled with a cell tracker BMQC, and viral p24 was examined with FITC-conjugated anti-p24 antibody. In order to distinguish cell-to-cell transmission from free virus particles, we used trans-wells to separate donor cells from target cells. As would be expected, few p24-positive cells were detected in the trans-well infection (Fig. 2.3.6 A), indicating that free HIV-1 particles did not lead to significant infection of target cells. We then added HIV-1 fusion inhibitor T-20 into the mixed cell population and did not observe significant infection of target cells (Fig. 2.3.6 B), confirming that the cell-to-cell virus transmission shown in Figure 6C is viral envelope-dependent. When the control SupT1 cells (without IFITM1 expression) were used as the target cells in the transmission assay, the HIV-1(Mut4) and Vpu34/EnvG367E mutants

were 3 to 4 fold more efficient at cell-to-cell transmission than the wild type virus or the Vpu34 and EnvG367E mutants (Fig. 2.3.6 C). We next used IFITM1-expressing SupT1 cells as target cells, and observed that IFITM1 did not markedly affect HIV-1 cell-to-cell transmission (Fig. 2.3.6 D). Again, Vpu34 and EnvG367E mutations together enhanced the transmission of viruses from donor cells to IFITM1-expressing target cells (Fig. 2.3.6 D). In order to validate these data, we measured the amount of HIV-1 late cDNA in the mixed of donor and target SupT1 cells at time 0 and 8 hours after mixing. The increases of HIV-1 late cDNA over a 8-hour period of co-culture are 4.6 and 3.7 folds for HIV-1(Mut4) and Vpu34/EnvG367E, but are less than 2-fold for wild type HIV-1, Vpu34 and Env367E (Fig. 2.3.6 F), which suggests a much higher cell to cell transmission efficiency for HIV-1(Mut4) and Vpu34/EnvG367E. Therefore, we conclude that the Vpu34 and EnvG367E mutations rescue HIV-1 replication in IFITM1-expressing SupT1 cells through promoting cell-to-cell virus transmission.

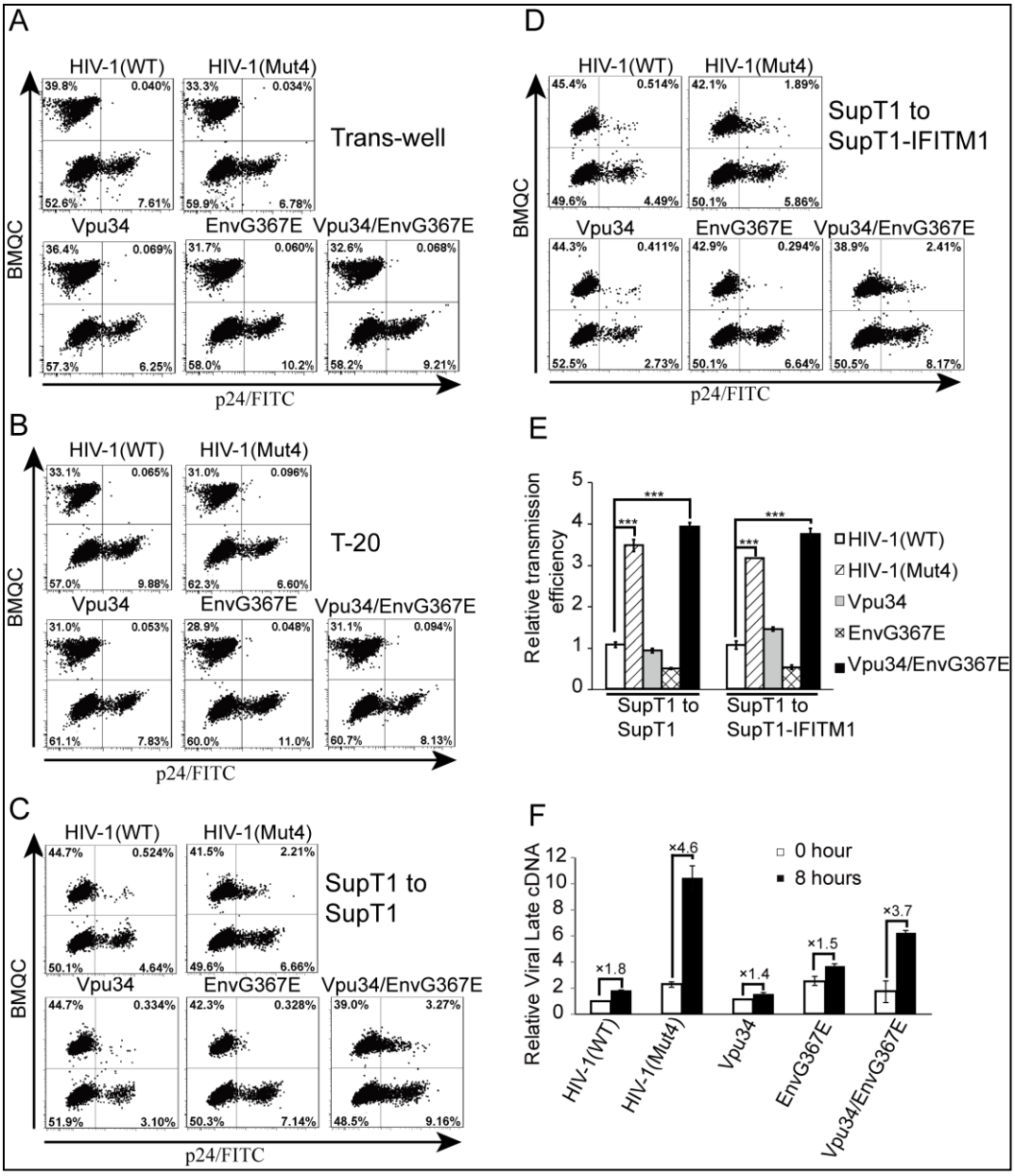


Figure 2.3.6: The Vpu34 and EnvG367E mutations enhance HIV-1 transmission between SupT1 cells.

SupT1 cells were first infected with HIV-1 or its mutants (equivalent to 50 ng of viral p24) for forty hours before being washed with complete RPMI1640 media and used in the following experiments. (A) The HIV-1 infected donor cells and the BMQC-labeled target cells were separated by trans-wells that allow free virus infection. The infected cells were harvested 8 hours after and stained with FITC-conjugated anti-p24 antibody and scored by flow cytometry. (B) HIV-1 fusion inhibitor T-20 was added to the mixture of donor and target cells to block HIV-1 cell-to-cell transmission. Cells were harvested 8 hours after incubation and stained for viral p24. (C) SupT1 cells were first infected with the wild type or mutated viruses before mixing with the BMQC-labeled target SupT1 cells. After 8 hours, the infected target cells were stained with FITC-conjugated anti-p24

antibody and scored by flow cytometry. (D) The infected SupT1 cells were mixed with the BMQC-labeled IFITM1-expressing SupT1 cells to assess the effect of IFITM1 on HIV-1 cell-to-cell transmission. (E) The relative transmission efficiency was calculated for each experiment with the value of wild type HIV-1 transmission arbitrarily set as 1. The results of three independent transmission experiments are averaged and shown in the bar graph. The p values were calculated and the significance is indicated by *** (<0.001). (F) Cell to cell transmission of the wild type and mutant viruses was determined by measuring viral late cDNA amounts. The donor and target cells were collected before mixing (time 0) and 8 hours after mixing. Total DNA was extracted and HIV-1 late cDNA was measured by real-time PCR. The GAPDH DNA was also quantified by real-time PCR and the results serve as the internal control for each sample. The amount of late DNA of the wild type HIV-1 at time 0 is arbitrarily set as 1. The results shown are the average of three independent infections.

2.3.6 The effect of Vpu34 and EnvG367E on cell-to-cell transmission is cell type-specific.

Since the IFITM1-resistant mutations arose in SupT1 cells by selection, we wished to examine the replication capacity of the escape virus in other CD4+ T cell lines. Surprisingly, in contrast to its higher than wild type level replication in SupT1 cells, the HIV-1(Mut4) mutant, which carried all escape mutations, was unable to grow in CEM, Jurkat, and PM-1 cells (Fig. 2.3.7 A), suggesting that this escape mutant has adapted to SupT1 cells for efficient replication. We noticed that SupT1 cells express relatively higher level of CD4 than CEM, Jurkat and PM-1 cells (Fig. 2.3.7 B), which may partially underlie the much more efficient replication of EnvG367E-containing HIV-1 in SupT1 cells.

We next examined the cell-to-cell transmission efficiency of HIV-1(Mut4) between CEM cells, and found that HIV-1(Mut4) was unable to spread, consistent with its inability to grow in CEM cells (Fig. 2.3.8 A). This transmission deficiency was caused by the EnvG367E mutation, because both EnvG367E and Vpu34/EnvG367E were incompetent in cell-to-cell transmission, yet the Vpu34 mutant spread more efficiently than the wild type virus (Fig. 2.3.8 A). To dissect whether this transmission deficiency is

of donor or target cell-effect, we performed additional cell-to-cell transmission assays using SupT1 as donor and CEM cells as target cells, or vice versa. The results showed that the HIV-1(Mut4), EnvG367E, and Vpu34/EnvG367E mutants transmitted efficiently only when SupT1 served as target cells (Fig. 2.3.8 B-D), suggesting that the target cell type determines the cell-to-cell transmission efficiency of the EnvG367E-containing HIV-1 mutants. Altogether, these data suggest that the Vpu34 and EnvG367E mutations were selected to circumvent IFITM1 restriction in a cell type-specific manner.

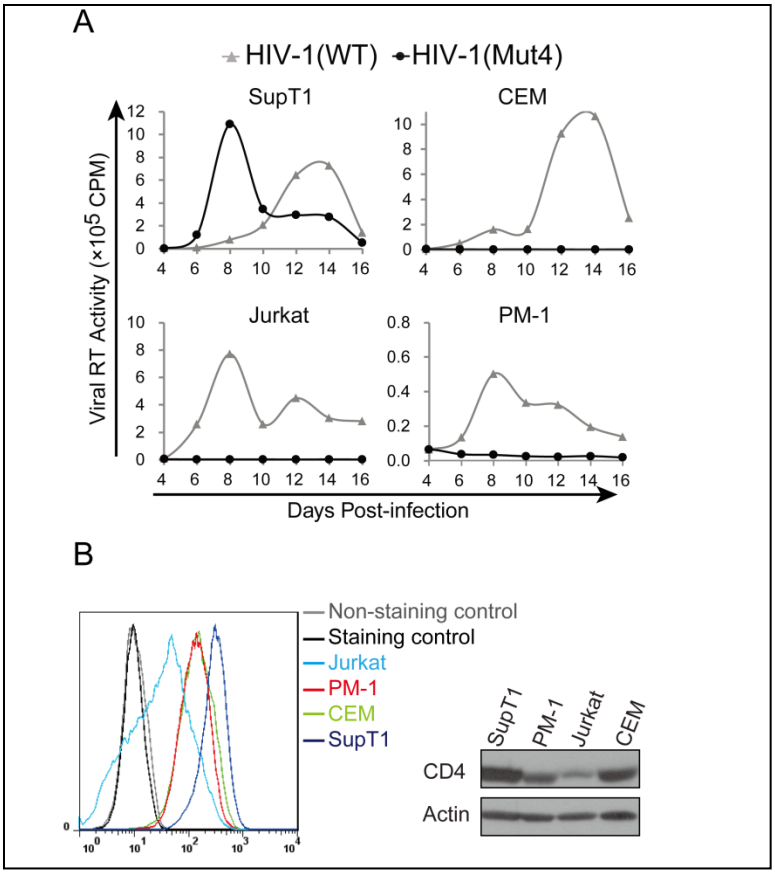


Figure 2.3.7: Replication of the wild type HIV-1 and the HIV-1(Mut4) mutant in SupT1, CEM, Jurkat and PM-1 cells.

(A) Levels of virus production were monitored by measuring viral reverse transcriptase activity in the supernatants at various time intervals. Results shown represent three independent infections. (B) Cell surface CD4 in SupT1, CEM, Jurkat and PM-1 cells was stained with anti-CD4 antibody and its level was determined by flow cytometry. The total levels of cellular CD4 were measured by Western blotting.

