Human IFITM2 inhibits SIV_{AGM} entry

Jin Qian

Department of Medicine, Division of Experimental Medicine

McGill University, Montreal

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Abstract

Interferon-inducible transmembrane proteins (IFITMs) restrict entry of many pH-dependent enveloped viruses such as Influenza A virus, dengue virus, hepatitis C virus, Ebola virus, and even non-enveloped virus such as Reovirus. These proteins are believed to have two transmembrane or intramembrane domains and prevent viral membrane fusion without relocating the virus to other sites or changing the pH of the endosome environment. Previously, our group reported that IFITMs inhibit human immunodeficiency virus type 1 (HIV-1), a virus that does not require access to endosome for entry. In this study, we set to provide more evidence for restriction at entry of a pH-independent virus and explore the mechanism of inhibition. Simian immunodeficiency virus strains that infect African green monkey (SIV_{AGM}) are found to be significantly restricted by human IFITM2. SIV strain from sooty mangabey is partially restricted, while strain from macaque is unaffected. The restriction of SIV_{AGM} by human IFITM2 occurs at entry. Interestingly, IFITM2 restricts these viruses better than IFITM3 does, despite their high homology at the amino acid level. We found that 2 amino acid residues at the N-terminal domain are responsible for the higher restriction efficiency by IFITM2. We also cloned AGM IFITMs to test whether the monkey proteins can inhibit HIV-1. Surprisingly, AGM do not have IFITM2 (similar to macaque), but AGM IFITM3 inhibits all tested strains including SIV_{MAC} better than it inhibits HIV strains. These findings have therefore expanded the pH-independent viruses that are inhibited by IFITM proteins and provide a new avenue to explore the antiviral actions of IFITM.

Résumé

Les protéines transmembranaires inductibles par interféron (IFITM) limitent l'entrée de nombreux virus enveloppés dont l'entrée dépend du pH, tels que les virus de la grippe, le virus de la dengue, le virus de l'hépatite C, le virus Ebola, et même des virus non enveloppés tels que le reovirus. Ces protéines ont deux domaines transmembranaires ou intramembranaires et peuvent empêcher la fusion des membranes virales sans altérer le site d'entrée ou de modifier le pH de l'environnement des endosomes. Notre groupe a rapporté auparavant que les protéines IFITM pouvaient aussi inhiber le virus de l'immunodéficience humaine de type I (HIV-1), un virus qui ne nécessite pas l'accès aux endosomes lors de l'entrée. Dans cette étude, nous avons décidé à la fois de fournir plus de preuves quant à la restriction d'un virus dont l'entrée est indépendante du pH mais également d'explorer le mécanisme d'inhibition.

Les souches de virus d'immunodéficience simienne (SIV) qui infectent les singes verts d'afrique (SIV_{AGM}) se trouvent être considérablement inhibées par les protéines IFITM humaines. Les souches de SIV provenant des mangabeys (SIV_{SMM}) sont partiellement affectées, tandis que la souche de SIV infectant le macaque (SIV_{MAC}) est résistante aux protéines IFITM. De plus, nous avons démontré que les protéines IFITM humaines inhibent l'étape d'entrée de SIV_{AGM} et que ces virus sont plus affectés par IFITM2 que par IFITM3. Nous avons aussi constaté que les acides aminés situés à l'extrémité N-terminale sont responsables de l'inhibition plus importante d'IFITM2 par rapport à IFITM3. Nous avons également cloné les gènes IFITM issus des singes verts d'Afrique pour tester s'ils peuvent inhiber HIV-1. De manière surprenante, les singes verts d'Afrique n'ont pas de IFITM2, mais IFITM3 issu de ces mêmes singes, inhibe toutes les souches examinées, y compris SIV_{MAC}, plus efficacement qu'il inhibe les souches de HIV. Ces résultats ont donc élargi les virus pH-indépendant qui sont inhibés par des protéines IFITM et fournir une nouvelle avenue à explorer concernant les actions antivirales de IFITM.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
AGM	African green monkey
APOBEC3G	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
AZT	Azidothymidine
BST2	Bone marrow stromal antigen 2
CA	Primate lentivirus capsid
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CMP	Count per minute
CpG	C—phosphate—G
CID	Conserved intracellular domain
CPZ	Pan troglodytes
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DDOST	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit (non-catalytic)
DENV	Dengue virus
DHX9	ATP-dependent RNA helicase A
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EBOV	Ebola virus
ECL	Enhanced chemoluminescence
ER	Endoplasmic reticulum
ESCRT	Endosomal Sorting Complexes Required for Transport
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
FUS	Fused in sarcoma
GALNT2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyl-transferase 2
GALT	Gut-associated lymphoid tissue
gp	glycoprotein

GPI	Glycophosphatidylinositol
HAART	Highly Active Antiretroviral Therapy
HCV	Hepatitis C virus
HDAC	Histone deacetylases
HEK	Human embryonic kidney cell
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HR1	C-terminal heptad repeats
HR2	N-terminal heptad repeats
HSPA9	heat shock 70kDa protein 9
HSV	Herpes simplex virus
IAV	Influenza A virus
IFN	Interferon
IFITM	Interferon inducible transmembrane
IN	Retrovirus integrase
IRF	Interferon Regulatory Factor
ISG	Interferon stimulated gene
ISG15	IFN-stimulated protein of 15 kDa
ISRE	IFN-stimulated response element
IST	Incompletely spliced transcript
JAK1	Janus activated kinase 1
KB	kilobase
KD	Knockdown
LASV	Lassa virus
LCMV	Lymphocytic Choriomeningitis Virus
LEDGF/p75	lens epithelium-derived growth factor
LTR	Long terminal repeat
MA	Primate lentivirus matrix
MAC	Macaca mulatta
MACV	Machupo virus
MARV	Marburg virus
MDA5	Melanoma Differentiation-Associated protein 5
MLV	Murine leukemia virus
mRNA	Messenger RNA
MT	Microtubule
MTOC	Microtubule organizing center
MVC	Maraviroc
NC	Primate lentivirus nuclear capsid

NCBI	National Center for Biotechnology Information
Nef	Negative Regulatory Factor
NELF-E	Negative elongation factor subunit E
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NP	Influenza virus nuclear protein
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NS	Non-structural
OAS	2',5'-oligoadenylate synthetase
ORF	Open reading frame
OWM	Old World Monkey
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Primer-binding site
pDC	Plasmacytoid dendritic cell
PI	Post-infection
PIs	Protease Inhibitors
PIC	Pre-integration complex
PKR	dsRNA-dependent protein kinase
pol	Viral polymerase
ppt	Polypyrimidine tract
PR	Primate lentivirus protease
PRR	Pattern-recognition receptor
PrEP	Pre-exposure prophylaxis
P/S	Penicillin/Streptomycin
P-TEFb	Positive transcription elongation factor
PVDF	Polyvinylidene fluoride
REOV	Reovirus
Rev	Regulator of viral expression
RIG-I	Retinoic acid inducible gene I
RI-MUHC	Research Institute of the McGill University Health Centre
RIPA	Radioimmunoprecipitation assay buffer
RLU	Relative light units
RNA	Ribonucleic acid
RNase L	Ribonuclease L
RPMI	Roswell Park Memorial Institute medium
RRE	Rev responsive element

rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
RTC	Reverse transcription complex
RVF	Rift Valley fever virus
SAMHD1	SAM domain and HD domain-containing protein 1
SARS	Severe acute respiratory syndrome
sCD4	Soluble CD4
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
ssRNA	Single-standed RNA
STAT	Signal Transducers and Activators of Transcription
STD	Sexually transmitted diseases
SIV	Simian immunodeficiency virus
SMM	Sooty mangabey
SP1	Primate lentivirus spacer peptide 1
SP2	Primate lentivirus spacer peptide 2
SU	Surface unit
TAR	Transactivation response region
Tat	Transactivator
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TLR	Toll-like receptor
TM	Transmembrane
TRIM5 α	Tripartite motif-containing protein 5 α
tRNA	Transfer RNA
UNAIDS	Joint United Nations Programme on HIV and AIDS
V3	Third variable loop of HIV-1 gp120
VAP-A	Vesicle-membrane-protein-associated protein A
VFA	Virion fusion assay
vgRNA	Viral genomic RNA
Vif	Viral infectivity
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Vesicular stomatitis virus glycoprotein

WNV	West Nile virus
WT	Wildtype
YFP	Yellow fluorescent protein
YFV	Yellow fever virus

Chapter I: Literature Review

1.1. HIV pandemic

1.1.1 Year 2013

There are more HIV infected individuals than ever in history, which is both good news and bad news (Figure 1). The good part about it is that HIV related death has been steadily decreasing from 2.3 million in 2005 to 1.7 million in 2011. However, the bad part is that HIV is still spreading, albeit at a slower rate, with 2.5 million new infections in 2011 compared to 3 million in 2001. This brings the number of infected individual to 34 million worldwide. (136)

We are two years away from the deadline set by the *United Nations Millennium Declaration* back in 2000 to halt and reverse the spread of HIV. The goal of universal access to HIV care for infected individuals by 2010 has been changed to that of providing access to 15 million people in need by 2015 in the *2011 Political Declaration* (7). Although there could be another pushback, progresses are being made in the combat against HIV. This is mostly reflected from the fading of terror instigated by HIV back in late 20th century, at least in the parts of the world with access to anti-retroviral therapy (ART). HIV is, after all, a pathogen with close to 100% mortality, if untreated. In low- and middle-income countries, social barriers toward HIV care are gradually being removed as movements to protect women and reduce stigma and discrimination associated with HIV have gained momentum (136). Prevention strategies are also deployed for high-risk population in an increasing number of countries.

If the recent trend in terms of number of people acquiring HIV and dying from acquired immunodeficiency syndrome (AIDS) continues, the number of HIV positive individuals will stabilize at 40 million in 20 years. As HIV remains in the body until death and the majority of the infected individuals will be still aged below 50 years old by then, a

significant part of the global health capacity will be consumed to manage HIV positive population, unless a cure is found.

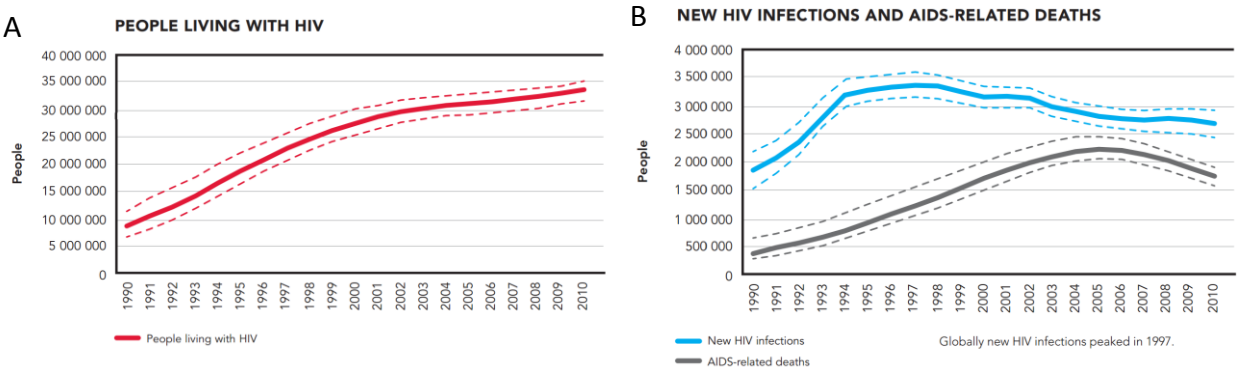

Figure 1. Global HIV trends from 1990 to 2010 (137). Global trend of A. number of infected individuals (red), B. new HIV infections (blue), and AIDS-related deaths (black). Dotted lines denote estimation range.

1.1.2 Discovery and origin

In 1981, opportunistic infection, commonly found in immunosuppressed patients, discovered in an unusually high number of young adults in the United States caught medical researchers' attention (23, 34). During the course of next five years, two independent laboratories isolated viruses responsible for the outbreaks and named it human immunodeficiency virus (HIV) (13, 49).

As HIV has an average incubation time of eight years before the syndromes surface (39), HIV should have come to North America during early 70s. Based on phylogenetic evidence, HIV-1 group M subtype B (to be elaborated below), the strain responsible for the pandemic in North America and European countries, came from Africa to Haiti between 1962 and 1970 (54). After spreading and diversifying for three years in Haiti, one of the strains moved to the United States and initiated the outbreak. Other parts of the world are impacted by a different strain. HIV-1 group M subtype C spread from Southern African to Asia via India (88, 120).

1.1.3 Group/subtypes and their distribution

There are many HIV strains. HIV is classified into 2 types using phylogenetics. HIV type 1 (HIV-1) is further divided into 4 groups with M being the major group and N, O, and P in the minor groups. The global pandemic is caused by viruses from group M, as aforementioned. There are currently nine identified subtypes within this group: A, B, C, D, F, G, H, J, and K (134). Subtype B is the most prevalent in America and many European countries (figure 2), and as a result, is the most widely studied. However, the strain with highest prevalence is subtype C (figure 2, inset): Sub-Saharan Africa remains the most heavily infected regions with 4.9% of the population being HIV subtype C positive, which accounts for 69% of people living with HIV worldwide. Moreover, the pandemic associated with this virus is expanding in Central Asia (136). In addition to the pure subtypes, there are chimeric strains generated when an individual is infected by two or more subtypes. More than 40 circulating recombinant forms (CRFs) have been identified to date (134).

HIV-1 group N, O, and P are largely confined to Central Africa, more specifically, Cameroon and its surrounding countries. Group O strains accounts for less than 1% of all HIV positive individuals (92, 99), while there are only 13 cases of group N infection (125). Group P is even scarcer, with only two cases identified so far (138).

HIV type 2 (HIV-2) is the second type. However, it is considerably different from HIV-1 in terms of origin and pathogenic ability. Due to its lower transmission rate, it is epidemic only in West Africa (31). So far, 1 to 2 million people are estimated to be infected by this virus (56). As with HIV-1, HIV-2 is also classified into several groups: group A and B are causing the pandemic in the area, while the other groups (C-H) have only been identified in single individuals (121).

1.1.4 Transmission

HIV is a sexually transmitted disease (STD). Although other routes of transmission such as fusion of contaminated blood and sharing injection needles are more effective, the

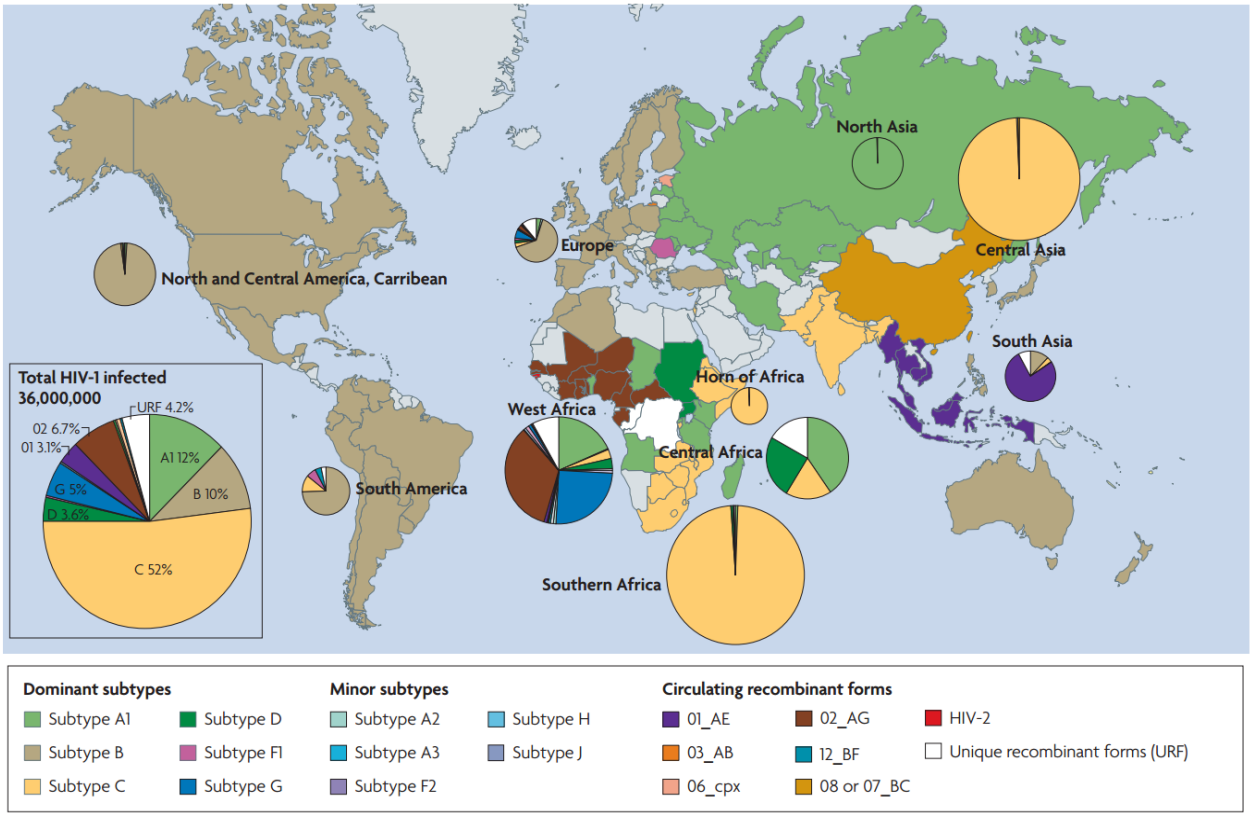

Figure 2. Global HIV-1 group M subtypes prevalence (6)**.** The size of the circles reflects disease burden, while the size of the pie reflects the relative prevalence of various subtypes (in color code) within the indicated region. Inset represents the prevalence of global total. There is not sufficient data on countries colored in grey.

majority of transmissions occur through sexual intercourse. Babies can be born HIV positive from infected mothers. In addition, breast milk also contains HIV virus and can infect newborns that are breastfed.

As with many STD, HIV is present in high concentration in semen and vaginal fluid. Knowledge on the molecular and biological mechanism of transmission has been limited until recently. During sexual intercourse, the majority of virus is blocked at the mucosal layer, a formidable innate immune barrier against pathogens. Only one or a few viruses manage to cross the mucosal layer and establish a productive infection (75, 115). These so called founder virus then expand locally and diversify during systemic dissemination. This suggests a bottleneck in the transmission of the virus. In fact, 1000 coital acts are required

for 1 transmission on average (143). The efficiency is factored by donor viral load, as transmission rate is 7 to 8 times higher from donors who got infected within 5 months and those who are in advanced stage (32, 143). On the recipient side, availability of $CD4^+$ T cells carrying C-C chemokine receptor type 5 (CCR5), a co-receptor needed for viral entry into cells, at the mucosal site can affect the rate of transmission (155). The availability of macrophages and dendritic cells (65) as well as inflammatory conditions could also increase the rate of acquiring HIV .

1.1.5 Treatments

Since the start of HIV/AIDS pandemic, drugs have been constantly developed and improved. In 1987, the first anti-viral drug, azidothymidine (AZT), was approved to treat HIV patients. It was indeed life-saving, as AIDS, a disease with 100% mortality, suddenly become manageable. However, the drug has numerous side effects and virus quickly build up resistance against the drug, as large viral population size and high immune cell turnover speed up viral evolution. As a result, more potent drugs with less side effects and less susceptibility to resistance are developed. They are now used in combination to further reduce the probability of developing resistance. Nevertheless, resistance can still happen. Recently, more drugs targeting different steps in the viral life are used in combination therapy. Up to now, there are over 25 drugs approved by the U.S. Food and Drug Administration (FDA) for HIV treatment (table 1) (40). If resistance develops or side-effects become intolerable, patients can switch from one drug to another.

