

# Novel Restriction of HIV-1 by Chimeric Human APOBEC3A

By

2017

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Submitted to the graduate degree program in Microbiology, Molecular Genetics & Immunology  
and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy.

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Date Approved: July 2017

## Abstract

The human apolipoprotein B mRNA editing enzyme catalytic peptide-like 3 (APOBEC3; A3) family of proteins are a family of seven cytidine deaminases (A3A, A3B, A3C, A3D, A3F, A3G and A3H) that restrict certain viral infections. These innate defense factors are best known for their ability to restrict the replication of human immunodeficiency virus type 1 (HIV-1) lacking a functional Vif protein (HIV-1 $\Delta$ vif) through the deamination of cytidine residues to uridines during reverse transcription, ultimately leading to lethal G-to-A changes in the viral genome. The best studied of the A3 proteins has been A3G because of its potent activity against HIV-1 $\Delta$ vif. However, one member of this family, A3A, has biological properties that make it unique among the A3 proteins.

Despite potent restriction activity when expressed in myeloid cells, human A3A (hA3A) is generally known lacking inhibitory function towards HIV-1 $\Delta$ vif. Previous studies in our laboratory have shown that rhesus macaque A3A (rhA3A) is capable of restricting both simian–human immunodeficiency virus lacking vif gene (SHIV $\Delta$ vif) and human immunodeficiency virus lacking vif gene (HIV-1 $\Delta$ vif) to a greater extent than hA3A. Chimeric studies by substituting amino acids 25–33 (AC loop 1 region) from rhA3A into hA3A showed that this region is critical in determining the restriction pattern against lentiviruses.

Further studies identified three Old World Monkey A3As that were able to restrict HIV-1 replication. Restriction assays with various A3As from hominids, New World monkeys, and Old World monkeys found that agmA3A and colA3A restricted the replication of SHIV $\Delta$ vif and that all three Old World monkey A3A proteins (agmA3A, colA3A, and rhA3A) restricted HIV-1 $\Delta$ vif to some extent. More impressively, it is also showed that the A3A protein from the black and white colobus monkey (mantled guereza; *Colobus guereza*; colA3A) potently inhibited

not only HIV-1 $\Delta$ *vif* replication but also wild-type HIV-1 in producer cells. Since previous studies indicated the importance of AC loop 1 region in determining the restriction ability and our sequence alignment analysis showed that A3A proteins from *Mandrillus sphinx* (mndA3A) and *Cercopithecus neglectus* (debA3A) had an AC-loop1 region that was almost identical to colA3A, we tested the restriction ability of mndA3A and debA3A. The restriction assay showed that both debA3A and mndA3A restricted HIV-1 similar as colA3A.

Next, we used chimeric protein and amino acid deletion studies to show that AC loop 1 region, especially the <sup>27</sup>WVS<sup>29</sup> motif, is the key determinant for HIV-1 and AAV-2 restriction. First, by fusing the first 100 amino acids of colA3A and the C-terminal region of hA3A (h<sub>1-100</sub>colA3A), or the first 100 amino acids of hA3A and the C-terminal region of colA3A (col<sub>1-100</sub>hA3A), we found that the N-terminal domain of colA3A contains the determinants for HIV-1 restriction. To further confirm that, we truncated the C-terminal off and constructed the mutant proteins that expressed only the N-terminal 100 amino acids (colA3A<sub>1-100</sub>, mndA3A<sub>1-100</sub>, debA3A<sub>1-100</sub>). The truncated colA3A, mndA3A and debA3A proteins were all able to restrict HIV-1 replication. We then narrowed the region down by examining chimeric proteins with the N-terminal 75, 50 and 33 amino acids of colA3A fused to hA3A (col<sub>1-75</sub>hA3A, col<sub>1-50</sub>hA3A, and col<sub>1-33</sub>hA3A). The results indicated that the first 33 amino acids harbor the critical determinants for HIV-1 restriction. As previous experiments indicate the importance of the AC loop 1 region, a chimeric hA3A containing the AC Loop1 region of mndA3A (mnd<sub>25-33</sub>hA3A) was made. Restriction assays revealed that the mnd<sub>25-33</sub>hA3A chimera was able to restrict HIV-1, indicating that the AC Loop1 region of mndA3A was critical to its restriction properties. As the hominids have a three amino acid deletion in this region of A3A, we constructed a chimeric hA3A with the WVS motif and found that this insertion completely restored the antiviral activity of hA3A. In

addition, insertion of “WVS” or AC Loop-1 region into hA3A also changed the restriction pattern of adeno-associated virus 2 (AAV-2). Therefore, we conclude that this WVS motif is the key for both retrovirus and parvovirus restriction.

As we found that the WVS motif in the AC loop 1 region plays a key role in HIV-1 restriction, we determined the role of each of these three amino acids in inhibiting HIV-1 replication. We constructed a series of single amino acid substitutions and single/double deletion mutants in this region and performed restriction assays on these mutants. The results suggested that the nature (charge and hydrophobicity) of the three amino acids was not as important as the length for restriction of HIV-1. Finally, we confirmed this by substituting the WVS motif with either AAA or GGG, and found that both hA3A<sup>[27AAA<sup>29</sup>]</sup> and hA3A<sup>[27GGG<sup>29</sup>]</sup> were able to inhibit HIV to the level similar as hA3A<sup>[27WVS<sup>29</sup>]</sup>.

Finally, we explored the mechanism of hA3A<sup>[27WVS<sup>29</sup>]</sup> restriction of HIV-1 replication. First, we examined the viral replication in producer cells. We determined the viral protein synthesis in producer cells, using p24 and pulse-chase analysis, were similar in controls and cells expressing hA3A<sup>[27WVS<sup>29</sup>]</sup>. Next, we excluded the possibility of subcellular localization contributing to defective viruses production as hA3A and hA3A<sup>[27WVS<sup>29</sup>]</sup> were both expressed in cytoplasm and the nucleus at similar level in producer cells. Further, we examined the viral RNA packaging and found that viral RNA was packaged at similar levels in progeny viruses from both control and hA3A<sup>[27WVS<sup>29</sup>]</sup> expressing cells. In addition, we showed that the viral reverse transcriptase enzyme activity of the progeny viruses was not affected by the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>. We next examined restriction in target cells. We first confirmed that the viral replication was inhibited in target cells by showing that the viral protein biosynthesis was significantly lower in target cells using pulse-chase experiments. We

also excluded that canonical hypermutation contributed to the restriction. We found the levels of early and late reverse transcription products were decreased approximately 10-fold in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>. This was also reflected in decreased viral integration and viral transcripts in the target cell. Thus, we concluded that the restriction of HIV-1 by hA3A<sup>[27WVS<sup>29</sup>]</sup> occurred at the level of reverse transcription in the target cells.

In summary, we were able to restore the ability of hA3A to restrict HIV-1 and abolish the ability of hA3A to restrict AAV-2 by inserting only three amino acids in the AC loop 1 region. This reveals that the AC loop 1 region harbors key determinants for both retrovirus and parvovirus restriction, and provides promising preliminary data for further translational application such as gene therapy.

## Acknowledgments

It has been a life changing six years being a graduate student. I am full of gratitude as I won't be able to make it without all the wonderful support from the following individuals.

The first and biggest appreciation goes to my Ph.D. mentor, Dr. Edward B. Stephens. He has a very unique mentoring style that offered me plenty of space and independence. He showed me the beauty of science and helped me become more confident and efficient in work. He always discussed the hypothesis and experiment design with me to make sure I understood what I was doing and why I needed to do it. He also offered space for me to make mistakes and find alternative approaches and other possibilities. He was always there with suggestions when I got stuck. He trained me to take detailed experimental notes and always included corresponding controls to become a meticulous scientist. He encouraged me to analyze data from a critical view and not be afraid to think out of the box. He sponsored me to attend world-class meetings to present my work and meet excellent scientists from all over the world. He has a cool appearance that many people find a little intimidating at first, but when you spend more time with him, you find his inner warmer than you expected. He was and is very supportive during both of my pregnancies, not to mention changing the CO<sub>2</sub> tanks for me. Despite his busy schedule, he spent a lot of time and efforts editing my scientific writings including comprehensive proposals, papers, meeting abstracts and dissertation. His mentorship has made my Ph.D. study a very exciting and rewarding journey full of growth.

Dr. Jianming Qiu is a very productive and talented scientist. His passion for science and research makes him an impressive role model for me. His special cultural background enables him to understand my special challenges, struggles, and strengths as an international student. In addition, he has been very supportive in my long-term career development. He and

his lab members (Fang Cheng, Zekun Wang, Xuefeng Deng, Peng Xu, Weiran Shen and others) gave me enormous help during my research experience.

It is my great honor to have Dr. Joe Lutkenhaus, an amazing scientist, on my committee. I am very grateful for his pertinent and insightful suggestions to my project. His critical thinking and meticulousness encouraged me to advance my findings and explore further for deeper understandings. He and his lab members were always willing to help. I really appreciate Du, Park, and Seb for their tremendous support and earnest discussions.

Another wonderful member of my committee is Dr. Thomas M. Yankee. His continued guidance from my comprehensive exam to dissertation private defense kept me on the right track. His specialty on immunity offered me a unique perspective of understanding the interaction between host and pathogens. He is a very knowledgeable professor and a super nice person. I am grieved that he passed away. He will surely be missed by everyone that knows him.

I also want to express my sincere gratitude to Dr. Russell Swerdlow. His support started when I was a rotation student in his lab. Although his research has nothing to do with viruses, he is still willing to participate in my research progression and spent hours and hours discussing my project. He provided invaluable and unique advice from a completely different perspective.

My special thanks goes to Dr. Mary Markiewicz for willing to join my dissertation committee at the very last minute and in the very special situation. Her expertise on Immunology is a great addition to my understanding of viruses and hosts interactions.

I would also like to thank Dr. Wolfram R. Zückert for being a very supportive member on my comprehensive exam.



Thanks as well to the lovely Stephens lab members. Doing research is like riding a roller coaster, which is full of ups and downs. They made me feel that I am not alone, and there is so much fun in the lab! Special thanks to Dr. Kimberly Schmitt! Kim is an incredible senior and friend. She is a smart, hardworking lady that educated me on almost everything I need to “survive” in this lab. My thanks to her for answering my random questions even after she left. Miki is a great companion that taught me many techniques. Sicheng is a good helping hand. Thank you to Ankita, Sachii and Wyatt! Thanks for making lab such a fun and rewarding place! Their questions allowed me to reorganize what I know, recall what I learned, and rethink what I understand. I would also like to thank Dr. Autumn Ruiz. Although I did not get chance to meet her in person, I learned so much from her organized notes, detailed protocols and dissertation book.

I am grateful for the collaboration opportunities with Dr. Mario Santiago. The collaboration did not only broaden my horizon of what we can achieve together, but also offered me great opportunities to experience excellent teamwork, not to mention the extra publication contribution.

I want to express my sincere gratitude to our writing specialist, Dr. Andrés Rodríguez's, for his consistent encouragement and support. He is very professional, patient, and knowledgeable. His expertise, invaluable advices helped me get better and better in scientific writing. Most importantly, he is very good at identifying the shining point of your writing and makes me more confident in expressing myself in written language.

To my parents, I am lacking words to express my gratitude. Thank you for giving me life, surrounding me with endless love, and supporting my dream unconditionally. I love you

from the bottom of my heart. To my parents in law, thanks for taking care of Blaire when we are out to work in the day, thanks for all the delicious meals and make us feel homey.

To my dear husband, thanks for being the strongest and biggest support in my life. You cheer me up when I feel down, you clean up my mess when I do something stupid, you spoil me more than my parents do. You are the sweetest husband in the world!

To my daughter Blaire and son Prince, you are the sunshine in my life. I never feel so complete, so motivated and so needed before you were born. Thanks for the brightest smile I ever see and the sweetest hug I ever have!

To all my friends, forgive me for not being able to list your names one by one here. Thanks for those joyful parties that replenish me, thanks for the random chats that make me feel I am not alone, and thanks for all the wonderful suggestions, reminders and help! I am so blessed to meet you all!

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## **Chapter I : Introduction**

### **A HIV-1**

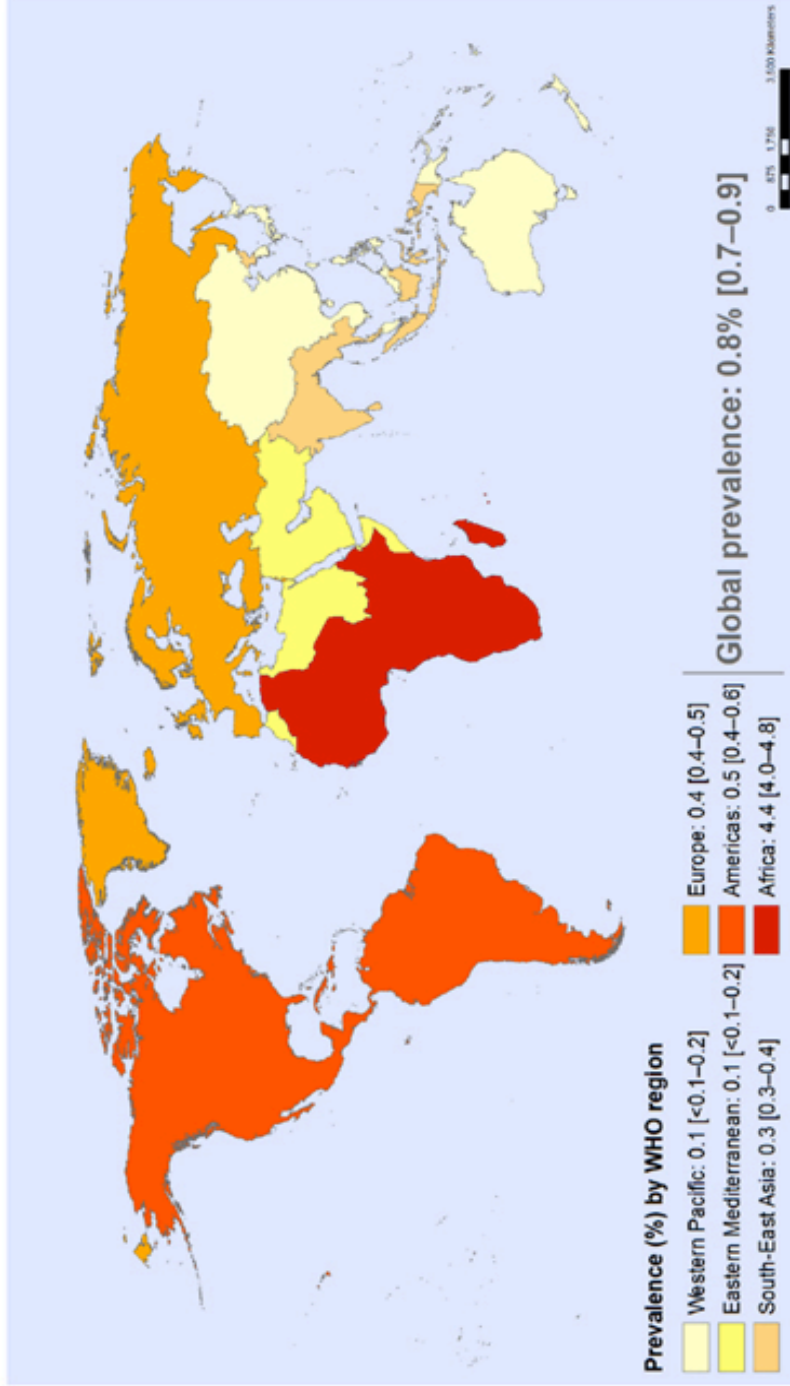
#### **1. HIV-1 epidemic**

Human immunodeficiency virus type 1 (HIV-1) infection of humans, which causes acquired immunodeficiency syndrome (AIDS), has been one of the most devastating pandemics and remains a global challenge (Douek, Roederer, & Koup, 2009; Weiss, 1993). The UNAIDS Global AIDS update 2015 from the World Health Organization (WHO) states that there are approximately 36.7 million [34.0 million–39.8 million] people currently living with HIV-1 with 70% of infected individuals residing in Sub-Saharan Africa. The global adult HIV prevalence up to 2015 is shown in Figure 1. This number has gradually increased due to emerging new infections and longer life spans of HIV-1 patients. Despite decades of global efforts in controlling this disease, 35 millions people have died of AIDS since the first case reported in 1981 (UNAIDS, 2015a, 2015b, 2016). HIV-1 remains leading cause of death worldwide and the number one killer in Sub-Saharan Africa. It also continues to be a major challenge in United States (U.S.), with approximately 1.2 million people living with HIV-1 in the U.S., with approximately 40,000 new infections per year, and roughly 18,000 deaths from AIDS related complications in 2015 (CDC, 2016). HIV-1 epidemic in US is shown in Figure 2. It has been estimated that about 16% of the HIV-1 positive people (17.1 million) worldwide are not aware of their infection due the asymptomatic early stage of the disease, which contributes to the dissemination of this disease.

#### **2. HIV-1 pathogenesis**

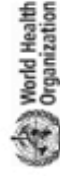
Figure 1. Global adult HIV prevalence, 2015. UNAIDS Global AIDS update 2015 from the World Health Organization (WHO) states that there are approximately 36.7 million [34.0 million–39.8 million] people currently living with HIV-1 with 70% of infected individuals residing in Sub-Saharan Africa.