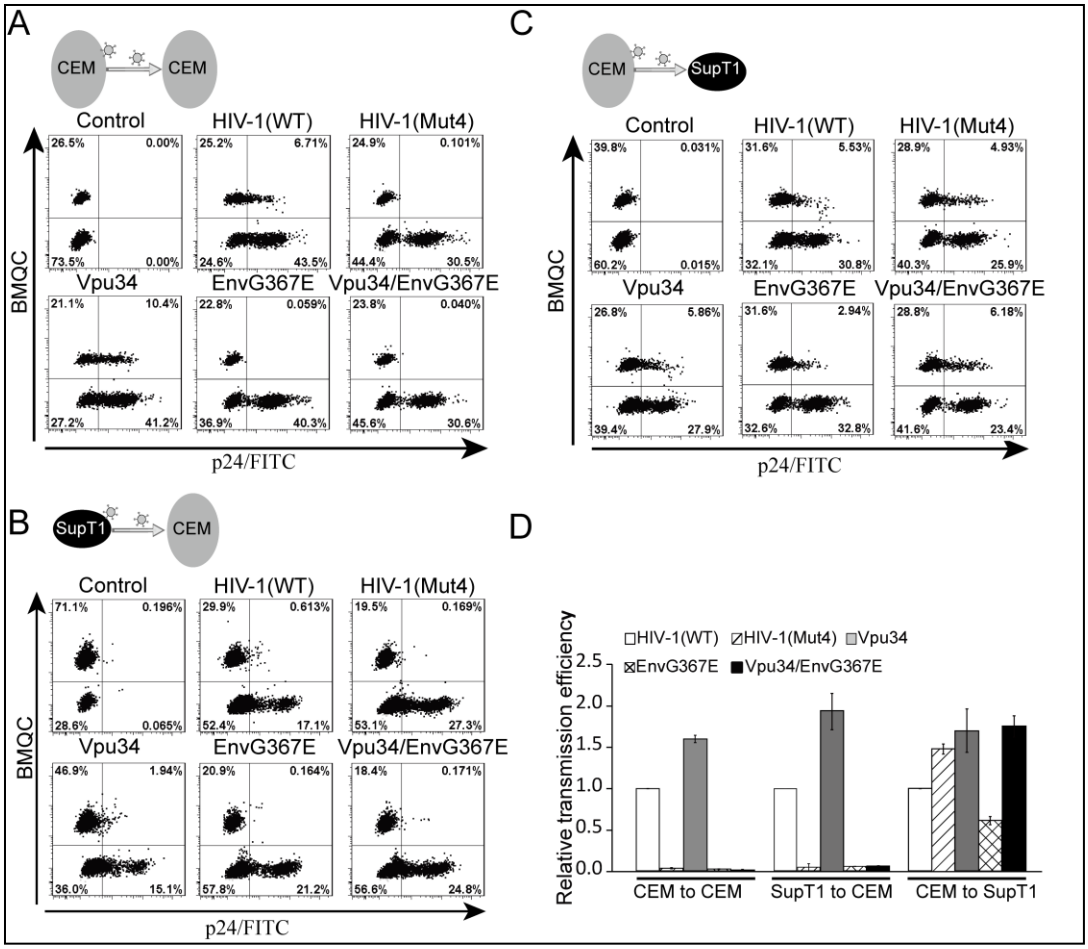


Figure 2.3.8: Cell-to-cell transmission of the HIV-1 wild type and mutants between different pairs of donor and target cells.

(A) Transmission of the wild type and mutated HIV-1 between CEM cells. (B) HIV-1 transmission from SupT1 to CEM cells. (C) HIV-1 transmission from CEM to SupT1 cells. (D) Results of three independent transmission experiments are summarized in the bar graph. The transmission efficiency of wild type HIV-1 is arbitrarily set as 1.

2.4 Discussion

In this study, we have identified two mutations Vpu34 and EnvG367E that act together to rescue HIV-1 from IFITM1 restriction. The Vpu34 mutation creates a translation stop codon at the amino acid position 35 (Fig. 2.3.1 C), resulting in the expression of the first 34 amino acids of Vpu that form the transmembrane domain. We ruled out the involvement of this 34-amino-acid Vpu fragment in antagonizing IFITM1, because eliminating the full-length Vpu expression by mutating the translation start codon AUG also enabled the EnvG367E mutation to rescue HIV-1 replication (Fig. 2.3.3). Together, our results indicate that the loss of Vpu and a mutation in Env (EnvG367E) are both critical for HIV-1 to evade IFITM1 restriction. We also detected the R311K mutation in the V3 loop of Env, but this mutation does not appear to contribute significantly to circumventing IFITM1. Nonetheless, mutating the V3 loop may represent one way for the virus to counter different inhibitory pressures. This scenario is supported by a recent study showing that feline immunodeficiency virus is able to escape from inhibition by a N-terminal fragment of TSG101 by acquiring a K410N mutation in the V3 loop of its Env protein [272].

Vpu plays two major roles in HIV-1 replication; one is to down-regulate the cell surface CD4, and another is to promote the virus release by counteracting tetherin [273]. Because the Vpu34 mutant replicated almost as efficiently as the wild type HIV-1 in SupT1 cells (Fig. 2.3.2), despite of its inability to down regulate the cell surface CD4 (Fig. 2.3.3 B), Vpu is dispensable for HIV-1 to replicate in SupT1 cells. This could result from the lack of endogenous tetherin expression in SupT1 [274], which alleviates the need for Vpu to antagonize tetherin. It is possible that, in the face of both tetherin and

IFITM1, both of which can be induced by IFN, HIV-1 would have to overcome these two restrictions by evolving pathways other than mutating Vpu alone.

Our data show that Vpu34 mutation alone is insufficient to antagonize IFITM1, it needs to act together with the EnvG367E mutation. The G367 amino acid in gp120 is highly conserved among different HIV-1 strains (HIV DATABASES, http://www.hiv.lanl.gov/content/index), as it serves as a key residue for CD4-binding (Fig. 2.3.1 D). Indeed, we found that the G367E mutation alone causes a 10-fold decrease in the infectivity of HIV-1 particles. Interestingly, this defect does not apparently affect the replication of the EnvG367E mutant in SupT1 cells, and Vpu34 and EnvG367E together enhance the viral growth in SupT1 cells as compared to the wild type virus. How can HIV-1 tolerate such a deleterious mutation as EnvG367E? One possibility is that SupT1 cells express relatively high level of CD4 (Fig. 2.3.7 B), which supports the EnvG367E mutant to efficiently transmit from cell to cell (Fig. 2.3.6). In addition, given that EnvG367E is unable to replicate in T cell lines other than SupT1, SupT1 cells may express a factor(s) that allow the replication of EnvG367E mutant.

We found that the Vpu34 and EnvG367E mutations did not overcome the defect in viral p24 expression caused by IFITM1 (Fig. 2.3.4). Instead, they enhanced the HIV-1 transmission between SupT1 cells by ~ 4-fold, indicating that the enhanced replication of these mutants in SupT1 cells are due to cell-to-cell transmission. It is possible that such a gain in cell-to-cell transmission allows HIV-1 to evade different types of restrictions. This Vpu34/EnvG367E-dependent pattern requires SupT1 cells as the target cells (Fig. 2.3.8), this suggests that certain features of the infected target cells promote the formation of virological synapse. These features may include high CD4 level at cell surface as a

result of losing Vpu and high level of the EnvG367E envelope that has a weak affinity for CD4 and therefore is less likely trapped at the ER as a result of CD4 binding. Alternatively, the Vpu34/EnvG367E mutant may be efficient in entry via cell-associated routes. It is currently unclear which of these possibilities promotes HIV-1 cell-to-cell transmission. It is interesting to note that a previous study by Gummuluru and colleagues has reported a Vpu mutation called Rap5, which is very similar to Vpu34 [275]. Rap5 was selected in a rapid turnover assay performed in Jurkat cells, resulting in a frameshift of Vpu and leading to the expression of its first 32 amino acids, which is remarkably similar to the Vpu34 mutation reported here. Importantly, Rap5 also rescued HIV-1 replication by promoting cell-to-cell spread of virus, which is related to accumulation of Rap5 viruses on the cell surface, despite that Rap5 had no apparent effect on the expression of Env. This mechanism was further supported by a later study showing that, in the absence of Vpu, tetherin promotes HIV-1 cell to cell transmission [267]. Although this virus particle retention mechanism may not operate for Vpu34 in SupT1 cells that do not express tetherin, the virus manages to evolve a second mutation G367E in envelope protein that, together with Vpu34, achieves higher transmission efficiency to escape IFITM1 inhibition. We observed that the EnvG367E virus caused a delayed cytopathogenecity during infection of SupT1 cells as compared to the wild type virus (data not shown), suggesting that the attenuated binding of Env367E to CD4 likely also diminishes cell fusion/killing and thereby allows the infected cells to produce more viruses.

IFITM1 has been shown to inhibit entry of a number of enveloped viruses, including IAV, Yellow Fever virus, SARS coronavirus, Ebola virus, etc [197, 205, 206, 221].

Interestingly, it does not restrict HIV-1 by impeding entry [203]. Instead, IFITM1 reduces HIV-1 Gag/p24 expression in virus producer cells, which results in diminished virus production. Interestingly, we found no evidence that the escape mutations would overcome this latter defect. While it is possible that the effect of IFITM1 on HIV-1 is cell type specific and virus-strain dependent [197], the identification of the escape mutations that promote HIV-1 cell-to-cell transmission, as reported here, demonstrates that HIV-1 can evolve a mechanism to evade IFITM1 restriction. We envision that similar mechanisms are also operative for other viruses that are restricted by IFITMs.

Chapter 3 – The envelope protein of HIV-1_{NL4-3} confers resistance to IFITM1

Preface

We observed that HIV-1_{BH10} was dramatically inhibited by IFITM1, whereas HIV-1_{NL4-3} was resistant. In order to identify the viral protein(s) mediating this resistance of HIV-1_{NL4-3} against IFITM1, we compared the protein sequences of HIV-1_{BH10} and HIV-1_{NL4-3}, and performed mutagenesis studies to identify the residues that determine this difference in susceptibility to IFITM1 inhibition between these two HIV-1 strains. The results showed that the viral envelope proteins account for this difference. Furthermore, we found that HIV-1_{NL4-3} envelope protein mediates higher efficiency in cell-to-cell transmission compared to HIV-1_{BH10} envelope, which may partially explain the resistance of HIV-1_{NL4-3} to IFITM1 inhibition.

3.1 Introduction

The genome of HIV-1 encodes structural proteins (Gag, Pol, Env), regulatory proteins (Tat and Rev), and accessory proteins (Vif, Vpr, Vpu and Nef). The accessory proteins have important roles in HIV-1 pathogenesis such as counteracting host restriction factors. Vpr arrests cell division at G2 phase, Nef downregulates cell surface CD4 [276] [277] [278]. Vif and Vpu counteract restriction factors APOBEC3G and tetherin (also called BST-2), respectively [152] [153] [168]. APOBEC3G is packaged into HIV-1 virus in the absence of Vif and modifies cytidine (C) to uridine (U) during viral reverse transcription, which causes hyper mutation in HIV-1 genome [146] [147] [148]. HIV-1 Vif binds to APOBEC3G and recruits the E3 ubiquitin ligase complex to ubiquitinate and degrade APOBEC3G [152] [153]. Tetherin tethers viral particles on the cell surface and prevents the release of newly generated virus particles by its GPI anchor and transmembrane region (TM) (reviewed in [164]). HIV-1 Vpu antagonizes tetherin by downmodulating tetherin from cell surface [168]. Other viruses have also evolved viral antagonists to counteract host restriction factors. For example, HIV-2 and some SIV strains have Vpx to antagonize SAMHD1 [160], Kaposi-Sarcoma Herpesvirus (KSHV) uses K5 protein to bind to tetherin and degrade it [169]. Viral structural proteins such as envelope protein have been reported to counteract host restriction factors. For example, Ebola and HIV-2 envelope proteins can overcome BST-2 restriction [170] [171] [172].

IFITM proteins inhibit a wide spectrum of viruses, including enveloped and non-enveloped viruses [197, 203, 205, 207, 222, 238, 239, 252, 253]. Among IFITM1, IFITM2 and IFITM3, IFITM3 is the most investigated with focus on restriction of

influenza A virus both *in vitro* and *in vivo* [197] [221] [226] [227]. Yet, the antiviral mechanism of IFITM3 is not completely understood [206] [230] [231]. IFITM1 restricts influenza virus to a lesser extent compared to IFITM3, whereas IFITM1 shows higher inhibition activity in the infection of Marburg virus, Ebola virus, SARS-CoV, and HIV-1 [221] [203]. The exact mechanism of IFITM1 inhibition is unknown, although it is reported that IFITM1 might change the fluidly of plasma membrane and endosome membrane and inhibits hemifusion during viral fusion process [206].

We previously reported that IFITM1 potently inhibits the replication of HIV-1_{BH10} in SupT1 cells, but it is unknown whether IFITM1 similarly inhibits other HIV-1 strains. In this study, we demonstrated the different sensitivity of two HIV-1 strains, HIV-1_{BH10} and HIV-1_{NL4-3}, to IFITM1 inhibition in SupT1 cells. Further studies revealed that envelope protein of HIV-1_{NL4-3} enables the resistance to IFITM1.

3.2 Materials and methods

3.2.1 Plasmids, cell lines and antibodies

The HIV-1_{BH10} and HIV-1_{NL4-3} proviral DNA clones were obtained from the NIH AIDS Reference and Reagent Program. The HIV-1 strains HIV-1_{BH10}/vpr+, HIV-1_{BH10}/nef+, HIV-1_{BH10}/vpr+/nef+, HIV-1_{NL4-3}/vpr+, HIV-1_{NL4-3}/nef+, Vpu40 and EnvA539V were engineered using the site-directed mutagenesis kit (Stratagene). All the restriction enzymes were purchased from Invitrogen. Dylight-649-conjugated anti-Flag antibody was purchased from Rockland, FITC-conjugated anti-HIV-p24 antibody from Beckman. G418 was purchased from Invitrogen, puromycin and doxycycline from Sigma.