That is the case for people living in developed countries. For those in middle- and low-income countries, the era of highly active antiretroviral therapy (HAART) just started (figure 3). Taking the example of sub-Saharan Africa, HIV positive population increased from 20.9 million to 23.5 between 2001 and 2011; however, less than one third of them have access to HAART by the end of 2011. One of the challenges in providing treatment is funding. With increasing number of relative inexpensive generic drugs, access to treatment in these parts of the world is increasing. (137)

Table 1. FDA approved drug for HIV infection. (40)

Class of drug	Generic Name
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	• lamivudine and zidovudine • emtricitabine, FTC • lamivudine, 3TC • abacavir and lamivudine • zalcitabine, dideoxycytidine, ddC • zidovudine, azidothymidine, AZT, ZDV • abacavir, zidovudine, and lamivudine • tenofovir disoproxil fumarate and emtricitabine • enteric coated didanosine, ddI EC • didanosine, dideoxyinosine, ddI • tenofovir disoproxil fumarate, TDF • stavudine, d4T • abacavir sulfate, ABC
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)	• rilpivirine • etravirine • delavirdine, DLV • efavirenz, EFV • nevirapine, NVP
Protease Inhibitors (PIs)	• amprenavir, APV • tipranavir, TPV • indinavir, IDV • saquinavir • saquinavir mesylate, SQV • lopinavir and ritonavir, LPV/RTV • Fosamprenavir Calcium, FOS-APV • ritonavir, RTV • darunavir • atazanavir sulfate, ATV • nelfinavir mesylate, NFV
Fusion Ihinbitors	• enfuvirtide, T-20
Entry Inhibitors	• maraviroc
Integrase Inhibitors	• raltegravir • dolutegravir

Hundreds and thousands of dollars have been spent in order to develop a cure for AIDS patients. However, there is still no effective way to cure AIDS. The reasons is that once HIV integrates itself in the cell genomic DNA, it will stay there until the cell dies. Many of these cells served as reservoir for HIV are long lived memory T cells. As they can last decades, HIV will also persist. Although modern anti-retroviral therapy can very effectively control viral load in circulation, they cannot touch the proviral DNA in cellular genome. Researchers are now looking into eradication of latent viral reservoir using histone deacetylase (HDAC) inhibitors (123). To date, there are very two reports of functional cure (28, 114), defined as sustained low to undetectable virus load without anti-retroviral therapy. In both cases, investigators suggested that early treatment might have contributed to the cure. There is currently no strict guideline on when to initiate treatment.

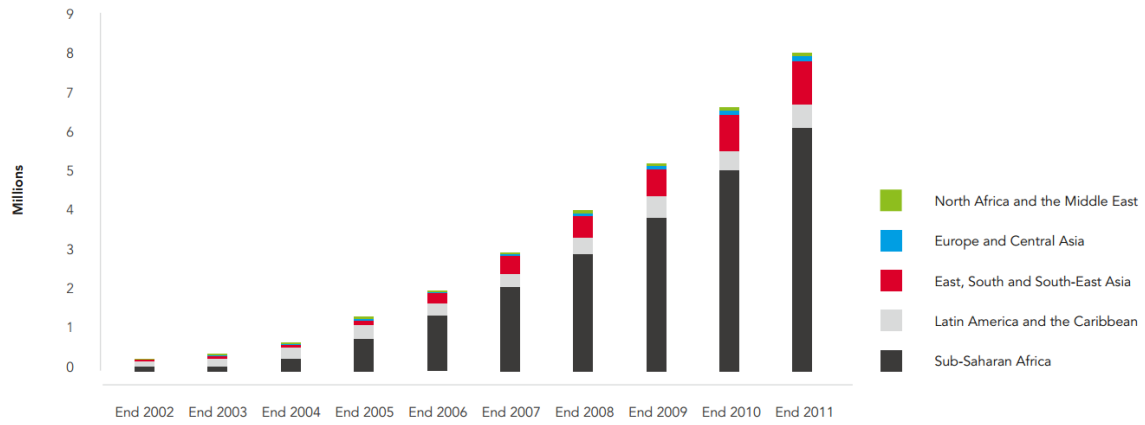

Figure 3. Number of people receiving antiretroviral therapy in low- and middle-income countries from 2002 to 2011 (137)**.** Each region is color-coded as in legend.

1.1.6 Preventions

When an important human virus is discovered, it is natural to think about making a vaccine for it. The necessity of an HIV vaccine is further underlined by the fact that there is no cure for the infection. The development began right after the etiological agent of AIDS was identified. The first two vaccines were designed to elicit conventional humoral response, where neutralizing antibody is produced against HIV surface protein, more specifically, glycoprotein 120 (gp120). However, the clinical trials did not demonstrate any

protective effect (45, 105). Antibodies were produced against the gp, but they do not neutralize the virus. Attention subsequently moved onto a conventional T cell based vaccine, but the trial also ended up in disappointment (20). Moreover, trial data suggest that there is an increase of risk of HIV acquisition in those who received the vaccine. Finally, the HIV vaccine community saw hope when the results from a 2003 trial (RV144 AIDS vaccine trial) were released (110). Although the rate of protection was modest (31%), new mechanisms of protection were revealed, providing further insights into HIV vaccine design and capacity of human immune system (103).

After 30 years of research with more than 15 million spent, we still do not have a vaccine for HIV. However, other means of prevention have been explored. First, as HIV is a sexually transmitted disease, condom is shown to provide 100% protection. This is precisely the reason the Joint United Nations Programme on HIV/AIDS (UNAIDS) is distributing and promoting condom usage in the most heavily infected areas. Second, since the rate of transmission is directly related to viral load, giving infected individuals ART can limit transmission to their sexual partners (29). This is also the case for mother-to-infant transmission. Third, circumcision can also reduce the rate of acquisition in men. Finally, due to the stalling in vaccine researches, pre-exposure propylaxis (PrEP) are being considered recently with many ongoing clinical trials (8). Taking ART drugs before engaging risky behavior could greatly reduce rate of transmission.

1.2. Primate lentivirus

1.2.1 Structure and genome organization

HIV belongs to the family of retroviridae and in the genus of lentivirus. The virus is roughly spherical and has a diameter of 120nm (Figure 4A). Its envelope is derived from lipid bilayer of infected cell it budded from. There are about 10 surface glycoproteins on the viral envelope (82). Each is constituted of trimers of gp120 surface unit (SU) docked onto trimers of transmembrane (TM) gp41 subunits. Immediately under the viral envelope aligns matrix protein (MA or p17). Viral protease (PR) used in processing group-specific

antigen (gag) is free floating along with gag spacer peptide SP1 (p1) and SP2 (p2) (not shown). The viral core is conical shaped and supported by processed gag subunit, p6 (not shown), and capsid protein (CA or p24). Inside, two copies of the single stranded RNA viral genome is protected with a coating of nucleocapsid (NC or p7) and associated with roughly 80 reverse transcriptases (RT) and integrases (IN). Functions of these proteins will be elaborated in the next section. Viral accessory proteins such as viral infectivity factor (vif), viral protein R/X (vpr/vpx), and negative regulatory factor (nef) are also included in the core.

Like many viruses, HIV packs as many genes as possible in the smallest space. There are in total 3 major genes and 6 smaller genes in a span of less than 10 kilobases (kb). The first major gene is the structural gene called gag. When the gene product is fully processed by viral protease, it yields MA, CA, NC, and p6, as described above. SP1 is between CA and NC and SP2 is between NC and p6 (not shown). The next major gene is pol, but it is not translated on its own. The gag and env genes are on two different reading frames as illustrated. In 5% of the case, a frameshift happens near the end of gag translation and ribosome shifts to the env gene reading frame, producing a gag-pol protein which can be processed into the gag subunits and env products: PR, RT, and IN. The last major gene encodes the envelope proteins, gp120 (SU) and gp41 (TM), after their precursor, gp160, is processed by cellular protease. Some of the regulatory genes and accessory genes on different frames overlap with each other to maximize nucleotide usage. They are produced as single proteins instead of polyproteins like the major genes. The genome is flanked by 5' and 3' long terminal repeat (LTR) on both ends, which are essential for reverse transcription.

1.2.2 Life cycle

The HIV life cycles is divided to early stage (1-6) and late stage (7-13) (figure 5). During early stage, the virus aim to infect its target cell and to remain within until the cell dies. During late stage, using cellular transcription and translation machineries, viral particles are made from integrated viral genome to infect more cells. Viral entry will be

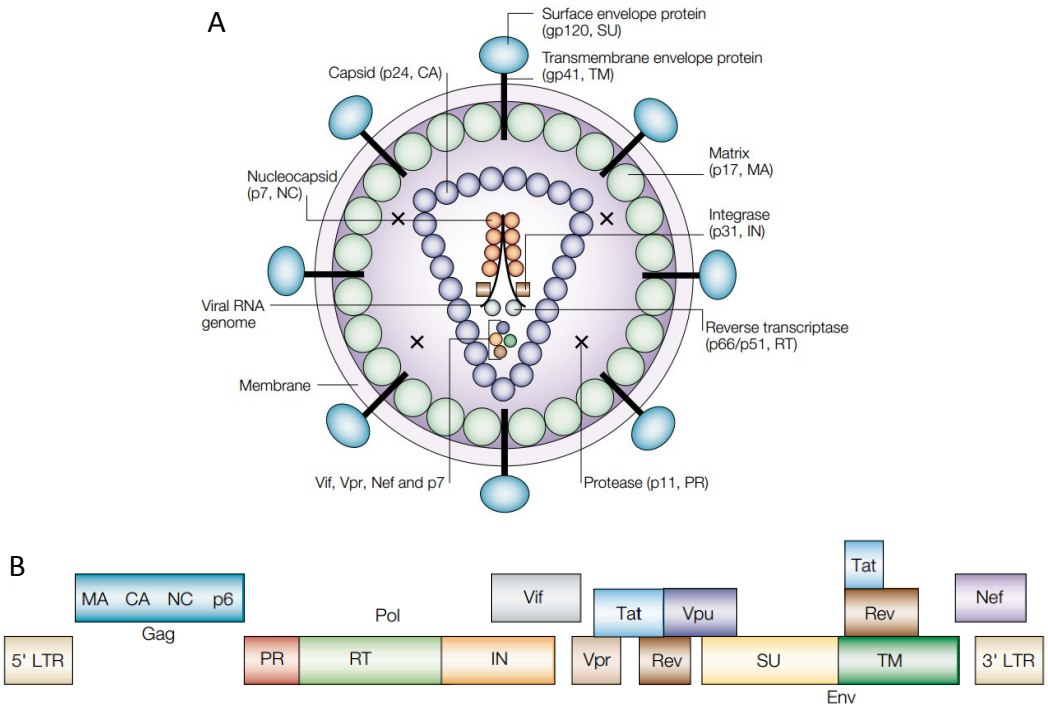

Figure 4. HIV structure and genome organization. Details on A) HIV structure and B) genome organization are given in section 1.2.1 Structure and genome organization. (112)

describe in more details, as the protein involved in this project is believed to intervene at this stage.

1.2.2.1 Entry

HIV starts entry with binding of gp120 to its receptor cluster of differentiation 4 (CD4), a common surface protein on a subgroup of T cells and on majority of dendritic cells and macrophages. This interaction stabilizes the virus on the cell plasma membrane. Once the main receptor is engaged, gp120 changes conformation revealing third variable (V3) loop – the co-receptor binding site. The recruitment of co-receptor, CCR5 or C-X-C chemokine receptor type 4 (CXCR4), by the V3 loop to the CD4-gp120 complex results in further conformational changes in the glycoproteins and exposure of the fusion peptide and N-terminal α-helix and C-terminal segment (or heptad repeat 1 and heptad repeat 2

respective) of gp41 (35). This intermediate state termed prehairpin is vulnerable to HR2 analogue inhibitors such as T20. As the HR1 and HR2 fold on each other in an antiparallel

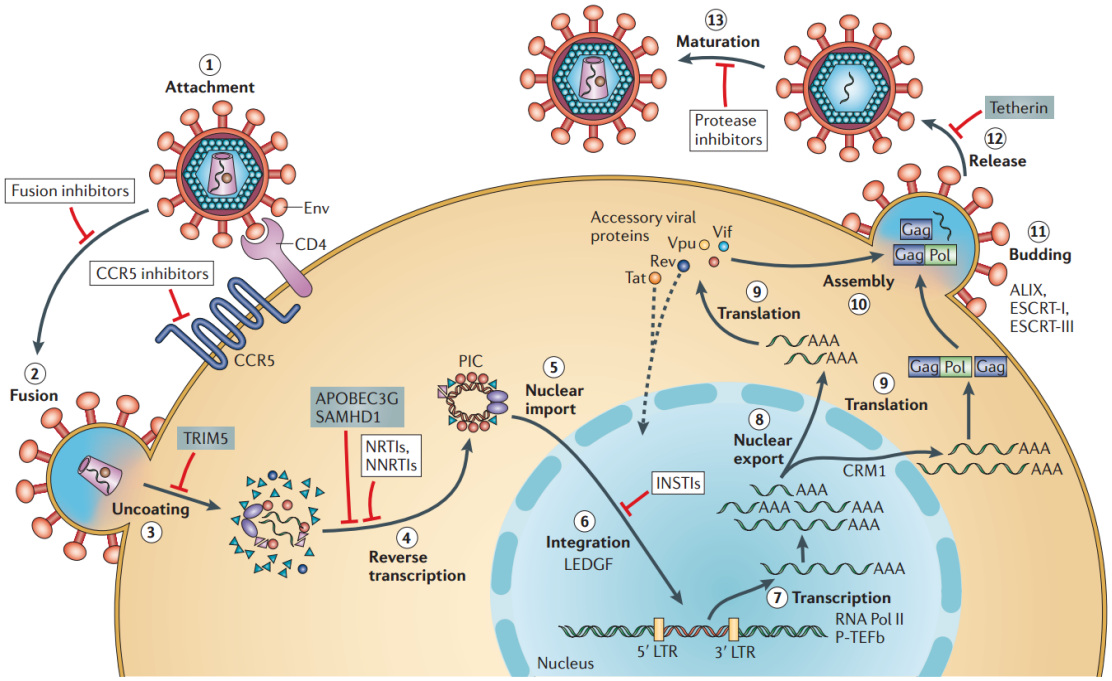

Figure 5. HIV life cycle and steps where cellular factors or drug inhibitors acts (36). See 1.2.2 Life cycle for detail on viral life cycle and 1.3.2 Anti-HIV restriction factors for the cellular factors.

manner into six-helix bundle, the viral and cellular membrane are brought into close proximity and results in fusion.

Membrane dynamic has been studied to further understand this process. A single protrusion from both viral and cellular membranes coming into close proximity of each other initiates the fusion event (figure 6) (149). The increased hydrophobicity on the protruded tip promotes lipid mixing at that location. The expansion of this so called "hemifusion stalk" (79) results in hemifusion diaphragm. Fusion pore can be formed from this diaphragm or direction from the stalk. Formation of six-helix bundle release more energy than any other conformational changes in the gp120/gp41. Thus, it is very tempting to believe that the energy needed to go through this fusion process is provided by the folding of six-helix bundle. However, details lacks on where the protrusion is formed and

how the protrusion is initiated. Electron tomography evidence further fuels the confusion, showing that multiple trimeric units are aligned in the interface between viral membrane and plasma membrane (126). Whether all or only one trimeric unit is needed is not clear. There is also no evidence in regard to whether the fusion peptide is inserted into the plasma membrane before or during the folding of six-helix bundle. Knowledge on the mechanism of pore formation is scarce at best. One report suggests that the membrane proximal external region is involved (98). Due to high energy requirement in this particular step, it has been postulated that cellular factors are involved to assist the virus. Harmon *et al.* provide evidence supporting that Abl and the Wave2 signaling complex assists pore formation through actin remodeling (60).

There is minor disagreement whether the fusion process occur on the cell surface, i.e. plasma membrane, or in the endosomal compartment. Many evidences support the former hypothesis. First, HIV is a pH-independent virus. Many pH-dependent viruses travel to the early or late endosomal compartment, because low pH is needed to trigger the conformational change in envelope. HIV has no such requirement (93). Second, expression of envelope protein on HIV-susceptible cells can induce syncytia formation. Third, internalization of CD4 is not needed for entry (89). Finally, cortical actin below plasma membrane can prevent HIV from moving toward nucleus if not rearranged during entry (150). However, a recent article published in a respected journal by Miyauchi demonstrates using time-resolved single virus imaging that HIV can enter cells through endocytosis (95). There are also evidences suggesting that neutralization of endosomal compartment prevents internalized HIV particles from being degraded (117), while forcing the virus through endosomal parthway will result in lysosomal degradation (43). So, endocytosis of HIV could happen, the question is whether it will lead to productive infection. After all, the investigations are meant to improve our understanding on what happens *in vivo*. Some believes that HIV could use the endosomal compartment as shelter from drug or immune system pressure and it could also use the cells as a vehicle to be carried elsewhere to initiate infection (101).

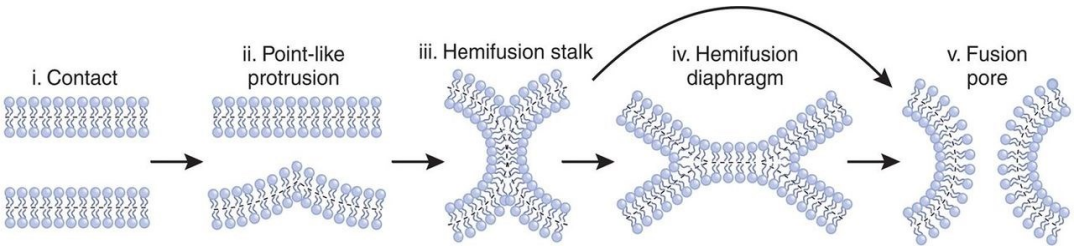

Figure 6. Fusion intermediate (16). Details are given in section 1.2.2.1 Entry.

1.2.2.2 Trafficking, uncoating, and reverse transcription

The viral core is released into the cytoplasm after fusion complete. The core needs to be trafficked to nuclear pores as reverse transcription and uncoating occurs. Although the RT process has been scrutinized *in vitro* due to the enzyme being a drug target, recent advance in cellular imaging techniques start to enable the study of intracellular trafficking (4). Evidences suggest that, similar to reovirus, adenovirus, herpes simplex virus (HSV), and influenza virus, HIV use intracellular highway – the microtubule (MT) network – to move from plasma membrane toward MT-organizing centres (MTOCs) which is close to nucleus (4, 94). The core subsequently moves slowly along actin filament to, presumably, get to nuclear pores (4). However, no evidence on direct interaction between the core and dynein motor complex has been shown to date, partially due to the difficulties in studying the HIV core.

The uncoating process of the core has been the subject of intensive investigation. There are three models. In the first one, upon fusion, the core is rapidly disassembled. Evidences supporting this model are the absence of significant amount of capsid protein associated with the ribonucleoprotein complex as well as the failure to observe core structure by transmission electron microscopy (58). The second model states that the uncoating process complete gradually during migration toward the nucleus, as different sizes of the core have been observed. The last model states that uncoating only happens after reverse transcription is completed and when the core reaches the surface of nucleus. Many lines of evidence support this model. The use of RT inhibitors is able to retain the complete core near the nucleus surface (5). In addition, tripartite motif 5-alpha (TRIM5α), a

cellular restriction factor that accelerates core dissociation, can abrogate RT (128). Mutant CA that reduces stability of the core also leads to abortive infection (46). Finally, capsid interaction with a component of the nuclear membrane gateways is essential for nuclear import (118). With improvements in live-cell imaging, it will be easier to study the uncoating and trafficking of viral core.

The process of reverse transcription is independent of uncoating (33) and has been studied both in a cellular and cell free context. The enzyme starts with an RNA template and finish with a doubled stranded DNA ready for integration (figure 7) (67). During the event, this unit is called reverse transcription complex (RTC). The process starts with the binding of a primer, in the case of HIV-1, trRNA Lys3, to the primer binding site (PBS). Synthesis of the first few nucleotide proceeds slowly and the enzyme eventually speeds up and go through U5 and direct repeat (R), which serves as a bridging sequence when the newly synthesized DNA flip to the 3' end of the RNA genome. The RT enzyme carries RNase H activity, which degrades RNA in a RNA-DNA complex. It is not determined if the degradation of 5' end R and U5 occurs as the DNA strand is being synthesized, but it is know that co-degradation is not required (135). The R region on the DNA could anneal onto either the current template or the other copy of RNA genome carried within the same HIV particle. The enzyme completes the synthesis of the rest of DNA strand while the RNA template is being degraded. However, polypurine tract (ppt), resistant to degradation, is left intact and it is used as the primer for the synthesis of the second DNA strand. The polymerization stops when the enzyme is 18 nucleotides into the tRNA primer, which generates a new PBS. On the first DNA strand, tRNA is cleaved off except the last ribonucleotide adenosine. At this stage, a second ppt close to the middle of the genome could initiate polymerization of second DNA strand in addition to the one started from 3' ppt (not shown). Next, a second strand transfer occurs: the new PBS on the second DNA strand anneals to the 3' end of the first DNA strand, which was synthesized based on the PBS from the RNA genome. At this stage, each strand can use the other one as template to complete the double stranded DNA. If the second polymerization even from central ppt occurred, this would result in a DNA flap at the ppt. The flap seems to be important for

nuclear import. Both ends are now identical and they are called long terminal repeat comprised of U3, R, and U5. The end product will be integrated into cellular genome and become a provirus.