## Adult HIV prevalence (15–49 years), 2015 By WHO region



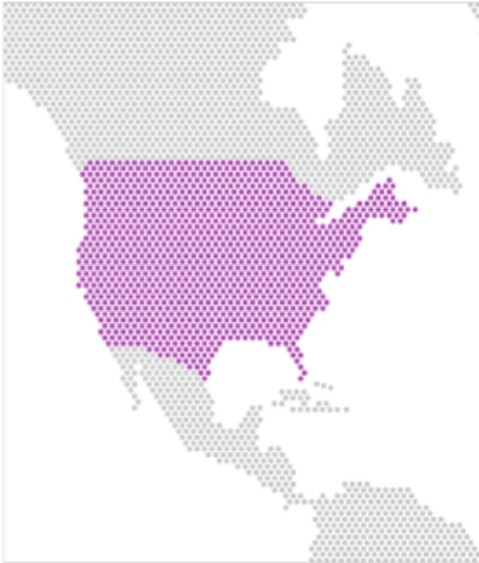
The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.

Data Source: World Health Organization  
Map Production: Information Evidence and Research (IER)  
World Health Organization



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Figure 2. HIV diagnoses in US. Approximately 1.2 million people are currently living with HIV-1 in the U.S., with nearly 40,000 new infections per year, and 13,000 deaths from AIDS-related complications in 2015 (UNAIDS, CDC, ONAP).



## **USA**

**1.2 million people living with HIV (2015)**

**0.4%-0.9% adult HIV prevalence (2012)**

**39,513 new HIV infections (2015)**

**12,333 AIDS-related deaths (2014)**

**37% ART treatment coverage (2011)**



HIV-1 is a single-stranded, positive-sense, enveloped RNA lentivirus. There are two types of HIV that have been characterized: HIV-1 and HIV-2. HIV-1 can be subdivided into four groups: M (main), O (outlier), N (non-M/non-O), and P (pending the identification of further human cases). HIV-1 group M was initially discovered and is the cause of the majority of HIV-1 deaths globally. The HIV-1 group M viruses predominate and are responsible for the AIDS pandemic (more than 90% of HIV/AIDS cases) and they can be further subdivided into subtypes A, B, C, D, F, G, H, J and K based on genetic sequence data (Gilbert et al., 2003). Group O is usually found in West-central Africa. It is mostly found in Cameroon, where 2% of HIV-positive samples were from Group O (Peeters et al., 1997). Group N was isolated from a dead patient in 1998. This variant stood out since it reacted with an envelope antigen from SIVcpz rather than with those of Group M or Group O (Mourez, Simon, & Plantier, 2013). Group P is the most recently identified HIV-1 subtype that has greater similarity to a simian immunodeficiency virus recently discovered in wild gorillas (SIVgor). The patient shows no signs of AIDS and remains untreated (News, 2009). HIV-2 is less virulent and transmissible. It can be further subdivided into A to H. The global distribution of HIV subtypes prevalence is shown in Figure 3.

HIV-1 infects various cellular components of the immune system that are central to many immune responses, including CD4<sup>+</sup> T cells, macrophages, and dendritic cells. Figure 4 is an artificially colored scanning electron micrograph of an HIV-1 particle (in green) budding from a cultured lymphocyte.

The clinical course of HIV-1 infected typical progressor can be divided into three distinct phases as shown in Figure 5. These include the acute seroconversion (primary stage of infection), asymptomatic stage of infection (clinical latency), and finally AIDS phase of disease. During the primary phase, infected cells carry the viruses to regional lymph nodes within days of

Figure 3. Global prevalence of all HIV-1 genetic subtypes and recombinants. Subtype C is the predominant subtype of the epidemic (Arien et al., 2007 and Hemelaar et al., 2006).



Figure 4. An artificially colored scanning electron micrograph of an HIV-1 particle (in green) budding from a cultured lymphocyte (CDC/ C. Goldsmith, P. Feorino, E. L. Palmer, W. R).

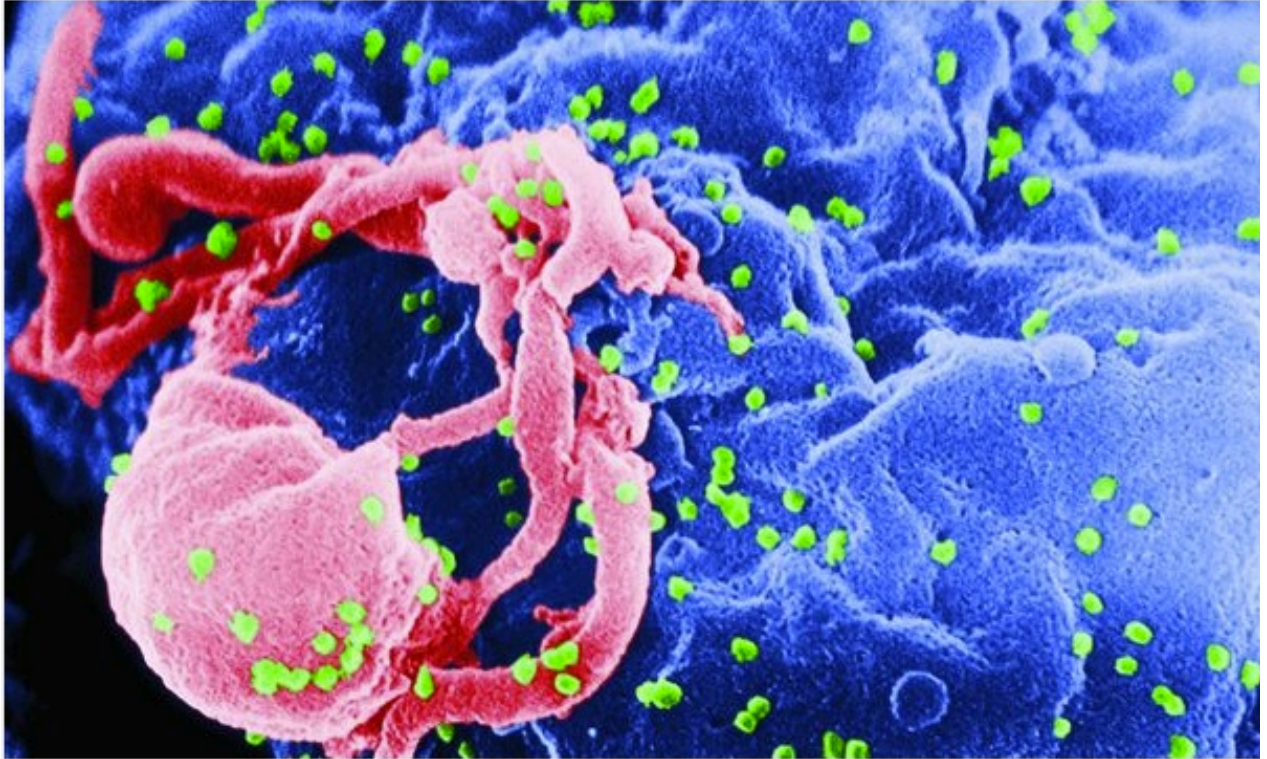
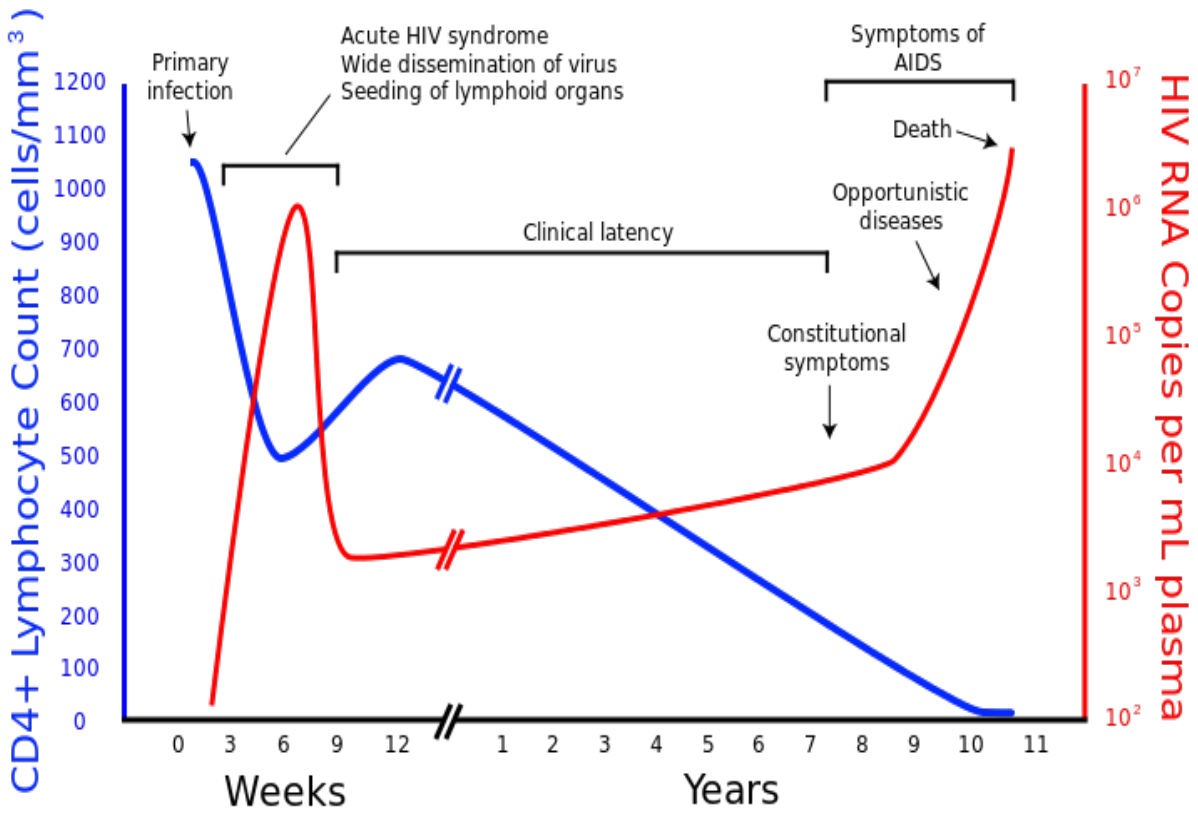


Figure 5. The clinical course of HIV-1 infection. A typical progressor (untreated) includes the acute seroconversion (primary stage of infection), asymptomatic stage of infection (clinical latency), and finally AIDS phase of disease (Adapted from Pantaleo, G *et al.* 1993 *New England Journal of Medicine*).



infection. During this period, virus undergoes exponential replication and the number of CD4<sup>+</sup> T cells declines. Concurrently, virus circulates via the bloodstream to different tissues of the body including lymphoid organs such as the lymph nodes, spleen, and gut associated lymphoid tissue (GALT), brain, and kidney. The formation of reservoirs and insertion of the proviral genome into the host chromosomes are some of the reasons why this virus is so difficult to eradicate. The high viremia during the primary phase of infection results in immune responses with anti-HIV-1 antibodies and CD8<sup>+</sup> T-cell responses. Concurrently with the development of immune response, the plasma viral loads decrease, and the circulating CD4<sup>+</sup> T cell levels rebounds. The primary stage of infection varies from weeks to months. The asymptomatic phase is associated with clinical normality with little or no symptoms. The immune response against HIV-1 during this stage is vigorous and able to keep the viral load at a “relatively” steady level. However, the level of circulating CD4<sup>+</sup> T cells steadily decline over time. When the CD4<sup>+</sup> T-cell counts drop to less than 200/ $\mu$ L, it marks the initiation of the AIDS phase of the disease. An infected individual is also classified as being in the AIDS phase of disease if they are HIV-1 positive and have an AIDS-defining condition. In a typical progressor who is untreated, the median time from infection to the onset of AIDS is approximately 10 years (Stevenson, 2003). At this point, the immune system has been exhausted and the patients become susceptible to various opportunistic infections (OIs). The common OIs of AIDS patients are listed in Table 1.

HIV-1 infection leads to the depletion of CD4<sup>+</sup> T cells through several mechanisms. Infected cells can be directly killed by replicating virus or be eliminated due to recognition by CD8<sup>+</sup> cytotoxic lymphocytes. HIV-1 can also kill uninfected bystander cells, causing them to die through apoptosis (Cox & Siliciano, 2014; Finkel et al., 1995; Garg, Mohl, & Joshi, 2012; Roshal, Zhu, & Planelles, 2001). When the levels of CD4<sup>+</sup> T cells decline below a critical level



Table 1 Opportunistic Infections of AIDS patients

Candidiasis of bronchi, trachea, esophagus, or lungs
Invasive cervical cancer
Coccidioidomycosis
Cryptococcosis
Cryptosporidiosis, chronic intestinal (greater than 1 month's duration)
Cytomegalovirus disease (particularly CMV retinitis)
Encephalopathy, HIV-related
Herpes simplex: chronic ulcer(s) (greater than 1 month's duration); or bronchitis, pneumonitis, or esophagitis
Histoplasmosis
Isosporiasis, chronic intestinal (greater than 1 month's duration)
Kaposi's sarcoma
Lymphoma, multiple forms
Mycobacterium avium complex
Tuberculosis
Pneumocystis carinii pneumonia
Pneumonia, recurrent
Progressive multifocal leukoencephalopathy
Salmonella septicemia, recurrent
Toxoplasmosis of brain
Wasting syndrome due to HIV
Progressive multifocal leukoencephalopathy
Salmonella septicemia, recurrent
Toxoplasmosis of brain
Wasting syndrome due to HIV

(200/ $\mu$ L), cell-mediated immunity becomes incapable of keeping common opportunistic infections under control. Ultimately, the elimination of CD4<sup>+</sup> T cells leads to the collapse of the immune system, resulting in a progressive susceptibility to various OIs, tumors, and ultimately death (Kumar, Abbas, Aster, & Robbins, 2013).

### 3. HIV-1 structure and genome

HIV-1 is an enveloped virus with a diameter of approximately 110-120 nm. Two identical, positive, single-stranded RNAs code for the nine viral genes essential for viral replication including reverse transcriptase, proteases, ribonuclease and integrase, are enclosed by a conical shaped capsid composed of 2000 copies of viral capsid p24 protein. This capsid is further packaged by a matrix protein composed of p17, which supports the integrity of the virion. The lipid envelope, which is derived from host cell plasma membrane during viral egress, contains the viral glycoprotein gp120/gp41 heterodimers that dictate viral tropism and virus entry (Chan, Fass, Berger, & Kim, 1997; Klein & Bjorkman, 2010). The structure of HIV-1 genome and virion is represented in Figure 6.

The HIV-1 RNA genome consists of two long terminal repeats (LTR) at both ends, and nine genes (*gag*, *pol* and *env*; six accessory regulatory genes: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in HIV-2)). The *gag*, *pol* and *env* encode for precursor molecules that are further cleaved by viral or cellular proteases into structural proteins, which are required for production of infectious virions. The Tat and Rev proteins are considered regulatory proteins, whereas Vpu, Vif, Vpr and Nef counter host defense mechanism. The corresponding functions and localization of these proteins are outlined in Figure 7.

Figure 6. Structure of HIV-1 Genome and Virion. HIV-1 is an enveloped virus with a diameter of approximately 110-120 nm. Two identical, positive, single-stranded RNAs code for the nine viral genes essential for viral replication including reverse transcriptase, proteases, ribonuclease and integrase, are enclosed by a conical shaped capsid composed of 2000 copies of viral capsid p24 protein. This capsid is further packaged by a matrix protein composed of p17, which supports the integrity of the virion. The lipid envelope, which is derived from host cell plasma membrane during viral egress, contains the viral glycoprotein gp120/gp41 heterodimers that dictate viral tropism and virus entry.