3.2.2 Virus infection

HIV-1 stocks were produced by transfection of human embryonic kidney cell line (HEK293T) with HIV-1 proviral DNA (wild type and mutated). Culture supernatants were clarified by passing through the 0.2 μm filter (VWR) to remove the cell debris. The amounts of viruses were determined by measuring viral p24 (CA) levels using the HIV-1 p24 Antigen Capture Assay kit (Cat. 5447, ABL Inc.).

Virus infection was measured in three assays. 1) Infect the TZM-bl indicator cells that express CD4/CXCR4/CCR5 and contain the HIV-1 LTR-Luc reporter [264]. TZM-bl cells were first seeded into 24-well plates (4 $X10^4$ cells/well) one day before virus infection. Forty hours after infection, cells were lysed with 1 X passive lysis buffer (Promega) and the levels of luciferase activity were measured using the luciferase assay kit (Promega). 2) Short-term infection of the SupT1 cells. SupT1 cells were first exposed to virus equivalent to 200 ng viral p24 antigen. Forty hours after infection, the infected cells were washed with cold 1 X phosphate-buffered saline and fixed with 1% paraformaldehyde. The infected cells were stained with FITC-conjugated anti-HIV-1 p24 antibody and scored by flow cytometry. 3) Long-term infection of SupT1 cells. SupT1 cells were infected with virus equivalent to 10 ng viral p24. Viral replication was monitored by measuring levels of viral reverse transcriptase activity in supernatants over various time intervals.

3.2.3 Co-transfection of IFITM1 and HIV-1 DNA

HEK293T cells were plated in 6-well plates (0.5×10^6 cells/well) 24 hours before transfection. Then an amount of 200ng plasmid of wild type or mutated HIV-1 DNA and 0, 50, 100 or 200ng IFITM1 plasmids were co-transfected into the cells. Viruses were harvested 48 hours after transfection. Virus amounts in supernatants were determined by

measuring the RT activity or by infecting the TZM-bl indicator cells.

3.2.4 Cell-to-cell transmission assay

SupT1 cells were first infected with HIV-1 particles bearing VSV-G protein. VSV-G protein was used to effectively increase the infection rate of donor cells. The subsequent virus transmission from donor cells to target cells was mediated by HIV-1 Env. Forty hours post infection, the infected cells (designated as donor cells) were washed with complete medium prior to mixing with un-infected SupT1 cells (designated as target cells) that had been labeled with cell tracker BMQC (Invitrogen). After 8 hours, the cell mixtures were fixed with 1% paraformaldehyde and permealized with 0.1% Triton X-100. After staining with FITC-conjugated anti-HIV-1 p24 antibody, the p24-positive donor and target cells were scored by flow cytometry. As described in Chapter 2, in order to control for the infection by free HIV-1 particles, infections were also set up with trans-wells that separated the donor from target cells but allowed the free movement of HIV-1 particles. In addition, we performed control experiments by adding the HIV-1 fusion inhibitor T-20 (2 mM, obtained from the NIH AIDS Reference and Reagent Program) to the mixed donor and target cells in order to block HIV-1 envelope-dependent cell-to-cell fusion.

3.3 Results

3.3.1 HIV-1_{BH10}, but not HIV-1_{NL4-3}, is inhibited by IFITM1.

We previously reported that IFITM1 inhibits the replication of HIV-1_{BH10} and that IFITM1 does not affect the entry of HIV-1_{BH10} [203]. We then extended to test whether IFITM1 also inhibits the replication of other HIV-1 strains such as HIV-1_{NL4-3}. To this

end, we infected the IFITM1-expressing SupT1 cells with HIV-1_{NL4-3} or HIV-1_{BH10} equivalent to 10 ng viral p24 and monitored virus replication by measuring levels of viral reverse transcriptase activity. The results showed strong suppression of HIV-1_{BH10} replication by IFITM1; however, HIV-1_{NL4-3} replicated to similar levels in both the IFITM1-expressing SupT1 cells and control cells (Figure 3.3.1).

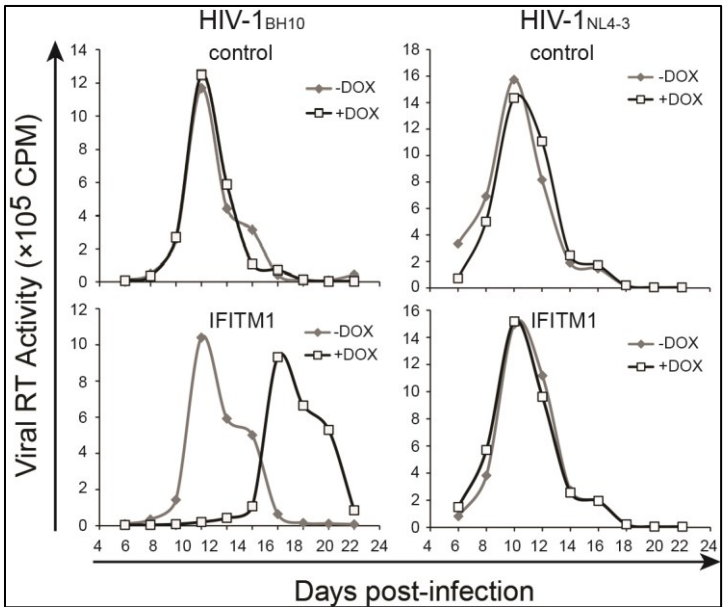


Figure 3.3.1: HIV-1_{BH10}, but not HIV-1_{NL4-3}, is inhibited by IFITM1.
Wild type HIV-1_{BH10} and HIV-1_{NL4-3} viruses were used to infect SupT1 cells with or without IFITM1 induction by doxycycline. Viral replication was determined by measuring the levels of viral reverse transcriptase activity in culture supernatants harvested every 2 days. Results shown represent three independent infection experiments.

3.3.2 The Vpr and Nef proteins do not have a role in overcoming IFITM1.

Next we wished to determine which viral protein(s) allowed HIV-1_{NL4-3} resistance of IFITM1 in SupT1 cells. We first tested the Vpr and Nef proteins because HIV-1_{BH10} expresses truncated Vpr and Nef whereas HIV-1_{NL4-3} codes full-length version of these two viral proteins (Figure 3.3.2 A). We therefore restored the full-length Vpr and Nef by repairing the mutations and generated constructs termed HIV-1_{BH10}/vpr+, HIV-1_{BH10}/nef+ and HIV-1_{BH10}/vpr+/nef+. We also generated HIV-1_{NL4-3} constructs that do not express

Vpr or Nef, including HIV-1_{NL4-3}/vpr- and HIV-1_{NL4-3}/nef-. We then used these mutated viruses to challenge IFITM1-expressing SupT1 cells. The results showed that viruses HIV-1_{BH10}/vpr+, HIV-1_{BH10}/nef+ and HIV-1_{BH10}/vpr+/nef+ were inhibited by IFITM1 to a degree similar to that the wild type HIV-1_{BH10} was inhibited (Figure 3.3.3 A). Consistent with this observation, mutating either Vpr or Nef did not affect the resistance of HIV-1_{NL4-3} to IFITM1 (Figure 3.3.3 A). These data suggest that Vpr and Nef are unable to counter IFITM1.

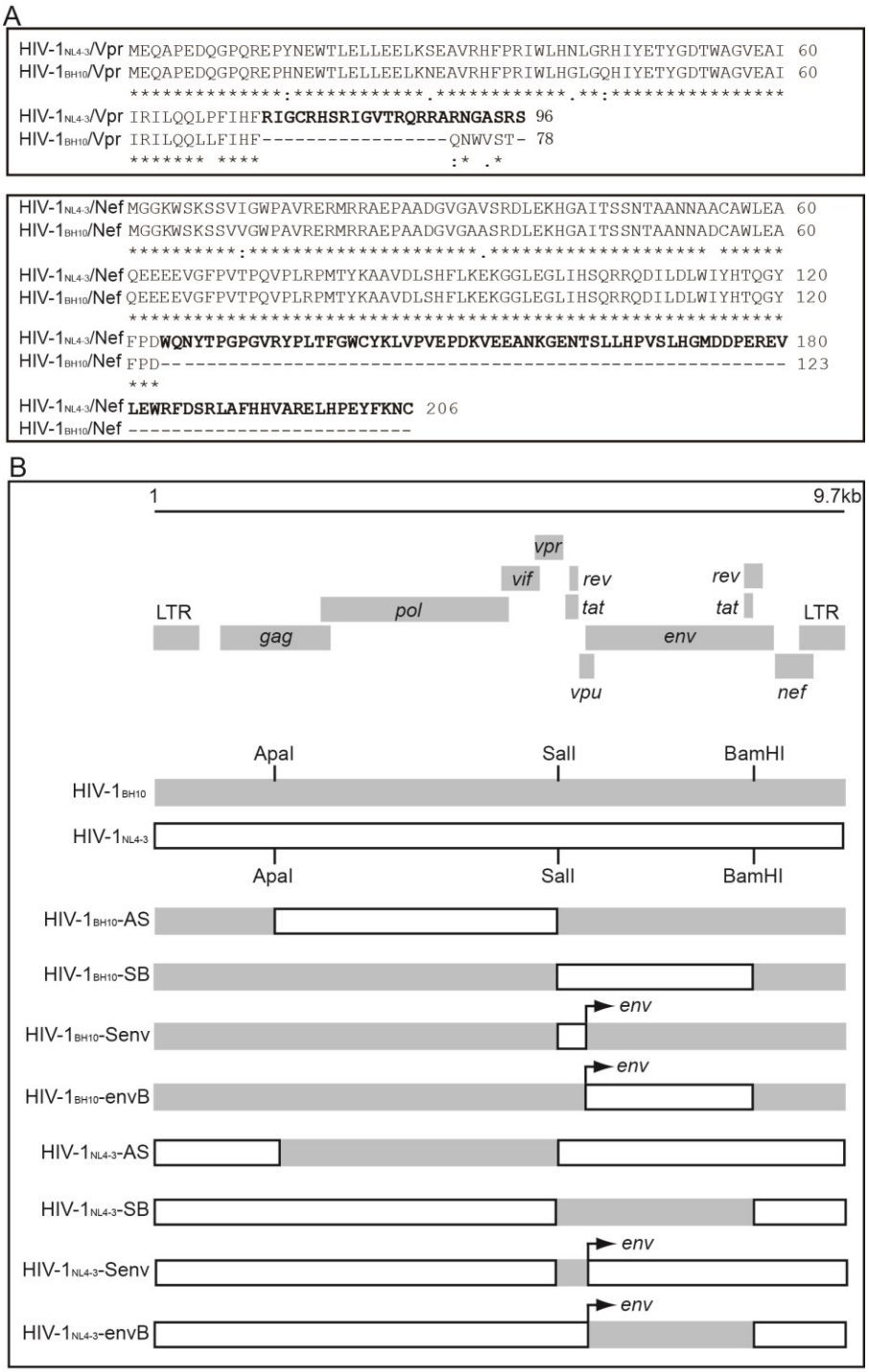


Figure 3.3.2: Differences of Vpr and Nef proteins between HIV-1_{BH10} and HIV-1_{NL4-3} and the construction of HIV-1_{BH10}/HIV-1_{NL4-3} chimeras.

(A) HIV-1_{BH10} and HIV-1_{NL4-3} are different in the expression of proteins Vpr (upper) and Nef (lower); (B) Schematic representation of HIV-1 genome and the construction of HIV-1_{BH10}/HIV-1_{NL4-3} chimeras: HIV-1_{BH10} was shown in grey bar and HIV-1_{NL4-3} in blank box, both genomes were separated by three restriction enzymes - Apa Ⅰ, Sal Ⅰ,

BamHⅠ and the start codon of *env*. Four constructs based on HIV-1_{BH10} (HIV-1_{BH10}-AS, HIV-1_{BH10}-SB, HIV-1_{BH10}-Senv and HIV-1_{BH10}-envB) were generated by having different regions – AS, SB, Senv and envB, respectively, from HIV-$1_{NL4-3;}$ on the contrary, constructs based on HIV-1_{NL4-3} (HIV-1_{NL4-3}-AS, HIV-1_{NL4-3}-SB, HIV-1_{NL4-3}-Senv, HIV-1_{NL4-3}-envB) were made by having the corresponding regions from HIV-1_{BH10}.