There are two significant events that can happen during the RT. First, due to the poor fidelity and the lack of proof reading mechanism of the enzyme, wrong nucleotide could be incorporated resulting in mutation. The rate of mutation of the viral genome is about 1.4×10^{-5} per nucleotide per replication cycle (1), which is contributed by both RT enzyme and cellular RNA polymerase II, an enzyme which also lacks proof reading mechanism. The second event that can happen is DNA recombination. During the process of RT, template switching during strand transfer could result in chimeric DNA. In order to achieve this, the two RNA genome packages into the same virion must be different. There are many factors that could influence the rate of recombination: the rate of co-infection by two different strains, subtypes of the two strains, secondary RNA structure, break in the RNA template which depends on the relative activity of polymerase and RNase H in the RT enzyme (67). Both events can lead to mutations allowing HIV to escape from immune system and drug pressure and increase fitness in the human population.

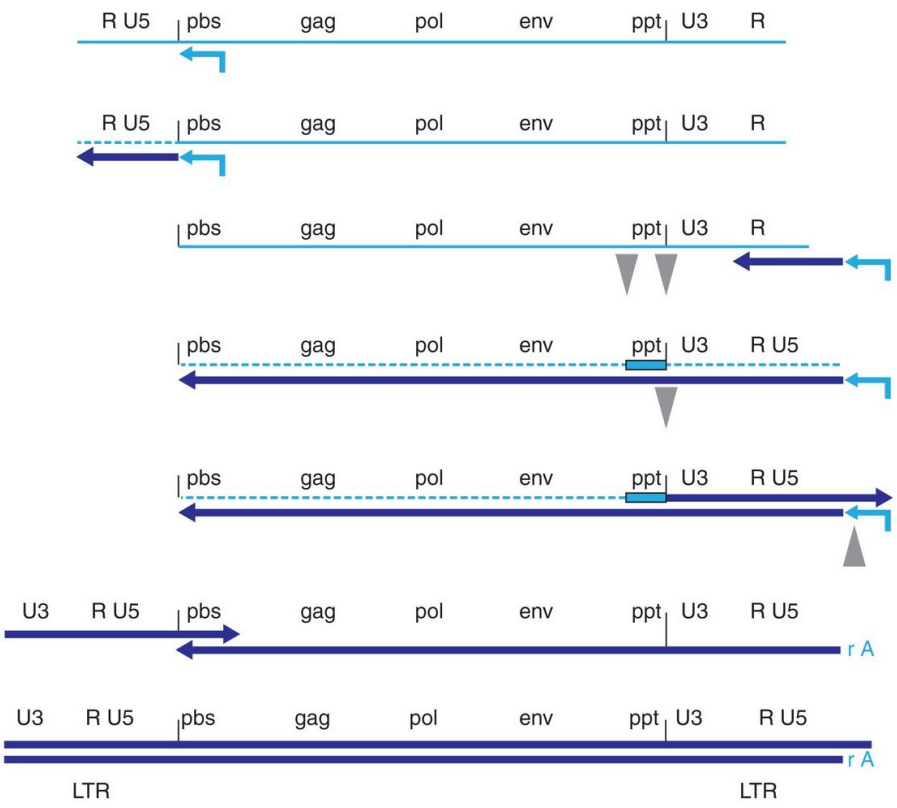

Figure 7. HIV reverse trans-cription (67)**.** Details are given above.

1.2.2.3 Nuclear translocation and integration

Lentivirus is known to be able to infect non-dividing cells in contrast to retrovirus like murine leukemia virus (MLV). After the RTC is transformed into a pre-integration complex (PIC) with integrase associated with both ends of the double-stranded DNA, HIV will cross the nuclear membrane and find an area of open chromosome to integrate itself into the host genome. Nuclear import is necessary for the virus to infect macrophages and resting T cells. This even is definitely an active energy dependent transport, as the nuclear pore can only allow passive transport of molecules with up to 9nm diameter and PIC is estimated to be around 50nm (130). Although the exact mechanism has not been fully elucidated, several viral and cellular factors are found to be involved in this process. Mixed reports suggest that the karyophilic sequence on MA, CA, and IN proteins may or may not contribute to import (130), while PIC with central ppt DNA flap mutant seems to accumulate at the nuclear pore (153). Interaction between MA, vpr, IN and importin-α as well as between vpr and many nucleoporins has been reported. Unstable phenotype associated with karyophilic sequences lead to discovery of lens epithelium-derived growth factor LEDGF/p75 import pathway (86). This host factor is associated with the PIC by interacting with IN and is also involved in integration.

Integration *in vitro* needs no more than the integrase itself (figure 8). Two pairs of homodimer clamp onto the end of the doubled stranded DNA and bring it to the site of integration. The IN first removes two nucleotides from the 3' end of each strand. The exposed 3' ends attack two phosphodiester bonds separated by five nucleotides on the target DNA. The two unpaired 5'

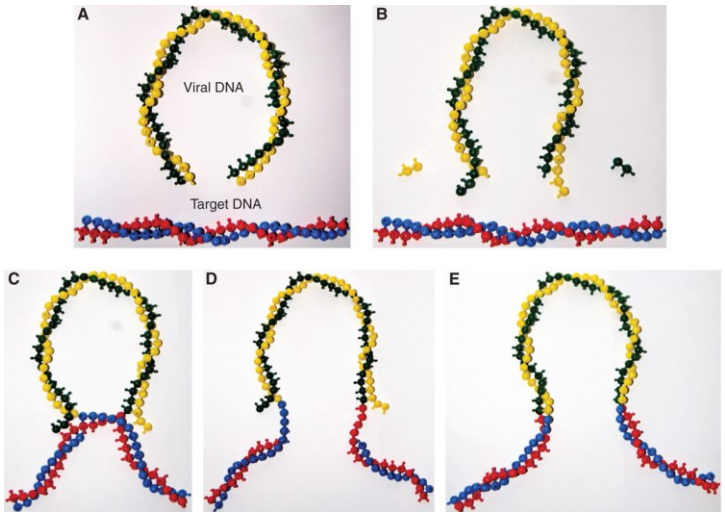

Figure 8. HIV integration (1). Details are given on the left.

nucleotides on each end of viral DNA are then removed and cellular repair machineries will fill up the gaps. The integrated DNA is two basepairs less on each end than the DNA from PIC and the five basepairs sandwiched by the IN is duplicated.

One aspect of integration is the location in cellular genome. Initially, it was believed that open chromosome is preferred over close chromosome due to ease of access. However, *in vitro* evidence suggests that DNA on nucleosome is targeted (106). It was later found that bending of DNA facilitates integration. The target sites *in vivo* are active transcription units (119). This is slightly different from the patterns of other retrovirus. Recent studies on LEDGF solved this puzzle. It was found that this cellular factor is tightly associated with IN. LEDGF being a transcriptional co-activator tether PIC to these active transcription units and allows efficient integration (67). This completes the early phase of HIV viral life cycle.

1.2.2.4 Production of viral proteins and genome

Once integrated into the host genome, HIV is called a provirus. In order to produce new viral particles to infect other cells, the proviral DNA needs to be transcribed into genomic RNA and translated into various viral proteins. This is a tightly regulated process in which trans-activator of transcription (tat) and regulator of virion expression (rev) play essential roles.

HIV proviral DNA brings its own promoter in the LTR, which includes many DNA regulatory elements (111). Despite being highly organized and very efficient in transcriptional initiation, the elongation is very slow due to suppression by cellular factors such as negative elongation factor NELF-E. However, once the amount of tat produced from the initial inefficient transcription reaches a certain threshold, viral RNA accumulation will increase dramatically. Tat returns to nucleus and bind to an RNA secondary structure called transcription transactivation-responsive region (TAR) on the transcript that is being slowly elongated. This structure can recruit NELF-E as well as inactive positive transcription elongation factor (P-TEFb) and other transcription factors. Tat activates P-TEFb by displacing regulatory proteins from P-TEFb, which in turn hyperphosphorylates C-terminal domain (CTD) of RNA polymerase II and of Spt5, and NELF-E. Phosporylated

NELF-E will leave the elongation complex (48), while RNA pol II and Spt5 with phosphorylated CTD will increase processivity of the complex (74). This results in a positive feedback loop with more tat being produced and higher efficiency in elongation. Conversely, if the initial tat level is too low due to closure of chromatin at the promoter, latency will establish (21). The restriction on chromatin can be relieved by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor activated by stress and immune activation, which leads to reactivation of HIV from latency (17).

Due to the presence of introns in the new transcript, the viral RNA will be retained in the nucleus and degraded unless it is fully spliced by cellular splicing machineries. It can then be exported through the nuclear pore complex as any other cellular mRNA. This fully spliced mRNA encodes tat, rev, and nef. Both tat and rev will return to nucleus after being translated. Tat functions were described above. Rev helps with the export of incompletely spliced transcript (IST) encoding env and vpu and full length transcript encoding all proteins. As with tat and its RNA target, tar, rev bounds to a RNA secondary structure called rev-response element (RRE) close to the center of env gene on the transcript (91). The binding of the first rev leads to recruitment of additional rev where oligomerization expands from the RRE site toward both ends of the IST (44, 90). Rev can then interact with exportin 1 with its nuclear export signal (NES) and the transcript is brought to the cytoplasm (44). Once in the cytoplasm, the exportin 1-rev-IST complex dissociates and rev re-enters nucleus to export more transcripts (62). In contrast to tat, reducing rev level will not reduce the amount of fully spliced transcripts. As rev level rises, unspliced mRNA will eventually be exported before it can be touched by splicing machineries. The unspliced mRNA can encode any viral protein and serve as viral genome to be packaged into new viral particles.

The polycistronic nature of the IST and secondary RNA structure facilitates protein production and downstream events in the viral life cycle. One example is the production of gag-pol poly-protein (74). A -1 frameshift occurs when ribosome is near the end of gag open reading frame (ORF). Ribosome will slip along a hexanucleotide sequence

(UUUUUUA) and bump into a stem-loop structure. The -1 frameshift moves ribozyme onto the pol ORF. This happens in 5% of the cases, which gives a gag/pol ratio of 20:1. More structural proteins are needed than the RT enzyme. This frameshift mechanism allows the virus to have two coding sequences on the same transcript.

Envelope protein is translated similarly as other membrane proteins. The protein is synthesized on the ER membrane as gp160 and glycosylated. The oligosaccharide side chains are further modified as the env protein travel through the trans-golgi network, where cellular proteases process the protein into gp120 and gp41 (59).

1.2.2.5 Assembly, budding, and maturation

The next step in the viral life cycle is the assembly of these newly produced proteins into particles. Viral envelope is produced at endoplasmic reticulum and is transported to the cell surface via trans-golgi network and endosomal pathway. Trafficking of gag and gag-pol to the plasma membrane is less understood, and so is the trafficking of the viral genomic RNA (vgRNA) (129). Current data suggest that the gag proteins do not polymerize in the cytoplasm but instead remain as monomer or form dimers. The vgRNA can dimerise (96) and associate with these gag proteins in cytoplasm (80).

The assembly is mainly mediated by gag. The MA domain will bind to micro-domain rich in saturated fatty acid by the virtue of a myristoyl group near the N-terminal domain (113). Env is also recruited to these micro-domains thanks to the long intracellular domain of gp41 (129). Env and gag would then interact with each other, although this interaction is not absolutely necessary for packaging (19). The NC domain binds to 5' UTR of a pair of dimerized vgRNA. This segment of RNA includes essential packaging sequence ψ, which ensures that only full length vgRNA is packaged, as none of the spliced forms contain the necessary elements in the 5' UTR (30). The recruitment of vgRNA reconfigure gag into the extended form (109), facilitating proper gag-gag interaction and oligomerization. $tRNA^{Lys}$ required by RT is recruited by gag-pol polyprotein (77). Accessory proteins such as vpr, vif, and nef are packaged via p6 domain on gag (78). Finally, gag polymerizes and forms a radial lattice, stabilized by interaction of CA-SP1 domain (140).

As gag polymerizes coat the inner leaflet of plasma membrane and forms a dome shape, viral particle budding occurs. P6 domain on gag hijacks the cellular ESCRT (Endosomal Sorting Complexes Required for Transport) pathway to mediate budding (127). The ESCRT machinery uses spiraling filaments to constrain the neck of budding virus (51). As gag cannot continue to polymerize during this stage, it results in a gap in the lattice typically seen in immature viral particles from electron microscopy slides. As the virus is budding off the surface, PR starts to process gag and gag-pol. The requirement of maturation for infectivity is well-documented and has been exploited as drug target. It is not well understood how the process starts, as PR within gag-pol is inactive before budding to prevent premature processing. Once activated, PR homodimerizes and cleaves various domains according to cleavage rate dictated by amino acid sequence from SP1/NC, SP1/p6 and MA/CA, to NC/SP2 and CA/SP1 (102). Each cleavage activates certain features or induces conformational change, preparing the particle for infection.

1.2.2.6 Cell-cell transmission

In addition to the conventional cell entry, cell-cell transmission is an important method in initial viral dissemination *in vivo*. The advantage of this type of infection is that, instead of diffusing and being diluted in the extracellular environment, viral particles are concentrated onto the target cell and immediately infect it. Being protected from extracellular environment can also avoid unwanted immune activation. The types of interaction that have been studied are T cell to T cell, macrophage to T cell, and dendritic cell to T cell. Viruses are polarized at the cell-cell interface called virological synapse. In T cells, virus budding is concentrated in the interface (104). As virus buds, CD4 on the uninfected cell could be engaged. Endocytosis of immature particles follows and virus could stay in endosomal compartment for maturation. In the case of macrophages, viruses are made in deep invagination of plasma membrane and are brought to the interface once it comes into contact with an uninfected cell (57). Mature DC infects T cells using virus bound on dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) in compartments positive for late endosome (42). Nonetheless, whatever the type of

interaction is, the process of entry, RT, integration, transcription and production of viral genes product, assembly, and budding are likely to be the same as cell-free virus infection (129).

1.2.3 Pathophysiology

HIV cause acquired immunodeficiency syndromes (AIDS). From the moment of infection to clinical symptoms of AIDS, it could take 10 to 20 years, depending on the individuals. This earns HIV the name lentivirus, which stands for "slow virus". Yet, the virus is very active during the entire process. The pathogenesis could be divided in several phases (figure 9) (27).

The first phase is called eclipse, as no viermia and immune response could be detected. The virus establishes an infection from the initial site of entry by replicating locally in $CCR5^+$ T cells, the primary target cell type, and spreading to other lymphoid organs as cell-free virus or using DCs and macrophages as a carrier. This could last one to two weeks.

Next is the acute phase spanning about three weeks. This typically associated with a drop in $CD4^+$ T cell counts accompanies the peak of viral copy number in the blood (10^7/ml). The virus reaches various mucosal lymphoid tissues, such as Gut-associated lymphoid tissue (GALT) where copious amount of $CCR5^+$ T cells are available. Viral production increases exponentially with the availability of target cells. Peripheral lymphoid tissues such as lymph nodes are also infected, albeit to a less extent due to scarcity of target cells. Although mutation occurs, there is no extensive genetic diversification in the viral population. Nevertheless, at the level of individual virus, mutations that lead to escape from immune system and/or drug pressure do arise during this stage. Then, acquired immunity starts to mount: antibodies against viral antigens and cytotoxic effector $CD8^+$ T cells try to contain and eliminate the virus and infected cells. This leads to a 100-fold drop in the numbers of viral particles in circulation, with partial recovery of $CD4^+$ T cells. The exponential increase in viral copy is probably due to both the initial uncontrolled replication from previous phase and the sudden increase in availability of target cell from

immune activation. The drop is due to viral control by the immune system and depletion in target cells. Clinically, the infected individual could have non-specific symptoms such as fever and enlarged lymph nodes. There is no way to tell if the individual is infected by HIV or not. As a result, HIV is rarely treated timely.

Chronic phase or clinical latency follows. During this phase, there is no overt symptom. Depending on the individual, this could take from months to 20 years with a medium of 8 years. The viral copy number remains stable (10^5/ml) with a steady rate of viral production, new infection, and immune cell death. On the other hand, the number of $CD4^+$ T cells decreases. Destruction of GALT continues, leading to microbial translocation into the circulation. This contributes to systemic immune activation. The longer an infected individual waits before initialization of HAART, the harder it is for mucosal lymphoid tissues to regenerate. Despite constant cytotoxic T lymphocytes and antibodies production, the majority of them are unspecific and non-neutralizing against circulating viral strains. The immune system seems to be unable to catch up with mutant variants that escaped from immune controls.

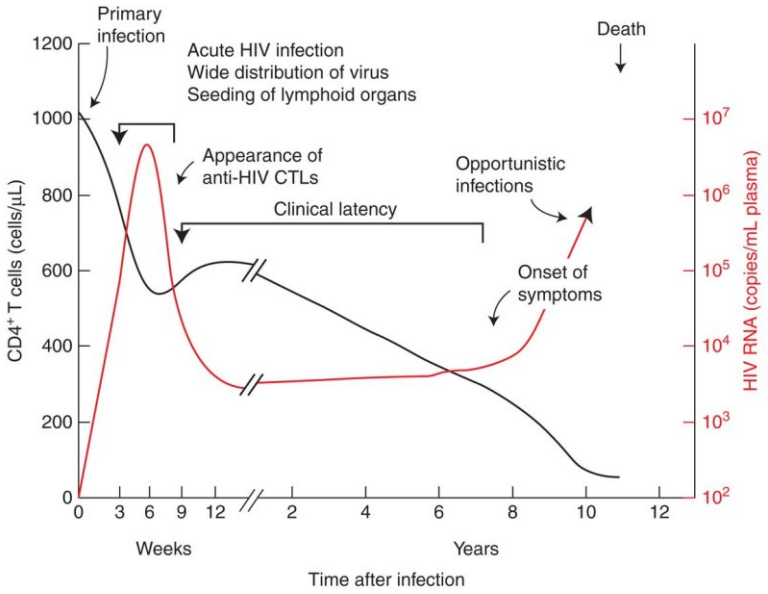

Figure 9. Progression toward AIDS from HIV-1 infection (27). Black line indicates $CD4^+$ T cells number, while red line indicates virus copy number per ml in blood. See above for more details.

Unable to eliminate the virus, the immune system eventually wears down, leading to the AIDS phase. Opportunistic infections and carcinoma start to appear as the immune system becomes dysfunctional with low number of $CD4^+$ T cells. The viral copy number will rise again before the individual dies due to other pathogens and cancers, but not the virus itself.

1.2.4 Origin and cross-species transmission

Ever since the beginning of the pandemic, investigators acquired an interest in how the HIV pandemic started. Major advances are made using phylogenetic epidemiology (figure 10). In addition, certain SIV strains seem to be non-pathogenic in their natural host. This further fueled the interests in the origin of HIV and primate lentivirus, as understanding of differences between hosts and viral strains could lead to better preventive strategies or even a cure for HIV.

As aforementioned, the HIV-1 group M strains causing pandemic in the U.S. originates from Africa. It was later identified and confirmed that strains from this group originate from an SIV virus in *Pan troglodytes troglodytes* (SIV_{cpzPtt}) in central Africa (76). However, even chimpanzees have just been recently infected, before the split of *Pan troglodytes* into the four subspecies less than 1 million year ago (63). SIVcpz sequences suggest that SIVs from two old world monkeys, greater spot-nosed monkey (*Cercopithecus nictitans)* and red-capped mangabeys (*Cercocebus torquatus*), crossed species barrier and recombined in chimpanzee (9). Earlier events of cross-species transmission are not known. Presence of distinct endogenous prosimian lentivirus in two lemur species (52, 53) and the absence of SIV infection in Asian old world monkeys (OWM) suggest that primate lentivirus existed for at least 4 million years and the cross-species transmission from prosimian to monkeys occurred after the split between Asian and African OWM, up to 6 to 10 million years ago (38).

There are also other groups in HIV-1, as outlined above. Evidences suggest that they rise from independent cross-species transmission. Group N is also believed to come from SIV_{cpzPtt}, although it originates from a different community of chimpanzees in Cameroon

(76, 139). Group P strains are passed in gorilla before being transmitted to human according to phylogenetics, while the origin of group O is unknown. The original ape communities in west central Africa giving rise to these two HIV-1 groups are not found yet. HIV-2, on the other hand, originates from sooty mangabey, and each group within this subtype represents a single cross-species transmission event (50).

How cross-species transmission occurs and establishes in new host population is not well understood. Primate lentivirus, like other viruses, is a parasitic entity and needs to interact with cellular machineries for replication. When it attempts to cross the species barrier, it must be able to use cellular proteins of the new host for replication as well as to counteract the host restriction factors (121). So far, HIV-1 group M is the predominant HIV-1 strain in human population. Evidences suggest that tetherin (BST2), a human restriction factor, is the reason behind the dominance of group M over all other groups as well as HIV-2. BST2 is a transmembrane protein that prevents viral release. BST2 from non-human primates are mostly antagonized by a SIV accessory protein, nef, via binding of cytoplasmic tail (70). However, BST2 from human has a five amino-acids deletion, rendering these SIV nef ineffective (116). HIV-1 group M successfully gained anti-BST2 function in another viral accessory protein, vpu, via interaction between transmembrane domains, while other groups from HIV-1 strains did not (116). Similarly, HIV-2 group A, one of the most prevalent HIV-2 strains, uses env to counter BST2, while other HIV-2 groups do not have a mechanism to anta-gonize this cellular factor (83).