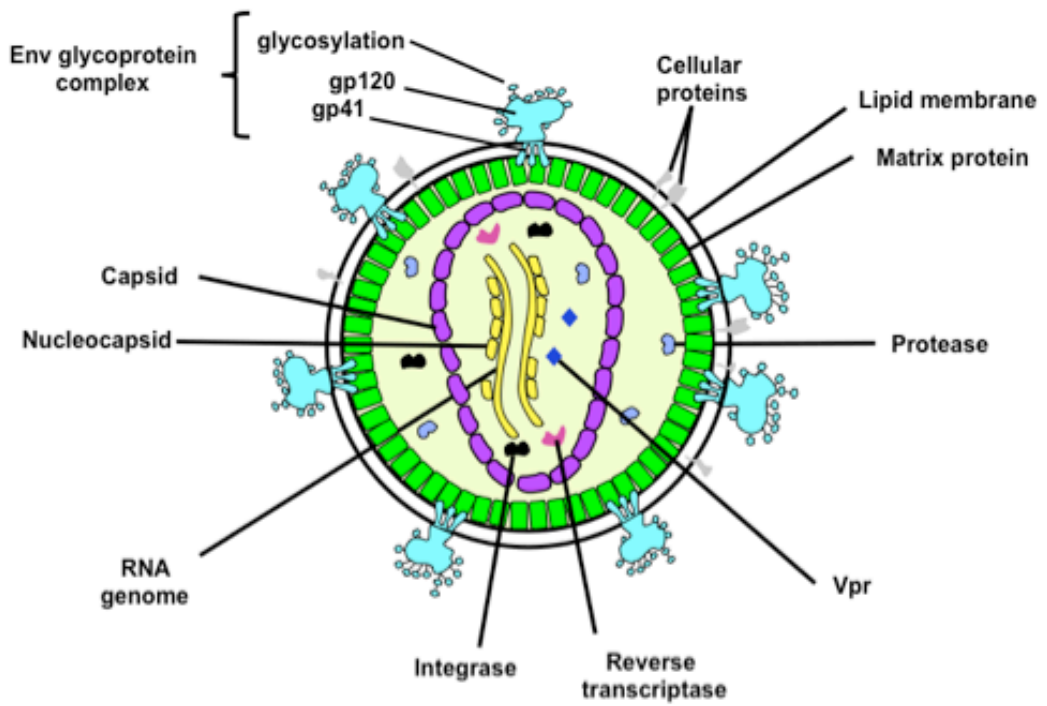
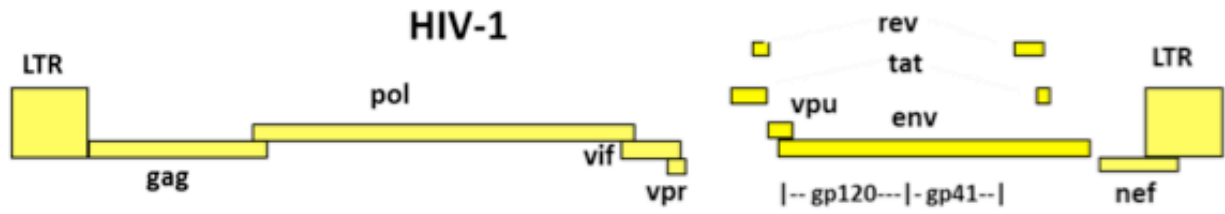


Figure 7. HIV proteins and their size, function, and localization (HIV Sequence Compendium 2008).

Name	Size	Function	Localization
<b>Gag</b>			
MA	p17	membrane anchoring; env interaction; nuclear transport of viral core (myristylated protein)	virion
CA	p24	core capsid	virion
NC	p7	nucleocapsid, binds RNA	virion
	p6	binds Vpr	virion
<b>Pol</b>			
PR	p15	Gag/Pol cleavage and maturation	virion
RT	p66, p51	reverse transcription, RNase H activity	virion
RNase H	p15		virion
IN	p31	DNA provirus integration	virion
Env	gp120/gp41	external viral glycoproteins bind to CD4 and secondary receptors	and secondary receptors
Tat	p16/p14	viral transcriptional transactivator	primarily in nucleolus/nucleus
Rev	p19	RNA transport, stability and utilization factor (phosphoprotein)	primarily in nucleolus/nucleus shuttling between nucleolus and cytoplasm
Vif	p23	promotes virion maturation and infectivity	cytoplasm (cytosol, membranes), virion
Vpr	p10-15	promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M	virion nucleus (nuclear membrane?)
Vpu	p16	promotes extracellular release of viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-1 and SIVcpz)	integral membrane protein
Nef	p27-p25	CD4 and class I downregulation (myristylated protein)	plasma membrane, cytoplasm, (virion?)

**Gag (group specific antigen)**

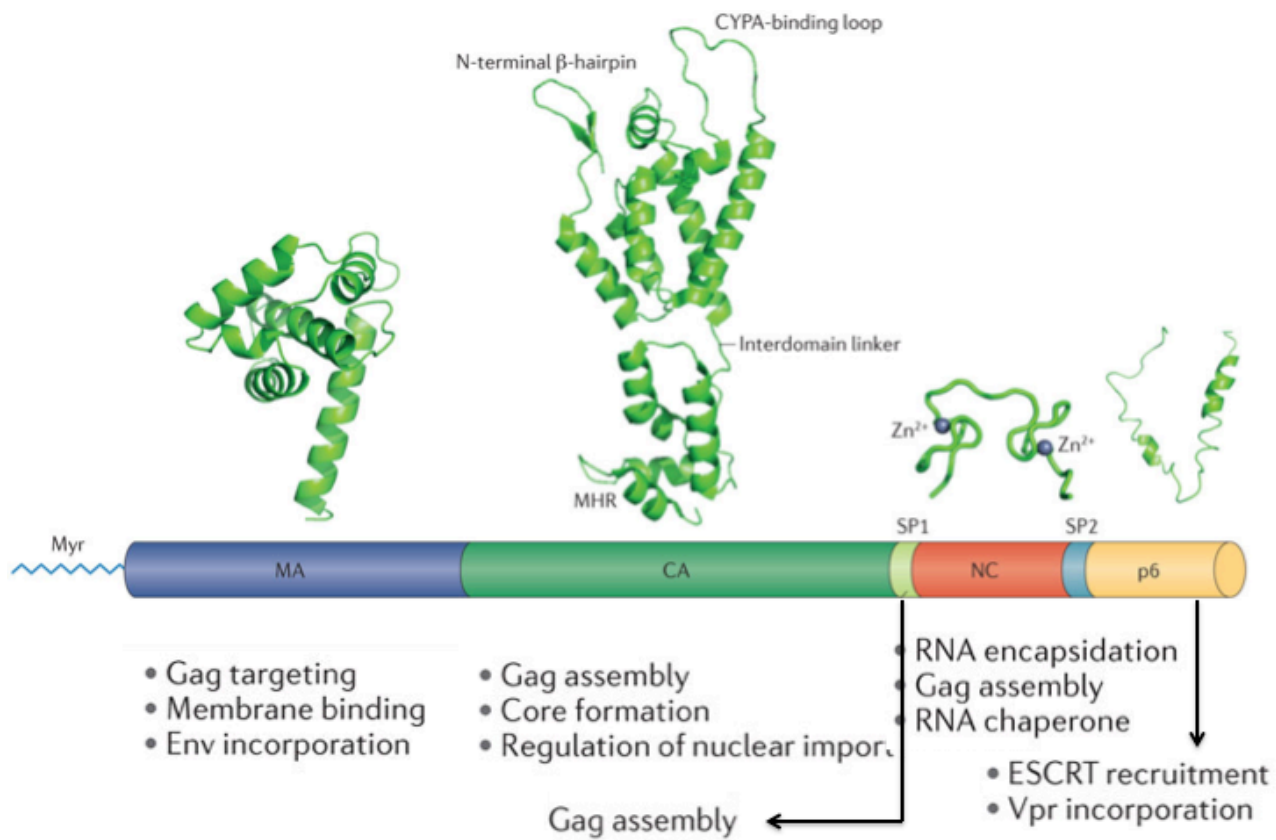
The Gag polyprotein is the main structural protein of HIV-1. Gag is translated from the unspliced full-length viral mRNA transcript of 9kb. Approximately 95% of the translation products from the full length viral RNA is the Gag precursor. A ribosomal frameshift signal at the 3' end of the gag results in 5% of translation products as Gag-Pro-Pol. After translation, the Gag polyprotein hijacks the endosomal-sorting complexes required for transport (ESCRT) system (Carlton & Martin-Serrano, 2009; Hurley, 2010; Hurley & Hanson, 2010) which interacts with other cellular trafficking proteins, and packages two copies of viral genome to form a spherical array, interspersed by Gag and Gag-Pro-Pol polyproteins at the budding site of the cell membrane (Bell & Lever, 2013; Lever, 2007). During maturation, the 55kDa Gag polyprotein is cleaved into four components matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and C terminus p6 (Briggs & Krausslich, 2011; Briggs et al., 2009; Engelman & Cherepanov, 2012; Ganser-Pornillos, Yeager, & Sundquist, 2008; Xue, Mizianty, Kurgan, & Uversky, 2012). The HIV-1 Gag structure and related functions are shown in Figure 8.

**MA (matrix protein)**

The MA protein originates from the full-length p55 precursor and is cleaved into the mature p17. The folded MA protein consist of five  $\alpha$ -helices, a short  $3_{10}$  helix ( $3_{10}$  helix is a type of secondary structure found in proteins and polypeptides. It is the fourth most common type observed, following  $\alpha$ -helices,  $\beta$ -sheets and reverse turns. The amino acids in a  $3_{10}$ -helix are arranged in a right-handed helical structure, with three residues per turn, and ten atoms per ring), and a three-strand mixed  $\beta$ -sheet (Massiah et al., 1994). MA can form trimers and into higher ordered hexamers (Alfadhli, Barklis, & Barklis, 2009; Alfadhli, Huseby, Kapit, Colman, & Barklis, 2007). The MA protein supports the integrity of the virion by interacting with the

Figure 8: HIV Gag structure and functions. High-resolution structures of the major domains of Gag: matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The major functions of each domain are also listed: MA is involved in Gag targeting, membrane binding (requires N terminal myristylation of MA), and envelope (Env) glycoprotein incorporation; CA participates in Gag assembly, conical capsid core formation, and regulation of the nuclear import of viral DNA. The CA<sub>NTD</sub> contains a  $\beta$ -hairpin and cyclophilin A (CYPA)-binding loop that is rich in proline; the CA<sub>CTD</sub> contains the major homology region (MHR). CA<sub>NTD</sub> and CA<sub>CTD</sub> are connected by a short, flexible interdomain linker which is not shown. Spacer peptide 1 (SP1) serves in Gag assembly; NC contains two zinc-finger domains that allow it to participate in RNA encapsidation, Gag assembly, and acts as an RNA chaperone; p6 is involved in ESCRT recruitment and in Vpr incorporation (Freed, E. O. (2015) HIV-1 assembly, release and maturation. *Nature Reviews Microbiology*, 13(8), 484-496).





cytoplasmic tail of the Env gp41 and facilitating the incorporation of Env into the virion. As part of the Gag polyprotein, MA attaches Gag to the plasma membrane for budding, and also helps recruit other viral and host factors. Co-translational myristoylation of the Gag protein at the penultimate glycine residue of MA plays an important role in targeting Gag protein to the particle assembly sites-plasma membrane (Morikawa, Hockley, Nermut, & Jones, 2000). The N-terminal myristoylated MA domain myr (+)MA mediates membrane binding by triggering the interactions with phosphatidylinositol 4,5-bisphosphate in the membrane (Chukkapalli & Ono, 2011; Freed, 2015; H. Li, Dou, Ding, & Spearman, 2007; Ono, 2009). In addition, it has been identified that myristoylation is essential for Gag-Gag multimerization in living cells (H. Li et al., 2007). When cleaved, it fills the space between the lipid envelope and the capsid of the virion (Datta et al., 2007; Ghanam, Samal, Fernandez, & Saad, 2012; Hurley, Boura, Carlson, & Rozycki, 2010).

### **CA (capsid protein)**

The capsid protein (p24) originates from p55, p41, and p25. The CA is the most abundant protein in the virion with approximately 2000 copies of p24 per virion. It forms both hexameric and pentameric rings, which are essential for production of the conical shaped mature core (Campbell & Hope, 2015; Mateu, 2009, 2013; X. Meng et al., 2012; Pornillos et al., 2009; Pornillos, Ganser-Pornillos, & Yeager, 2011; von Schwedler, Stray, Garrus, & Sundquist, 2003). The capsid protein contains two domains that are connected by a flexible hinge: an N-terminal domain (CA<sub>NTD</sub>) containing seven  $\alpha$ -helices followed by a proline rich loop and a C-terminal domain (CA<sub>CTD</sub>) containing a  $3_{10}$  helix followed by a strand of four  $\alpha$ -helices (C. Tang, Ndassa,

& Summers, 2002; Worthylake, Wang, Yoo, Sundquist, & Hill, 1999). They form hexameric or pentameric rings with the CA<sub>NTD</sub> facing the outer side towards the MA protein to stabilize the structure, while the CA<sub>CTD</sub> faces towards the inside providing the base of the mature conical capsid core. The hinge interface forms a binding pocket for various host factors. The capsid packages the viral genome and associated enzymes that are required for the next round of viral replication. This not only provides an enclosed space that allows an efficient initiation and elongation of reverse transcription, but also protects the resulting linear dsDNA from being recognized by host factors. Recently, investigators showed that the CA is involved in the nuclear translocation of viral genome and is a key protein that allows HIV-1 to infect non-dividing cells (Yamashita & Emerman, 2004, 2006; Yamashita, Perez, Hope, & Emerman, 2007).

#### **NC (nucleocapsid protein)**

The nucleocapsid protein is a small protein of the Gag polyprotein, only 55 amino acids in length. It is made up of two zinc finger domains (Cys-X<sub>2</sub>-Cys-X<sub>4</sub>- His-X<sub>4</sub>-Cys), which are connected by a basic domain, RAPRKKG (Godet et al., 2012). Despite its small size, the NC plays several important roles in viral replication including the: 1) support of the structural interaction between Gag proteins for assembly (de Rocquigny et al., 2014; K. Lu, Heng, & Summers, 2011; Muriaux & Darlix, 2010); 2) recognition of nucleic acids for packaging; 3) contribution to the viral synapse formation and cell-cell transmission (Llewellyn, Hogue, Grover, & Ono, 2010); and 4) facilitation of viral budding by hijacking ESCRT (endosomal sorting complexes required for transport) machinery (Dussupt et al., 2009).

The p6 domain is derived from the C terminus of Gag and consists of 52 amino acids. It produces two proteins: 1) the inframe Gag p6; and 2) the -1 frameshifted Gag-Pol p6 (p6\*) (Bell & Lever, 2013). The p6 is structurally more stable and functionally important. Helix 18 of p6 (<sup>432</sup>SFRSG<sup>466</sup>) is responsible for TSG101 binding, and the helix 19 of p6 (<sup>481</sup>KELYPLTSLRSL<sup>492</sup>) provides the binding sites for ALIX and Vpr proteins. TSG101 is “a ubiquitin (Ub)- conjugating E2 enzyme variant (UEV) protein involved in regulation of intracellular trafficking, transcriptional regulation, and cell cycle control” (VerPlank et al., 2001a). The direct association of p6 and TSG101 reveals that cellular trafficking machinery plays a role in virus budding and maturation. In contrast, the p6\* appears to be a poorly structured protein and is unable to bind those factors (Xue et al., 2012). The p6 protein is involved in many important aspects of viral replication, including virus budding, viral maturation (F. H. Yu, Chou, Liao, Huang, & Wang, 2015), recruiting TSG101 for Gag ubiquitination, and determining the size of the virus particle. (Bell & Lever, 2013)

**Pol (DNA polymerase):**

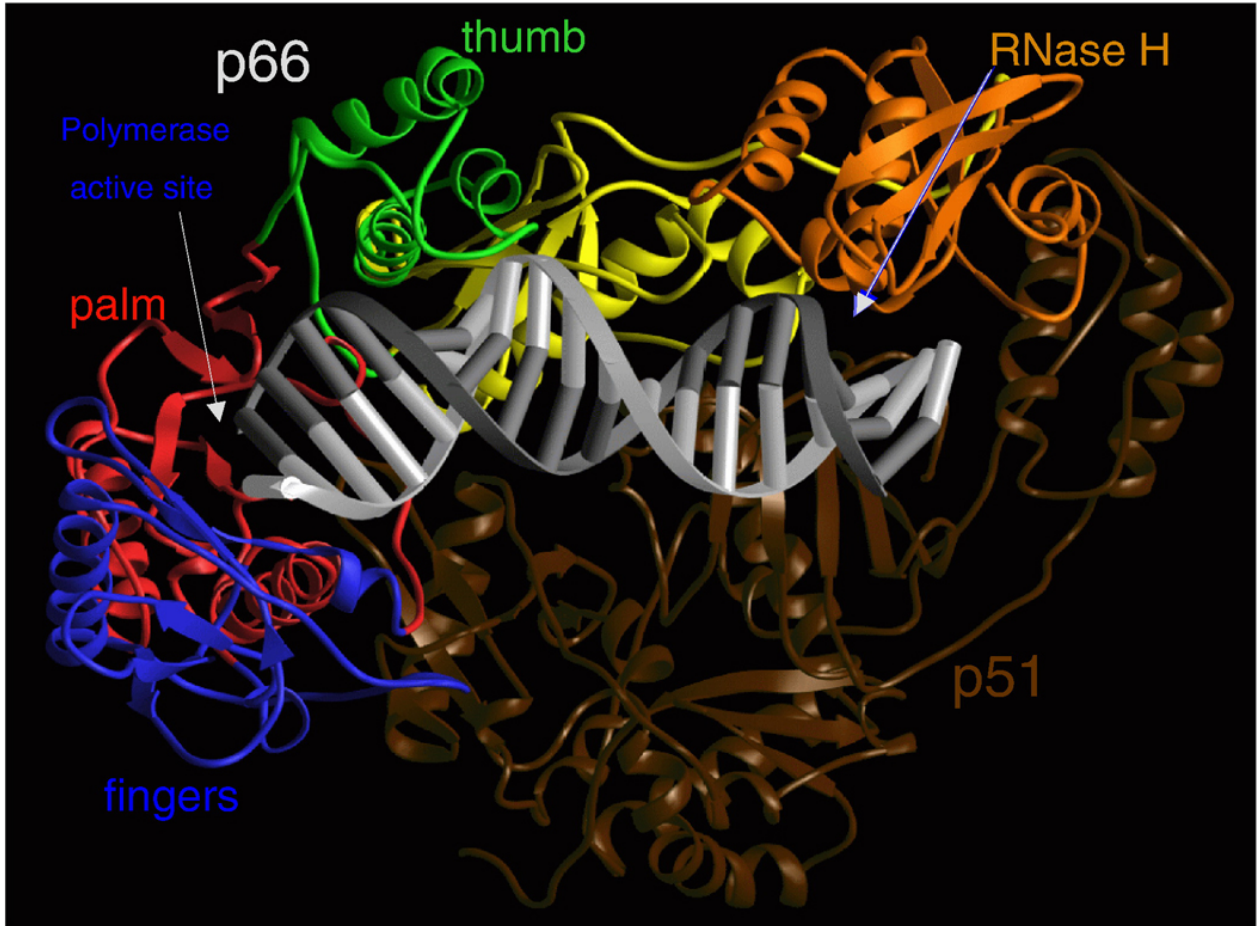
The Pol polyprotein is synthesized as a part of a larger polyprotein, Pr160GagPol, which results from the ribosomal frameshifting during Pr55Gag translation (1/20 molecules). It encodes a polyprotein which harbors the precursor of the protease (PR), reverse transcriptase (RT), RNase H, and the integrase (IN). The structure and function of each protein resulting from Pol polyprotein cleavage is discussed below:

**RT (Reverse Transcriptase):**

The RT of HIV-1 is an asymmetric heterodimer made with two related subunits, p66 and p51. The larger subunit, p66, is 560 amino acids in length and contains the active sites for enzymatic activities of both reverse transcriptase and RNase H. The smaller subunit, p51, is 440 amino acids in length and supports the structure of the enzyme (Jacobomolina et al., 1993; Kohlstaedt, Wang, Friedman, Rice, & Steitz, 1992; Sarafianos et al., 2009). The crystal structure of HIV-1 (RT) was resolved and the ribbon representation of HIV-1 RT in a complex with nucleic acid is shown in Figure 9. The polymerase domain in p66 is made up of four subdomains: the “fingers” (residues 1–85 and 118–155), “palm” (residues 86–117 and 156–236: the palm contains three catalytic carboxylates-D110, D185, and D186 that bind two divalent ions  $Mg^{2+}$ ), “thumb” (residues 237–318), and the “connection” (residues 319–426)” (Jacobomolina et al., 1993; Kohlstaedt et al., 1992). The smaller subunit p55 also folds into these four subdomains at different amino acid positions. The four polymerase subdomains and RNase H subdomain of p66 form the nucleic-acid binding cleft while the connection and thumb subdomains of p51 form the floor of the binding cleft. Therefore, the viral genome is able to contact both the polymerase and the RNase H active sites in the binding cleft.

Reverse transcriptase reversely transcribes viral RNA into double stranded DNA (dsDNA). Like other RT molecules, the HIV-1-RT does not have an exonucleolytic proofreading function, resulting in an average error rate of 1/1700 nucleotides synthesized. Some hotspots have an error rate as high as 1/70 nucleotides (Roberts, Bebenek, & Kunkel, 1988). It is considered to be the least accurate reverse transcriptase known. This error-prone viral replication offers a unique advantage for HIV-1, by providing an enormous genetic potential and allowing them to escape immune responses, to adapt to changes in cellular and species tropism, and to

Figure 9. Ribbon representation of HIV-1 RT in a complex with nucleic acid. The p66 subunit is shown in the upper part: fingers as blue, palm as red, thumb as green, connection as yellow, and RNase H as orange subdomains of the p66 subunit are shown in blue, red, green, yellow, and orange, respectively. The p51 subunit is at the bottom and is shown in dark brown. The template DNA is shown in light gray and primer DNA strands is shown in dark gray (Sarafianos, S. G, et al. (2009) Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. *Journal of Molecular Biology*, 385(3), 693-713).



resist various antiviral drugs (Domingo & Holland, 1997; Rambaut, Posada, Crandall, & Holmes, 2004). However, this benefit comes at a cost. HIV-1 is believed to replicate at the threshold of an error catastrophe (E. C. Holmes, 2003; R. A. Smith, Loeb, & Preston, 2005). Thus, manually increasing the mutation rate to beyond the error threshold can limit viral replication.

### **RNase H**

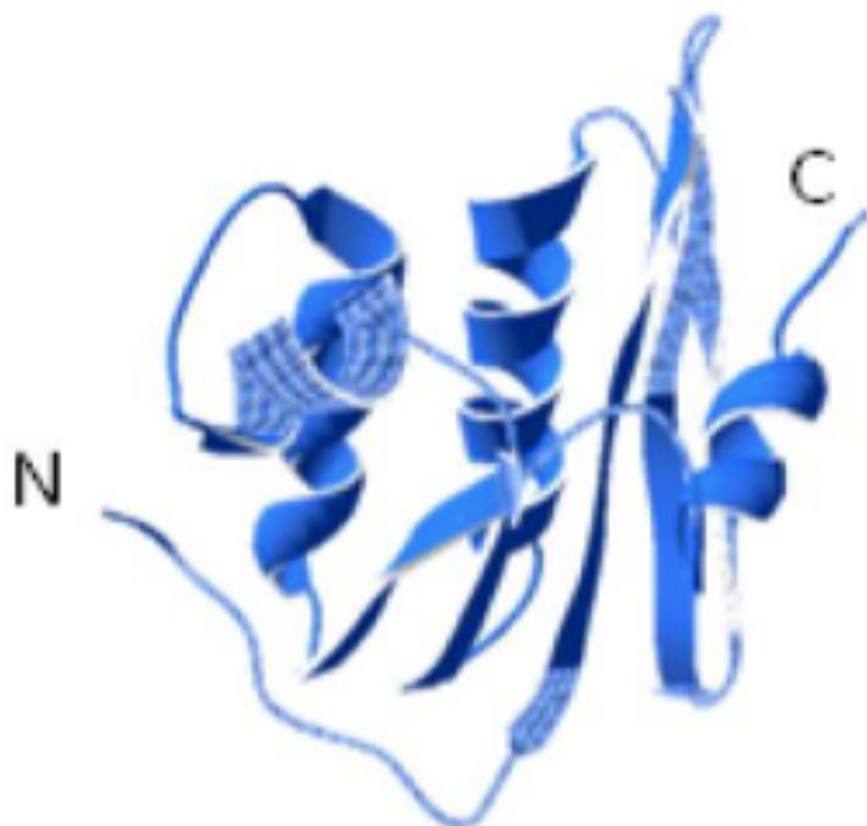
RNase H is located at the C terminus of the p66 protein. It consists of a five-stranded  $\beta$ -sheet surrounded by a distribution of four  $\alpha$ -helices (Beilhartz & Gotte, 2010; T. J. Schmitt, Clark, & Knotts, 2009). It is an endonuclease that cleaves the 3'-O-P bond of RNA in a DNA/RNA hybrid. This function is important for removal of the t-RNA primer, RNA template, and polypurine tract (PPT) to allow elongation, strand transfer, and the completion of reverse transcription. This will be discussed later in greater detail. The ribbon representation of RNase H is shown in Figure 10.

### **IN (integrase):**

The IN is an essential viral enzyme that facilitates the insertion of the double stranded linear viral DNA into the host chromosome. It contains three canonical domains: the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD), which are connected by flexible linkers that are susceptible to proteolysis. The NTD has a His<sub>2</sub>Cys<sub>2</sub> motif that binds to zinc; the CCD is the core component that has the catalytic DDE motif and shares similar structure with other polynucleotidyl transferases; the CTD has a Src-homology-3 (SH3)-



Figure 10. Ribbon representation of HIV-1 RNase H structure. It contains a five-stranded  $\beta$ -sheet which is surrounded by a distribution of four  $\alpha$ -helices (Beilhartz, G. L., et al. (2010) HIV-1 ribonuclease H: structure, catalytic mechanism and inhibitors. *Viruses*, 2(4), 900-926).

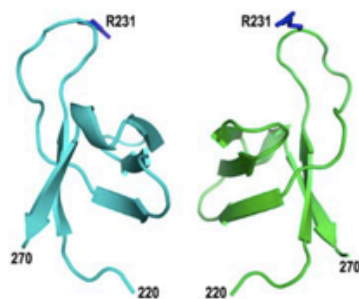
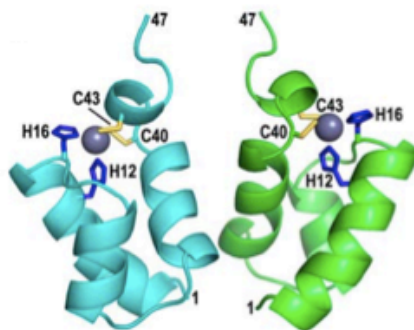
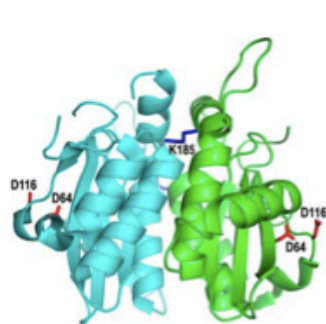
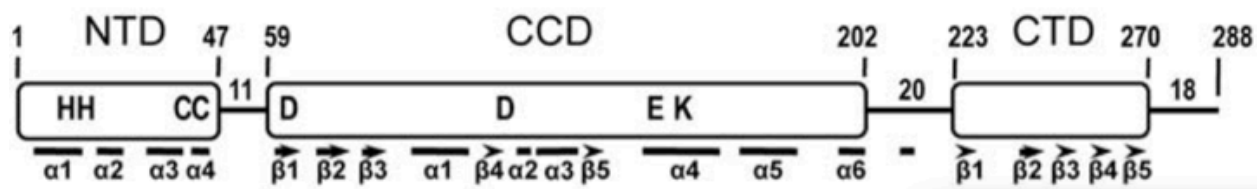


like fold that nonspecifically binds DNA (SH3 domain consists five or six  $\beta$ -strands arranged as two tightly packed anti-parallel  $\beta$  sheets). The domain organization and structure of HIV-1 domain is represented in Figure 11. IN forms a tetramer in the presence of viral DNA. This stable complex enables the integration of viral genome into host chromosome. IN interacts with a variety of host factors, including barrier-to-autointegration factor (BAF), lamina-associated polypeptide (LAP)-2a, integrase interactor 1 (INI1), lens epithelium-derived growth factor (LEDGF/p75), and cleavage and polyadenylation specificity factor subunit 6 (CPSF6) (Craigie & Bushman, 2012; Marini et al., 2015; Sowd et al., 2016a). In addition to the key function of viral integration, mutation studies have revealed that IN also plays a role in nuclear transport of PIC, reverse transcription, particle assembly & release, and viral maturation (Asante-Appiah & Skalka, 1999; Ceccherini-Silberstein et al., 2009; T. K. Chiu & Davies, 2004; Craigie, 2001; d'Angelo, Mouscadet, Desmaele, Zouhiri, & Leh, 2001; Engelman & Cherepanov, 2014; Feng, Larue, Slaughter, Kessl, & Kvaratskhelia, 2015; Grandgenett, Pandey, Bera, & Aihara, 2015; Hill, Tachedjian, & Mak, 2005; Nair, 2002; Pommier, Pilon, Bajaj, Mazumder, & Neamati, 1997; Van Maele, Busschots, Vandekerckhove, Christ, & Debysse, 2006; Zeinalipour-Loizidou, Nicolaou, Nicolaidis, & Kostrikis, 2007).

### **PR (Protease)**

The PR is a retroviral aspartyl protease (retropepsin) that cleaves polyproteins at designated sites in a highly ordered sequence to induce structural arrangement. This maturation step is indispensable in converting immature, non-infectious into infectious virions. HIV-1 PR folds into a four-stranded antiparallel  $\beta$ -sheet by two identical monomers whose dimerization is

Figure 11. HIV integrase domain organization and domain structures. IN contains three canonical domains: the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD), which are connected by flexible linkers that are susceptible to proteolysis. The NTD has a His<sub>2</sub>Cys<sub>2</sub> motif that binds to zinc; the CCD is the core component that has the catalytic DDE motif and shares similar structure with other polynucleotidyl transferases; the CTD has a Src-homology-3 (SH3)-like fold that nonspecifically binds DNA (SH3 domain consists five or six  $\beta$ -strands arranged as two tightly packed anti-parallel  $\beta$  sheets) (Engelman, A, et al. (2014) Retroviral integrase structure and DNA recombination mechanism. *Microbiology Spectrum*, 2(6), 1).



mediated by its N- and C-termini, as shown in Figure 12. PR allows HIV-1 to use a single set of transcriptional and translational control elements in a defined stoichiometry. This space-saving strategy offers a great advantage for HIV-1 whose genome has a limited size. It allows HIV-1 to produce a variety of proteins required for particle formation, replication and spreading using a single set of transcriptional and translational control elements. (Blundell et al., 1990; Davies, 1990; Fitzgerald & Springer, 1991; Huang et al., 2014; Konvalinka, Krausslich, & Muller, 2015; Louis, Ishima, Torchia, & Weber, 2007; Scharpe et al., 1991; J. Tang, Lin, Co, Hartsuck, & Lin, 1992; vonderHelm, 1996).

### **Env (Envelope)**

The HIV-1 Env glycoprotein is translated from a singly spliced, bicistronic *vpu/env* mRNA on the rough endoplasmic reticulum (RER) into a precursor polyprotein, known as gp160. The Env precursor is glycosylated with N- and O-linked oligosaccharide side chains. Monomers of gp160 form trimers in ER and these trimers are subsequently transported to the Golgi complex. In the Golgi complex, the conserved K/R-X-K/R-R motif is recognized by cellular furin or furin-like proteases resulting in cleavage of gp160 into gp120 and gp41 (Checkley, Luttge, & Freed, 2011; Freed, Myers, & Risser, 1989; Mccune et al., 1988). Following cleavage, gp120 and gp41 interact through noncovalent bonds and form a heterotrimeric HIV-1 glycoprotein spike. The domains and structure of HIV-1 env are depicted in Figure 13.

During virus assembly, gp120/gp41 heterotrimeric spikes are incorporated into the lipid bilayer of the virion and are responsible for receptor recognition, binding, and fusion for the next round of replication. In addition, the Env-receptor interaction is crucial in forming

Figure 12. Ribbon diagram of the HIV protease dimer: one monomer in orange and the other in blue. Each monomer folds into a four-stranded antiparallel  $\beta$ -sheet and dimerizes through their N- and C-termini (Venkatakrisnan, B, et al. (2012). Mining the protein data bank to differentiate error from structural variation in clustered static structures: an examination of HIV protease. *Viruses*, 4(3), 348-362).

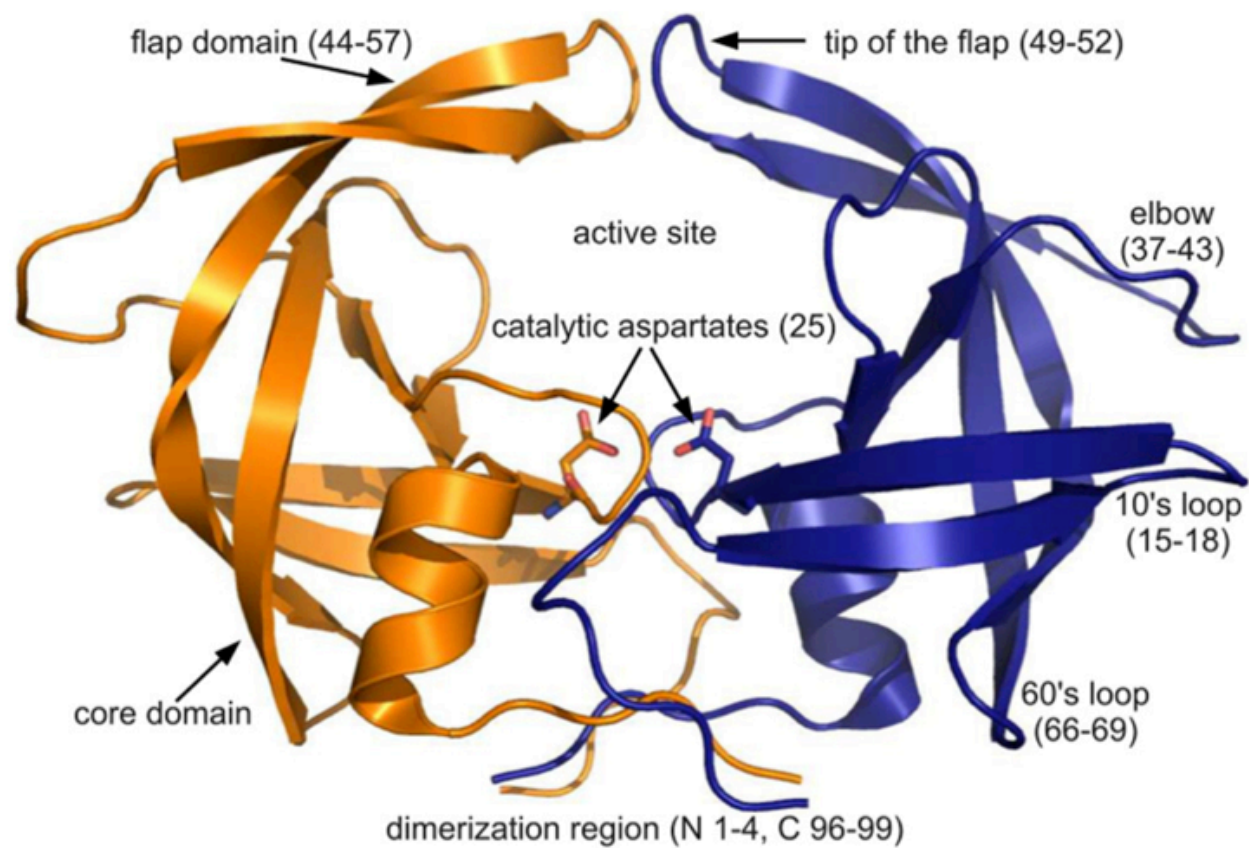




Figure 13. (A) The structure of HIV-1 envelope glycoprotein (Env). (B) Schematic diagram of trimeric HIV-1 Env with sites of epitopes for broadly neutralizing antibodies. Following cleavage, gp120 and gp41 interact through noncovalent bonds and form a heterotrimeric HIV-1 glycoprotein spike (Haynes, B. F, et al. (2012) B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nature Biotechnology*, 30(5), 423-433).



virological synapses (VS), which contributes to the virus spread through direct cell-to-cell transmission (Acharya, Lusvardi, Bewley, & Kwong, 2015; Araujo & Almeida, 2013; Burton & Mascola, 2015; Checkley et al., 2011; Freed et al., 1989; Mccune et al., 1988; Merk & Subramaniam, 2013; O'connell, Kim, & Excler, 2014; Postler & Desrosiers, 2013; Schiffner, Sattentau, & Duncan, 2013; Ward & Wilson, 2015). The gp160 has also been reported to play a critical role in CD4 downmodulation through binding to newly synthesized CD4 in the ER and preventing their transport to the cell surface (Ruiz, Guatelli, & Stephens, 2010; Wildurn, Schindler, Munch, & Kirchhoff, 2006).