3.3.3 HIV-1 envelope protein modulates the susceptibility to IFITM1 restriction.

We next utilized three restriction enzyme recognition sites in HIV-1 genome to generate chimeric viruses of HIV-1_{BH10} and HIV-1_{NL4-3}. These three sites are recognized by ApaI, SalI and BamHI. The viruses thus generated were named HIV-1_{BH10}-AS, HIV-1_{BH10}-SB, HIV-1_{NL4-3}-AS, and HIV-1_{NL4-3}-SB (Figure 3.3.2 B). When these viruses were used to infect IFITM1-expressing SupT1 cells, the SB DNA fragment from HIV-1_{NL4-3} allowed HIV-1_{BH10} to become partially resistant to IFITM1 (Figure 3.3.3 A). Similarly, the same DNA fragment of HIV-1_{BH10} rendered HIV-1_{NL4-3} susceptible to IFITM1 suppression (Figure 3.3.3 B). No such effect was observed for the AS DNA fragment. The SB DNA fragment codes four viral genes, tat, vpr, vpu and env. We therefore generated four more chimeric viruses named HIV-1_{BH10}-Senv, HIV-1_{BH10}-envB, HIV-1_{NL4-3}-Senv and HIV-1_{NL4-3}-envB that contained a portion of the SB DNA sequence (Figure 3.3.2 B). The results of infection showed that the HIV-1_{BH10}-envB became partially resistant to IFITM1 and HIV-1_{NL4-3}-envB was subject to inhibition by IFITM1 (Figure 3.3.3 C). Exchanging the Senv DNA did not affect the susceptibility of either HIV-1_{BH10} or HIV-1_{NL4-3} to IFITM1 (Figure 3.3.3 C). Since the envB DNA codes for viral envelope protein, we conclude that HIV-1 envelope acts as one determinant of susceptibility to IFITM1 inhibition.

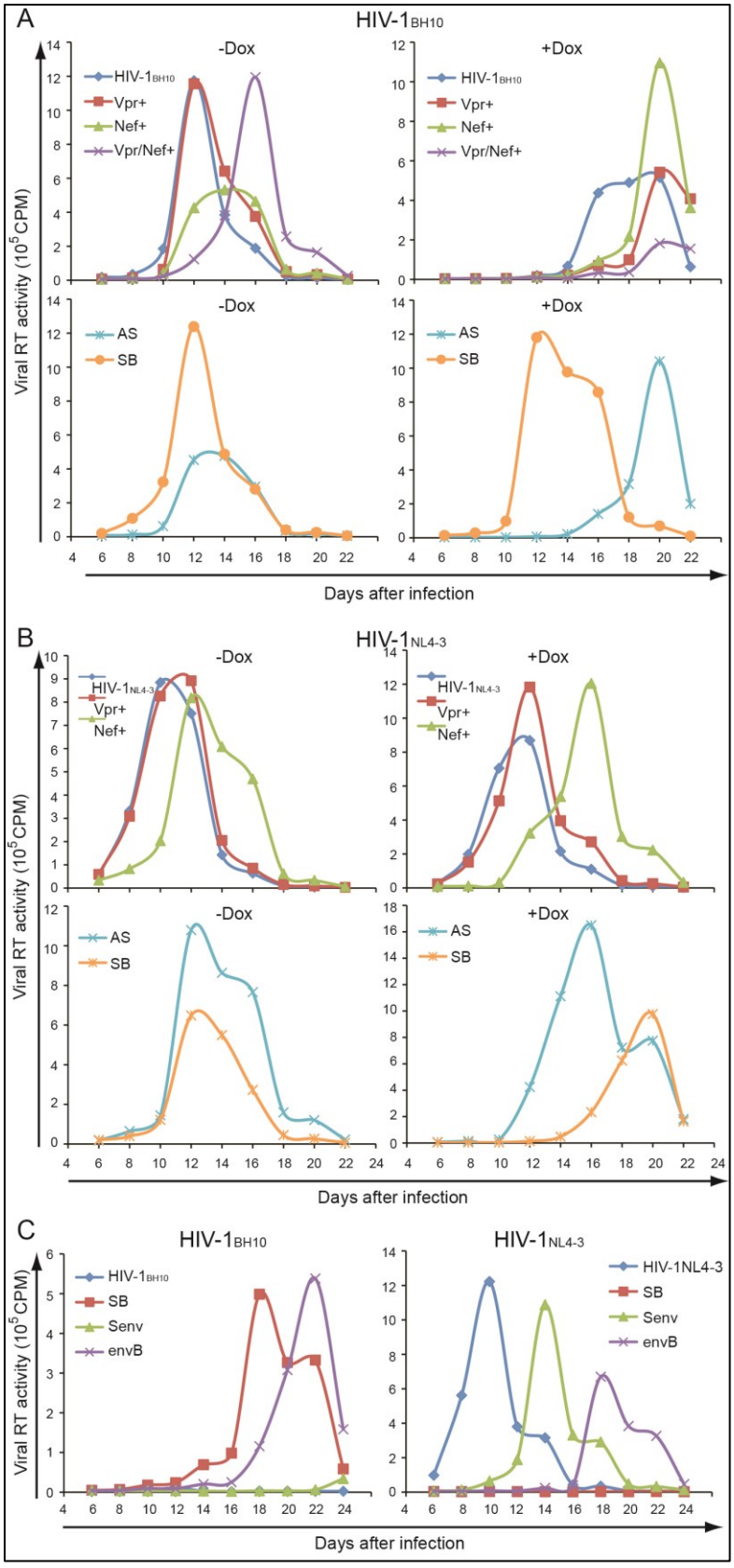

A
HIV-1BH10
-Dox
+Dox
HIV-1BH10
Vpr+
Nef+
Vpr/Nef+
AS
SB
Viral RT activity (10^5 CPM)
Days after infection
B
HIV-1NL4-3
-Dox
+Dox
HIV-1NL4-3
Vpr+
Nef+
AS
SB
Viral RT activity (10^5 CPM)
Days after infection
C
HIV-1BH10
HIV-1NL4-3
HIV-1BH10
SB
Senv
envB
HIV-1NL4-3
SB
Senv
envB
Viral RT activity (10^5 CPM)
Days after infection

Figure 3.3.3: HIV-1 envelope protein modulates the susceptibility to IFITM1 restriction
(A) Long-term infection of mutated HIV-1_{BH10} viruses having HIV-1_{NL4-3} Vpr or Nef proteins (upper A), and HIV-1_{BH10}/HIV-1_{NL4-3} chimeras based on HIV-1_{BH10} – HIV-1_{BH10}-AS and HIV-1_{BH10}-SB (lower A) in SupT1/IFITM1 cells induced with or without Doxycycline; (B) Long-term infection of mutated HIV-1_{NL4-3} viruses having HIV-1_{BH10} Vpr or Nef proteins (upper B), and HIV-1_{BH10}/HIV-1_{NL4-3} chimeras based HIV-1_{NL4-3} – HIV-1_{NL4-3}-AS and HIV-1_{NL4-3}-SB (lower B) in SupT1/IFITM1 cells induced with or without Doxycycline; (C) Long-term infection of all HIV-1_{BH10}/HIV-1_{NL4-3} chimeras based on HIV-1_{BH10} (left C) and HIV-1_{NL4-3} (right C) in IFITM1 expressing SupT1 cells.

3.3.4 IFITM1 diminishes the infectivity of HIV-1_{BH10} but not that of HIV-1_{NL4-3}.

We next investigated how the envelope protein of HIV-1_{NL4-3} overcomes the inhibition of IFITM1. We first co-transfected IFITM1 and HIV-1 DNA and measured the effect of IFITM1 on virus production and virus infectivity. Different amounts of IFITM1 were used for transfection in order to determine the quantity of IFITM1 that is required to achieve maximal inhibition of HIV-1. Levels of HIV-1 production were determined by measuring viral reverse transcriptase activity and by infecting the TZM-bl indicator cells. The infectivity of virus particles was calculated by dividing the luciferase activity (representing relative virus infectivity) by viral reverse transcriptase levels. The results in Figure 3.3.4 A show that the infectivity of both HIV-1_{BH10} and HIV-1_{NL4-3} was diminished by IFITM1 of high doses (0.1, 0.15 and 0.2 μg), whereas when 0.05 μg of IFITM1 DNA was used in transfection, only the infectivity of HIV-1_{BH10} decreased by 2.5 fold, which suggests a greater sensitivity of HIV-1_{BH10} to IFITM1 as compared to HIV-1_{NL4-3}. We then tested the sensitivity of chimeric viruses HIV-1_{BH10}-SB and HIV-1_{NL4-3}-SB to IFITM1 inhibition in this co-transfection assay. The results showed the marked resistance of HIV-1_{BH10}-SB to IFITM1 in contrast to the high susceptibility of HIV-1_{NL4-3}-SB (Figure 3.3.4 B), which is consistent with the phenotype of virus replication shown in Figure 3.3.3. Furthermore, exchanging the Senv DNA fragment between HIV-1_{BH10}

and HIV-1_{NL4-3} did not affect the sensitivity of the parental virus to IFITM1 (Figure 3.3.4 B). In contrast, the infectivity of the HIV-1_{BH10}-envB virus, that contains the Env sequence from HIV-1_{NL4-3}, was less affected by IFITM1 as compared to the wild type HIV-1_{BH10} (Figure 3.3.4 B), further supporting a role of viral envelope in resisting IFITM1.

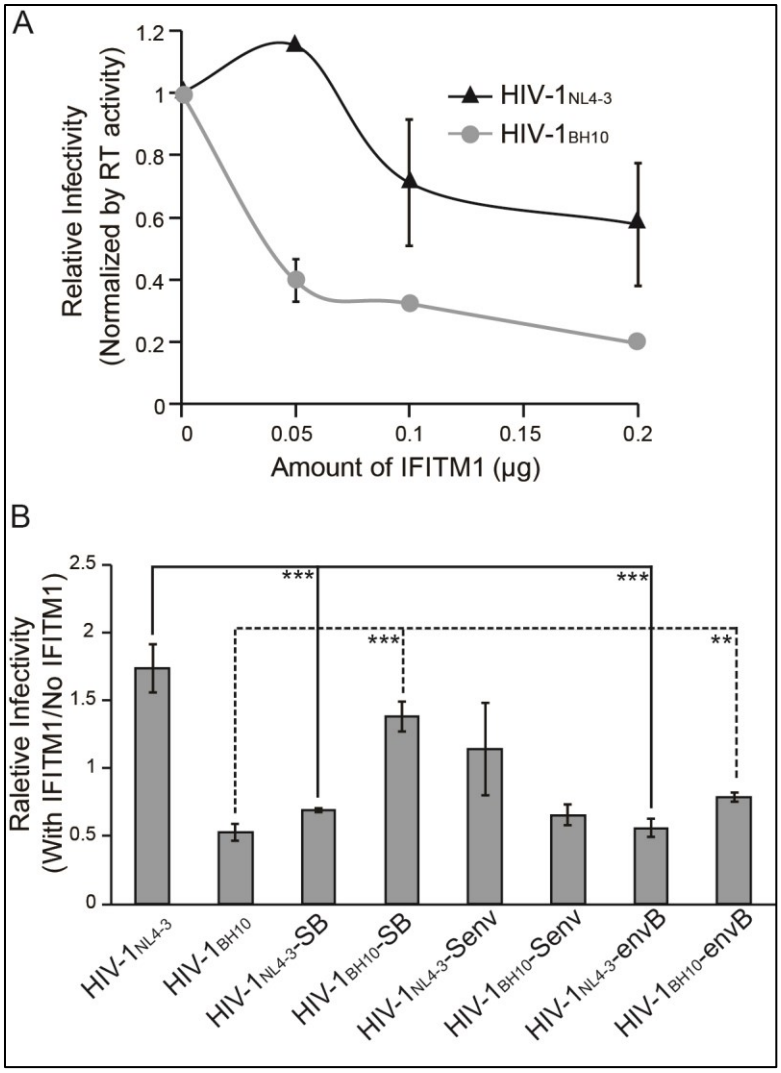


Figure 3.3.4: IFITM1 diminishes the infectivity of HIV-1_{BH10} but not that of HIV-1_{NL4-3}.

(A) 0.2μg proviral DNA plasmids of HIV-1_{BH10} or HIV-1_{NL4-3} was transfected into HEK293T cells together with 0, 0.05, 0.1 and 0.2μg plasmid of IFITM1; Supernatants containing virus were collected 48 hours after transfection and the amount of generated viruses was determined by measuring virus RT activity; Viral infectivity was measured by infecting TZM-bl cells; the relative infectivity was calculated by dividing the luciferase activity by viral reverse transcriptase levels and compared with the infectivity of HIV-1_{BH10} or HIV-1_{NL4-3} without co-transfection with IFITM1 (set the infectivity of control virus as 1). The results shown are the average of three independent experiments. (B) 0.2μg plasmids of wild type HIV-1_{NL4-3}, HIV-1_{BH10} and their chimeras were transfected into HEK293T cells together with or without 0.05μg plasmid of IFITM1. Relative infectivity was shown as the ratio between the infectivity of virus generated with and without IFITM1 co-transfection. Results of three independent experiments were summarized in the bar graph. The p values were calculated and the significance is indicated by ** (<0.01) and *** (<0.001).

3.3.5 The envelope protein of HIV-1_{NL4-3} allows higher cell-to-cell transmission efficiency than that of HIV-1_{BH10}.