1.3. Host defense against primate lentiviruses

During the course of evolution, viruses including lentiviruses constantly assault the mammalian species. Thus, mammals evolved defense mechanisms to counter them. In return, viruses evolve faster and circumvent the restrictions imposed by their hosts. At the molecular level, the host could target viral proteins for proteosomal degradation, while the virus responds by mutating a key residue to evade targeting. Existing viral or host protein could also acquire new functions to antagonize each other. This virus-host co-evolution has been going for millions of years (148). One of the key players of this arms race is the

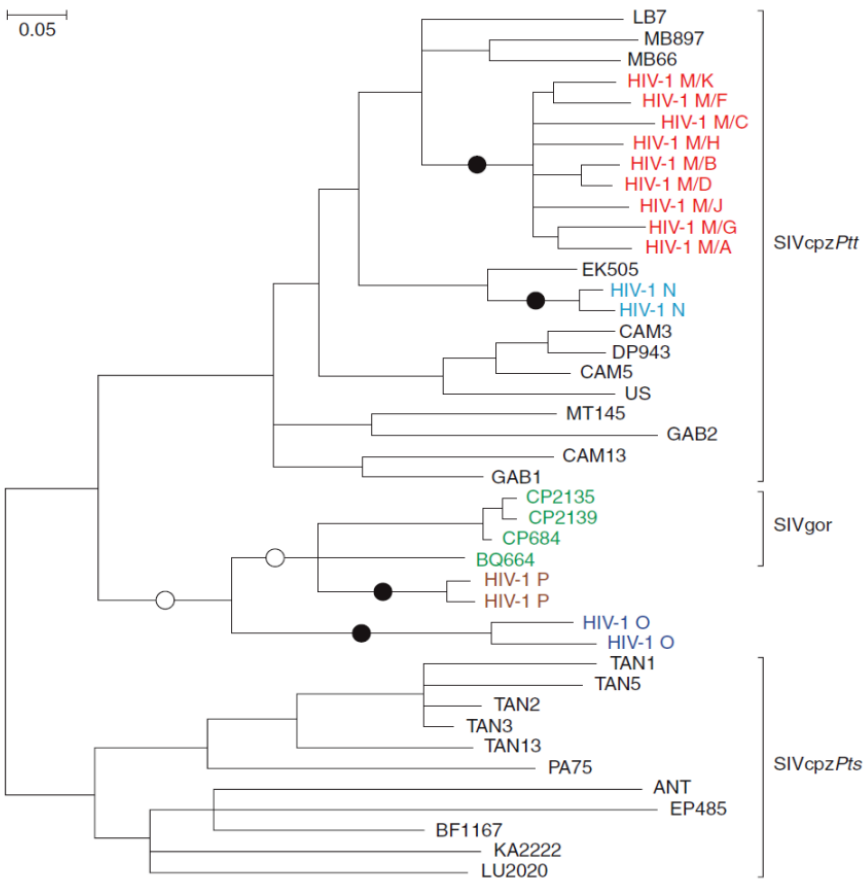

Figure 10. Phylogenetic trees of HIV-1 groups and SIV strains based on a section of pol gene (121). Black circles indicate cross-species trans-mission to from ape to human, while empty circles indicate possible transmission from chim-panzee to gorilla.

interferon system. This non-specific and broadly acting system has been demonstrated many times for its importance in protecting human against pathogens such as Influenza (12). A few members of this system are also known for their anti-HIV/SIV activity and have been the focus of researches in primate lentivirus.

1.3.1 The interferon system and antiviral state

The interferon system is the most important anti-viral arm of our innate immunity. It can effectively limit viremia of many common viral infections during early clinical phase, while the adaptive immunity mounts to clear the infections. To this end, the interferon

system is designed to accomplish two tasks: reduce the viral replication in already infected cells and prevent neighboring cells from being infected. As type I interferons (IFNs) are more relevant in the context of viral infection than type II and type III interferon, only the mechanism of type I IFNs will be discussed.

First, various cellular sensors on the plasma membrane and in cytoplasm, collectively termed pathogen-recognition receptors (PRRs), detect components of invading viruses (132). Toll-like receptor 3 (TLR3) and TLR7 can detect double-stranded (dsRNA) and single-stranded RNA (ssRNA) in endolysosome respectively; TLR9 can detect unmethylated DNA with CpG motifs; retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are cytoplasmic and detect various forms of dsRNA. Ligand binding will trigger downstream signal transduction, leading to phosphorylation and nuclear translocation of interferon-regulatory factor (IRF) (66). There are two IRFs essential in inducing antiviral state: IRF3 and IRF7. IRF3 are found in large amount but can only activate interferon-β (IFN-β) production when coupled with IRF7 as a heterodimer. IRF7 are found in much smaller amount initially. Thus, only IFN-β is produced in infected cells following detection. However, once this interferon binds to type I interferon receptors on infected and uninfected neighboring cells, IRF7 is upregulated and potently increases production of both IFN-α and IFN-β, leading to full-blown interferon inducible genes (ISGs) induction via classic JAK-STAT signaling and the establishment of antiviral state (66).

The ISGs will attempt to restrict every step in viral life cycle. Some blocks entry, especially in uninfected cells, others selectively block transcription and translation needed for replication of viruses. Some others can block release of new viral particles. Due to the unspecificity of this system, many types of virus can be inhibited, including lentivirus.

1.3.2 Anti-HIV restriction factors

In fact, HIV is one of the reasons certain ISGs become the focus of investigation. APOBEC3G, BST-2, TRIM5α from OWM, and SAMHD1 are the mostly studied in this regard. APOBEC3G can prevent new infection. This cellular cytidine deaminase is packaged into

viral particles via vgRNA and NC interaction. When the virus undergoes RT process, the enzyme causes deamination of cystidine leading to G to A hypermutation on the DNA product (61). The RT product is most likely unusable. BST-2 has a very unusual structure: an extracellular coil-coil domain is anchored onto the cellular membrane by an N-terminal transmembrane domain and a Glycophospha-tidylinositol (GPI) anchor on the C-terminal end. When one end is inserted into the viral membrane while the other end remains on the plasma membrane, it can block particles release by tethering them on the cell surface (100). TRIM5α can accelerate the viral core disassembly by an unknown mechanism and in turn blocks the RT process (128). Lastly, SAMHD1, a deoxynucleoside triphosphate triphosphohydrolase (55), blocks RT process in non-cycling cells such as macrophages and DCs. However, recent evidences suggest that phosphorylation of the protein, instead of its dNTPs hydrolysis activity, dictates its ability to inhibit HIV (146).

Recently, our group identified another anti-HIV restriction factor: interferon inducible transmembrane protein (IFITM) (87).

1.4 Interferon inducible transmembrane protein

The first IFITM protein that has been discovered is IFITM1, from a neuroblastoma interferon screening in 1984 (47). It was called as Leu-13 back then. IFITM2 and IFITM3 soon followed. They are found at basal level in many organ tissues (107) and can be further induced by interferon treatment, although the level of upregulation differs between each other. After the protein sequences have been deduced, the IFITMs are predicted to have an intracellular domain with N-terminal and C-terminal tails facing lumen or extracellular space.

1.4.1 The IFITM genes and structure

The IFITM gene family is currently classified into three groups (64). Members in the first group are involved in cellular defense. Different species carry different number of IFITMs in this group. The genes are closely clustered within a region on the chromosome. There are three immune-related members in human: IFITM1, IFITM2, and IFITM3. All three

of them are found on chromosome 14 within a region of 37kb. They contain interferon-stimulated response element (ISRE) in the 5' UTR and, thus, are expressed upon interferon treatment (84). The second group is mainly involved in bone maturation. There is only one member in this group - IFITM5 or bone related IFITM-like protein - and it is found in almost all vertebrates. On the chromosome, this gene is located near the immune related members. The third group also has only one member - IFITM10 - and it is the most conserved IFITM protein across all vertebrates. It is not located on the same chromosomes as the other IFITM proteins and not much is known about this protein.

Structural analysis suggests that IFITM proteins have two transmembrane (TM) domains with a highly conserved intracellular domain (CID). The first TM and the CID make up the CD225 domain, which is conserved between about 300 proteins. However, there are experimental evidences that suggest an alternative structure (see section 1.4.3.2).

1.4.2 Roles in cancer, embryogenesis, and bone development

Before the many studies on the anti-viral effect of IFITMs, researches have been focused on the anti-tumor effect of the proteins (124). Some reports suggest that IFITM can limit tumor growth, while other reports suggest the opposite. There is no conclusive evidence of IFITMs *in vivo*. The confusion also exists in regard to their role in embryogenesis. A report in Developmental Cell demonstrated how IFITM1 and IFITM3 are responsible for homing of certain cells during embryogenesis (133). However, mice with their entire *ifitm* locus knocked out have no overt developmental defect and they can reproduce just like their wild-type littermate (81). IFITM5 roles in bone development are also being studied. A mutation in 5' UTR is involved in a congenital bone disorder (25, 131).

1.4.3 Antiviral functions

In 2009, Brass et al. reported that IFITMs inhibit influenza A virus, West Nile virus, and yellow fever virus (18). Other groups followed closely. Guo's group also identified IFITMs as ISGs that inhibit dengue virus (DENV) and West Nile virus (WNV) entry (72). Further reports by Brass' group added more viruses to this list: Marburg virus (MARV),

Ebola virus (EBOV), and SARS corona virus (SARS) (68). In 2011, our group reported that the IFITM proteins also inhibit HIV-1_{BH-10} (87). This year, reovirus, a double stranded RNA non-enveloped virus, is added to the expanding list of viruses restricted by IFITMs (3).

As more viruses are found to be sensitive by IFITMs, a pattern emerged regarding the restricted stage in the viral life cycle as well as the type of viruses that are sensitive to these proteins. The consensus of the field is that IFITMs affect viral entry, more specifically, the release of genetic material into the cytosol. Each IFITM also exhibits different restriction phenotype toward different viruses. For instance, IFITM3 is the most potent out of 3 for IAV, while IFITM1 is the most potent for SARS-coronavirus.

However, disagreement exists for whether HIV-1 is inhibited. siRNA against IFITM3 did not rescue HIV-1_{IIIB} in HeLa-CD4 (18). From the list of affected and non-affected virus, Brass' group suggests that only viruses that need to go through endosomal pathway will be affected by IFITMs, while viruses such as MLV and HIV that fuse on the cell surface should not be affected. Furthermore, removing the requirement for access to acidified compartment for SARS corona virus or reovirus by pre-digesting the virus with trypsin or pseudotyping with pH-independent reovirus envelope, respectively, renders these viruses resistant to IFITM restriction (3, 14, 68). Nevertheless, arenaviruses tested by the group such lymphocytic choriomeningitis virus (LCMV), Lassa virus (LASV), and Machupo virus (MACH) undergoes pH-dependent entry in endosome and yet, these viruses are not sensitive toward IFITMs. Although it is possible that these viruses can use alternative entry site or evade IFITM restriction. The more likely source for the discrepancy is the cell line used. All our work showing IFITMs phenotype on HIV-1 was done in T cell lines. When we used TZM-bl for siRNA knockdown of IFITMs, Brass' result is confirmed: HIV-1_{BH-10} was not rescued by single knockdown of each IFITMs. Instead, a 2-to 3-fold increase in infection was observed only when all 3 IFITMs are simultaneously depleted (87).

Since IFITMs are ISGs, their significance in interferon restriction against viruses is often assessed. For IAV, IFITM3 is the only member that was considered for knockdown, since it is the most potent one when overexpressed. Indeed, complete knockdown of

IFITM3 completely abolished protection of IFN-γ against IAV (18, 41). Both IFITM1 and IFITM3 induced by IFN-β are important in restricting MARV and EBOV (68). SARS knockdown was done in K562 which express high level of endogenous IFITM1 (68). At low dose (100UI/ml) of IFN-α, shRNA knockdown of IFITM3 abrogated the inhibitory effect on reovirus replication in HeLa cells (3). In the case of WNV and YFV, knockdown was done in a cell line expressing high level of endogenous IFITM3, instead of using IFN treatment. The infection of WNV and YFV envelope pseudotyped viruses was greatly enhanced (18). Knockdown of IFITMs in HeLa demonstrate that these proteins are responsible for more than half of the effect of IFN-α (5UI/ml) against RVF virus (97).

1.4.3.1 *In vivo* evidences and clinical significance

In vivo evidence supporting IFITMs restriction of IAV was obtained in mice studies. Deletion of IFITM3 resulted in greater morbidity and mortality (37). People who carry a truncated IFITM3 splice variant are more susceptible to IAV infection resulting in hospitalization. Indeed, the deletion of the first 21 amino acid resulted in loss of restriction *in vitro* (37, 145). Deletion of the entire IFITM locus has the same phenotype as deletion of IFITM3: mice lost weight and died faster than those in the control group (10).

The most intriguing *in vivo* evidence of IFITMs restriction comes from a mice IAV model (141). The authors demonstrated that IFITM3 helps resident memory $CD8^+$ T cells (T_{RM}) specific to the antigens from primary infection to survive in subsequent challenges. This constitutive expression of IFITM3 is due to hypomethylation of the promotor. IFITM3-deficient T_{RM} also failed to protect naïve mice from challenges when transferred from an immunized mouse.

IFITM1 can inhibit HCV replication (108). HCV is, so far, the only virus that is known to specifically down regulate IFITM protein. Patients who respond to interferon treatment have higher level of IFITM1 compared to non-responders (147). It was found that HCV is able to induce microRNA-130A to downregulate IFITM1 in liver biopsy from HCV patients (15). Removal of this microRNA *in vitro* increased IFITM1 level. Considering that interferon

is the only effective treatment of HCV, ISG like IFITM1 could be playing a role in restricting HCV.

Other clinical evidence of IFITMs on human pathogens include the hospitalization rate of a European population carrying a T/C SNP in IFITM3 and the rate of acquiring tuberculosis in children in a Chinese population (122). Another SNP rs12252-C common in Han population also increase the risk of IAV severe infection by 6 fold (154).

1.4.3.2 Working mechanism of anti-viral function of IFITMs

Despite all the work done on IFITMs in different models such as IAV, SARS, and JRSV, the mechanism of inhibition remains elusive, partly because IFITMs are membrane proteins which are in general hard to study.

It is shown that IFITMs can block viral entry by preventing the release of viral core and genomic material into the cytoplasm. It is also known that these proteins do not interact with viral receptors, which is consistent with the fact that a wide range of viruses with different receptors are inhibited by IFITMs. The only exception is the HCV receptor, CD81, where interaction has been demonstrated. IFITMs do not prevent pH-dependent viruses such as IAV and reovirus from accessing the acidified compartments (3, 68). The presence of IFITM3 expands endosomal and lysosomal compartment, although its significance is unknown.

So far, only two studies provided some hints on the mechanism (figure 12). IFITM3 apparently interacts with (vesicle-membrane-protein-associated protein A) VAP-A to increase intracellular and intravesicular cholesterol level (2). Disruption of cholesterol homeostasis is often detrimental for viral entry. It has also been suggested that IFITMs can alter membrane fluidity to prevent viral entry (85). Using a pH-dependent virus, the authors demonstrated that IFITMs specifically affect the hemifusion step, where negative curvature is needed for the fusion of outer leaflets between two membranes. Other evidences also suggest that IFITMs affect the properties of the endosome. Apparently,

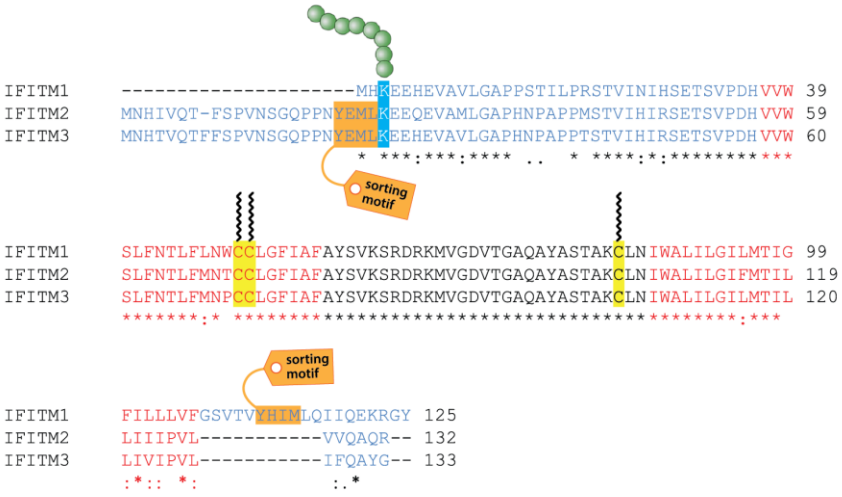

Figure 11. Human immune related IFITMs sequence alignment. Amino acid sequences are aligned and color-coded. N-terminal and C-terminal are blue; the two intra-membrane domains are red; the conserved intracellular domain is black. Y20 of the YEML sorting motif on IFITM2 and IFITM3 can be phosphorylated. C71, C72, and C105 can be S-palmitoylated. K24 can be ubiquitinated for degredation. YHIM could be a potential sorting motif on IFITM1.

interaction between IFITM3 and v-ATPase is required for acidification of endosomal and lysosomal compartment (144).

There exist other attributes essential to the antiviral effect of IFITMs. For instance, the location of the proteins can dictate whether IFITMs will block viruses that go through the endocytic pathway. Y20 of IFITM3 is responsible for its endosomal localization (figure 11) (71). Mutation of this residue redistributes IFITM3 in the cell homogenously and abrogates its antiviral activity against IAV, DENV, and VSV (37, 71, 73, 151). The tyrosine residue can be phosphorylated and appear to be key for intracellular localization of various proteins. However, this mutant is still efficient in inhibiting HIV-1, a virus that does not need to go through the endosomal pathway to initiate entry (71). Membrane vs. cytoplasmic localization is largely determined by S-palmitoylation of cysteine residues. C71, C72, and C105 on IFITM3 are S-palmitoylated (figure 11) (151, 152). This modification

is also important for the anti-IAV function of the protein. Mutation of these residues to alanine de-clusterizes IFITM3 and reduce its expression level (151), but it did not alter the trafficking and localization (152). Multimerization is also a feature of IFITMs. Using Co-IP, IFITMs are demonstrated to interact with each other (unpublished data). F75 and F78 on IFITM3 do not play a role in intracellular localization. Instead, they are essential for multimerization between IFITM3s and anti-IAV function (73).

Since IFITMs have not been reported to have enzymatic activity and do not affect receptors themselves, the mechanism of inhibition could lie in their structure. Therefore, many studies looked at the topology of IFITMs. IFITMs were first proposed to be transmembrane proteins, as there are two hydrophobic stretches of amino acids predicted by software. N-tagged IFITM3 are detected to similar extent on intact and permeabilized cells by FACS (145). However, later experimental evidences suggest other possible topologies for IFITMs. The first one is an intramembrane topology. Residues on the N-terminal domains such as Y20 and K24 have to be present in cytoplasm in order to be phosphorylated or ubiquitinated (71, 151). Furthermore, addition of residues that can be glycosylated to the N-terminal and C-terminal ends did not result in change in the mobility of IFITM3 (151). Glycosylation is a process that occurs only on the luminal face of a protein. These evidences support an intramembrane topology model of IFITMs. A recent report suggests a third topology, with N-terminal in the cytoplasm while the C-terminal in the lumen or extracellular space (11).

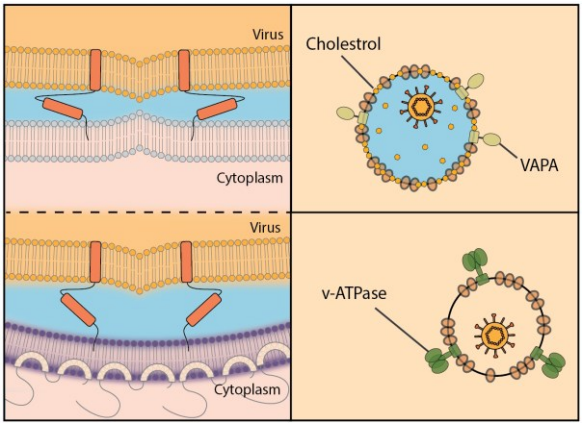

Figure 12. Possible mechanisms of restriction at entry. IFITMs could block viral-host membrane fusion by increasing the negative curvature during hemifusion possible due to its structure. Alternatively, IFITM3 could increase intracellular and intravesicular cholesterol level. IFITMs could also affect acidification of the endosomal compartment.