### **gp120**

The gp120 consists of five variable domains (V1-V5) interspersed with five relatively constant domains (C1-C5) (Willey et al., 1986). The conserved domains are key determinants that bind the CD4 receptor, while the variable domain such as V3 loop is involved in coreceptor specificity. Eighteen Cys residues form nine intramolecular disulfide bridges that are essential to the formation of the Env tertiary structure. The gp120 is highly glycosylated, especially by N-linked glycans, which aids HIV-1 escaping from the host immune surveillance, folds into a functional tertiary structure, and facilitates viral binding to susceptible target host cells (H. L. Li et al., 2008; Montefiori, Robinson, & Mitchell, 1988; Raska & Novak, 2010). Following binding to the host cell receptor CD4, gp120 undergoes a remarkable degree of refolding. When gp120 bound to CD4, it consists of an “inner” domain and an “outer” domain linked by a four-stranded “bridging sheet”, allowing CD4 to directly contact 26 residues in gp120 (the most important residues for this interaction are Phe43 and Arg59 of CD4 and Asp368, Glu370, and Trp427 of gp120 (Ryzhova et al., 2002)) and to expose the co-receptor binding sites (Kwong et al., 1998).

## **gp41**

The other component of the Env heterodimer is gp41, which consists three major domains: an extracellular domain (or ectodomain), a transmembrane domain (TMD), and a C-terminal cytoplasmic tail (CT). The extracellular domain contains the major fusion determinants that include the hydrophobic fusion peptide which forms the fusion pore, a polar region, heptad-repeat regions HR1 and HR2 (their interaction with each other drives the fusion process by forming a stable six-helix bundle that brings the viral and cell membranes close enough for fusion), and a highly conserved membrane-proximal external region (MPER) (Chan et al., 1997; Checkley et al., 2011; M. Lu, Blacklow, & Kim, 1995; Merk & Subramaniam, 2013; Postler & Desrosiers, 2013). The gp41 TMD consists of a  $\alpha$ -helix that spans the membrane once and anchors Env in the lipid bilayer. The gp41 has a very long CT that is approximately 150 amino acids and is involved in many functions, including “Env incorporation into virus particles, virus infectivity, cell-surface Env expression, and Env-induced fusion” (Checkley et al., 2011).

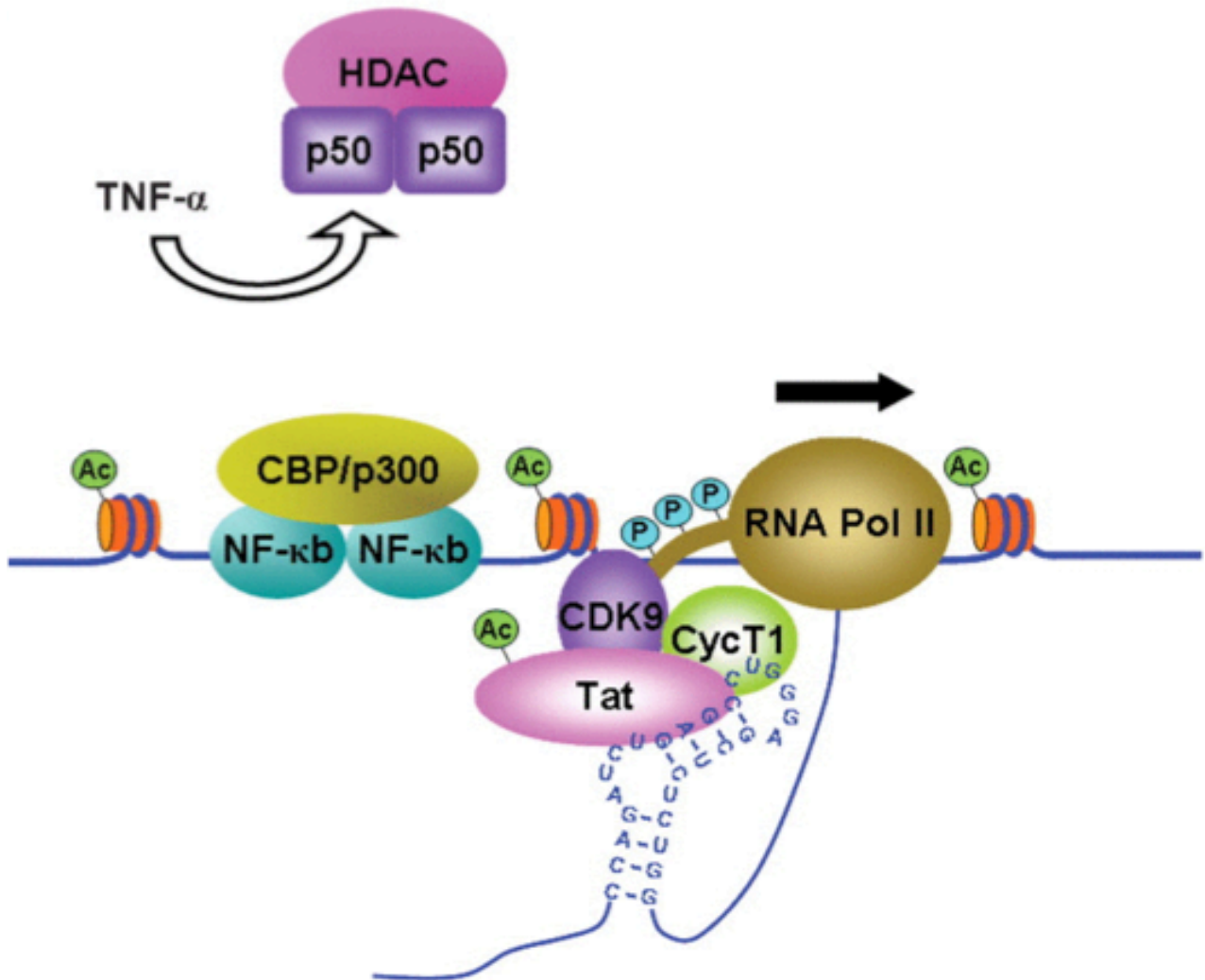
## **Tat (Trans-Activator of Transcription)**

Tat is an early regulatory protein that stimulates viral transcript elongation several hundred-fold by binding to the stem-loop structure of the viral RNA known as the trans-activating response element (TAR). This is achieved through: 1) recruiting cellular chromatin modifying complexes and histone modifying enzymes to relieve the repression on viral long-terminal repeat (LTR); 2) phosphorylating the RNA polymerase II, transcription factors Sp1 and CREB, the  $\alpha$  subunit of eIF2 $\alpha$ , and NF- $\kappa$ B; and 3) facilitating the elongation of viral transcripts, as summarized in Figure 14. In addition to activating viral transcription, Tat modulates the expression of host genes by up-regulation of several cytokines, CCR5 and CD25 (Mayol,

Munier, Beck, Verrier, & Guillon, 2007; Zheng, Yang, Lu, & Salvato, 2005). It down-regulates expression of major histocompatibility complex (MHC) class I by repressing the activity of an MHC class I gene promoter up to 12-fold (Howcroft, Strebel, Martin, & Singer, 1993; Matsui, Warburton, Cogswell, Baldwin, & Frelinger, 1996). This regulation of host gene expression can result in apoptosis induction/inhibition and immune suppression (Romani, Engelbrecht, & Glashoff, 2010). Additionally, Tat plays an important role in viral reverse transcription by promoting the placement of tRNA<sup>Lys3</sup> onto viral RNA, and suppressing reverse transcription at late stages of the viral replication. Moreover, Tat has been implicated in AIDS-associated cancer progression and neurotoxicity (Johri, Mishra, Chhatbar, Unni, & Singh, 2011; King, Eugenin, Buckner, & Berman, 2006; W. X. Li, Li, Steiner, & Nath, 2009).

HIV-1 Tat can be secreted by productively infected cells and can be taken up by neighboring non-infected cells through a paracrine/autocrine mechanism (Dahiya, Nonnemacher, & Wigdahl, 2012). The extracellular form of Tat also directly interacts with the cell surface receptors, such as cellular heparan sulfate proteoglycans and CXCR4. This interaction promotes the expression of HIV-1 chemokine co-receptors in uninfected cells, which favors susceptibility to HIV-1 infection (Brigati, Giacca, Noonan, & Albini, 2003; Chiozzini & Toschi, 2016; Gatignol, 2007; Gibellini, Vitone, Schiavone, & Re, 2005; Hetzer, Dormeyer, Schnolzer, & Ott, 2005; Johri et al., 2011; King et al., 2006; W. X. Li et al., 2009; Romani et al., 2010).

Figure 14. The role of HIV-1 Tat in the synthesis of full-length viral mRNAs. In the presence of stimulation factors such as TNF- $\alpha$ , the latency inducing complex, p50 and HDAC1, are relieved from the viral LTR. The cellular chromatin modifying complexes and histone modifying enzymes such as CBP/p300 complex then binds to the promoter and acetylates the surrounding histones. The complex also acetylates Tat and the acetylated Tat interacts with CDK9 and cyclin T1, to recruit the stem-loop structure, TAR. The P-TEFb complex and Tat also activates the phosphorylation of RNA polymerase II in CTD. After initiation, Tat contributes to elongation of viral transcripts by facilitating the activated polymerase to synthesize full-length transcripts from the relieved template (Romani, B, et al. (2010) Functions of Tat: the versatile protein of human immunodeficiency virus type 1. *Journal of General Virology*, 91(1), 1-12).



## Rev

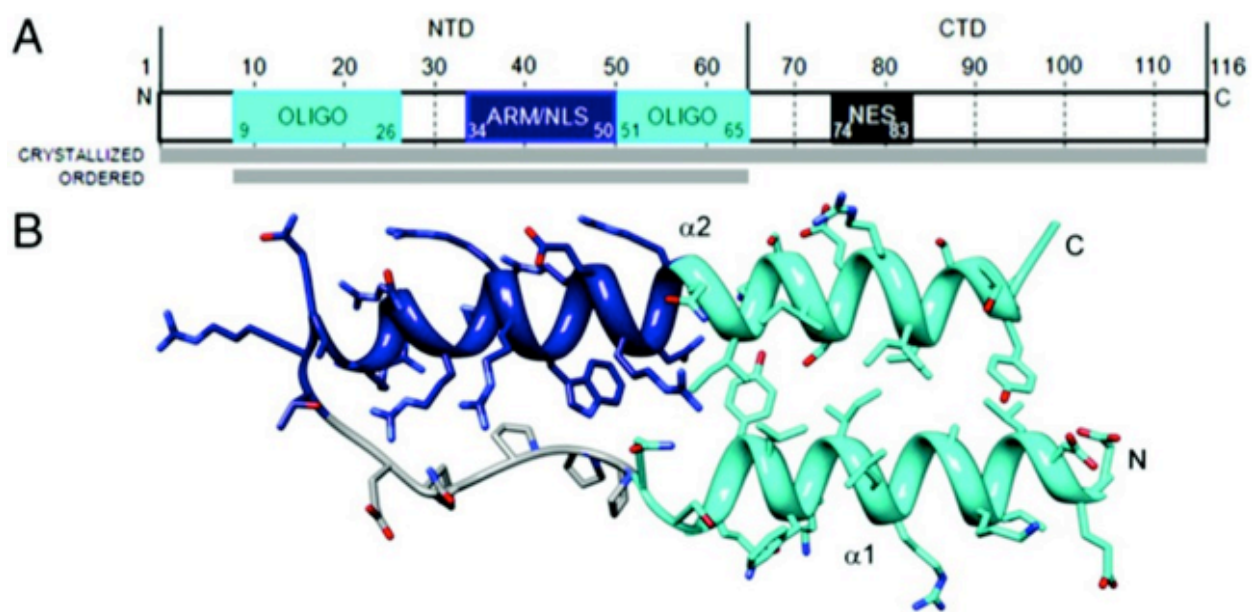
The Rev is a 13-kDa protein of 116 amino acids. There are two critical domains that are important to its nuclear import and export functions. The first is an arginine-rich motif (ARM) located at the N-terminus. The second domain is a Rev-activation domain (nuclear export signal or NES) located at the C-terminus. The domains and structure of HIV-1 Rev are shown in Figure 15. The ARM is responsible for tetramerization of Rev proteins, which is essential for its proper binding to the Rev response element (RRE). The nuclear export signal of Rev allows the intron-containing viral RNA to exit the nucleus into the cytoplasm. Rev exports and increases levels of the mRNAs that encode structural proteins and (Gag, Pol, and Env) in the cytoplasmic, and down-regulates the expression of doubly spliced mRNA species that encode the regulatory proteins (Rev and Tat) through a negative feedback loop (Hammariskjold et al., 1989; Hope, 1999; Kjems, Calnan, Frankel, & Sharp, 1992; Malim, Mccarn, Tiley, & Cullen, 1991; Pollard & Malim, 1998; Rausch & Le Grice, 2015; Vercruyssen & Daelemans, 2013; Zapp, Hope, Parslow, & Green, 1991).

## Nef (Negative Regulatory Factor)

Nef, 27–35 kDa, is N-myristoylated. It is predominantly localized in the cytoplasm of infected cells but is also recruited to plasma membrane. Nef is not essential for viral replication *in vitro*. However, Nef is a virulence factor *in vivo* and is a key contributor in the disease progression. Nef facilitates AIDS pathogenesis by: 1) down-regulating CD4 levels on cell surface by endocytosis and lysosomal degradation; 2) down-regulating major histocompatibility class I (MHC-I) molecules on the cell surface; 3) enabling immune evasion from host



Figure 15. HIV-1 Rev domain and structure. (A) Linear domain organization of Rev: oligomerization motifs are shown in cyan, arginine-rich motif /nuclear localization sequence (ARM/NLS) shown in navy blue, and nuclear export signal (NES) shown in dark gray. (B) Ribbon representation of Rev monomer showing a helix-loop-helix motif with coplanar helices (DiMattia, M. A, et al. (2010) Implications of the HIV-1 Rev dimer structure at 3.2 Å resolution for multimeric binding to the Rev response element. *Proceedings of the National Academy of Sciences*, 107(13), 5810-5814).



recognition by manipulating host cell vesicular transport, cytoskeleton dynamics, cell mobility and T cell receptor signaling; and 4) enhancing viral particle infectivity independent of CD4 modulation (Abraham & Fackler, 2012; Arien & Verhasselt, 2008; Basmaciogullari & Pizzato, 2014; Baur, 2004; Das & Jameel, 2005; Foster, Denial, Temple, & Garcia, 2011; Foster & Garcia, 2007, 2008; Geyer, Fackler, & Peterlin, 2001; M. Harris, 1999; Jere, Fujita, Adachi, & Nomaguchi, 2010; Joseph, Kumar, & Mitra, 2005; Olivetta, Arenaccio, Manfredi, Anticoli, & Federico, 2016; Pawlak & Dikeakos, 2015; Percario, Ali, Mangino, & Affabris, 2015; Pereira & daSilva, 2016; Quaranta, Mattioli, Giordani, & Viora, 2009; Roeth & Collins, 2006; Wei, Arora, Foster, Sodora, & Garcia, 2003; Witkowski & Verhasselt, 2013).