Given that HIV-1_{BH10} are able to acquire IFITM1 escape mutations in *vpu* and *env* that act by increasing the virus cell-to-cell transmission, we speculated that HIV-1_{NL4-3} may become resistant to IFITM1 by a similar mechanism. To test this hypothesis, we performed virus cell-to-cell transmission assay in SupT1 and SupT1/IFITM1 cells using the wild type or chimeric viruses of HIV-1_{BH10} and HIV-1_{NL4-3} as described in Chapter 2: 2.3.6. The results in Figure 3.3.5A reveal a 4-fold higher cell-to-cell transmission efficiency of HIV-1_{NL4-3} in SupT1 cells than that of HIV-1_{BH10}. Exchanging the SB viral DNA fragment between these two viruses enabled HIV-1_{BH10}-SB virus having 2-fold higher cell to cell transmission efficiency than the HIV-1_{NL4-3}-SB virus (Figure 3.3.5 A). The same phenotype was observed when the chimeric viruses contained only the envB sequence, which demonstrates that the envelope protein sequence determines the cell-to-cell transmission efficiency and this function of viral envelope protein contributes to the susceptibility of HIV-1 to IFITM1 restriction. We also used IFITM1-expressing SupT1 cells as target cells, in the cell-to-cell transmission studies. IFITM1 expression in target cells did not markedly affect HIV-1 cell-to-cell transmission. Again, the envelope protein of HIV-1_{NL4-3} enhanced the cell-to-cell transmission efficiency of HIV-1_{BH10} (Figure 3.3.5 B). Therefore, we conclude that HIV-1_{NL4-3} has higher virus cell-to-cell transmission efficiency than HIV-1_{BH10} in SupT1 cells and this difference in cell-to-cell transmission results from the difference in viral envelope protein. Moreover, IFITM1 does not markedly reduce the cell-to-cell transmission efficiency of either HIV-1_{NL4-3} or HIV-1_{BH10} in SupT1 cells.

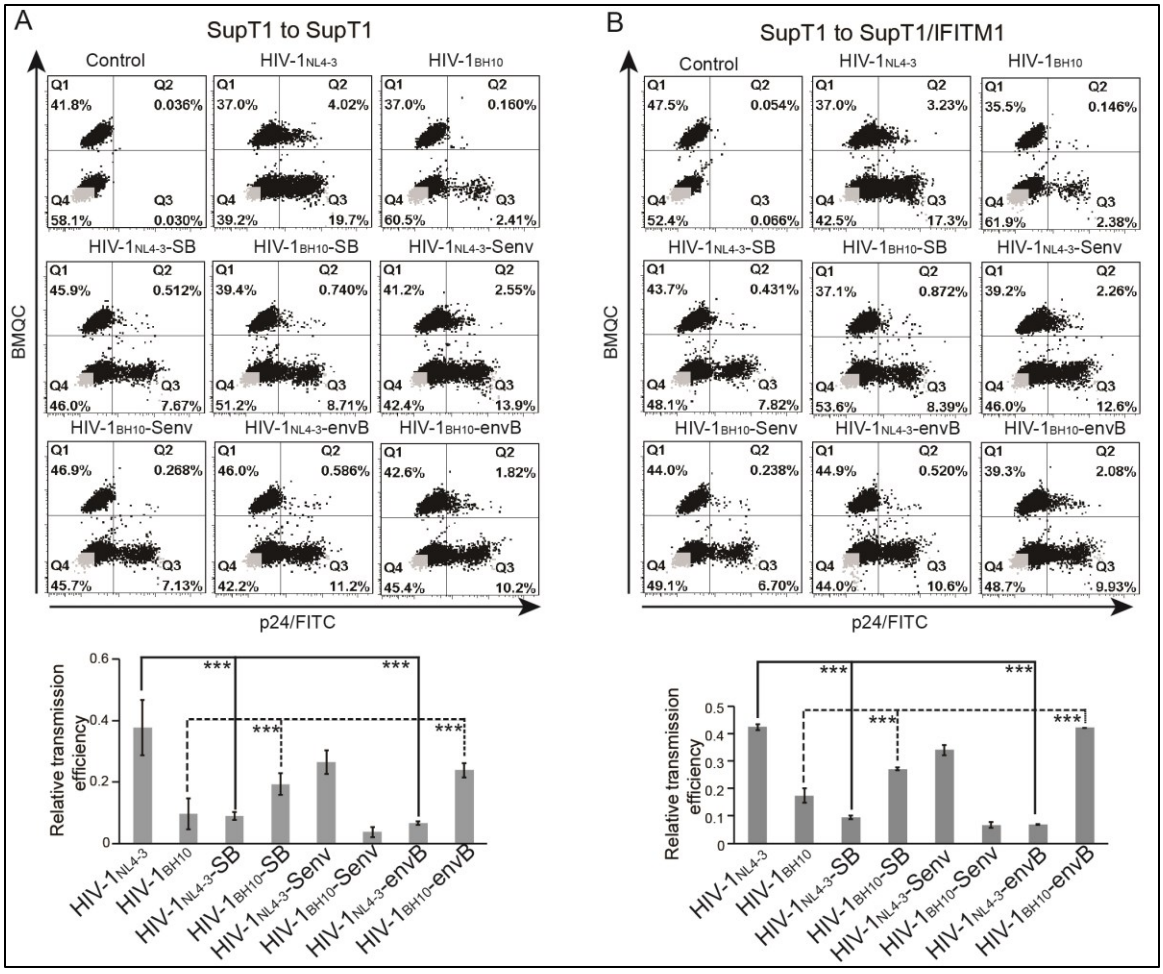


Figure 3.3.5: The envelope protein of HIV-1_{NL4-3} allows higher cell-to-cell transmission efficiency than that of HIV-1_{BH10}.

SupT1 cells were first infected with the VSVG pseudotyped viruses of wild type HIV-1_{BH10} and HIV-1_{NL4-3} and their chimeras (equivalent to 50 ng of viral p24) for forty hours before being washed with complete RPMI1640 media and used as donor cells in the following experiments. (A) SupT1 donor cells were mixed with the BMQC-labeled target SupT1 cells. After 8 hours, the infected target cells were stained with FITC-conjugated anti-p24 antibody and scored by flow cytometry (upper A). The relative transmission efficiency was calculated for each experiment. The results of three independent transmission experiments were averaged and shown in the bar graph. The p values were calculated and the significance was indicated by *** (<0.001) (lower A). (B) The infected SupT1 cells were mixed with the BMQC-labeled IFITM1-expressing SupT1 cells to assess the effect of IFITM1 on HIV-1 cell-to-cell transmission (upper B). The results of three independent transmission experiments were averaged and shown in the bar graph. The p values were calculated and the significance is indicated by *** (<0.001) (lower B).

3.4 Discussion

This study was inspired by the observation that two closely related HIV-1 subtype B strains BH10 and NL4-3 were differentially inhibited by human IFITM1, which leads a valuable tool to identify viral component(s) in NL4-3 that is able to overcome IFITM1 inhibition. Results of mutagenesis and virus replication studies revealed a role of viral envelope protein in assisting the evasion from IFITM1. Exchanging the Env sequences between HIV-1_{BH10} and HIV-1_{NL4-3} reversed the susceptibility of the parental strain to IFITM1 inhibition. This role of HIV-1 Env protein corroborate our recent report showing that HIV-1_{BH10} was able to evolve in IFITM1-expressing SupT1 cells to become resistant to IFITM1 through mutating viral Env and Vpu proteins [228].

We found that HIV-1_{NL4-3} is 3-fold more efficient in transmitting from cell-to-cell as compared to HIV-1_{BH10} (Figure 3.3.5). This advantage is lost when the Env sequence in HIV-1_{NL4-3} was changed to that of HIV-1_{BH10}. Similarly, HIV-1_{NL4-3} Env protein stimulated the cell-to-cell transmission of HIV-1_{BH10} by 3-fold. We propose that this greater ability of cell-to-cell transmission assists HIV-1_{NL4-3} to overcome IFITM1 inhibition in spread infection. Through a similar mechanism, HIV-1_{BH10} overcomes IFITM1 inhibition by acquiring escape mutations in Vpu and Env that together enhance cell-to-cell transmission [228]. It remains to be tested whether HIV-1 can employ such a mechanism to overcome the inhibition of IFITM2 and IFITM3 as well as other host restriction factors.

Our data suggests that the envelope protein of HIV-1 can determine the sensitivity to IFITM1. Although human IFITM1 does not affect the entry of either HIV-1_{BH10} or HIV-1_{NL4-3} [203], inserting the envelope sequence from HIV-1_{NL4-3} into HIV-1_{BH10} enables the

latter virus to grow in human IFITM1-expressing SupT1 cells (Figure 3.3.3). One possibility is that the HIV-1_{NL4-3} envelope confers a growth advantage to the virus, which helps to overcome the replication deficit caused by IFITM1. Alternatively, the envelope protein of HIV-1_{NL4-3}, not that of HIV-1_{BH10}, has the ability of antagonizing human IFITM1, in analogy to the counteracting activity of the envelope protein of HIV-2, but not HIV-1, toward tetherin [170].

It remains to be determined how many different HIV-1 strains that human IFITM1 can inhibit. One possibility is that the chance of finding these human IFITM1-sensitive HIV-1 strains may be low if IFITM1 does exert significant pressure *in vivo* on HIV-1 replication. In the context of this scenario, HIV-1 should have evolved measures to counter IFITM1 restriction. Since both HIV-1_{BH10} and HIV-1_{NL4-3} are strains that have been propagated in cultured human T cell lines, both strains have adapted to the *in vitro* culture conditions and as a result, they may lose the original viral sequences that confer resistance to IFITM1. This may have happened to HIV-1_{BH10}.

Chapter 4 –The C-terminal sequence of IFITM1 regulates its anti-HIV-1 activity

Preface

IFITM1 differs from IFITM2 and IFITM3 by a relatively longer C-terminal sequence. Yet, it is unknown what role this C-terminal sequence plays in the anti-HIV-1 activity of IFITM1. To address this question, we first deleted sequences from the C-terminus of IFITM1 and generated mutants Δ(108-125), and Δ(117-125), and then stably expressed these IFITM1 mutants in SupT1 cells. Although HIV-1_{NL4-3} was resistant to the wild type IFITM1, this virus was profoundly inhibited by each of the two IFITM1 mutants that lack the C-terminal sequence of various lengths. Next we found that these IFITM1 mutants inhibited HIV-1_{NL4-3} via impairing virus entry. Further mutagenesis studies mapped the key amino acid residues to 117-QII-119. Interestingly, HIV-1_{NL4-3} was able to evolve in tissue culture and became resistant to the Δ(117-125) mutant through mutating Vpu and Env proteins. Taken together, these data reveal a role of the C-terminal sequence of IFITM1 in regulating the ability of IFITM1 to affect HIV-1 entry.

4.1 Introduction

Human interferon-induced transmembrane (IFITM) proteins have been reported to inhibit the infection of a wide spectrum of viruses including influenza A virus (IAV), flavivirus (West Nile virus, Dengue virus, Yellow Fever virus), filovirus (Ebola virus, Marburg virus), SARS coronavirus, vesicular stomatitis virus (VSV), hepatitis C virus (HCV), reovirus, Rift Valley fever virus, as well as human immunodeficiency virus type 1 (HIV-1) [197, 203, 205, 207, 222, 238, 239, 252, 253]. Among the 5 members of IFITM proteins in human, the antiviral activity of IFITM1, IFITM2 and IFITM3 was mostly investigated.

IFITM1 has a shorter N terminal region and a relatively longer C terminal region as compared to IFITM2 and IFITM3. The N termini of IFITM2 and IFITM3 have the YEML motif that regulates protein endocytosis and trafficking [209] [212]. Mutating this sequence relocates IFITM3 to the plasma membrane and as a result, impairs its ability to inhibit virus entry. The subcellular location of IFITM1 is different from that of IFITM2 and IFITM3. IFITM1 is preferably in the plasma membrane or early endosome, whereas IFITM2 and IFITM3 are most seen in late endosomes or lysosomes [201] [202] [203] [203] [204] [205]. IFITM1, IFITM2 and IFITM3 differ in their antiviral functions. For example, Filoviruses, SARS-CoV and HIV-1 are more sensitive to the inhibition by IFITM1, influenza virus, Yellow fever virus and Hepatitis C virus are more inhibited by IFITM2 and IFITM3 [221] [222] [197].

IFITM proteins have unique membrane topologies. Early studies suggested that IFITM proteins are transmembrane proteins with extracellular/luminal N- and C-termini [206] [207]. With the discovery of the phosphorylation of an N-terminal residue Tyr-20 and the

ubiquitination of K24 [208] [209] [210], the possible cytoplasmic localization of the N-terminal region was suggested. A recent study by Bailey *et al.* [211] demonstrated that IFITM3 is a type Ⅱ transmembrane protein with cytosolic N-terminus and extracellular/luminal C-terminus.

IFITM1 has a relatively longer C-terminal region. No specific function has been reported for this region. Removing this region does not affect the ability of IFITM1 to inhibit HIV-1_{BH10} replication. Since the HIV-1_{NL4-3} strain is resistant to wild type IFITM1, we expected similar resistance of HIV-1_{NL4-3} to the C-terminal truncated IFITM1. To our surprise, the Δ(108-125) and Δ(117-125) mutants, that were deleted of 18 and 9 amino acids from the C-terminus of IFITM1, blocked the replication of HIV-1_{NL4-3} in SupT1 cells. This observation suggests a new role of the C-terminal sequence in regulating the anti-HIV-1 activity of IFITM1.