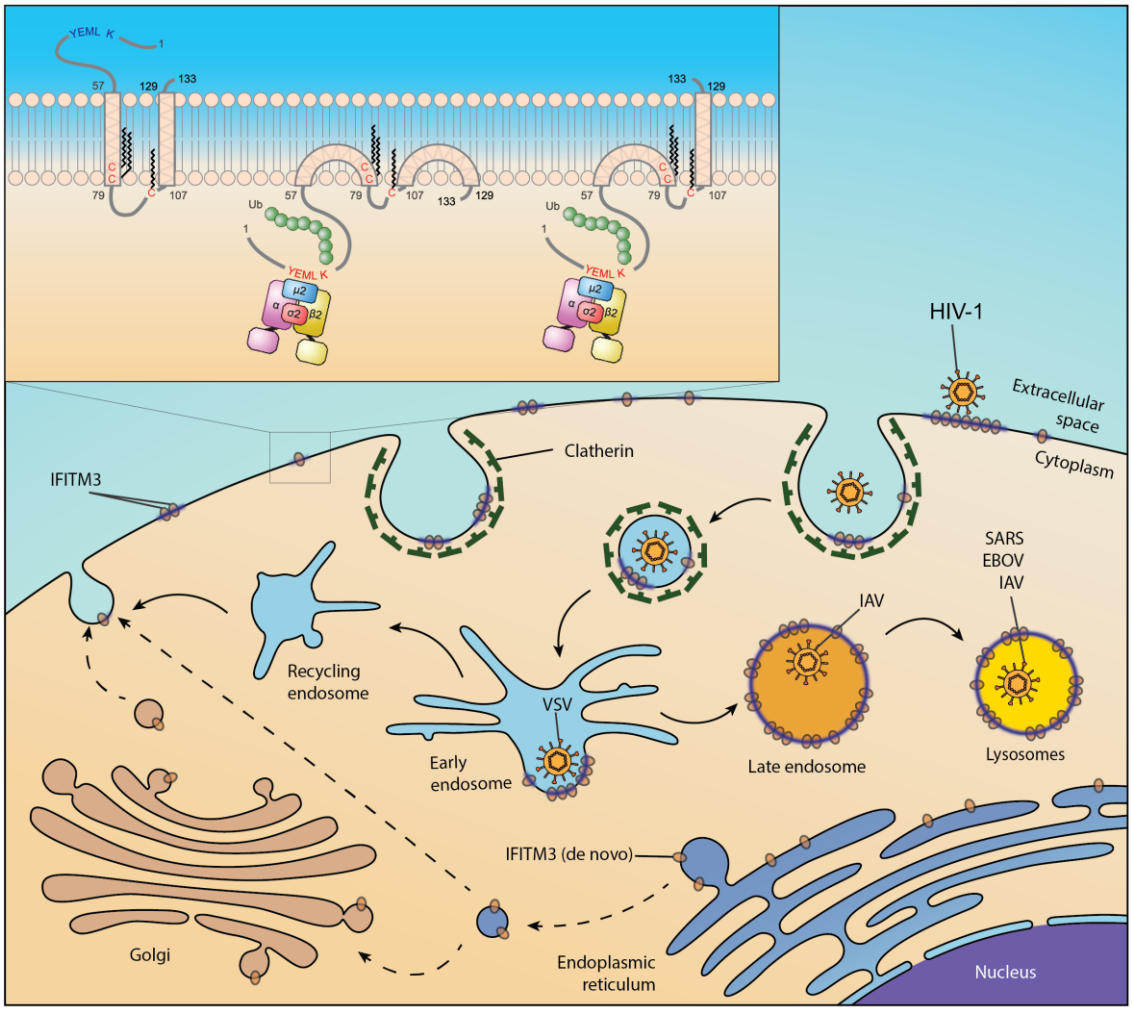

Figure 13. Working model of IFITM proteins in restriction of viruses. IFITM3 is used as an example. IFITM3 is produced on rough endoplasmic reticulum (ER). Then the protein is transported, possibly through the trans-Golgi network or directly, to the cell surface. The targeting to IFITM3 to the acidified endosomal compartment is achieved by the virtue of the Y20 residue. There, IFITM3 will exert its entry inhibitory activity on incoming viruses. Inset: so far, three topologies have been reported. The first one is the transmembrane topology where the protein pass through membrane twice, forming a U shape. The second one is the intramembrane configuration where the N-terminal, conserved intracellular domain, and the C-terminal are all facing the cytoplasm. The third one is a hybrid with the N-terminal tail in the cytoplasm while the C-terminal tail in the luminal or extracellular space.

Finally, viral entry is not the only step in viral life cycle IFITMs can intervene. IFITM1 potently inhibits HIV-1_{BH-10} at post-entry stage (87). IFITMs have been shown to decrease gag expression in a codon-dependent manner (26). It was also found that S-palmitoylation is not needed to restrict HIV-1 (26).

1.5 Project rationale

Previous evidences suggest that human IFITM1, IFITM2, and IFITM3 proteins (hIFITMs) can inhibit HIV-1_{BH-10}. In order to convincingly demonstrate their restriction activity against HIV, a few primate lentivirus strains were tested in cell lines overexpressing hIFITMs. Next, restriction activity at HIV/SIV entry was tested using virion fusion assay, as these proteins are known to inhibit entry of Influenza and other viruses. During the course of the project, SIV strains from AGM were shown to be inhibited by hIFITM2. We are interested in whether IFITMs play a role in cross-species transmission. IFITM proteins from African Green Monkey were cloned from AGM cell lines, and their activity was tested against the SIV/HIV strains in a similar manner to hIFITMs.

On the other hand, insights into the mechanism were also sought. First, chimeric IFITM2/IFITM3 proteins were made, due to their differential restriction ability. Second, possible IFITMs cellular binding partners were also investigated using affinity purification followed by mass spectrometry.

Chapter II: Materials and Methods

2.1 Cell lines and culture condition

Suspension cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% Penicillin/Streptomycin (P/S) (Invitrogen), while adherent cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% FBS and 1% P/S. For SupT1 cells expressing human IFITM proteins under doxycycline inducible promoters (87), Tet system approved FBS (Clontech) was used instead of regular FBS. Selection drugs were 2μg/ml puromycin and 1mg/ml neomycin (Sigma). For TZM-bl overexpression or knockdown cell-lines, 1mg/ml neomycin was used. To make TZM-bl lines, cells were spin-inoculated for 45 minutes at 12°C at 1800rpm in CS-6R (Beckman Coulter) with VSV-G pseudotyped retroviral particles containing construct of gene of interest in the presence of 5μg/ml polybrene (Sigma). Cell-lines were put under selective pressure 2 days later and were selected for a week before phenotyping using Western blot and infection assays.

2.2 Production of viruses

HIV-1_{YU-2} (1350), HIV-$1_{89.6}$ (3552), $SIV_{CPZ1.910}$ (11496), $SIV_{AGM-tan}$ (3444), $SIV_{AGM-sab}$ (2614), and $SIV_{MAC-1A11}$ (2736) proviral clones were obtained through NIH AIDS Reagent Program. HIV-$1_{A/G}$, HIV-1 subtype C, and HIV-2_{ROD} clones were generously provided by Dr. Mark Wainberg. $SIV_{SMM-E543}$ clone was generously provided by Dr. James Witney.

Both pseudoviruses to make cell-lines and viruses to conduct infection assays were made from 293T transfection. All manipulations of virus were done in biohazard safety laboratory level 3 (P3). For the generation of pseudoviruses, MLV gag/pol construct, proper retroviral vector expressing gene of interest, and VSV-G construct were co-transfected at 1:1:0.2 ratio in HEK293T plated 24 hours ago in P/S free medium. A ratio of 2.5 to 5 by weight between lipofectamine (Invitrogen) or polyethylenimine (Polysciences) and plasmid was used. 16 hours post transfection, culture medium was replaced. Viruses were collected 48 hours post-transfection. Cell debris was removed by spinning the

medium at 4°C at 3000rpm in CS-6R for 15 minutes and supernatant was aliquoted in microfuge tubes and stored at -80°C.

Viruses used in human IFITMs knockdown (KD) cell-lines were produced from co-transfection of pLP1 (HIV-1 gag/pol), pLP2 (HIV-1 rev), pLP (VSV-G), and pLKO.1-puro or desired KD construct (Sigma MISSION) at 1:1:0.3:1 ratio. There are 5 KD constructs for each of the 3 human IFITM proteins: IFITM1 TRCN0000057498 to TRCN0000057502, IFITM2 TRCN0000118117 to TRCN0000118121, and IFITM3 TRCN0000118022 to TRCN0000118026.

HIV/SIV proviral clones were transfected to generate WT viruses. Viruses with BlaM-vpr for virion fusion assay were generated simply by co-transfection of proviral clones with pCMV-BlaM-Vpr construct (obtained from Dr. Warner C. Greene) at 3:1 ratio. The resulting virus was then concentrated by ultracentrifugation at 4°C at 35krpm for 1 hour in Ultra-Clear centrifuge tubes placed in a SW41 TI rotor (Beckman Coulter), resuspended in RPMI or DMEM medium depending on the cells to be infected, and aliquoted and stored as above.

2.3 Western Blot and antibodies

Cells were washed twice with PBS before being lysed on ice in radioimmunoprecipitation assay buffer (RIPA) or in NP-40 buffer. Samples were lysed in NP-40 and protein concentration was normalized by Bradford Assay on GENESYS 10S UV-Vis. Samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche). Membranes were blocked in 5% milk in PBS or 5% BSA in TBS and incubated with proper antibodies before being treated with enhanced chemiluminescence (ECL) substrate (PerkinElmer) for exposure.

Human α-IFITM antibodies (1:1000) are from ProteinTech: mouse α-IFITM1 (60074-1-Ig), rabbit α-IFITM2 (12769-1-AP), and rabbit α-IFITM3 (11714-1-AP). Other primary antibodies used are mouse monoclonal α-Flag (Sigma-Aldrich) (1:5000), mouse

monoclonal α-β-Tubulin (Santa Cruz Biotechnology) (1:5000), and rabbit α-rab5 (Cell Signaling #9385S). Secondary antibodies are either horseradish peroxidase-linked donkey anti-rabbit IgG or sheep anti-mouse IgG (GE Healthcare) (1:5000).

2.4 Luciferase assay

TZM-bl was used in luciferase assay. Original TZM-bl line as well as cell lines over-expressing human and AGM IFITMs were used. Cells were plated on day 1 in 24-wells plates (BD Bioscience); viruses were added on day 2; medium was replaced on day 3; on day 4, supernatant was removed, cells were washed by Phosphate-Buffered Saline (PBS) (Invitrogen) and lysed in 1X passive lysis buffer (Promega). The plates were either stored at -80°C for measurement at a later time or put on shaker for 20 minutes at room temperature for complete lysis. 10µl of lysate supernatant was mixed with 30µl of luciferase substrate (Promega) in a microfuge tube and sample was read for 10 seconds on GloMax® 20/20 Luminometer (Promega). Data was exported to excel for analysis.

For testing of co-receptor usage, cells were pre-incubated with AMD3100 or Maraviroc for an hour prior to infection. The protocol is the similar to the standard infection described above, except that the medium was not changed on day 3.

2.5 Reverse transcriptase assay

RT assay was initiated in P3 by technician with appropriate licenses. 10µl of medium containing virus from each sample was put into 96-wells plate. 40µl of reaction cocktail was added. The cocktail was made of appropriate buffer, Triton-X to extract RT enzyme from virus, poly-adenosine RNA template (Midland Certified Reagent Company), and tritium based dTTP (Perkin Elmer) as substract for the reaction. The plate was incubated in Heracell incubator for 3 hours. Reaction was stopped and nucleic acids were precipitated by adding 150µl of 10% trichloroacetic acid (TCA) (Millipore) and incubate at 4°C for 30 minutes. The mixture was then transferred into MultiScreen filter plate (Millipore). dTTP substrate was removed with solvent by vacuum, and the filter carrying nucleic acids were washed twice with TCA and once with 95% alcohol before being

transferred into scintillation vials (Diamed). 3 mL of liquid scintillation cocktail (MP Biomedicals) was added to each vials and β decay was measured liquid scintillation counter Wallac 1410 (PerkinElmer). Data was entered into excel for analysis.

2.6 Flow cytometry

SupT1 or TZM-bl cell lines expressing Flag-tagged hIFITMs were washed twice in PBS, fixed in 1% paraformaldehyde, permeabilized in IC permeabilization buffer (Invitrogen), stained with Dylight-649 conjugated α-Flag antibodies (Rockland 200-343-383), and run on FACScalibur (BD bioscience). Data was further processed in Microsoft Excel.

2.7 Virion fusion assay (VFA)

Viruses containing BlaM-vpr were generated as described above. VFA was performed as described previously (87). Briefly, TZM-bl cell lines were plated 16 hours before infection in 24-wells plate. On the day of infection, supernatant was removed and virus was added in the presence of 5μg/ml polybrene and spun at 15°C at 1800rpm for 45 minutes. The plate was placed back into culture incubator for 3 hours to allow viral entry to complete. Room temperature CO_2-independent medium (Invitrogen) was used to wash away unbound viruses. CCF2-AM substrate containing medium was added to the cells and the plate was incubated in dark at room temperature (15°C) for an hour. Cells were washed again, and loaded with developing medium containing 10% FBS and incubated in dark at room temperature for 16 hours, allowing the enzyme to process the substrate. On the next day, cells were lifted using 0.05% trypsin (Invitrogen), washed twice with 2% FBS in PBS, fixed in 3.7% formaldehyde, and processed in LSRFortessa cell analyzer (BD Bioscience). Data was analyzed in Flowjo and further processed in excel.

2.8 Monkey IFITM genes cloning

AGM cell lines, Vero and COS-7, were used to clone agmIFITM genes. Cells were washed with PBS and lysed in TRIzol (Invitrogen). Total RNA was extracted from upper aqueous layer and precipitated using isopropanol. Pellet was washed with 75% ethanol,

air-dried, and resuspended in RNase-free water. 2 primer sets for IFITM1 and 2 sets for IFITM2 and IFITM3 were designed to amplify mRNA transcripts: IFITM1 set 1 forward 5'-CAACAGGGGAAAGCAGGGCTC-3' and reverse 5'-GTCATTGTGGACAGGTGTGTGGG-3'; IFITM1 set 2 forward 5'-CAACACTTCTTTCCCCAAAGCCAG-3' and reverse 5'-CTGTATCTAGGGG CAGGACCAAG-3'; IFITM2 and 3 set 1 forward 5'-GGGAAAGGGAGGGCCCACTGAG-3' and reverse 5'-GTGTGTGAGGATAAAGGGCTG-3'; IFITM2 and 3 set 2 forward 5'-CCCACTAA CCCGACCACCGCTG-3' and reverse 5'-GGGCAGAGCTCCTGGCCTGAATG-3'; Using Titan One Tube RT-PCR System (Roche), desired transcripts from both Vero and COS-7 were reverse transcribed and amplified in MJ Mini Personal Thermal Cycler (BIO-RAD). Amplicons were verified on gel by size, extracted using QIAEX II gel extraction kit (Qiagen), and sequenced (McGill University and Génome Québec Innovation Centre). Primers for subcloning were designed based on the sequence. BamHI site and Flag-tag are added to the 5' end, while EcoRI site was added to the 3' end: IFITM1 forward 5'-GACTGGATCCATGGATTACA AGGATGACGACGATAAGCACAAGGAGGAGCACGAGGTGTC-3' and reverse 5'-CAGTGAATTCC TAGTAACGCTGTTTTTCCTGTAC-3'; IFITM3 forward 1 5'-GACTGGATCCATGGATTACAAGGA TGACGACGATAAGATGAACCACACGGTCCAAACCGTC-3', forward 2 5'-GACTGGATCCGATTAC AAGGATGACGACGATAAGATGAACCACACGGTCCAAACTGTC-3', and only one reverse primer 5'-CAGTGAATTCCTATTGATGGGCTTGGAAGATCAA-3'. PCRs were done on the RT-PCR products. The amplicons and pQCXIP vector were digested by proper enzyme, gel purified, ligated (Invitrogen) for an hour, transformed into DH5-α (Invitrogen). Bacteria were recovered for an hour in S.O.C. medium (Invitrogen) in a 37°C shaker, plated on 100μg/ml ampicillin (Sigma) agar plates, and incubated at 37°C. Plasmids are purified (Qiagen), sequenced again to confirm the constructs, and ready for co-transfection or generation of cell lines.

A macIFITM1 and two macIFITM3 gene sequences are available from National Center for Biotechnology Information (NCBI) database. Coding sequences were obtained and, as in agmIFITMs, BamHI site and Flag-tag were added to the 5' end, while EcoRI site was added to the 3' end. Final sequences were sent to GeneArt® Gene Synthesis (Invitrogen). Synthesized construct were subcloned as described above.

2.9 Immunoprecipitation

SupT1 cell-lines with inducible Flag-tagged IFITM proteins and 293T transiently transfected with overexpression constructs were used. IFITM1 SupT1 cell line was treated with 100ng/ml of doxycycline (Sigma), with or without 500U/ml of interferon-α2b (Jewish General Hospital) for 16 hours, while IFITM2 and IFITM3 cell lines were treated with 500ng/ml of doxycycline, with or without IFN-α2b. For 293T cells, 2μg of each IFITM construct was transfected per 10cm dish. 24 hours later, medium was replaced and 500U/ml of IFN-α2b was added. Negative controls include untreated cell-lines containing each of the genes and IFN-α2b treated cells with control vector only. 24 hours post-IFN-treatment, cells were harvested for co-immunoprecipitation (co-IP).

Cells were spun down at 1500g for 5 minutes, washed with PBS once, and lysed in 1ml of 1% NP-40, 1% TritonX-100, 20mM Tris pH8.0, 150mM NaCl, 2mM EDTA, 10% glycerol with Complete Mini protease inhibitor cocktail (Roche) for 20 minutes on ice. Cell lysate was spun at 4˚C for 30 minutes at 20,000g. 50 μl of affinity gel with flag antibody (Sigma) was washed once with 1ml of Tris buffer saline (TBS) to remove glycerol, mixed with lysate in a 2ml epperndorf in a total volume of 1.5ml and incubated overnight at 4˚C on an orbital shaker. Beads were washed twice with 500μl of TBS on the next day. Elution was done with 100μl of flag peptide at 500ng/μl overnight on orbital shaker. On the next day, beads were then spun down and supernatant were carefully transferred into another tube for analysis. Samples were verified by α-Flag and α-β-tubulin antibodies on Western Blot.

2.10 Mass spectrometry and analysis

Samples were delivered on dry ice to proteomic facility at Research Institute of the McGill University Health Centre (RI-MUHC) and processed by facility personnel as described. Elutes were loaded onto 4% stacking SDS-PAGE in order to concentrate the samples. The gel was stained, de-strained, cut out, reduced with DTT in ammonium bicarbonate, and alkylated with iodoacetamide, in-gel digested overnight, and vacuum dried. The dried samples were re-suspended in the appropriate buffer before injected onto

the Agilent Q-TOF 6550. Data were extracted with Peak Distiller (Matrix Science), and searched against the human database using the Mascot search engine ('trans-proteomic pipeline') and analysis was performed in Scaffold.

For analysis, proteins appearing in the two negative controls were filtered out, and the list was crossed between each sample to generate a Venn diagram showing common binding partners between the IFITM proteins as well as binding partners irrespective of IFN treatment.