### **Vpr (Viral Protein R)**

Vpr is a 14 kDa protein that is 96 amino acids in length. It forms three  $\alpha$ -helical secondary structures. Unlike other accessory proteins of HIV-1, Vpr is packaged into the core of the mature virions at approximately 275 molecules per virion through a direct interaction with the C-terminal p6 region of the Gag precursor p55. Vpr regulates the nuclear import of HIV-1 pre-integration complex (PIC), thus facilitates virus replication in non-dividing cells, such as macrophages. It also modulates the fidelity of reverse transcription, and induces cell cycle arrest at G2/M and causes apoptosis in proliferating cells leading to immune dysfunction. Additionally, Vpr causes metabolism alternations similar to the Warburg effect in cancer cells such as changes in glycolysis, gluconeogenesis, the pentose phosphate pathway, TCA cycle, mitochondrial functions, and hypoxia-inducing factors level (James, Nonnemacher, Wigdahl, & Krebs, 2016). Finally, Vpr has been shown to contribute to the neuropathogenesis of HIV-1 infection. Vpr has been found to exist in an extracellular form in the cerebrospinal fluid (CSF) from HIV-1-infected

patients. Vpr has been shown to directly affect neurons and induce the release of neurotoxic factors, cytokines, and chemokines from astrocytes and microglial cells (the resident macrophage in the brain) (Andersen & Planelles, 2005; Guenzel, Herate, & Benichou, 2014; James et al., 2016; Kogan & Rappaport, 2011; Le Rouzic & Benichou, 2005; G. Li, Bukrinsky, & Zhao, 2009; Pandey et al., 2009; Romani & Engelbrecht, 2009; Sharifi, Furuya, & de Noronha, 2012; Strebel, 2013; Thieu et al., 2009; Tungaturthi, Sawaya, Ayyavoo, Murali, & Srinivasan, 2004; Tungaturthi et al., 2003; Zhao, Li, & Bukrinsky, 2011).

### **Vpu (Viral Protein Unique)**

The Vpu protein is found exclusively in HIV-1 and select simian immunodeficiency virus (SIV) isolates. Vpu is an oligomeric, type I membrane protein with a size of 16 kDa consisting 81-86 amino acids. Vpu and Env are translated from the same bicistronic mRNA in a Rev-dependent manner, through leaky scanning of ribosomes. Vpu contains an N-terminal leader sequence that also serves as a hydrophobic transmembrane domain while the cytoplasmic tail (a 54 residues, hydrophilic domain) contains a high level of charged residues. The transmembrane domain has a stable helical structure with a small tilt angle to the plane of the membrane (Park, De Angelis, Nevzorov, Wu, & Opella, 2006). The cytoplasmic domain forms two  $\alpha$ -helices which are connected through a flexible segment that contains two conserved kinase II phosphorylation site S(S/T-X-X-E/D). These two conserved serine residues are critical for the interaction between Vpu and the human  $\beta$ -transducin-repeat containing protein ( $\beta$ -TrCP), which is directly involved in the degradation of CD4 (Bour & Strebel, 2003; Hout et al., 2004; Montal, 2003).

Vpu facilitates the degradation of CD4 in the endoplasmic reticulum via inducing the degradation of CD4 molecules bound by Env (gp160) and targeting it for proteolysis in the cytosolic ubiquitin-proteasome pathway (Lenburg & Landau, 1993; Lindwasser, Chaudhuri, & Bonifacino, 2007). This targeted degradation not only prevents newly synthesized CD4, but also liberates gp160, allowing transport to the cell surface (A. Andrew & Strebel, 2010; Binette & Cohen, 2004; Dube, Bego, Paquay, & Cohen, 2010; Ruiz et al., 2010). In addition, Vpu promotes viral particle release from the plasma membrane of infected cells. The enhancement of virion release is independent of CD4 destabilization and requires the transmembrane domain of Vpu. Vpu contributes to viral egress by down-regulating the restriction factor BST-2 (Neil, Zang, & Bieniasz, 2008; Van Damme et al., 2008). The detailed interaction between Vpu and BST-2 will be expanded in later chapters. Additionally, Vpu down-regulates lipid-antigen presenting protein CD1d in antigen-presenting cells, and NTB-1 in natural killer cells. (A. Andrew & Strebel, 2010; Binette & Cohen, 2004; Bour & Strebel, 2003; Dube et al., 2010; Ewart, Sutherland, Gage, & Cox, 1996; Gonzalez, 2015; Guatelli, 2009; Hout et al., 2004; Hsu, Seharaseyon, Dong, Bour, & Marban, 2004; Moll, Andersson, Smed-Sorensen, & Sandberg, 2010; Montal, 2003; Neil et al., 2008; Ruiz et al., 2010; Schubert et al., 1996; Shah et al., 2010; Strebel, 2014; Van Damme et al., 2008).

**Vif (Viral Infectivity Factor):**

Vif is a 23 KDa phosphorylated protein with 192 amino acids that is produced at a high level in the late stage of viral life cycle. Vif primarily resides in the cytoplasm and cellular membrane, co-localizing with Gag. Vif is indispensable for viral replication in non-permissive primary CD4<sup>+</sup> T cells, macrophages, CD4<sup>+</sup> T cell lines, and some leukemic T-cell lines that have

the host restriction factor APOBEC3G. Vif will be discussed later in great details along with the APOBEC family proteins (Goila-Gaur & Strebel, 2008; Kim, 2015; Kremer & Schnierle, 2005; Lake et al., 2003; Miller, Presnyak, & Smith, 2007; Rose, Marin, Kozak, & Kabat, 2004; Stanley et al., 2008; Strebel, 2003, 2007).

#### 4. HIV-1 life cycle

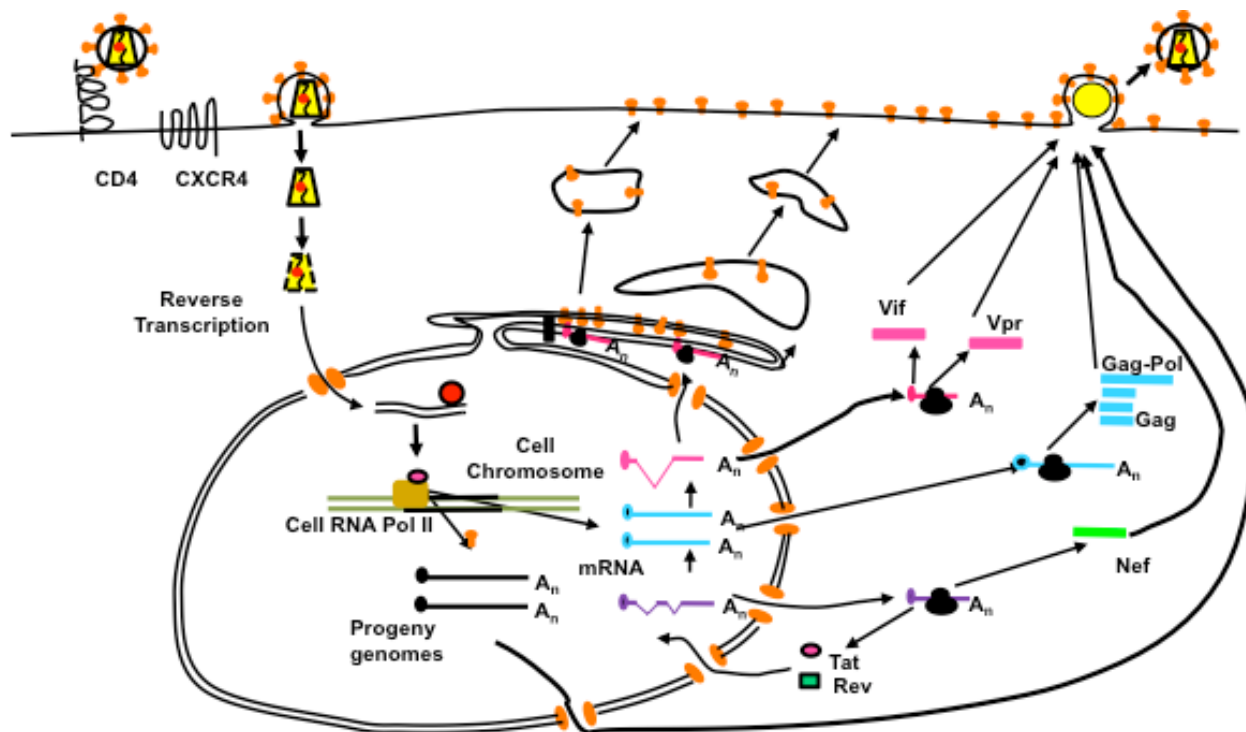
The HIV-1 replication cycle includes the following steps: 1) entry; 2) reverse transcription; 3) integration; 4) transcription and translation; 5) assembly; 6) budding; and 7) maturation. The HIV-1 replication cycle in CD4<sup>+</sup> T cells is shown in Figure 16

##### *Virus Entry:*

HIV-1 entry into susceptible cells can occur through three mechanisms including the classical binding/attachment and fusion, endocytosis, and direct cell-cell transmission via viral synapse fusion.

**Binding/attachment and fusion:** In this mechanism, the initial attachment between Env and cell surface receptors can be nonspecific, such as to surface heparin sulfate proteoglycans, or more specific such as  $\alpha 4\beta 7$  integrin and DC-SIGN (Arthos et al., 2008; Pohlmann et al., 2001). This attachment is not essential but facilitates Env coming into close proximity to the viral receptor and co-receptors for further binding. Env is a heavily glycosylated trimer of gp120 and gp41 heterodimers. Initially, gp120 binds to CD4 receptor on T cell and undergoes a conformational change resulting in a repositioning of bridging sheet and V3 loop. This leads to the exposure of their chemokine binding domain and allows it to interact with target chemokine receptors-like CXCR4 on T cells and CCR5 on T cells and macrophages. This more stable attachment triggers the exposure of the hydrophobic gp41 fusion peptide, which penetrates the cell membrane, assembles into a six-helix bundle (6HB), and activates the membrane fusion.

Figure 16. HIV-1 replication cycle in CD4<sup>+</sup> T cells. This schematic picture shows the typical HIV-1 replication cycle in CD4<sup>+</sup> cells. First, the Env protein gp120 will binds to CD4 receptor on T cells and undergo conformational change which will expose their chemokine binding domain and allows them to interact with target chemokine receptors like CXCR4 or CCR5. This more stable attachment will initiate gp41 (the transmembrane protein) to unfold and penetrate cell membrane and allows for fusion and subsequent entry of the viral capsid. After entry into cells, the viral RNA and reverse transcriptase in the capsid begin reverse transcription. This will result in a double strand viral DNA. The integrase escorts the PIC to nucleus and the viral DNA is integrated into host chromosome. Transcription process initially results in the early synthesis of regulatory HIV-1 proteins such as Tat and Rev. Tat will binds to the transactivation response element (TAR) and stimulates the transcription of longer RNA transcripts. Rev assists in this process, as well as ensures the export from nucleus to cytoplasm of the correctly processed messenger and genomic RNA. The viral RNA and proteins are and Env is concentrated at the plasma membrane leading to viral egress. The final maturation step is cleavage of the Gag and Gag-Pro-Pol precursor by PR to generate a mature virion.





The stabilization of the membrane fusion allows the subsequent entry of the viral capsid. However, this cell free spread has several disadvantages: First, the Env heterotrimers require multiple receptors available on cell surface for an effective binding. Second, free virions encounter more immunological challenges such as neutralizing antibody (Overbaugh & Morris, 2012). Finally, it is rate limiting compared to direct cell-to-cell transmission (Ambrose & Aiken, 2014; Brandenburg, Magnus, Regoes, & Trkola, 2015; Klasse, 2012; Melikyan, 2014; Micewicz & Ruchala, 2013; Wilen, Tilton, & Doms, 2012a, 2012b). The schematic representation of HIV-1 entry through binding and fusion is shown in Figure 17.

**Endocytosis:** In the second mechanism, HIV-1 can also enter cells by endocytosis. The whole virus particles are internalized into cells through clathrin-dependent, dynamin-dependent endocytosis (Hela cells and lymphoid CEMss cells), or macropinocytosis (macrophage) (Daecke, Fackler, Dittmar, & Krausslich, 2005; de la Vega et al., 2011; Gobeil, Lodge, & Tremblay, 2013; Miyauchi, Kim, Latinovic, Morozov, & Melikyan, 2009; von Kleist et al., 2011). Following the engulfment of the intact virions, the viral nucleocapsid is released into the cytoplasm and begins reverse transcription. The HIV-1 entry through endocytosis is shown in Figure 18. This strategy offers several advantages. It minimizes the exposure of virus antigens to the immune system by hijacking cellular endocytic machinery; and, it allows quick transport of the virus to a perinuclear space where the virus appears to fuse with endosome (Miyauchi et al., 2009; Miyauchi, Marin, & Melikyan, 2011), facilitating reverse transcription and nuclear entry of PIC (Carter, Bernstone, Baskaran, & James, 2011; Chauhan & Khandkar, 2015; Chauhan, Tikoo, Patel, & Abdullah, 2014; Daecke et al., 2005; Hamamoto & Yamamoto, 2010; Permanyer, Ballana, & Este, 2010).

Figure 17. HIV-1 entry through binding and fusion. The Env protein gp 120 will bind to CD4 receptor on cells and undergo conformational change which will expose their chemokine binding domain and allows Env to interact with target chemokine receptors like CXCR4 or CCR5. This stable attachment will initiate gp41 (the transmembrane protein) to unfold and penetrate cell membrane, which allows for fusion of the cellular and viral membranes.