4.2 Materials and methods

4.2.1 Plasmids, cell lines and antibodies

The tetracycline-inducible IFITM1, IFITM2 and IFITM3 SupT1 cell lines were generated as previously described [203]. The HIV-1_{NL4-3} proviral DNA clone was obtained from the NIH AIDS Reference and Reagent Program. The mutations Vpu34, EnvA539V, Vpu34/A539V were engineered using the site-directed mutagenesis kit (Stratagene). The anti-Flag and anti-β actin antibodies were purchased from Sigma, anti-tubulin antibody from Santa Cruz biotechnology, anti-HIV-1 p24 antibody from ID Lab Inc., Dylight-649-conjugated anti-Flag antibody from Rockland, FITC-conjugated anti-HIV-p24 antibody from Beckman. G418 was purchased from Invitrogen, puromycin and doxycycline from Sigma.

4.2.2 Virus infection

HIV-1 stocks were produced by transfecting the human embryonic kidney cell line (HEK293T) with 4μg HIV-1 proviral DNA in 10-cm dishes using polyethylenimine (PEI). The supernatants were clarified by passing through the 0.2 μm filter (VWR) to remove the cell debris. Amounts of viruses were determined by measuring viral p24 (CA) levels using the HIV-1 p24 Antigen Capture Assay kit (Cat. 5447, ABL Inc.)

Virus infection was measured in three assays. First, infection of the TZM-bl indicator cells that express CD4/CXCR4/CCR5 and contain the HIV-1 LTR-Luc reporter [264]: TZM-bl cells were first seeded into 24-well plates (4 $X10^4$cells/well) one day before virus infection. Forty hours after infection, cells were lysed with 1 X passive lysis buffer (Promega) and the levels of luciferase activity were measured using the luciferase assay kit (Promega). Second, short-term infection of the SupT1 cells: SupT1 cells were first exposed to viruses equivalent to 200 ng viral p24 antigen, free virus was washed off 16 hours late. Forty hours after infection, the infected cells were washed with cold 1 X phosphate-buffered saline and fixed with 1% paraformaldehyde. The infected cells were stained with anti-HIV-1 p24 antibody and scored by flow cytometry; the supernatants of the infected cells were collected for the TZM-bl infection to determine the infectivity of newly generated viruses. Third, long-term infection of SupT1 cells: SupT1 cells were infected with virus equivalent to 10 ng viral p24. Viral replication was monitored by measuring the levels of viral reverse transcriptase activity in supernatants over various time intervals.

4.2.3 HIV-1 virion fusion assay

The experiment was performed as described previously [203, 266]. Briefly, 3 μg of

HIV-1 DNA was co-transfected with 1 μg pCMV-BlaM-Vpr DNA into 293T cells. Supernatants containing virions were filtered with 0.22 μm filter and then concentrated by ultracentrifugation at 100,000xg for 1 hour at 4°C. Pelleted viruses were suspended with DMEM, quantified for viral RT activity, aliquoted and stored at -80°C. For virion fusion assay, SupT1 cells were infected with same amounts of wild type HIV-1 and mutant viruses by spinoculation for 45 minutes at room temperature, followed by incubation for 2 hours at 37°C. Cells were then washed with CO2-independent medium (Invitrogen), mixed with 100 μl loading solution (CCF2/AM substrate, Invitrogen) for 1 hour at room temperature in the dark. After washing off the loading solution, cells were incubated in 200 μl of development medium in dark for 16 hours at room temperature. Cells were washed and fixed with 3.7% formaldehyde. The cleavage of CCF2/AM was measured by flow cytometry.

4.2.4 Western blotting

Cell lysates were separated in 1% sodium dodecyl sulfate-12% polyacrylamide gels (SDS-PAGE) by electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche). The membranes were blocked with 4% skim milk (in 1 X phosphate-buffered saline) and further probed with anti-Flag (1:5,000), anti-β actin (1:5,000) or anti-tubulin (1:5,000) antibodies. After a further incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare), the protein bands were visualized by exposure to X-ray films following a brief treatment of the membranes with the ECL (enhanced chemiluminescence) reagents.

4.2.5 Select for the escape viruses

HIV-1_{NL4-3} was used to infect SupT1 cells that stably express IFITM1 mutant Δ(117-125). When cytopathogenic effect and high levels of viral RT activity were detected 3

weeks after infection, we used these newly produced viruses to infect fresh Δ(117-125)-expressing SupT1 cells. After five such passages, HIV-1 replication reached the peak level at 6 to 8 days. We then harvested SupT1 cells that were infected by the highly replicable HIV-1 and extracted the total cellular DNA using the DNeasy Blood & Tissue kit (Qiagen). Viral genomic DNA was amplified with three primer pairs to cover the entire genome. The PCR products were sent to McGill University and Quebec Innovation Center for sequencing. We also grew HIV-1 in the control SupT1 cells that did not express Δ(117-125) for the same period of time, and viral genomes were similarly amplified by PCR, and sequenced.

4.3 Results

4.3.1 IFITM2 and IFITM3 inhibit the replication of HIV-1_{NL4-3} in SupT1 cells

We first performed the short-term infection experiments using HIV-1_{NL4-3} to infect SupT1 cells expressing IFITM1, IFITM2 or IFITM3 (Figure 4.3.1 B). IFITM2 and IFITM3 diminished the infection of HIV-1_{NL4-3} by 2-fold, no effect was seen for IFITM1 (Figure 4.3.1 A). This inhibition of HIV-1_{NL4-3} by IFITM2 and IFITM3 was further reflected by the much delayed replication of HIV-1_{NL4-3} in SupT1 expressing IFITM2 or IFITM3 (Figure 4.3.1 C). HIV-1_{NL4-3} virus could replicate to similar levels in SupT1 and SupT1/IFITM1 cells with or without Doxycycline (500 ng/ml) treatment (Figure 4.3.1 C).

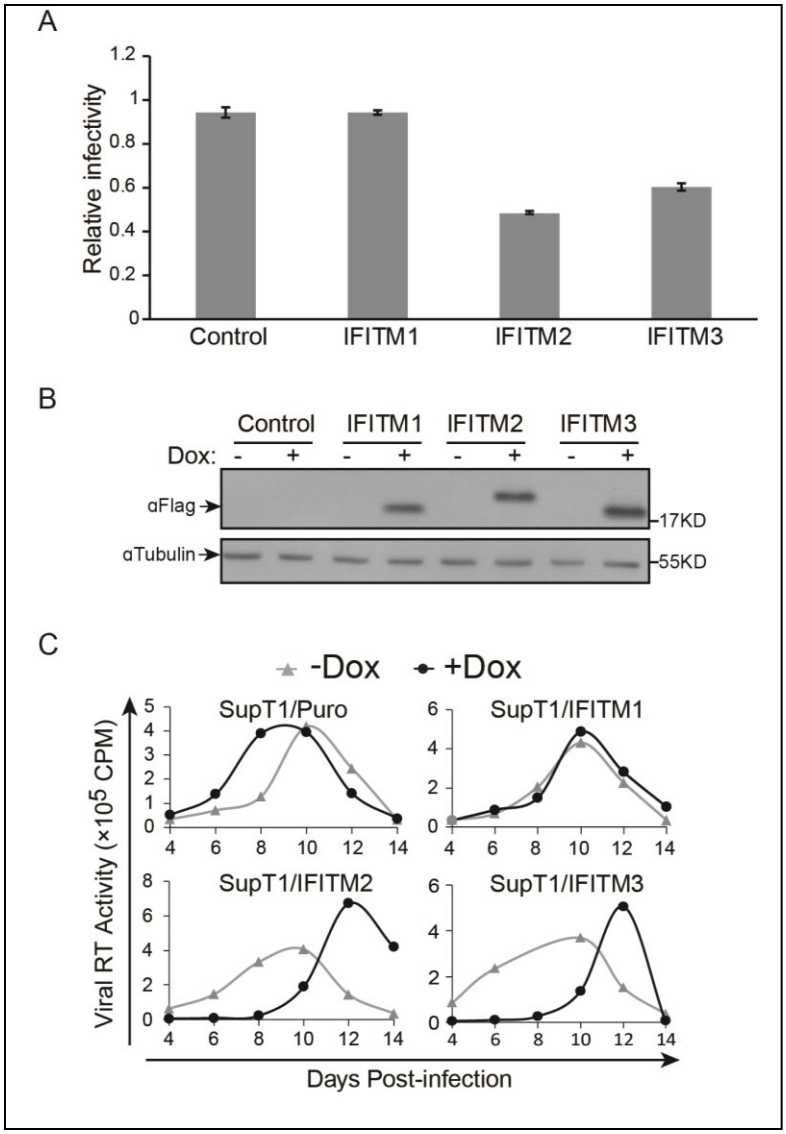


Figure 4.3.1: IFITM2 and IFITM3 inhibit the replication of HIV-1_{NL4-3} in SupT1 cells.

(A) Virus of HIV-1_{NL4-3} was used to infect SupT1 cells with or without IFITM1, IFITM2 and IFITM3 induction. Forty hours after infection, the infected cells were stained with FITC-conjugated anti-p24 antibody and scored by flow cytometry. Results of three independent infections are summarized in the bar graph. (B) Expressing level of doxycycline induced IFITM1, IFITM2 and IFITM3 in SupT1 cell lines was determined by Western Blot. (C) HIV-1_{NL4-3} virus was used to infect SupT1 cell lines with or without IFITM1, IFITM2 and IFITM3 induction by doxycycline. Viral replication was determined by measuring levels of viral reverse transcriptase activity in culture supernatants. Results shown represent three independent infection experiments.

4.3.2 Deleting the C-terminal sequence allows IFITM1 to inhibit the replication of HIV-1_{NL4-3}.

We have previously shown that deleting the first 21 amino acids did not affect the ability of IFITM3 to inhibit HIV-1[209]. This result suggests that the inability of IFITM1 to inhibit HIV-1_{NL4-3} is a result of its relatively longer C-terminal region as compared to IFITM2 and IFITM3. To test this, we deleted the C-terminal sequence of IFITM1 and generated two mutants named Δ(117-125) and Δ(108-125) (Figure 4.3.2 A). We then used HIV-1_{NL4-3} to challenge SupT1 cells that stably express either Δ(117-125) or Δ(108-125). The results showed that HIV-1_{NL4-3} replication was blocked by either of these two mutants (Figure 4.3.2 B). This suggests that the C-terminal sequence of IFITM1 prevents IFITM1 from inhibiting HIV-1.

Since both IFITM2 and IFITM3, but not IFITM1, are able to impair HIV-1 entry, we speculated that the Δ(117-125) and Δ(108-125) mutants have gained the function of inhibiting HIV-1 entry and therefore are also able to suppress the replication of HIV-1_{NL4-3}. This possibility was tested by performing the Blam-Vpr virion fusion assay. Indeed, both Δ(117-125) and Δ(108-125) mutants significantly diminished HIV-1_{NL4-3} entry as opposed to the wild type IFITM1 that exerted no effect on this regard (Figure 4.3.2 C). Taken together, these data indicate that removing as few as 9 amino acids from the C-terminus allows IFITM1 to inhibit HIV-1 entry as well as HIV-1 replication.