Chapter III: Results

3.1 Human IFITM2 restricts SIV_{AGM} and SIV_{SMM}

Lu *et al.* demonstrated the restriction of HIV-1_{BH10} by human IFITM1, 2, and 3 proteins (87). We would like to confirm this result and show that this restriction is not limited to this particular strain of HIV-1. Preliminary studies suggest that SIV_{AGM} is markedly restricted by IFITM2. To further investigate this phenotype, we selected a panel of HIV and SIV strains: two strains from HIV-1 subtype B: NL4.3 is a common laboratory strain, while YU-2 is a primary isolate; HIV-$1_{A/G}$, a circulating recombinant formed between subtype A and G; $SIV_{CZP1.9}$, a strain reconstructed from fecal samples of *Pan troglodytes schweinfurthii*; two strains of SIV_{AGM}, isolated from *Chlorocebus sabaeus* and *Chlorocebus tantalus*; HIV-2_{ROD}, a subtype A primary isolate; $SIV_{MAC\text{-}1A11}$, a strain derived from passage of SIV_{SMM} in rhesus macaques; an SIV_{SMM} strain that has been passaged in macaques twice. Due to the differences in the tropism of the strains, we selected TZM-bl indicator cell to generate stable cell lines expressing hIFITMs (figure 14B). This is a HeLa-CD4 cell line with overexpression of both CCR5 and CXCR4 co-receptor, thus can be infected by lentiviruses of any tropism. It also has *lacZ* and *Luc* gene under tat control. Luciferase activity, which correlates to the amount of tat, can be quickly measured using proper substrate. TZM-bl overexpression cell lines were infected with the panel of serial diluted lentivirus and measure for luciferase activity. The relative light unit (RLU) would reflect the degree of infection by lentiviruses from entry to tat production and will not be able to reveal the effect of hIFITMs on later stages of viral life cycle. The value was plotted on log scale (figure 14A). There are two major observations. First, the potency of restriction of each IFITM proteins differs. Although the fold of inhibition in some strains might not be statistically significant, IFITM2 is more potent than the other two proteins. IFITM3 restriction is moderate compared to IFITM2, while IFITM1 is similar or weaker than IFITM3. Second, some strains are more sensitive to hIFITMs restriction than other strains. $SIV_{AGM\text{-}tan}$ is the

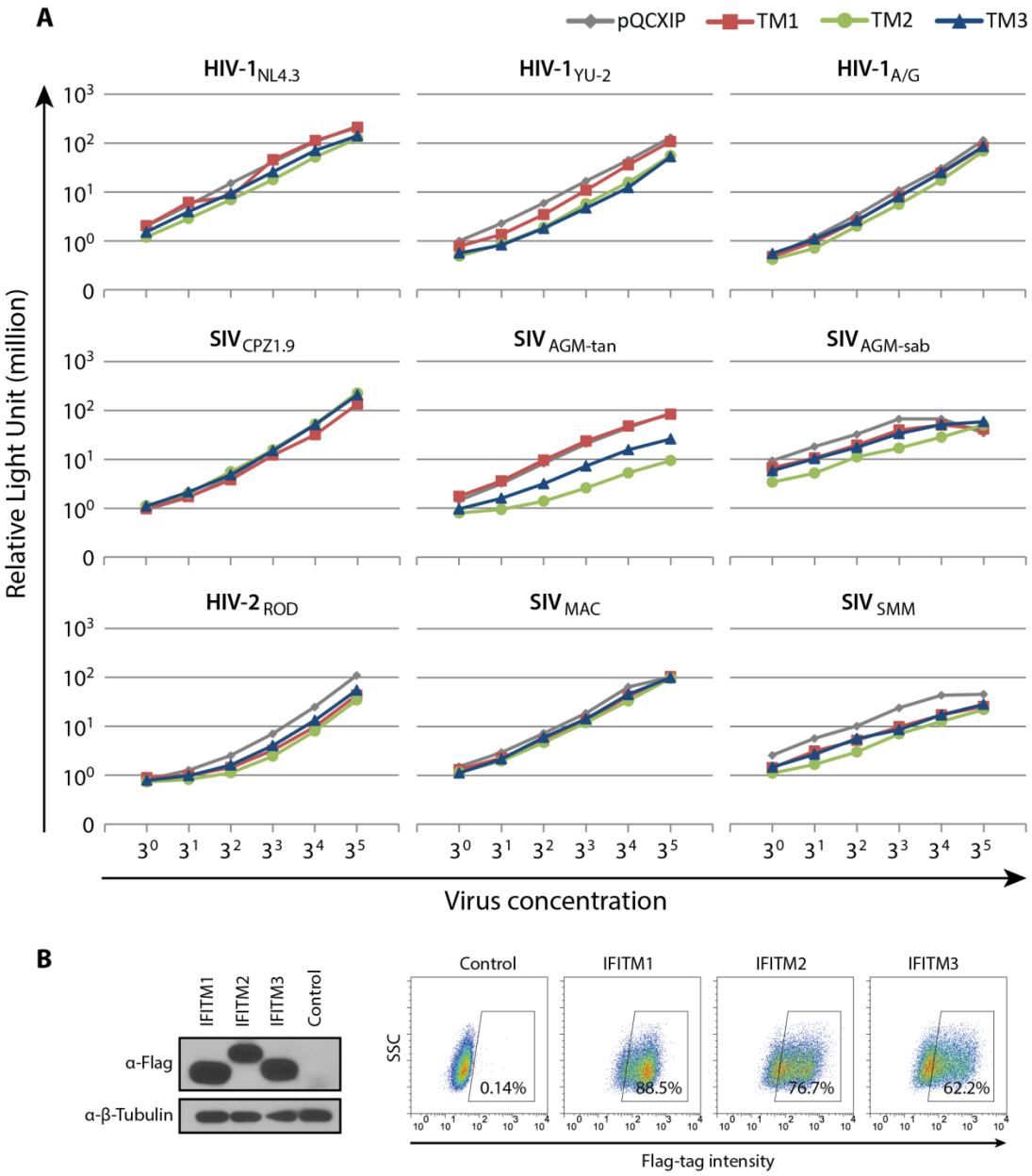

Figure 14. Infection of TZM-bl overexpressing hIFITMs. A. TZM-bl cell lines were infected with HIV-$1_{NL4.3}$, HIV-1_{YU-2}, HIV-$1_{A/G}$, $SIV_{CPZ1.9}$, $SIV_{AGM-tan}$, $SIV_{AGM-sab}$, HIV-2_{ROD}, SIV_{MAC}, and SIV_{SMM}. Viruses were serial diluted over a range of 2 logs to prevent saturation with 3-fold dilution between each point. Relative light unit was measured 48 hours post-infection (PI) and is shown in log scale. This is a representative graph out of 3 independent repeats where each data point is the average of two samples. B. Flag-tagged hIFITMs expression level in TZM-bl cell lines using Western Blot and flow cytometry.

most susceptible to hIFITMs, consistent with preliminary data. $SIV_{AGM\text{-}sab}$, SIV_{SMM}, and HIV-$1_{YU\text{-}2}$ are moderately inhibited by hIFITMs, followed by HIV-$1_{NL4.3}$, HIV-$1_{A/G}$, and HIV-2_{ROD}, while $SIV_{CPZ1.9}$ and SIV_{MAC} are resistant.

3.2 Human IFITM2 restricts SIV_{AGM} and SIV_{SMM} at entry

hIFITMs are known to restrict entry of Influenza virus, West Nile Virus, Dengue Virus, and vesicular stomatitis virus entry (18). Lu *et al*. also showed that IFITM2 and IFITM3 inhibit HIV-1_{BH10} entry (87). We decide to test if this is also the case for SIV_{AGM} strains and to test whether the restriction demonstrated by luciferase assay can be accounted by inhibition at entry stage. To this end, we selected HIV-$1_{NL4.3}$, $SIV_{AGM\text{-}tan}$, $SIV_{AGM\text{-}sab}$, SIV_{MAC}, and SIV_{SMM} to perform virion fusion assay in the same TZM-bl cell lines (Figure 15). The virus particles contains BlaM-vpr, an enzyme that can cleave β-lactam ring of a fluorescence resonance energy transfer (FRET) based substrate, CCF2. When the fusion pore is formed, CCF2 could diffuse into the core and be processed by the enzyme. Pore enlargement and uncoating do not affect the enzymatic activity (22). Thus, disruption of CCF2 FRET measured by flow cytometry should correlate with pore formation and but not completion of entry process (i.e.: release of core into the cytoplasm). Consistent with the trend seen in the luciferase assay, IFITM2 poses the most potent restriction at entry to strains that are sensitive (figure 15 C, D). IFITM3 is less potent compared to IFITM2. The trend in sensitivity of the strains is also consistent with the luciferase data: $SIV_{AGM\text{-}tan}$ and $SIV_{AGM\text{-}sab}$ are more susceptible, while HIV-$1_{NL4.3}$ and SIV_{MAC} are not restricted at entry. Despite the large amount of viruses used (equivalent to over 1million counter per minute (CMP) from RT assay), the entry process in this context is still receptor dependent, as HIV-$1_{NL4.3}$ and $SIV_{AGM\text{-}sab}$ entry is blocked by co-receptor antagonists specific to each of the two viruses (figure 15 C, D). Maraviroc (MVC) inhibits entry of strains with R5 tropism, while AMD3100 (AMD) inhibits strains with X4 tropism. HIV-$1_{NL4.3}$ is an X4 strain and it is only inhibited by AMD, while $SIV_{AGM\text{-}sab}$ is only inhibited by MVC, as it is an R5 strain.

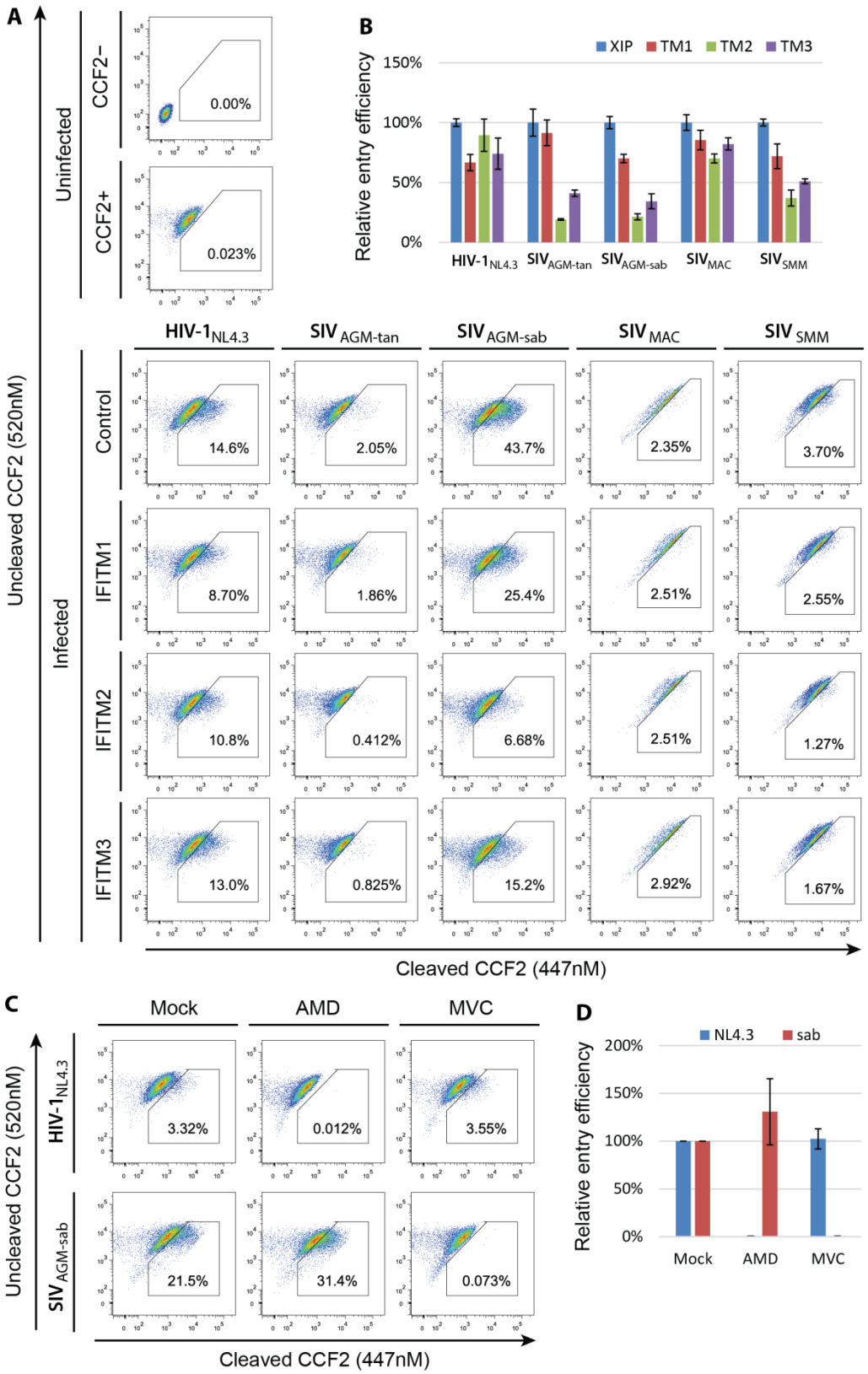

Figure 15. Restriction at entry in TZM-bl overexpressing hIFITMs. **A**. Virion fusion assay was

3.3 SIV_{AGM} are less sensitive to co-receptor antagonists

In order to gain insights into the mechanism of hIFITMs restriction at entry, we decided to look for correlates of this restriction. First, we want to look at co-receptor dependency by titrating MVC and AMD from 2nM to 2μM in luciferase assay. HIV-$1_{NL4.3}$, $SIV_{AGM\text{-}tan}$, $SIV_{AGM\text{-}sab}$, SIV_{MAC}, and SIV_{SMM} are selected. Strains insensitive to hIFITMs such as HIV-$1_{NL4.3}$ and SIV_{MAC} are more sensitive compared to strains sensitive to hIFITMs such as $SIV_{AGM\text{-}tan}$, $SIV_{AGM\text{-}sab}$, and SIV_{SMM} (figure 16).

3.4 IFITM2 N-terminal domain contributes to the restriction phenotype

As we are investigating which step(s) of the entry is affected, we are also interested in which residue(s) of the hIFITMs is crucial for anti-SIV activity. We decided to investigate the IFITM2 protein, as it is the most potent out of the three isoforms. We noticed that IFITM2 and IFITM3 are very similar (90% by amino acid sequence), yet there is a significant difference between their efficiency in inhibiting $SIV_{AGM\text{-}tan}$ and $SIV_{AGM\text{-}sab}$ (figure 14A). There are in total 12 different residues and most of them are in the N-terminal and C-terminal domain (figure 17A). We decided to swap these domains on the two proteins and examine if any of the regions are responsible for the inhibition. N-terminal domain was further split into two parts, as it is much longer than the C-terminal end. Six chimeric proteins constructs were synthesized (C1 to C6 as illustrated in figure 17A). TZM-bl cell lines overexpressing these proteins were infected with $SIV_{AGM\text{-}tan}$ and $SIV_{AGM\text{-}sab}$ and measured for luciferase unit. Switching the first part of the N-terminal region switched the

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performed in TZM-bl cell lines expressing hIFITMs with HIV-$1_{NL4.3}$, $SIV_{AGM\text{-}tan}$, $SIV_{AGM\text{-}sab}$, SIV_{MAC}, and SIV_{SMM}. Shift in the population toward right (cleaved CCF2 @ 447nM) reflects efficiency of pore formation. One of the triplcate from a representative data set out of three independent repeats is shown here. All data are summarized in bar graph with standard deviation in **B**. **C**. Co-receptor dependency of HIV-$1_{NL4.3}$ and $SIV_{AGM\text{-}sab}$ in virion fusion assay was tested using 2μM of MVC and AMD3100. One out of three graphs from a single data set is shown. The data set is summarized in bar graph with standard deviation in **D**.

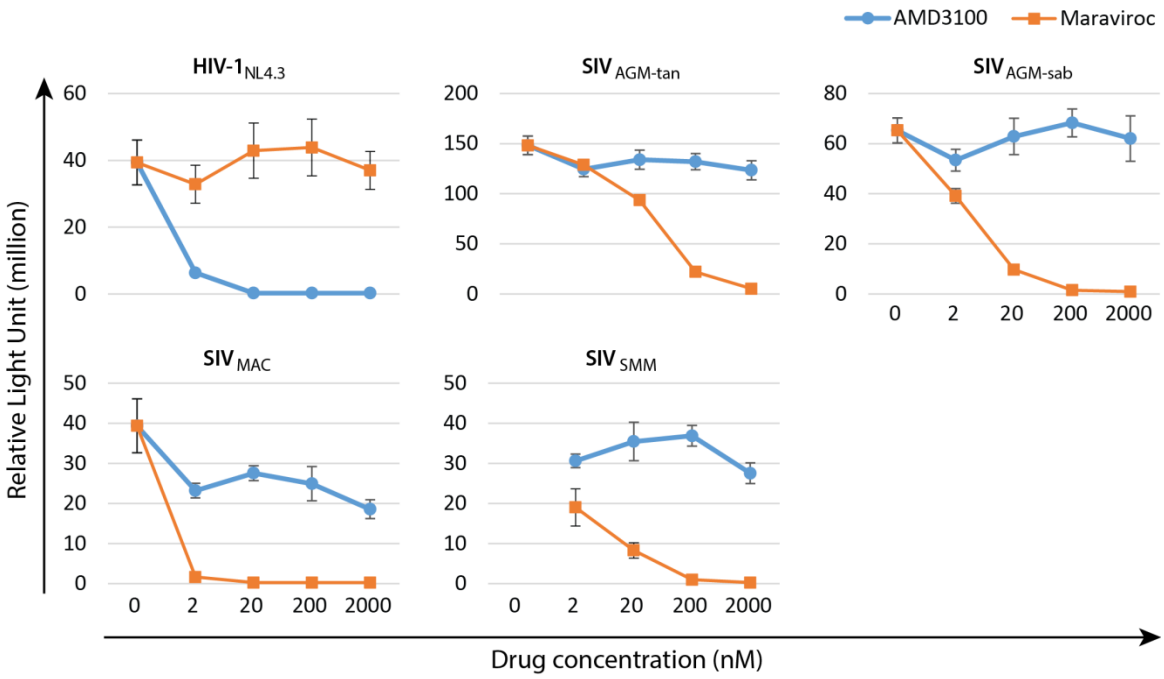

Figure 16. Co-receptor dependency of HIV-$1_{NL4.3}$, $SIV_{AGM-tan}$, $SIV_{AGM-sab}$, SIV_{MAC}, and SIV_{SMM}. MVC and AMD were titrated from 2nM to 2μM in luciferase assay. Infection was performed without changing medium one day post-infection. This is a single data set with triplicated samples showing standard deviation. Results are independently confirmed by Dr. Yann Le Duff.

phenotype between IFITM2 and IFITM3, while switching the second part of N-terminal region or the C-terminal region did not produce significant difference (figure 17C). There are two differences in the first part: IFITM2 has an isoleucine, while IFITM3 has a threonine on position 4; IFITM3 has an extra phenylalanine on position 8 compared to IFITM2. We speculate that one of the two or both positions could be important for the restriction. We decide to mutate the two proteins at these positions to find out the essential residues. Four chimeric constructs were made and the proteins are stably expressed in TZM-bl cell lines (figure 18A, B). Infection of $SIV_{AGM-tan}$ and $SIV_{AGM-sab}$ in the presence of any of the four mutants is restricted to a similar degree as WT IFITM2 (figure 18C).

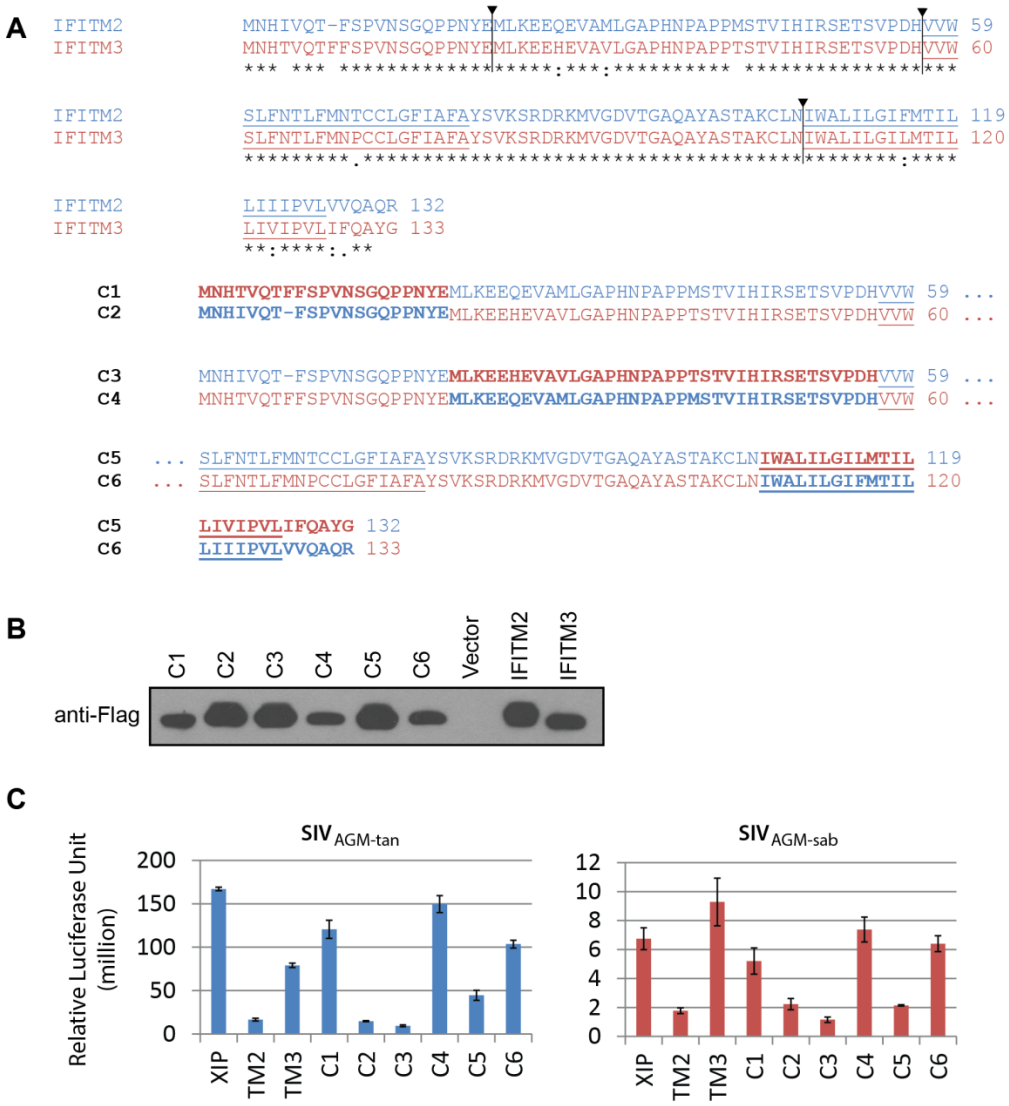

Figure 17. Inhibition of SIV_{AGM} by chimeric IFITM2 and IFITM3 proteins. A. Protein sequence alignment between IFITM2 (blue) and IFITM3 (red) and the switched domains are shown. Transmembrane domains are underlined. Switched domains are bolded and follow the same color code as WT proteins. C1, C3, and C5 are based on IFITM2, while C2, C4, and C6 are based on IFITM3. Chimeric constructs were synthesized and cloned into pQCXIP vector and corresponding TZM-bl cell lines were produced. B. The protein expression level detected by Western Blot. Samples protein concentration was normalized by Bradford Assay. C. Cell lines were infected by $SIV_{AGM\text{-}tan}$ and $SIV_{AGM\text{-}sab}$ and luciferase value in million was measured 2 days PI. This represents one out of three independent repeats each with three samples per data point. Standard deviation error bar is shown.