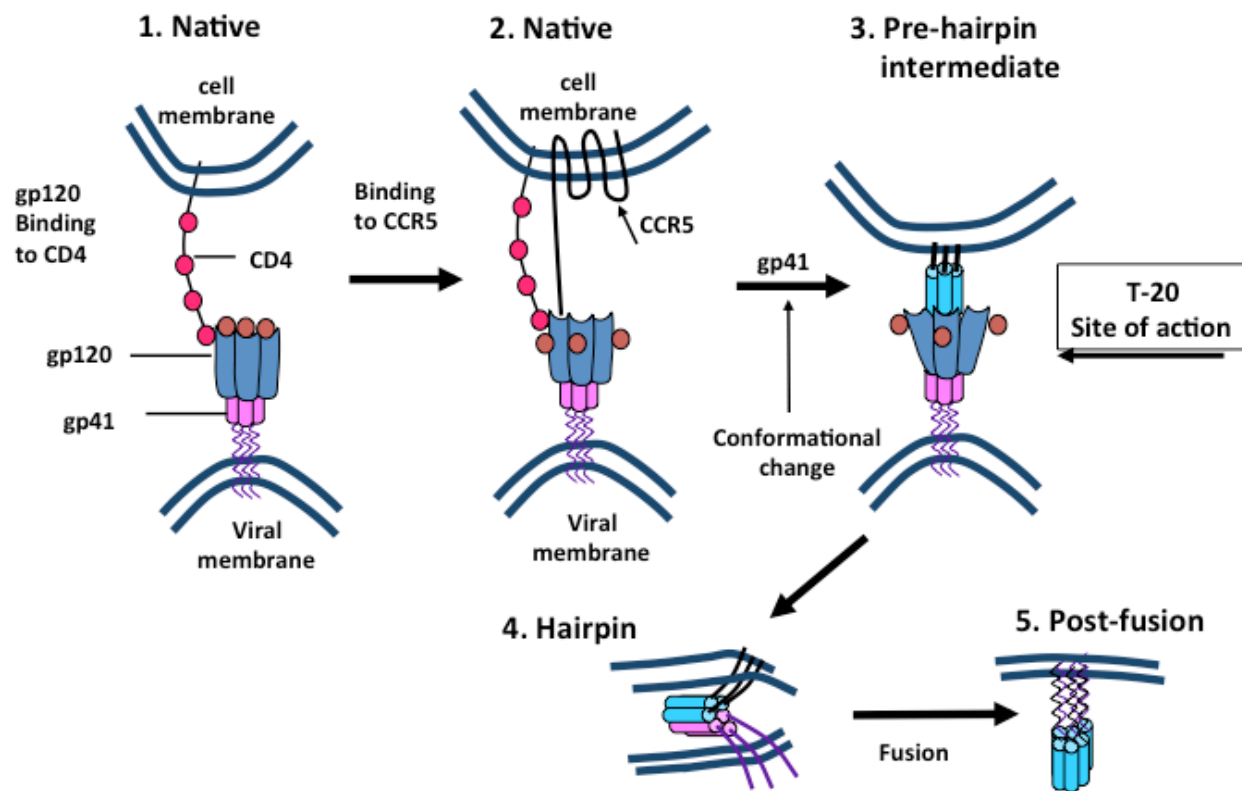
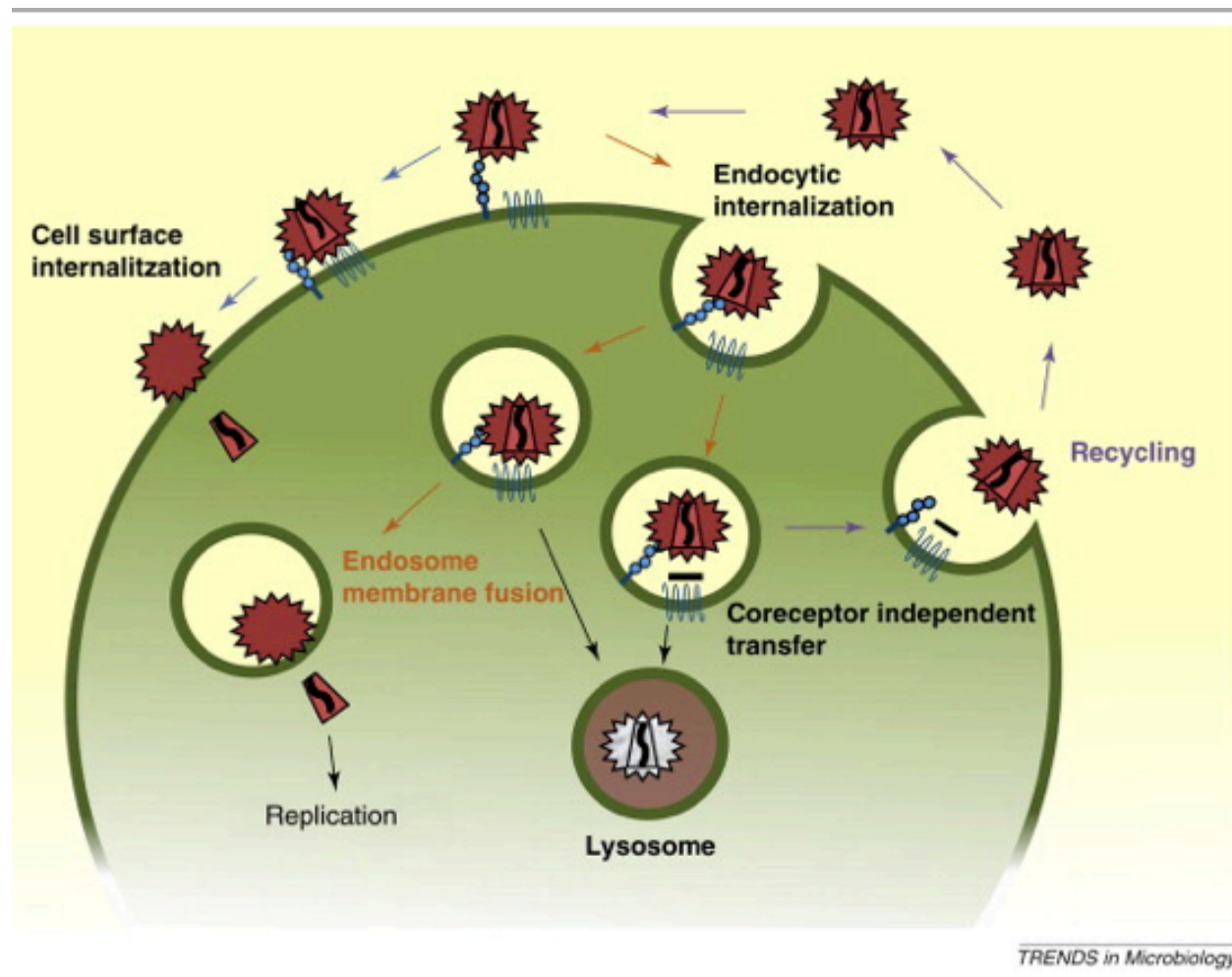
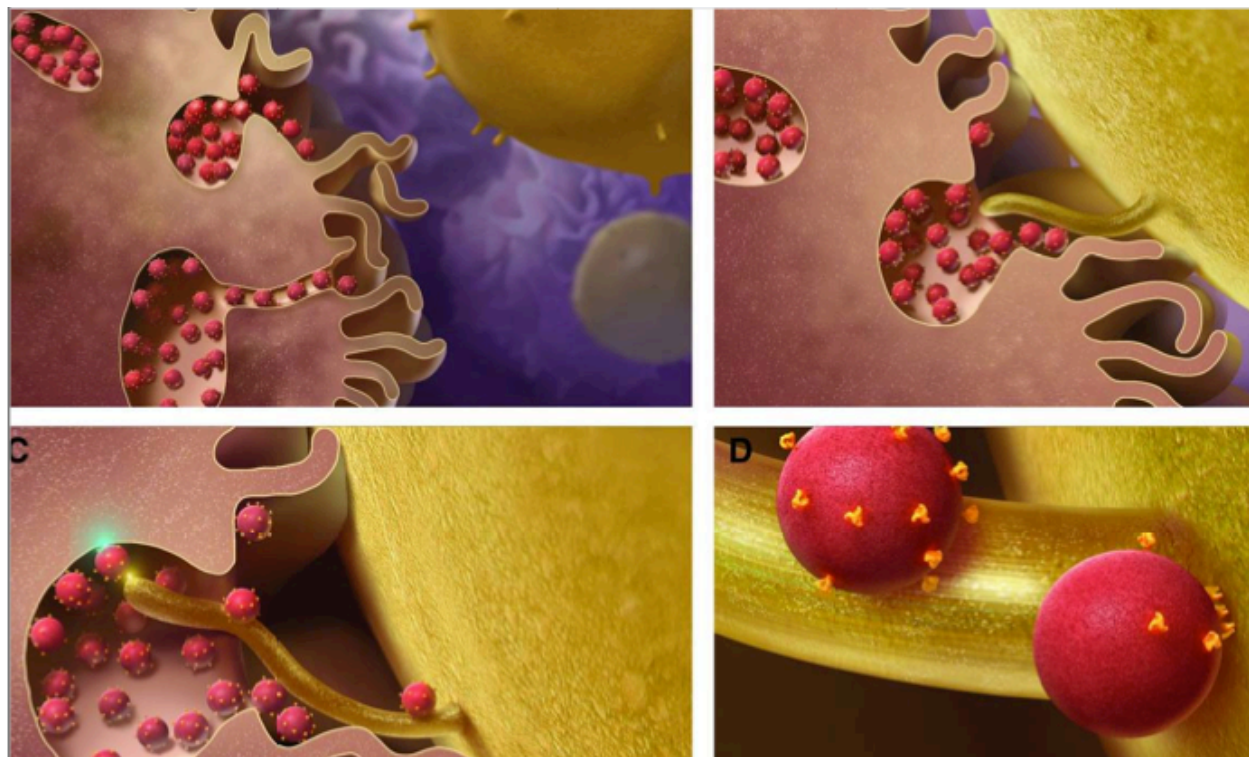


Figure 18: HIV-1 entry through endocytosis. After binding of HIV to the CD4 receptor, the virus can enter target cells through endocytosis. HIV particles are internalized into cells through clathrin-dependent, or dynamin-dependent endocytosis (cell surface internalization). Following the engulfment of the intact virions, coreceptor engagement could result in fusion of the viral and endosomal membrane and release the viral nucleocapsid into the cytoplasm for reverse transcription; Alternatively, in the absence of the appropriate coreceptor or coreceptor independent transfer, viruses may be recycled back to the extracellular medium (Permanyer, M, et al. (2010). Endocytosis of HIV: anything goes. *Trends in Microbiology*, 18(12), 543-551).



**Cell-to-cell transmission:** In the final mechanism of viral entry, retroviruses can be transmitted directly from an infected cell to receptor-expressing uninfected cells via viral synapse (VS) formation and nanotubes. HIV-1 replication is significantly stimulated in the presence of VS. VS has been characterized between T cells (Jolly, Kashefi, Hollinshead, & Sattentau, 2004a), between dendritic cells (DC) and T cells (Cameron et al., 1992; McDonald et al., 2003), and between macrophages and T cells (Groot, Welsch, & Sattentau, 2008). The formation of the HIV-1 VS is a multistep process that requires donor and target coming to close contact with each other. Generally, it is initially triggered by the binding of the HIV-1 envelope (Env) glycoprotein on the donor cells to the CD4 receptors expressed on the target cells. Noteworthy, Env/CD4 interactions are dispensable to form the DC-T cell VS, while the recognition and binding between cellular membrane adhesion proteins, such as ICAM-1-3 on the donor and the ligand LFA-1 on targets, are required (Rodriguez-Plata et al., 2013). In addition, lipid raft recruitment also plays an important role in VS formation (Jolly & Sattentau, 2005). Remodeling of adhesion molecules, actin cytoskeleton in both donor cells and target cells, and the repositioning of the microtubule-organizing center (MTOC) in donor cells contributes to the VS formation (Jolly, Welsch, Michor, & Sattentau, 2011b). Figure 19 depicts HIV-1 entry through cell to cell transmission (from DC to CD4<sup>+</sup> T cell). This direct cell-cell transmission: 1) is more rapidly and efficiently (1000-fold more efficient than free-virus transmission); and 2) minimizes immune exposure and promotes evasion from host system recognition. (Agosto, Uchil, & Mothes, 2015; Casartelli, 2016; Costiniuk & Jenabian, 2014; Jolly, Kashefi, Hollinshead, & Sattentau, 2004b; Jolly, Mitar, & Sattentau, 2007; Jolly, Welsch, Michor, & Sattentau, 2011a; Rudnicka et al., 2009)

Figure 19. HIV-1 entry through cell to cell transmission (from dendritic cell (DC) to CD4<sup>+</sup> T cell). (A) Virions are concentrated in compartment of DCs; (B) The membrane protrusions of CD4<sup>+</sup> T cells reach to DCs. (C) The CD4<sup>+</sup> T cell protrusions invade the virus-containing compartments and bind to viruses; (D) Viruses migrate along the protrusion to infect CD4<sup>+</sup> T cells (Wilén, C. B, et al. (2012). HIV: cell binding and entry. *Cold Spring Harbor Perspectives in Medicine*, 2(8), a006866).





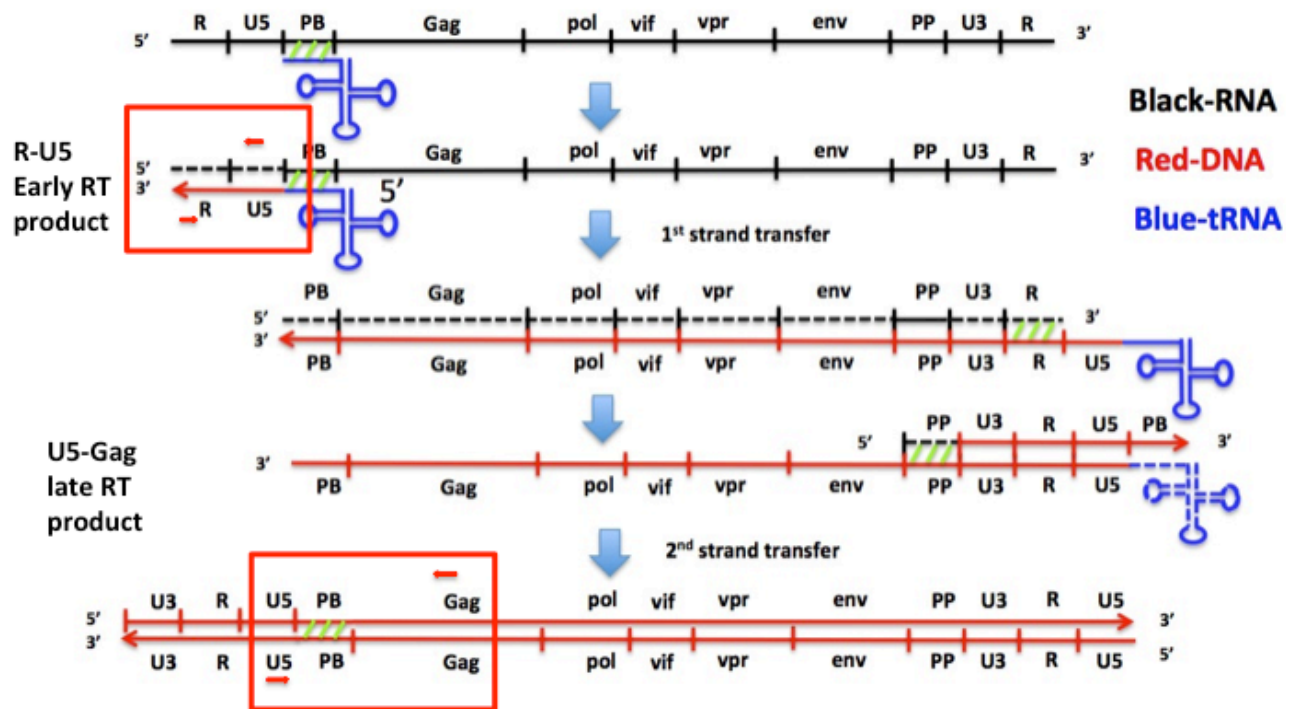
### ***Reverse Transcription of Viral Genome:***

Once the nucleocapsid enters the cytoplasm, it is partially uncoated, and the process of reverse transcription begins, which results in the two copies of single-stranded RNA converted into double-stranded linear viral DNA. Reverse transcription of HIV-1 is a complicated process involving two strand transfers. First, tRNA<sup>Lys3</sup> packaged in the virions is hybridized to a complementary region of the virus genome and is called the primer binding site or PB. Complementary DNA is synthesized to the U5 (non-coding region) and R region (a direct repeat found at both ends of the RNA molecule) of the viral RNA. This priming initiates the synthesis of the minus strong stop DNA (R-U5). The reverse transcriptase pauses, and another domain on the reverse transcriptase enzyme, RNase H, degrades the 5' end of the RNA, which removes the U5 and R region. The minus strand strong stop DNA then 'jumps' and hybridizes to the complementary R region on the 3' end of the viral RNA, completing the first strand transfer. With exception of a region referred to as the polypurine tract (PPT), the first strand of complementary DNA (cDNA) is extended, and the majority of viral RNA is degraded by RNase H. The PPT serves as a primer to initiate the synthesis of the second strand of DNA-plus strong stop DNA. Once the second strand of DNA finishes LTR and PB synthesis, the PPT and tRNA is degraded, and another 'jump' will occur, in which the PB from the second strand hybridizes with the complementary PB on the first strand. Both strands are further extended, resulting in a double-stranded linear viral DNA. The schematic process of reverse transcription is shown in Figure 20.

### ***Integration***

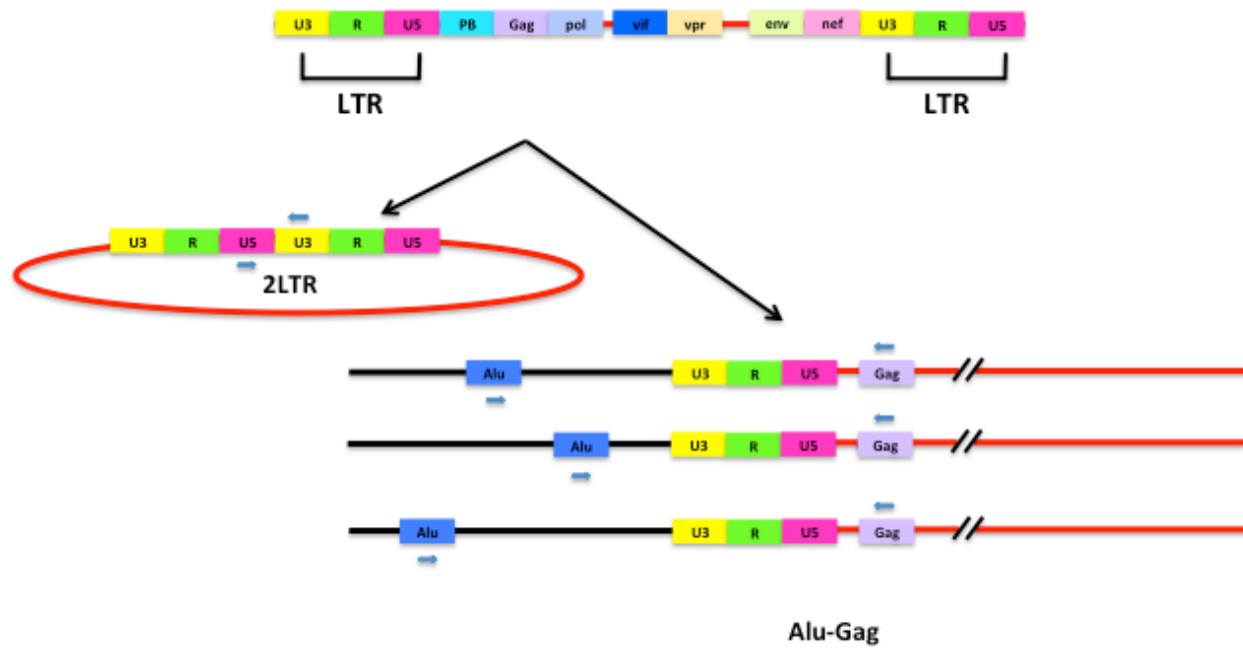
Following reverse transcription, the newly synthesized double stranded linear DNA

Figure 20. HIV-1 Reverse transcription. HIV reverse transcription is a complicated process that involves two strand transfers. In this Figure, I have used black to represent the viral original RNA and red as the newly synthesized DNA. The dashed lines means they are degraded by RNase H. A specific cellular tRNA, shown in blue, acts as a primer and hybridizes to a complementary part of the virus genome called the primer binding site or PB, which initiates the synthesis of the first piece DNA. Complementary DNA synthesized to the U5 (non-coding region) and R region (a direct repeat found at both ends of the RNA molecule) of the viral RNA. Another domain on the reverse transcriptase enzyme, RNase H, degrades the 5' end of the RNA which removes the U5 and R region. The primer then 'jumps' to the 3' end of the viral genome, which is referred to as the first strand transfer. The newly synthesised DNA strands hybridizes to the complementary R region on the RNA. The first strand of complementary DNA (cDNA) is extended and the majority of viral RNA is degraded by RNase H, except a region called PPT (polypurine tract). The PPT will serve as a primer and initiate the synthesis of second strand DNA. Once the second strand of DNA synthesized (LTR and PB), the PPT and tRNA will be degraded by RNase H and another 'jump' occur where the PB from the second strand hybridizes with the complementary PB on the first strand. Both strands are further extended, resulting in a double strand linear viral DNA which are ready to be integrated into the hosts genome by the enzyme integrase.



associates with integrase to form the preintegration complex (PIC). The PIC and other proteins are transported to the nuclear pore. It has been reported that HIV-1 Vpr facilitates nuclear entry of viral nucleic acids in nondividing cells, such as macrophages, and also causes a G2/M cell-cycle arrest (Aida & Matsuda, 2009; Vodicka, Koepp, Silver, & Emerman, 1998). The double-stranded viral DNA and integrase enter the nucleus, and the double stranded linear DNA is ligated into the host chromosome. In the first step (3' end processing), two nucleotides, in most cases are removed from each 3' end of the blunt-ended linear viral DNA by integrase. The resulting 3' end of the viral DNA in all cases terminate with the conserved CA-3' sequence. In the second step (DNA-strand transfer), these 3' ends, with the help of integrase, attack a pair of phosphodiester bonds on opposite strands of the target DNA, across the major groove. The resulting integration intermediate involves the 3' end of the viral DNA hybridized to the target DNA. The single-strand gaps and the two-nucleotide overhang at the 5' end of the viral DNA must be repaired by cellular enzymes to complete integration. The sites of joining the two target DNA strands are separated by five base pairs, resulting in a five base-pair duplication (Craigie & Bushman, 2012). The integration sites were originally thought to be random but more recent studies (Singh et al., 2015; Sowd et al., 2016b) have shown that cleavage and polyadenylation specificity factor 6 (CPSF6), and the host chromatin-binding factor LEDGF/p75, guide HIV-1 integration to preferred sites of the genes that are transcriptionally active. The interaction between host factors and PIC does not only determine the integration sites, but also cloaks the provirus from cellular pathogen-associated molecular pattern (PAMP) recognition. HIV-1 integration is shown in Figure 21.