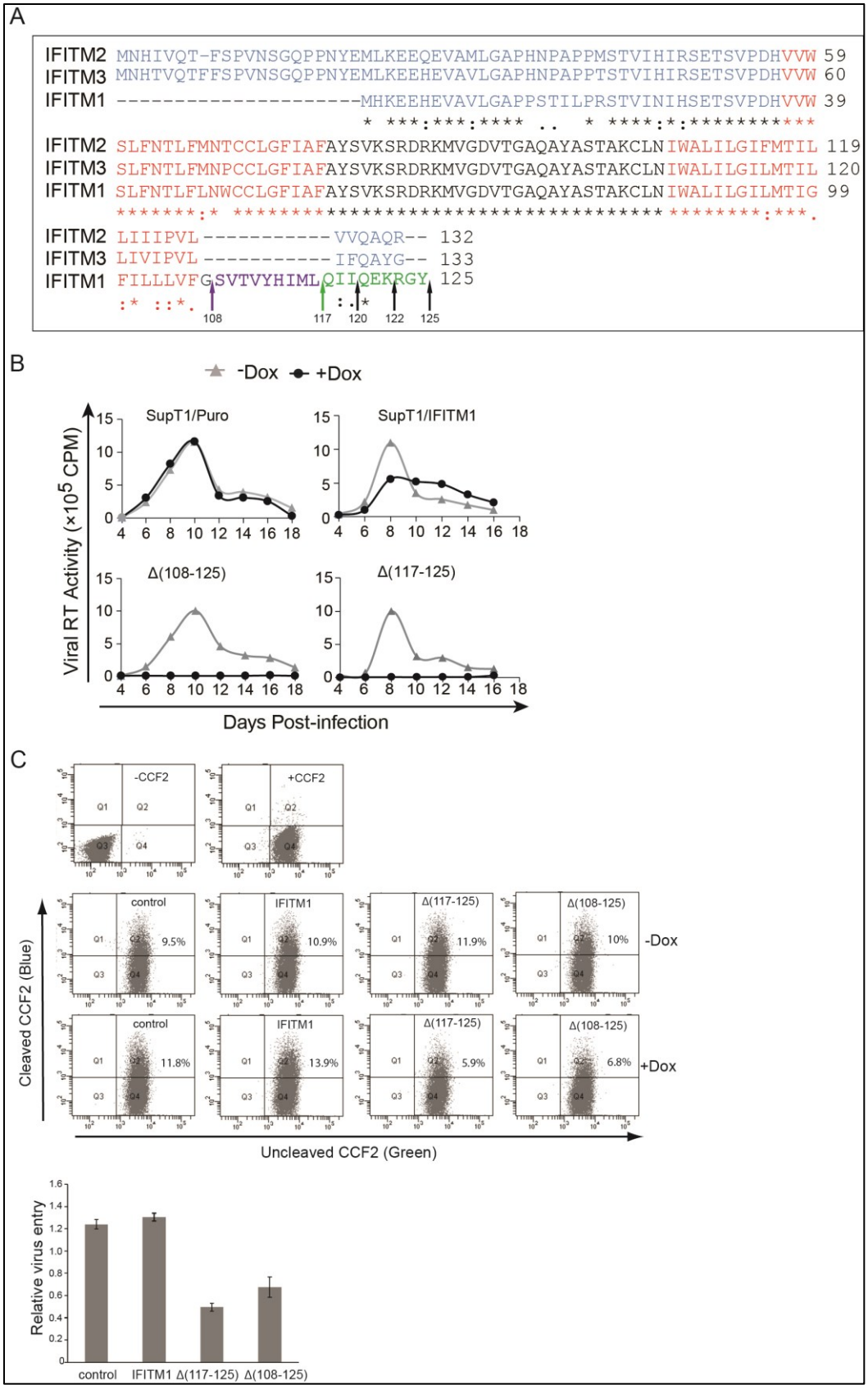

A
IFITM2 MNHIVQT-FSPVNSGQPPNYEMLKEEQEVAMLGAPHNPAPPMSTVIHIRSETSVPDHVVW 59
IFITM3 MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVPDHVVW 60
IFITM1 ----------MHKEEHEVAVLGAPPSTILPRSTVINIHSETSVPDHVVW 39
IFITM2 SLFNTLFMNTCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGIFMTIL 119
IFITM3 SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIL 120
IFITM1 SLFNTLFLNWCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIG 99
IFITM2 LIIIPVL----------VVQAQR-- 132
IFITM3 LIVIPVL----------IFQAYG-- 133
IFITM1 FILLLVFGSVTVYHIMLQIIQEKRGY 125
108
117
120
122
125
B
-Dox
+Dox
SupT1/Puro
SupT1/IFITM1
Δ(108-125)
Δ(117-125)
Viral RT Activity (×10^5 CPM)
Days Post-infection
C
-CCF2
+CCF2
control
IFITM1
Δ(117-125)
Δ(108-125)
9.5%
10.9%
11.9%
10%
11.8%
13.9%
5.9%
6.8%
-Dox
+Dox
Cleaved CCF2 (Blue)
Uncleaved CCF2 (Green)
Relative virus entry
control
IFITM1
Δ(117-125)
Δ(108-125)

Figure 4.3.2: Deleting the C-terminal sequence allows IFITM1 to inhibit the replication of HIV-1_{NL4-3}.
(A) Schematic representation of IFITM1, IFITM2 and IFITM3 amino acid sequences. Blue letters indicated the outer-membrane area, red letters indicated inner-membrane area and black letters indicated conserved intra-cellular domain. IFITM1 had longer C terminus than IFITM2 and IFITM3, C-terminal deleted constructs were generated by deleting the amino acids from 108 to 125(purple and green) and from 117 to 125 (green); triple amino acids mutations were generated by changing to alanine residues or deleting the amino acids of 117-QII-119, 120-QEK-122 and 123-RGY-125. (B) HIV-1_{NL4-3} virus was used to infect SupT1 cell lines with or without the expression of wild type and C – terminal deleted IFITM1 proteins (Δ(108-125) and Δ(117-125)). Viral replication was determined by measuring levels of viral reverse transcriptase activity in culture supernatants. Results shown represent three independent infections. (C) The virus entry efficiency of HIV-1_{NL4-3} in SupT1 cells with or without the expression of wild type and C terminus deleted IFITM1 proteins was examined by Blam-Vpr virion fusion assay. The cleavage of CCF2/AM by Blam-Vpr was measured by flow cytometry. Results of three independent infections are summarized in the bar graph.

4.3.3 Mutating the 117-QII-119 sequence enhances the ability of IFITM1 to inhibit HIV-1 replication.

We next tried to determine which of the 9 amino acids that were deleted in the Δ(117-125) mutant played a key role in modulating the anti-HIV-1 activity of IFITM1. We first mutated each amino acid in the 117-QIIQEKRGY-125 sequence to alanine and generated 9 mutants (Figure 4.3.2 A). We then stably transduced SupT1 cells with each of these mutants and then challenged these cells with HIV-1_{NL4-3}. Unfortunately, none of these 9 mutants was able to markedly inhibit the replication of HIV-1_{NL4-3} in SupT1 cells (Figure 4.3.3 A). One possibility might be that more than one residue of these 9 amino acids are involved in modulating the anti-HIV-1 ability of IFITM1. To test this, we mutated three adjacent amino acids either by deletion or substitution with alanines (Figure 4.3.2 A). The results of HIV-1_{NL4-3} replication experiments showed that the Δ(117-119) and (117-119)-AAA mutants markedly suppressed the replication of HIV-1_{NL4-3}, although the inhibition was not as strong as that by Δ(117-125) (Figure 4.3.3 B). Together, these data suggest the important role of the 117-QII-119 in regulating the anti-HIV-1 activity of IFITM1.

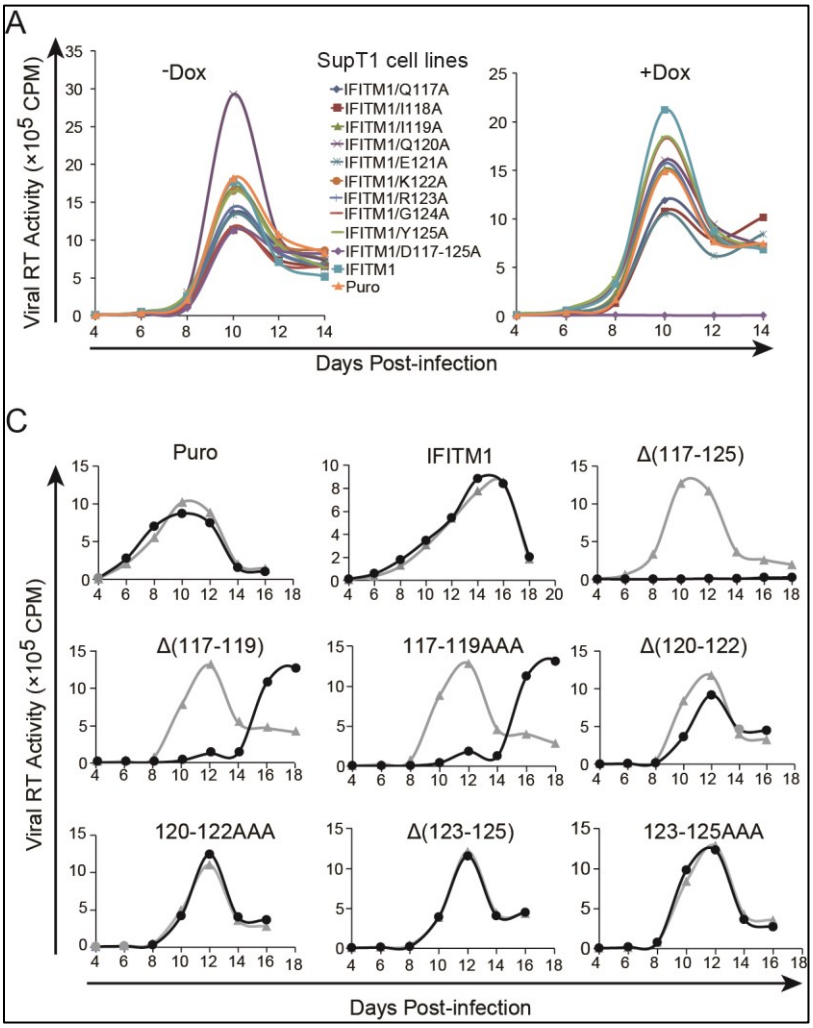


Figure 4.3.3: Mutating the 117-QII-119 sequence enhances the ability of IFITM1 to inhibit HIV-1 replication.

(A) HIV-1_{NL4-3} virus was used to infect SupT1 cell lines with or without the induction of wild type and single amino acid mutated IFITM1 proteins. Viral replication was determined by measuring levels of viral reverse transcriptase activity in culture supernatants. (B) HIV-1_{NL4-3} virus was used to infect SupT1 cell lines with or without the induction of wild type and triple amino acid mutated IFITM1 proteins (Δ(117-119), (117-119)-AAA, Δ(120-122), (120-122)-AAA, Δ(123-125) and (123-125)-AAA). Viral replication was determined by measuring levels of viral reverse transcriptase activity in culture supernatants. Results shown represent three independent infections.

4.3.4 HIV-1_{NL4-3} mutates Vpu and Env to escape the inhibition by Δ(117-125)

We next performed virus evolution experiments to investigate whether and how HIV-1_{NL4-3} escape from the inhibition by Δ(117-125) in SupT1 cells. Indeed, after a long-term culture of HIV-1_{NL4-3} in Δ(117-125)-expressing SupT1 cells, cytopathogenic effect was observed and high levels of viral reverse transcriptase activity was measured in the culture supernatants, which indicates the emerge of escape viruses. We then amplified viral DNA from the infected SupT1 cells and sequenced for escape mutations. Two mutations were identified. One inserted a stop codon at the 40th amino acid position in Vpu and caused Vpu truncation. The other one changed Alanine at 539 to Valine in Env. We then inserted these two mutations, Vpu40 and EnvA539V, either individually or together into HIV-1_{NL4-3} and tested whether these two mutations are able to confer resistance to Δ(117-125) (Table 4.3.1). The Vpu40 or EnvA539V alone marginally increased the replication of HIV-1_{NL4-3} in Δ(117-125)-expressing SupT1 cells (Figure 4.3.4 A). Together, these two mutations allowed virus replication to peak at day 12 in Δ(117-125)-expressing SupT1 cells as compared to the replication peak at day 8 in control SupT1 cells, which suggests a partial restoration of virus replication. Given that HIV-1_{BH10} also mutates Vpu and Env to escape from IFITM1, mutating these two viral proteins is likely the common pathway that HIV-1 adopts to evolve resistance to IFTIM inhibition.

		Mutations	
		Vpu34	EnvA539V
NL4-3 DNA clones	Vpu40/A539V	√	√
	Vpu40	√	
	A539V		√

Table 4.3.1: The mutated HIV-1_{NL4-3} clones

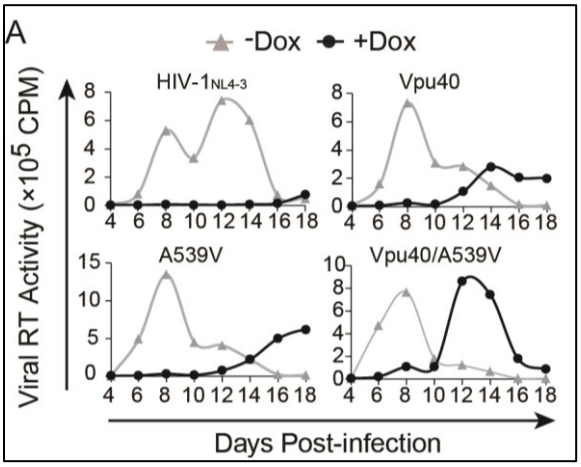


Figure 4.3.4: HIV-1_{NL4-3} mutates Vpu and Env to escape the inhibition by Δ(117-125)
(A) Replication of wild type and mutated HIV-1_{NL4-3} viruses (HIV-1_{NL4-3}, Vpu40, A539V and Vpu40/A539V) in SupT1 cells with or without the induction of Δ(117-125). Viral replication was determined by measuring levels of viral reverse transcriptase activity in culture supernatants. Results shown represent three independent infections.

4.4 Discussion

In this study, we observed that IFITM2 and IFITM3, but not IFITM1, inhibited the replication of HIV-1_{NL4-3} in SupT1 cells. We successfully demonstrated that the C-terminal sequence of IFITM1 modulates the anti-HIV-1 ability of IFITM1. Removing the last 9 amino acids from the C-terminus allowed IFITM1 to block HIV-1_{NL4-3} replication as a result of the gained function of impairing HIV-1 entry. We further observed that mutating the 117-QII-119 sequence led to marked suppression of HIV-1_{NL4-3} replication, suggesting the importance of this short motif in modulating the anti-HIV-1 ability of IFITM1.