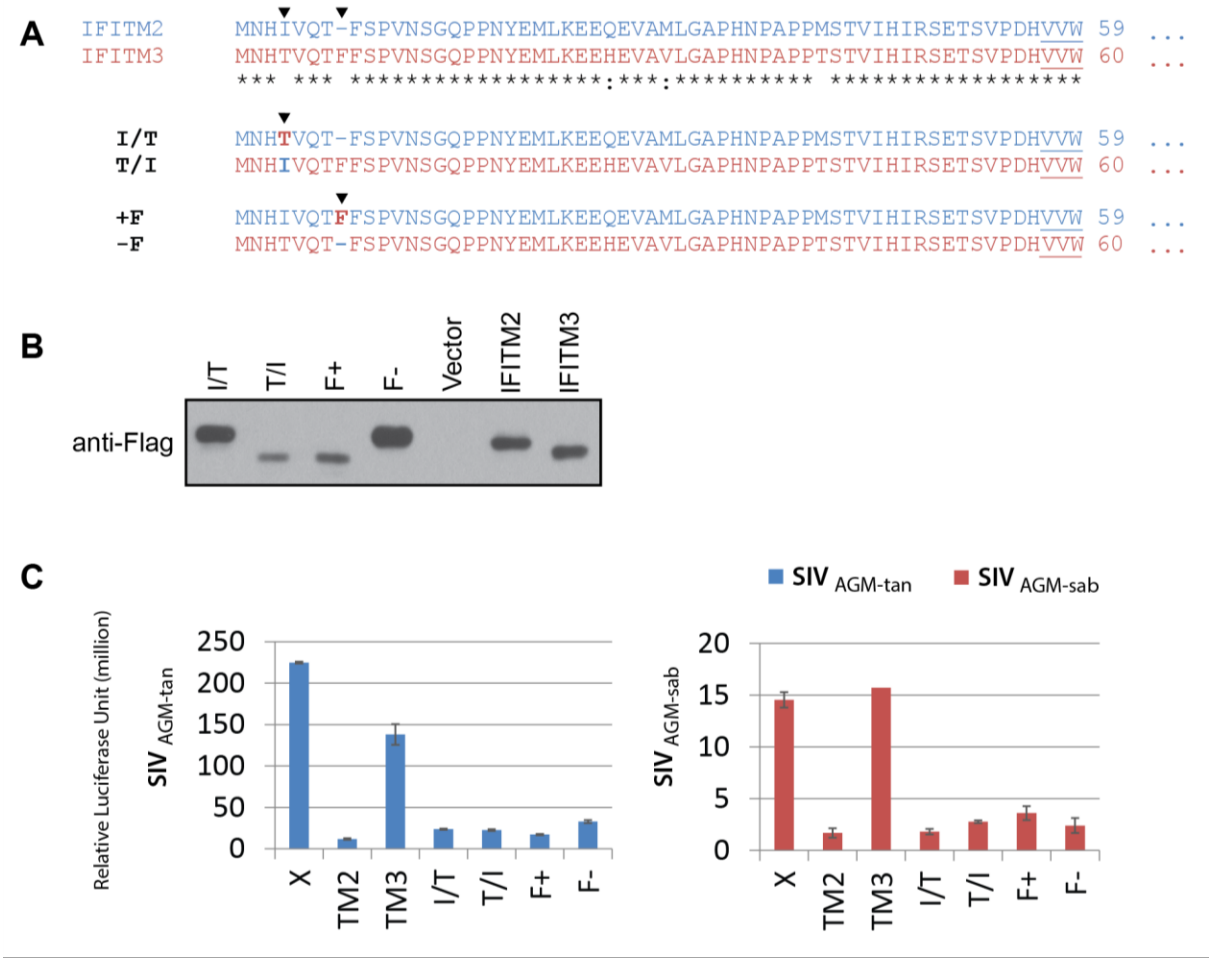

Figure 18. Inhibition of SIV_{AGM} by chimeric IFITM2 and IFITM3 proteins. A. N-terminal protein sequence alignment between IFITM2 (blue) and IFITM3 (red) is shown and position 4 and 8 are highlighted. 4 constructs are made using site directed mutagenesis. I/T and +F are based on IFITM2, while T/I and –F are based on IFITM3. B. The protein expression level detected by Western Blot. Samples protein concentration is normalized by Bradford Assay. C. Cell lines are infected by $SIV_{AGM\text{-}tan}$ and $SIV_{AGM\text{-}sab}$ and luciferase value in million is measured 2 days PI. This represents one out of three independent repeats each with three samples per data point. Standard deviation error bar is shown.

3.5 AGM IFITMs inhibits SIV strains

Restriction factors such as TRIM-5α and APOBEC3G from different species have different restriction phenotype against different lentivirus strains as a result of virus-host arm race. IFITM proteins could have gone through similar path during the course of evolution. We would like to first test if there are differences in the potency of IFITMs in restricting various viral strains. SIV_{AGM}, but not HIV, are markedly reduced by human IFITM2 (figure 14A). We suspect the opposite could be true for the AGM ortholog. Since AGM genome is not publicly available yet, we decide to clone the IFITM genes from AGM cells. COS-7 and Vero cells are selected. To clone agmIFITM1, we aligned 5' and 3' untranslated regions of the gene between human, chimpanzee, pongo, and macaque. Two forward and two reverse primers are designed based on conserved sequence in order to generate four different RT-PCR products. RT-PCR products from COS-7 have much stronger band compared to Vero, so only COS-7 amplicons are gel extracted and sent for sequencing. Two out of four are successfully sequenced and they turned out to be the same (figure 19A). When agmIFITM1 sequence is aligned with those from human, chimpanzee, pongo, and mac, IFITM1 across these 5 primate species are 85.6% identical (Figure 19A). Most of the differences are in the relatively less conserved region of IFITMs, i.e.: the N-terminal and C-terminal domains. agmIFITM1 shares even more sequence similarity with macIFITM1 (97.6%), as expected.

When we were designing IFITM2 and IFITM3 primers, we noticed that there is no IFITM2 gene in macaque. In the locus where IFITM2 should be, a second copy of IFITM3 is found. This raised doubts in whether AGM has IFITM2 or not. We designed the primers based on the conserved regions between IFITM2 and IFITM3 of human and chimpanzee and the two copies of IFITM3 from macaque. This ensures that, if AGM do have IFITM2 gene and transcripts, the primers will be able to amplify them. Again, four RT-PCR products from each cell lines are obtained. 2 products from COS-7 and 3 products from Vero are selected based on band intensity. Some primer combinations produced 2 bands around 500bp. We decide to extract them separately along with other RT-PCR products totaling 7 extractions. DNA is cloned into vector and amplified in bacteria. In order to not miss

possible IFITM2 construct, we picked 8 colonies per plate. 21 out of 44 positive clones are sent to sequencing and 14 of them are successfully sequenced (figure 19B). Amino acid sequences of all agmIFITM2/3 clones are identical except at position 22 and 38. On position 22, 5 clones have isoleucine, while 9 clones have methionine. On position 38, 4 clones have histidine, while 10 clones have leucine. These different variants might be cell-line dependent (table 2). Primer choice might also play a role in the amplification of variants with isoleucine at position 22: 4 out of 5 clones with isoleucine are based on the second forward primer. However, it is not statistically significant due to the small number of samples. There are in total 7 M/L, 3 I/L, 2 I/H, and 2 M/H variants. Clone V2-2_2 has a serine instead of glycine at position 15 compared to all other clones. We suspect that the G/S mutation is a mistake during cloning or sequencing and did not investigate it further.

When aligned with IFITM2 and IFITM3 from human, chimpanzee, and mac, it becomes clear that the clones we obtained from AGM cell lines are IFITM3. They all have valine at position 8, compared to IFITM2 proteins that have the typical gap on the alignment. IFITM3-mac* is the sequence of the IFITM3 gene that shares the same locus with IFITM2 in human. It also has a valine at position 8. The other copy of IFITM3 as well as IFITM3 from other human and chimpanzee has a phenylalanine. The next observation is that, just like IFITM1, IFITM3 transmembrane and intracellular domains are almost perfectly conserved between these species.

A

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IFITM1-homo       MHKEEHEVAVLGAPPSTILPRSTVINIHSETSVPDHVVWSLFNTLFLNWCCLGFIAFAYS 60
IFITM1-chimp      MHKEEHEVAVLGAPPSTILPRSTVINIHSETSVPDHVVWSLFNTLFLNWCCLGFIAFAYS 60
IFITM1-pongo      MHKEEHEVTVLGAPPSTILPRSTVINIHSEISVPDHVVWSLFNTLFLNWCCLGFIAFAYS 60
IFITM1-mac        MHKEEHEVSVLGAPHSTILPRSTMINIQSETSVPDHIVWSLFNTIFLNWCCLGFIAFAYS 60
IFITM1-agm        MHKEEHEVSVLGAPHSTILPRSTMINIQSETSVPDHVVWSLFNTIFFNWCCLGFIAFAYS 60
                  ********:***** ********:***:** *****:*******:*:************

IFITM1-homo       VKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIGFILLLVFGSVTVYHIMLQIIQ 120
IFITM1-chimp      VKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIGFILLLVFGSVTVYHIMLQIIQ 120
IFITM1-pongo      VKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIGFILLLVFGSVTVYHVMLQIVH 120
IFITM1-mac        VKSRDRKMVGDVTGAQAYASTAKCLNISALIVGILMTIGFILLLVYGSVAIYHVMLQIVQ 120
IFITM1-agm        VKSRDRKMVGDVTGAQAYASTAKCLNISALIVGILMTIGFILLLVFGSVAIYHVMLQIVQ 120
                  ************************** ***:*************:***::**:****::

IFITM1-homo       EKRGY 125
IFITM1-chimp      EKRGY 125
IFITM1-pongo      EKRGY 125
IFITM1-mac        EKQRY 125
IFITM1-agm        EKQRY 125
                  **: *
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B

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V1-2_3          MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
V2-2_1          MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
V1-2_1          MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
V1-1-1_4        MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
V1-1-1_2        MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
C2-1-1_3        MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
C2-1-1_1        MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
C1-1_3          MNHTVQTVFSPVNSGQPPSYEILKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
C2-2_3          MNHTVQTVFSPVNSGQPPSYEILKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
C2-2_4          MNHTVQTVFSPVNSGQPPSYEILKEEHEVAVLGAPHNLAPPTSTVIHIRSETSVPDHVVW 60
V2-1-1_2        MNHTVQTVFSPVNSGQPPSYEILKEEHEVAVLGAPHNHAPPTSTVIHIRSETSVPDHVVW 60
V2-2_2          MNHTVQTVFSPVNSSQPPSYEILKEEHEVAVLGAPHNHAPPTSTVIHIRSETSVPDHVVW 60
C1-1_4          MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNHAPPTSTVIHIRSETSVPDHVVW 60
C1-2_8          MNHTVQTVFSPVNSGQPPSYEMLKEEHEVAVLGAPHNHAPPTSTVIHIRSETSVPDHVVW 60
IFITM3-mac      MNHTVQTFFSPVNSGQPPNYEMLKEEHDVAMMGAPHNPAPPTSTVIHIRSETSVPDHVVW 60
IFITM3-mac*     MNHTVQTVFPPVNSGQPPSYEMLKEEHEVAVLGAPHNPAPPMSTVIHIRSETSVPDHVVW 60
IFITM3-homo     MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVPDHVVW 60
IFITM3-chimp    MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPMSTVIHIRSETSVPDHVVW 60
IFITM2-homo     MNHIVQT-FSPVNSGQPPNYEMLKEEQEVAMLGVPHNPAPPMSTVIHIRSETSVPDHVVW 59
IFITM2-chimp    MNHIVQT-FSPVNSGQPPNYEMLKEEQEVAMLGAPHNPAPPMSTVIHIRSETSVPDHVVW 59
                *** *** *.****.***.**:****::**::*.*** *** ******************

V1-2_3          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
V2-2_1          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
V1-2_1          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
V1-1-1_4        SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
V1-1-1_2        SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C2-1-1_3        SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C2-1-1_1        SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C1-1_3          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C2-2_3          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C2-2_4          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
V2-1-1_2        SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
V2-2_2          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C1-1_4          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
C1-2_8          SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
IFITM3-mac      SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIL 120
IFITM3-mac*     SLFNTLFMNTCCLGFIAFAYSVKSRDRKMVGDLTGAQAYASTAKCLNIWALILGILMTIP 120
IFITM3-homo     SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIL 120
IFITM3-chimp    SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTIL 120
IFITM2-homo     SLFNTLFMNTCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGIFMTIL 119
IFITM2-chimp    SLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGIFMTIL 119
                *********.**********************:**********************:***

V1-2_3          LIVVPVLIFQAHQ 133
V2-2_1          LIVVPVLIFQAHQ 133
V1-2_1          LIVVPVLIFQAHQ 133
V1-1-1_4        LIVVPVLIFQAHQ 133
V1-1-1_2        LIVVPVLIFQAHQ 133
C2-1-1_3        LIVVPVLIFQAHQ 133
C2-1-1_1        LIVVPVLIFQAHQ 133
C1-1_3          LIVVPVLIFQAHQ 133
C2-2_3          LIVVPVLIFQAHQ 133
C2-2_4          LIVVPVLIFQAHQ 133
V2-1-1_2        LIVVPVLIFQAHQ 133
V2-2_2          LIVVPVLIFQAHQ 133
C1-1_4          LIVVPVLIFQAHQ 133
C1-2_8          LIVVPVLIFQAHQ 133
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IFITM3-mac          LIVVPVLIFQAHQ 133
IFITM3-mac*         LIVIPVLIYQAHR 133
IFITM3-homo         LIVIPVLIFQAYG 133
IFITM3-chimp        LIVIPVLIFQAYG 133
IFITM2-homo         LIIIPVLVVQAQR 132
IFITM2-Chimp        LVIIPVLVVQAQR 132
                    *:::***: **
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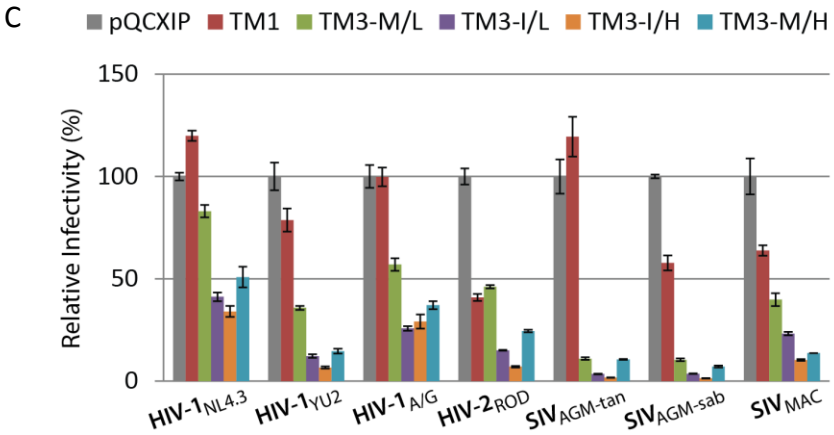

Figure 19. Alignment of AGM IFITMs and anti-HIV/SIV phenotype. A. agmIFITM1 amino acid sequence is aligned with those of IFITM1 from human, chimpanzee, pongo, and macaque. B. agmIFITM3 clones sequences are aligned with IFITM2 and IFITM3 from human, chimpanzee, pongo, and IFITM3 from macaque. Residue at position 8, where identification of IFITM2 and IFITM3 can be made, is highlighted in grey. Serine residue at position 15 in Clone V2-2_2 is highlighted. Residues at position 22 and 38 are highlighted: methionine in green, isoleucine in blue, leucine in cyan, histidine in yellow, and proline in red. All IFITM sequences except those of AGM are obtained from NCBI database. Homo: homo sapien; chimp: chimpanzee; mac: macaque. C. TZM-bl cell lines overexpressing agmIFITMs are infected with HIV-$1_{NL4.3}$, HIV-1_{YU2}, HIV-$1_{A/G}$, HIV-2_{ROD}, $SIV_{AGM\text{-}tan}$, $SIV_{AGM\text{-}sab}$, and SIV_{MAC}. Cells are collected and luciferase value is measured 2 days PI. The vector control, pQCXIP, is set as 100%. Error bar displays standard deviation.

Next, we expressed agmIFITM1 and the 4 variants of agmIFITM3 in TZM-bl cells. Infection was done using HIV-$1_{NL4.3}$, HIV-1_{YU2}, HIV-$1_{A/G}$, HIV-2_{ROD}, $SIV_{AGM\text{-}tan}$, $SIV_{AGM\text{-}sab}$, and SIV_{MAC} (figure 19C). A few observations can be made. First, similar to human IFITM1, agmIFITM1 is not efficient in restricting the tested strains. Second, agmIFITM3 are more

Table 2. 4 variants of IFITM3 according to A) primer combinations and B) cell line choice.

A

	ML	IL	IH	MH
F1-R1	2	1	0	1
F1-R2	2	0	0	1
F2-R1	2	0	1	0
F2-R2	1	2	1	0

B

	ML	IL	IH	MH
COS	2	3	0	2
Vero	5	0	2	0

potent in inhibiting all tested HIV and SIV strains compared to human IFITM3. Third, interestingly, each agmIFITM3 inhibits the viruses to a different extent, with I/H variant being the most potent, followed by I/L and M/H. The least potent agmIFITM3 is M/L, the most common variant. Fourth, contrary to what we have expected, agmIFITMs inhibits SIV strains better than HIV strains. Fifth, SIV_{MAC}, a strain that is insensitive to hIFITM3, is inhibited by the AGM ortholog, although SIV_{AGM} strains are the most sensitive to agmIFITM3, as in the case of hIFITM2 and hIFITM3.

3.6 IFITM proteins binding partners that could contribute to the restriction

In order to gain further insights into the anti-viral mechanism of hIFITMs restriction, we decide to find the cellular interaction partner of hIFITMs. SupT1 cell lines with IFITMs under doxycycline promoter are either treated with or without IFN-α2b for 16 hours. The negative control consists of a mixture of the three cell lines each containing one IFITM gene without doxycycline induction. A repeat is done using 293T cells transiently transfected with IFITMs constructs. Immunoprecipitation and mass spectrometry is carried out as outlined in Materials and Method.

We crossed the list of proteins in two ways to analyze the data. First, Venn diagram is generated for common binding partners between the three IFITM proteins with or without the presence of ISGs induced by IFN (figure 20). Proteins are color coded based on the intracellular localization. There are four major observations. First, the baits are not the

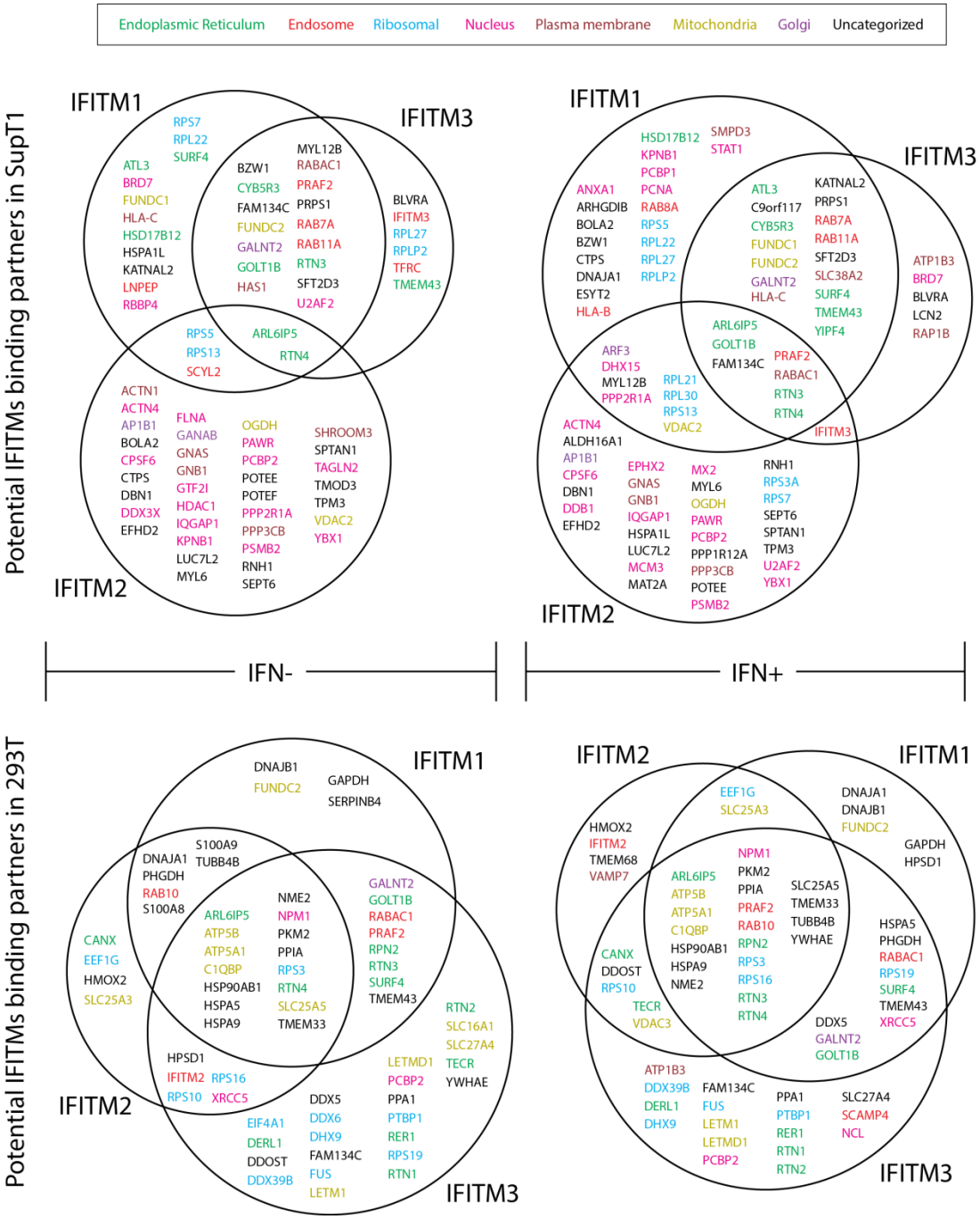

Figure 20. Potential IFITMs interaction partners. Co-IP samples of SupT1 and 293T expressing flag-tagged IFITM proteins in the presence or absence of IFN-α are processed by mass spectrometry. Proteins candidates are color-coded based on primary intracellular localization, except ribonucleoproteins (blue). Uncategorized proteins are in black. The list is crossed between the three proteins.

most common hit from the list. In fact, they are missing from the list in most of samples. Second, in the data set obtained from SupT1 cells, there are more common interaction partners between IFITM1 and IFITM3 compared to IFITM2 and IFITM3. This is surprising considering that IFITM2 and IFITM3 are very similar based on amino acid sequence as well as their antiviral patterns. Sample mishandling has been ruled out, as none of the IFITM2 exclusive binding proteins from SupT1 appeared in the IFITM3 samples from 293T. In addition, some of the IFITM1 and IFITM3 common binding partners from SupT1 samples reappeared as IFITM1 and IFITM3 shared binding partner from 293T samples. Third, there is considerable variation between the two data sets. IFITM2 has more exclusive binding partners in SupT1 than in 293T, while IFITM3 has more exclusive binding partners in SupT1 than in 293T. This is expected as the expression pattern could be different between T cell and epithelial cell. Finally, when treated with IFN, the number of binding partners common to all three proteins increased.

When we crossed the list between non-treated and IFN treated samples for each protein, we see that all three proteins lost some binding partners and gained other partners from IFN treatment. This is expected as IFN induce an antiviral state by changing the cellular expression pattern in many cell types including T cells and epithelial cells.

Next, we picked common binding proteins and proteins that appear in both sets and our collaborator ordered all available siRNAs (3 per gene, representated by blue, red, and green, except SLC27A4, RPS19, RPS16, and PPIA) in order to make an unbiased confirmation test. VAP-A is included as control. 48 hours post siRNA transfection, IAV stain WSN is used to infect 293T stably overexpressing IFITM3. Cells are fixed 8 hours PI and are processed by FACS for percentage of infected cells (figure 21). The fold inhibition from control siRNA (percentage from 293T-IFITM3 divided by percentage from 293T-vector) is divided by the fold from siRNA knockdown to get the relative IFITM3 inhibition efficiency. When a particular gene is knocked down, an increase in inhibition efficiency (such as in the case of VAP-A) would suggest that the gene hampers anti-viral activity of IFITM3. No qPCR

or Western Blot was done to confirm the knockdown. Hence an absence of phenotype does not rule out that the gene could affect IFITM3 antiviral activity.

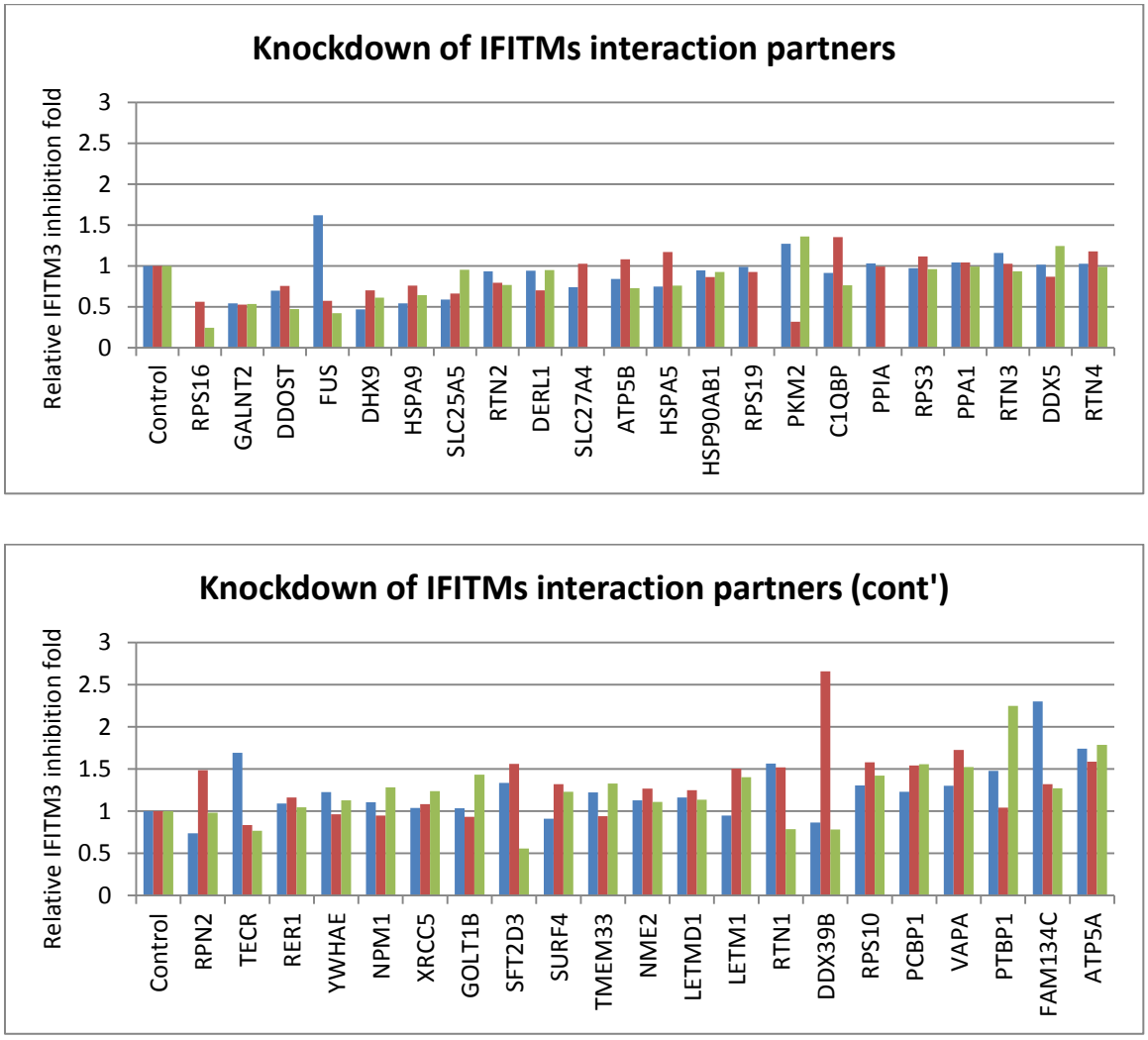

Figure 21. siRNA knockdown of potential IFITMs interaction proteins. Selected proteins are knocked down by siRNA in 293T expressing IFITM3. 8h PI by WSN, cells are washed and stained for NP and percentage of fluorescent positive cells is obtained by FACS. Fold inhibition in the presence of IFITM3 is divided by the fold inhibition in control vector. Each gene is knocked down by 3 different siRNAs (blue, red, and green).

VAP-A knockdown phenotype is consistent with published results: knockdown of VAP-A reduced IAV infection (2). However, we decide to focus on those genes that have synergistic effect with IFITM3 in inhibiting the virus. Some of the gene, when knocked down, reduced IFITM3 antiviral efficiency. RPS16 is one of them. However, the removal of this ribosomal protein stopped cell growth and affected the multiplicity of infection. This is also the case for the cytosolic protein PKM2 or pyruvate kinase, an enzyme key to glucose metabolism. On the other hand, DHX9 and FUS knockdown significantly reduced antiviral activity from IFITM3 without affect cell proliferation. IFITM3 is known to inhibit IAV at the entry stage, although it is possible that it also affect later stage of the viral life cycle.

Chapter IV: Discussion

Since the findings of IFITMs viral restriction reported by Brass *et al*. in 2009, many more studies investigated the effect of IFITMs on pH-dependent viruses at entry. So far, IAV, SARS, DENV, WNV, YFV, MARV, EBOV, VSV, REOV, RVFV, and HCV have been shown to be restricted by IFITMs. SARS is not strictly a pH-dependent virus, but its entry requires proteases present in the acidified compartment. Each virus is inhibited to a different extent by each of the 3 IFITM members. The only pH-independent virus reported to be affected by IFITMs is HIV-1. The current consensus is that IFITMs inhibit viruses that require access to endosomes.

The mechanism of inhibition also remains elusive. The current hypothesis is that the IFITMs change the properties of surface or vesicular membrane, abrogating the fusion between viral membrane and the cellular membrane. It also appears that the post-translational modifications are important for the localization of IFITMs, which in turn affects their antiviral activity.

In the current study, we provided more evidence for restriction of pH-independent viruses by IFITMs. We also investigated the mechanism of IFITM restriction using primate lentivirus as a model organism.

4.1 IFITM2 inhibits entry of pH-independent virus

We used a panel of HIV-1 and SIV viruses to assess the inhibitory activity of IFITMs on primate lentivirus in a luciferase system. HIV-1_{YU-2}, $SIV_{AGM-tan}$, $SIV_{AGM-sab}$, and SIV_{SMM} are inhibited by members of IFITM family to different extent, while HIV-$1_{NL4.3}$, HIV-$1_{A/G}$, and HIV-2_{ROD} are less affected. $SIV_{CPZ1.9}$ and SIV_{MAC} are largely insensitive to IFITMs (Figure 14).

After we demonstrated the restriction in luciferase assay, we used virion fusion assay to investigate whether the restriction of sensitive SIV strains occurs at entry. We found that $SIV_{AGM-tan}$, $SIV_{AGM-sab}$, and SIV_{SMM} are inhibited at viral entry, while HIV-$1_{NL4.3}$ and

SIV_{MAC} not affected (Figure 15). We also need to confirm this phenotype in a relevant cell type such as T cells.

4.2 Mechanism of inhibition

Following the publication of the study on IFITMs hemifusion by Liu's group, we decided to test whether IFITMs inhibits SIV entry in a similar fashion as they do to pH-dependent viruses: by inhibiting hemifusion stage of membrane fusion. However, we were not able to reproduce the data in our system (figure 23). Oleic acid (OA) induces negative curvature and has been shown to rescue infection from IFITM restriction (85). Chlorpromazine (CPZ) induces positive curvature and had no effect on IFITM restriction. In our experiment, only CPZ led to a minor increase in infection in both control lines and IFITM overexpression lines. There are a few key differences between the system used in the study and our system. First, we used a pH-independent virus, while the authors used a pH-dependent virus. Second, we used wild type virus in virion fusion assay, while Liu's group used cells expressing viral envelope protein and receptor in cell-cell fusion assay.

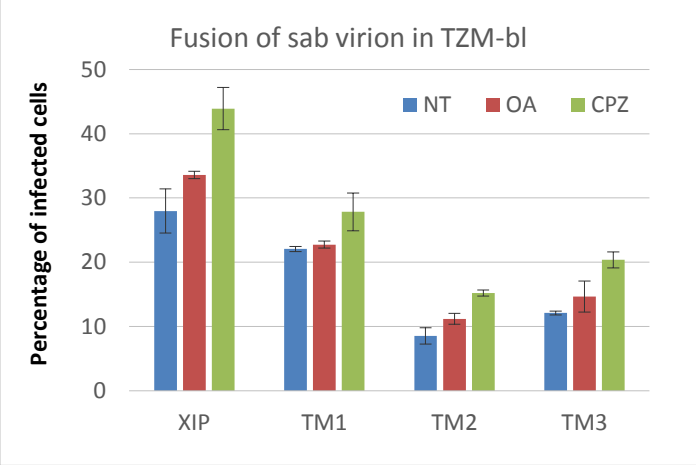

Figure 23. Effect of small lipids on IFITM restriction of $SIV_{AGM\text{-}sab}$. Virion fusion assay is performed in TZM-bl IFITM cell lines. Drug treatment is described in the original publication (85). OA: oleic acid; CPZ: chlorpromazine.

IFITMs inhibition of pH-dependent viruses does not depend on receptor of viral envelope. Nor do they influence the triggering mechanism (e.g. low pH and cathepsin activity) for fusion proteins. We decide to test whether this also applies to primate lentivirus. We used soluble CD4 as well as CXCR4 and CCR5 inhibitors to test whether strains affected by IFITMs bind to receptor and co-receptor similar to strains unaffected by

IFITMs. Entry of all tested viruses is inhibited to the same extent by sCD4 (data not shown). However, strains affected by IFITMs are more resistant to co-receptor blockade compared to strains insensitive to IFITMs (Figure 16). It is possible that the sensitive strains uses need very few co-receptors to trigger the conformational change in gp41. Alternatively, these strains could require less envelope proteins to complete membrane fusion. We have not investigated the effect of these inhibitors in the presence of IFITMs.

We noticed a difference in efficiency in inhibition of SIV_{AGM} by IFITM2 and IFITM3 in both luciferase system and virion fusion assay. Since they are very similar based on their amino acid sequence, we decide to produce chimeric proteins to find out which residues are important in restricting SIV_{AGM} (Figure 17). The examination of expression level of the chimeric proteins by the first Western Blot did not reveal a significant variation under ambient lighting. However, closer examination of a repeat of the Western Blot with different exposure time revealed significant difference in expression level (Figure 17B). Initially, we thought that use of trypsin to collect samples could degrade proteins. However, this is not the case, as the expression level based on Western Blot remains unchanged when cells are lifted using EDTA. What we could have done is to measure expression level using FACS. If only a small percentage of cells express the chimeric proteins in C1, C4, and C6, then we could gate out the positive cells and check the level of infection. John and colleagues did N-terminal domain swap between IFITM2 and IFITM3 as well, and the expression level is very different between these two chimeric proteins (73). They concluded that the NTD of IFITM2 and IFITM3 are not functionally interchangeable. Nevertheless, we decided to further investigate the chimeric proteins by swapping single residues (Figure 18), as the experimental results were obtained prior to confirmation from Western blot. The chimeric proteins are again expressed at different level. However, SIV_{AGM} are inhibited irrespective of the expression level. We conclude that the residue at position 4 as well as the phenylalanine could modulate the inhibition efficiency of SIV_{AGM}.

Since IFITMs have not been reported for enzymatic activity, we believe that other cellular proteins might contribute to the restriction of IFITMs. We decide to perform an

affinity purification of IFITMs followed by mass spectrometry in order to identify potential interaction partners (figure 20). Our collaborator then took the list of potential interacting proteins and performed siRNA knockdown in an IAV system. Restriction phenotype of IFITM3 was assessed when a protein is knocked down relative to knockdown control. Knockdown of RPS16, GALNT2, DDOST, FUS, DHX9, HSPA9, and SLC25A5 reduced IFITM3 inhibiton of IAV by 50% (Figure 21). As Western Blot was not performed to check knockdown efficiency, we might have missed other interesting binding partners. However, we will first investigate the candidate with knockdown phenotype.

As HIV-1 is the only pH-independent virus that is affected by IFITMs, it could be possible that the mechanism of inhibition is also different from the other viruses. Although IFITMs do not bind to receptors of pH-dependent virus, exception such as HCV exists. Our data on co-receptor usage suggest that IFITMs might involve, directly or indirectly, co-receptors in the restriction of primate lentivirus entry. In the siRNA knockdown list, many proteins with a synergistic phenotype with IFITM3 are enzymes that mediate protein glycosylation. Although IFITM3 is shown to be non-glycosylated (151), it could be transported through the trans-Golgi network and influence the enzyme in glycosylating other proteins, which in turn affects viral entry. One of these molecules could be the HIV co-receptor. Removal of the sole glycosylation site (N11) from CXCR4 allows both X4 common lab stains and R5 lab strains and primary isolates to infect efficiently (24, 142). This is done without affecting the co-receptor inhibitors efficiency (69), suggesting that the receptor conformation is not significantly altered by this post-translational modification. We could test whether IFITMs can influence co-receptor glycosylation simply by running a mobility assay.

4.3 IFITMs genes in Monkey and their inhibition activity

SIV_{AGM} are inhibited by human IFITM proteins (Figure 14). Based on the knowledge of the virus-host arm race for well-known primate lentivirus restriction factor such as APOBEC3G, TRIM5α, and tetherin, we were curious whether the HIV-1 can be inhibited by AGM IFITM proteins. We used macaque as a reference, as SIV_{MAC} are not affected by human

IFITMs. We found that, similar to macaque, there is no IFITM2 in AGM. Surprisingly, when we tested for their restriction phenotype, we found that AGM IFITM3 restricts SIV more efficiently than HIV (Figure 19C). SIV_{AGM} strains are still the most affected, as in the case of hIFITMs.

There are four variants of AGM IFITM3. The most frequent one is the less potent in restricting tested strains while the least common one is the most potent. The two most potent ones have an isoleucine on position 22, while the two least potent one has a methionine. It is interesting that they are in the middle of the YEML sorting motif. This shift from methionine to isoleucine will likely destroy the motif, leading to redistribution of the protein throughout the cell. Whether this change in localization could affect primate lentivirus restriction is not clear. Relocalization of human IFITM3 from endosomal compartment to cell surface slightly increased HIV-1_{BH10} infection (71). All other sequences in the alignment (mac, human, chimp) have the conserved methionine residue. On position 38, AGM IFITM3 has either leucine or histidine, while IFITM2 and IFITM3 from other species have a proline. This proline residue will form a tandem PxxP motif with P34 and P42. We can find out if this tandem motif is important by testing the macIFITm3.

We suspect that, similar to human IFITM2, AGM IFITM3 also restrict SIV at entry. AGM are known to carry SIV_{AGM} at high titer in their blood without reduction in memory T cell population. It is possible that IFITMs help memory T cells to survive in a similar manner as how mouse IFITM3 helped memory T cells in the lung during IAV infection (141).

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