We observed that IFITM2 and IFITM3, but not IFITM1, inhibited virus entry of HIV-1_{NL4-3} in SupT1 cells (Figure 4.3.1). IFITM2 and IFITM3 have a longer N terminus than IFITM1. This N-terminal region has the motif 20-YEML-23 that act as a endocytic signal through binding to the AP-2 proteins [209] [212]. Mutating 20-YEML-23 relocates IFITM3 to the plasma membrane and abrogates the ability of IFITM3 to inhibit influenza A virus. Interestingly, deleting the 20-YEML-23 motif does not prevent IFITM3 from inhibiting HIV-1, indicating that lack of this N-terminal sequence is not the reason why IFITM1 is unable to inhibit HIV-1. To our surprise, removing the C-terminal sequence of IFITM1 allows strong inhibition of HIV-1_{NL4-3}, which suggests a regulatory role of this C-terminal region in controlling the antiviral activity of IFITM1. Our observation is supported by the results of a recent study showing that deleting the C-terminal sequence of IFITM1 renders IFITM1 stimulatory in the infection of human coronavirus OC43 [225].

We performed detailed mutagenesis studies to determine the key amino acids within the last 9 amino acids (117 to 125) that prevent IFITM1 from inhibiting HIV-1_{NL4-3}. The

results revealed a partial role of the 117-QII-119 in this regard (Figure 4.3.3 B). We were unable to assign QII to a known protein motif with a known function. The fact that no amino acids account for the total phenotype seen for the C-terminal sequence of IFITM1 suggests that the secondary or tertiary structure of this specific region, not the specific amino acid sequence, determines the function.

In analogy to the escape of HIV-1_{BH10} from IFITM1 inhibition, we observed that HIV-1_{NL4-3} also mutated Vpu and Env to overcome the inhibition by IFITM1 mutant Δ(117-125). Both HIV-1_{BH10} and HIV-1_{NL4-3} acquired mutations in Vpu that caused the production of a truncated Vpu of 33 to 39 amino acids. The escape mutations in Env differ considerably between these two viruses. HIV-1_{BH10} has the G367E mutation that attenuates the binding of Env to receptor CD4 and therefore profoundly diminishes the infectivity of HIV-1_{BH10}. In the case of HIV-1_{NL4-3}, the A539 residue within HR1 of gp41 was changed to a valine. The same mutation was seen in HIV-1_{NL4-3} that escaped from the inhibition by a newly discovered restriction factor called MxB [181]. A539V was shown to stimulate the production of HIV-1_{NL4-3} from SupT1 cells and thus plays a compensatory role in rescuing virus infection. Although HIV-1_{BH10} and HIV-1_{NL4-3} mutate different sites of Env to counter the inhibition by IFITM1 and its mutant, these data support an active role of Env in overcoming host restrictions of HIV-1 infection.

Chapter 5 – General Discussion

One major contribution of this thesis is the elucidation of one strategy that HIV-1 utilizes to escape from IFITM1 inhibition *in vitro*, i.e. through mutating viral Vpu and Env proteins. This conclusion is supported by the results of three independent studies, 1) HIV-1_{BH10} acquired the Vpu36 and EnvG367E mutations to overcome IFITM1 inhibition (chapter 2); 2) HIV-1_{NL4-3} evades the restriction of IFITM1 mutant Δ(117-125) through accumulating Vpu40 and EnvA539V mutations (chapter 4); 3) The Env of HIV-1_{NL4-3} confers resistance to IFITM1 (chapter 3). Interestingly, Vpu and Env do not seem to directly correct the deficit caused by IFITM1, but rather stimulate viral replication by enhancing HIV-1 cell-to-cell transmission.

IFITM1 differs from IFITM2 and IFITM3 in inhibiting HIV-1_{BH10}. Although all three IFITM proteins diminish the expression of viral proteins in infected SupT1 cells and thereby reduce the production of virus particles, IFITM2 and IFITM3, but not IFITM1, hinder HIV-1 entry [203]. It is suspected that IFITM1 negatively affect HIV-1 gene expression. This possibility is supported by a study showing that overexpression of IFITM proteins in transfection experiments inhibit the levels of HIV-1 Gag, Vif and Nef in 293T cells [201]. Our results show that the Vpu34 and EnvG367E mutations did not restore the low level of viral protein expression in the presence of IFITM1, but rather enhanced the replication of HIV-1_{BH10} by stimulating virus cell-to-cell transmission.

It is still not clear how IFITM1 blocks HIV-1 replication. This study revealed a

regulatory role of the C-terminal sequences of IFITM1 in modulating its anti-HIV-1 activity. Deletion of the C-terminus sequence, especially the last 9 amino acids, allows IFITM1 to inhibit the entry of HIV-1. Our recent data (unpublished) also show that deleting the C-terminal sequence relocates IFITM1 to the plasma membrane, where HIV-1 completes fusion and entry. Therefore, the antiviral activity of IFITM1 may depend on its subcellular distribution. Moreover, the C-terminus deleted IFITM1, not the wild type form, inhibited the replication of HIV-1_{NL4-3}. It is worth noting that IFITM1 inhibits some viruses (influenza virus, Yellow fever virus etc.) less efficiently than IFITM3 does. Further comparing the C-terminus deleted IFITM1 with IFITM3 in inhibiting these viruses may help to further elucidate the antiviral activity of IFITM1. In this context, one group recently reported that the C-terminus deleted IFITM1 increased the infectivity of HCoV-OC43 but still inhibited influenza A virus and SARS-CoV [225].

No viral antagonist has been reported so far to counter IFITM proteins. Viral Env proteins are good candidates in this regard because their presence on viral membrane allows them to directly interact with IFITM proteins and thereby abrogate the antiviral function of IFITM. There have been precedents of the involvement of viral Env in countering host restriction factors. Examples are Ebola virus GP and HIV-2 Env that both are able to interact with the restriction factor tetherin and downmodulate tetherin from the cell surface [170] [171]. It remains to be tested whether HIV-1 Env, as well as the Env of other viruses, is associated with IFITM proteins and neutralize their antiviral function.

Our study revealed a new mechanism of IFITM1 inhibition, which involves impairing the infectivity of HIV-1_{BH10} particles. This likely results from the incorporation of

IFITM1 into HIV-1 particles as demonstrated by two recent studies [279] [280]. Since IFITM1 is membrane-bound, it is not surprising to see the association of IFITM1 with HIV-1 particles that are assembled on the plasma membranes. The challenge of these experiments will be how to demonstrate the specificity of IFITM1 incorporation into HIV-1 particles but not a result of IFITM1 presence in exosome vesicles that are often co-purified with HIV-1 particles. This mechanism of inhibition naturally extends to the restriction of other enveloped viruses not only by IFITM1, but also by IFITM2 and IFITM3. In the context of this antiviral mechanism, the Env of HIV-1_{NL4-3} appears to act as an antagonist of IFITM1 through overcoming the inhibition of HIV-1 infectivity, the infectivity of HIV-1_{NL4-3} is not affected by IFITM1 and since inserting HIV-1_{NL4-3} Env into HIV-1_{BH10} renders the chimeric virus resistant to IFITM1 at least partially because IFITM1 does not markedly affect the infectivity of this chimeric virus (Figure 3.3.3).

This mechanism of inhibition does not come as a surprise considering that IFITM proteins inhibit the entry of multiple viruses. For this latter activity, the experiments were designed to challenge IFITM-expressing cells with virus particles that have never been examined for the presence of IFITM proteins [221] [222] [223] [224]. These studies therefore conclude that IFITM proteins act in the target cells, especially in the late endosomes where the majority of pH-dependent viruses complete their entry, to block the entry of free virus particles [205] [230] [231]. With this inhibitory activity, it is only logical that when IFITM1, also IFITM2 and IFITM3, is present on the viral membrane, the entry of these virus particles will also be impaired [206] [279] [280].

HIV-1 differs from the other viruses that are also inhibited by IFITM1, 2 and 3 in that HIV-1 is the only virus whose entry is pH-independent (reviewed in [199] [213]). The typical pH-dependent virus is influenza A viruses that, following binding to its receptor on the cell surface and endocytosis, complete entry in late endosomes/lysosome where the low pH triggers viral membrane fusion with endosome membrane [281] [282]. In line with this entry mechanism, IFITM proteins are mainly found at late endosomes so they are well positioned to encounter the invading virus particles and block their entry. The fact that IFITM2 and IFITM3, as well as the C-terminally truncated IFITM1, also inhibit the entry of HIV-1 allude to the presence of these proteins on the plasma membrane where HIV-1 completes its entry without the need of low pH to trigger viral membrane fusion. This speculation is supported by the results of our recent study showing the presence of a small portion of IFITM3 on the plasma membrane [212]. Since IFITM proteins restrict virus entry at both the endosomes and the plasma membrane, a specific membrane environment may not be a prerequisite for IFITM proteins to exert their antiviral effects.

It remains to be determined how many different HIV-1 strains that human IFITM1 can inhibit. To find out the possible measures that HIV-1 develops to counter IFITM1 restriction, we have utilized the lab-adapted HIV-1 strains - HIV-1_{BH10} and HIV-1_{NL4-3} to perform the experiments. Both HIV-1_{BH10} and HIV-1_{NL4-3} have adapted to the *in vitro* culture conditions and they might lose the ability to escape from the inhibition of IFITM1. Then under certain pressures such as the expression of IFITM1, they may regain the ability to counter IFITM1. As we expected, the replication of HIV-1_{BH10} was dramatically

inhibited by the expression of IFITM1. Under the expression of IFITM1, HIV-1_{BH10} developed special countermeasures by mutating proteins Vpu and Env to overcome the restriction of IFITM1 (Chapter 2). Interestingly, the other lab-adapted HIV-1 strain HIV-1_{NL4-3} is refractory to the inhibition of IFITM1, which indicates that different HIV-1 strains have different sensitivities to IFITM1. The results of viral genome comparison showed that the amino acids differences in envelope protein determine their sensitivities to the restriction of IFITM1 (Chapter 3). Due to the universal expression of IFITM proteins, one CD4+ T cell line expressing only IFITM1 proteins but not the other known endogenous HIV-1 restriction factors (BST-2, APOBEC3G/F and SAMHD1, etc.) should be utilized to perform the evolutionary experiments. HIV-1 might develop multiple mutations to counteract the multiple pressures exerted by the different restriction factors. Indeed, loss of the expression of Vpu is critical for HIV-1_{BH10} to escape from the restriction of IFITM1 in SupT1 cells, which might not happen in BST-2 expressing T cell lines such as primary CD4 T cells.

It is undetermined whether the primary HIV-1 strains also utilize Vpu and Env to evade IFITM restriction. We are in the process of testing different primary HIV-1 strains, HIV-2 and SIV for their susceptibility to IFITM inhibition. The goal is to have a complete picture on the impact of IFITM proteins on primate lentiviruses. Since HIV-1 uses either CXCR4 or CCR5 as co-receptor, it will also be worth testing whether IFITM proteins have any preference to inhibit X4 or R5 strains. These studies will also extend to investigate the anti-HIV activity of IFITM proteins in primary cells such as CD4+ T cells and macrophages. IFITM proteins are unique ISGs because IFITM proteins are the only

one that impairs virus entry. This unique property endows IFITM proteins with the possible utilization in HIV intervention.

IFITM3 inhibits the infection of influenza virus more potently than IFITM1 and IFITM2 [221] [226]. People from Caucasian and Han Chinese containing synonymous single nucleotide polymorphism (SNP) rs12252 in the *ifitm3* gene are more susceptible for infection by influenza viruses [226]. SNP rs12252 in *ifitm3* expresses the N-terminal 21 amino acids deleted IFITM3 that loses anti-influenza activity [209] [212]. We found the deletion of last 9 amino acids from the C-terminus allowed IFITM1 to block HIV-1_{NL4-3} replication as a result of the gained function of impairing HIV-1 entry (Chapter 4). Since the *CCR5 Δ32* allele confers protection from HIV-1 infection [283], the possible polymorphism in IFITM1 C-terminus might also affect HIV-1 transmission or AIDS progression in HIV-1 infected individuals.

Contribution to general knowledge

Research in this thesis generated the following new knowledge in the context of HIV and host interactions:

For the first time, we discovered that HIV-1_{BH10} is able to acquire mutations to escape the restriction by IFITM1. The mutations on HIV-1_{BH10} viral proteins Vpu and envelope increased the virus cell-to-cell transmission efficiency in SupT1 cells and therefore restored HIV-1_{BH10} replication in the presence of IFITM1. This study also demonstrated that the expression of IFITM1 is pressure on the replication of HIV-1.

We identified the first HIV-1 strain called NL4-3 that is refractory to IFITM1. We further demonstrated viral Env as the viral determinant behind this resistant phenotype. HIV-1_{NL4-3} is able to counter the ability of IFITM1 to diminish HIV-1 infectivity and also mediates a much higher cell-to-cell transmission as compared to that of HIV-1_{BH10}.

For the first time, we discovered a role of the C-terminal region of IFITM1 in modulating the antiviral function of IFITM1. The results explain the previous puzzling observation that IFITM2 and IFITM3, but not IFITM1, are able to inhibit HIV-1 entry.

Overall, using HIV-1 as a virus model, we have explored how viruses may counter the restriction by IFITM proteins and discovered an important role of viral Env protein in this regard.

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