Figure 21. HIV-1 integration. First, the exonuclease activity of integrase trims two nucleotides from each 3' end of the linear viral DNA duplex. This is followed by a double-stranded endonuclease activity that cleaves the host DNA at the integration site, allowing hybridization of the viral DNA into the chromosome. Finally, a ligase activity generates a single covalent linkage at each end of the proviral DNA. It is believed that cellular enzymes that repair the integration site.



## *Transcription*

After provirus integrates into transcription-active cellular genes, cellular transcription factors, such as NF- $\kappa$ B, initiate the basal transcription of HIV-1 (Hiscott, Kwon, & Genin, 2001). However, the RNA polymerase II pauses shortly after promoter clearance (Toohey & Jones, 1989). Thus, majority of the initial transcripts are short and abortive, and full-length transcripts are infrequent. The first viral protein, Tat, is expressed at low levels from the rarely produced full-length transcripts that are multiply spliced (Lassen, Bailey, & Siliciano, 2004). The p300/CBP-associated factor (PCAF) acetylates Tat at lysine 28, and the acetylated Tat recruits the positive transcription elongation factor  $\beta$  (P-TEF $\beta$ ) from a large inactive complex containing 7SK snRNA, the methylphosphate capping enzyme MePCE, the La-related protein LARP7, and hexamethylene bis-acetamide inducible 1 (HEXIM1 protein) (Barboric et al., 2007; Krueger et al., 2008; Sedore et al., 2007). This results in the production of doubly spliced mRNAs that encode Tat and another HIV-1 regulatory protein, Rev. The Tat-P-TEF $\beta$  complex binds to the Transactivation Response element (TAR), while autophosphorylated CDK9 phosphorylates additional host factors and stimulates the transcription of full-length mRNA transcripts (Garber et al., 2000; Ott, Geyer, & Zhou, 2011). Tat is subsequently acetylated at lysine 50 by p300/CBP and a histone acetyltransferase hGCN5, and recruits host factors, such as P300/CBP-associated factor PCAF and SWItch/Sucrose Non-Fermentable (SWI/SNF) to further enhance HIV-1 transcription. The synthesis of full-length viral transcripts is elevated by more than 100-fold (Cullen, 1986; Mousseau, Mediouni, & Valente, 2015). Rev also facilitates this process to ensure the export of unspliced and incompletely spliced mRNAs that encode the structural proteins from the nucleus to the cytoplasm. Rev increases cytoplasmic levels of the mRNAs encoding both structural proteins (Gag, Pol, Env, Vif, Nef, Vpr) and non-structural protein Vpu, but decreases

the expression of the regulatory mRNAs that encode Rev and Tat through a negative feedback loop.

### ***Translation***

The initiation of HIV-1 mRNA translation does not use the typical ribosomal scanning mechanism since the exon-1 of HIV-1 contains several highly structured regions, such as TAR sequence, the primer binding site, the poly(A) hairpin, and RNA packaging sequence. Rather, HIV-1 uses an internal ribosome binding site (IRES) to initiate the translation of Gag precursor. It has also been reported that HIV-1 uses post-transcriptional elements (PCEs), which bind to cellular RNA binding proteins to enhance translation initiation. Ribosomal frameshifting is essential for the HIV-1 replication cycle. A (-1) shift occurs approximately 5% of the time in the translational reading frame to shift from the production of the Gag precursor reading frame to translate a Gag-Pro-Pol precursor. This frameshifting requires two upstream *cis*-acting sequence: a hexanucleotide “slippery” sequence (UUUUUUA) and a stem-loop pseudoknot structure located just 30 nts to the 3’ heptanucleotide sequence (UUUUUUA) (Karn & Stoltzfus, 2012).

### ***Assembly***

Assembly begins with the synthesis of the Gag polyprotein in the cytoplasm and its translocation to the site of assembly. The Gag precursor requires myristoylation of Gag-Gag multimerization in cells (H. Li et al., 2007). Each domain of Gag precursor (MA, CA, NC, p6) plays an important role in the translocation. The MA domain, via its myristic acid moiety, targets Gag to the plasma membrane and fosters incorporation of the viral Env glycoproteins into the progeny virions. The basic residues clustering on top of MA allows the protein to interact



electrostatically with the negatively charged inositol headgroup of phosphoinositide phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>), which is highly enriched in the inner leaflet of the plasma membrane (Ono, Ablan, Lockett, Nagashima, & Freed, 2004). Myristate exposure is triggered by the binding of PtdIns (4,5)P<sub>2</sub> to MA, and may promote the insertion of MA into the inner leaflet of the plasma membrane (Saad et al., 2006). Investigators have proposed that the interaction between MA and gp41 cytoplasmic tail of Env may play a central role in Env glycoprotein incorporation (Tedbury & Freed, 2014). The C-terminal domain of CA (CA<sub>CTD</sub>) contains a dimer interface and drives Gag multimerization during assembly. In addition, interaction between NC and RNA also promotes Gag multimerization (Burniston, Cimarelli, Colgan, Curtis, & Luban, 1999; Lingappa, Dooher, Newman, Kiser, & Klein, 2006). The NC recruits the viral RNA genome encapsidation and facilitates the assembly process with the dimer initiation signal (DIS) located at 5' UTR, inducing viral RNA dimerization. The RNA dimer then serves as the recognition unit for packaging into progeny virions. Retroviral RNA usually uses a psi site ( $\psi$ ) in the 5' UTR as the packaging signal. However, the 5' UTR of the HIV-1 genomic RNA contains several highly structured elements (K. Lu et al., 2011). It is more likely that the multiple folding conformations of 5' UTR, rather than a small, defined  $\psi$ -element, serve as the packaging signal for dimerized viral RNA. The NC contains two Cys-Cys-His-Cys zinc-finger-like (or 'zinc-knuckle') domains flanked by basic residues. These two domains increase the affinity of NC for RNA binding and NC functions as a nucleic acid chaperone (Levin, Guo, Rouzina, & Musier-Forsyth, 2005). The p6 domain hijacks the cellular endosomal sorting complex required for transport (ESCRT) apparatus and catalyses the membrane fission step to complete the budding process. The ESCRT system was originally found to be responsible for the biogenesis of multivesicular bodies (MVBs) and the sorting of ubiquitinated cargo into designed

endocytic organelles (O. Schmidt & Teis, 2012). It consists of a network of class E vacuolar protein sorting (Vps) proteins that is made up with components of ESCRT-I, -II and -III complexes (Usami et al., 2009; Votteler & Sundquist, 2013). Initially, ESCRT-I recognizes ubiquitinated protein cargo via its Tsg101 (tumor susceptibility gene 101) subunit. The initial recognition activates ESCRT-II and then leads to the assembly of ESCRT-III on the cytosolic side of endosomal membranes. HIV-1 hijacks this system by binding directly to this cellular budding machinery. This direct association requires two late-assembly domains (L domains) on the C-terminal p6 domain of HIV-1 Gag. The most important domain is called PTAP (Pro-Thr-Ala-Pro). PTAP motif is located near the N terminus of p6 and interacts directly with tumour susceptibility gene 101 (TSG101) - a component of ESCRT-I (Garrus et al., 2001; VerPlank et al., 2001b). Each of the four residues (Pro-Thr/Ser-Ala-Pro) directly interacts within an extended groove on the amino-terminal ubiquitin E2 variant (UEV) domain of TSG101. The second L domain on p6 is called LYPX<sub>n</sub> L (Leu-Tyr-Pro-Xaa<sub>n</sub>-Leu), which serves as a docking site for ALG-2 (apoptosis-linked gene 2)-interacting protein X (Alix) (Demirov, Ono, Orenstein, & Freed, 2002; Freed, 2015; Garrus et al., 2001; Martin-Serrano, Zang, & Bieniasz, 2001; B. Meng & Lever, 2013; Sundquist & Krausslich, 2012; VerPlank et al., 2001b; Votteler & Sundquist, 2013)

### ***Budding***

Following assembly of the immature Gag precursor lattice at the plasma membrane, the nascent particle must undergo a membrane fission event to be released from the cell surface. PTAP motif of p6 recruits ESCRT, a host system that plays a vital role in virus release. ESCRT has been observed to assemble into circular arrays or spirals. The assembled filaments may

create closed “domes” that constrict the opposing membranes and promote fission as they inward (Hanson, Roth, Lin, & Heuser, 2008; Shen et al., 2014). In addition, ESCRT has been proposed to provide energy for the membrane scission event by recruiting the AAA ATPase vacuolar protein sorting 4 (VPS4) (B. Meng & Lever, 2013). The HIV-1 budding and the associated ESCRT pathway is shown in Figure 22.

### ***Maturation***

HIV-1 virus particles egress from cells as non-infectious, immature particles and require the proteolytic activity of the viral protease (PR) for maturation. The HIV-1 protease is an aspartyl protease. Since the active site is located in a cleft at the dimer interface, the protease needs to be dimerized for function (Wlodawer & Erickson, 1993). In the immature particle, Gag molecules are packed in a radial manner. Following cleavage, “MA remains associated with the inner leaflet of the viral membrane, forming a discontinuous matrix shell that lacks long-range order” while the CA protein reassembles to form a conical shaped capsid core as represented in Figure 23 (Sundquist & Krausslich, 2012) (Frank et al., 2015; Keller et al., 2013).

## **5. Current HIV-1 Antiretroviral strategies**

In the early 1990s, the process of antiretroviral drug development increased due to the advent of: “(1) high throughput compound screens with virus-specific replication or enzymatic assays; (2) optimization of inhibitors using lead compounds based on homologous enzymes or targets; and (3) rational drug design modeled on the 3-dimensional structures of viral proteins” (Arts & Hazuda, 2012). The current antiretroviral drugs can be divided into six distinct classes:

Figure 22. Schematic depiction of ESCRT pathway recruitment and retrovirus budding. Cellular adaptors are underlined and ESCRT factors are shown in white background. Polyubiquitin chains are represented by connected yellow hexagons, solid arrows refer to known protein-protein interactions, dashed arrows denote inferred or indirect protein-protein interactions, and question marks indicate undetermined site(s) of ubiquitin attachment (Votteler, J, et al. (2013). Virus budding and the ESCRT pathway. *Cell Host & Microbe*, 14(3), 232-241).

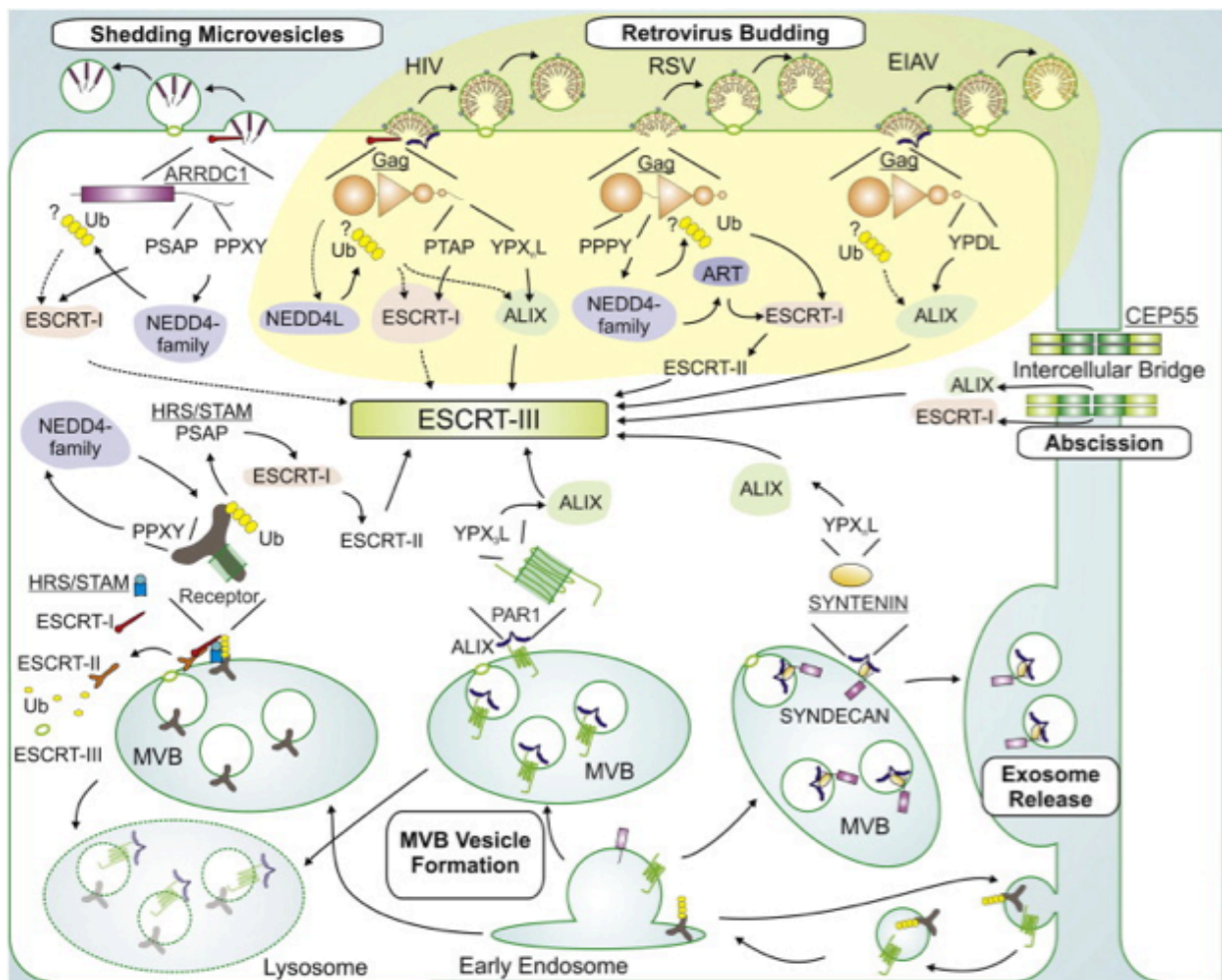
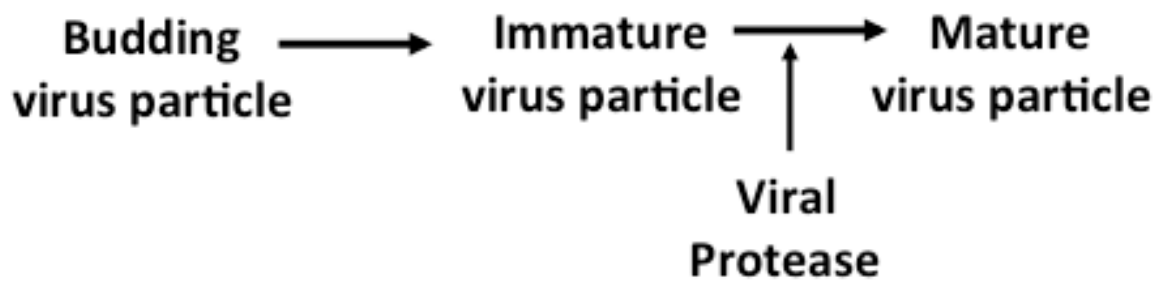
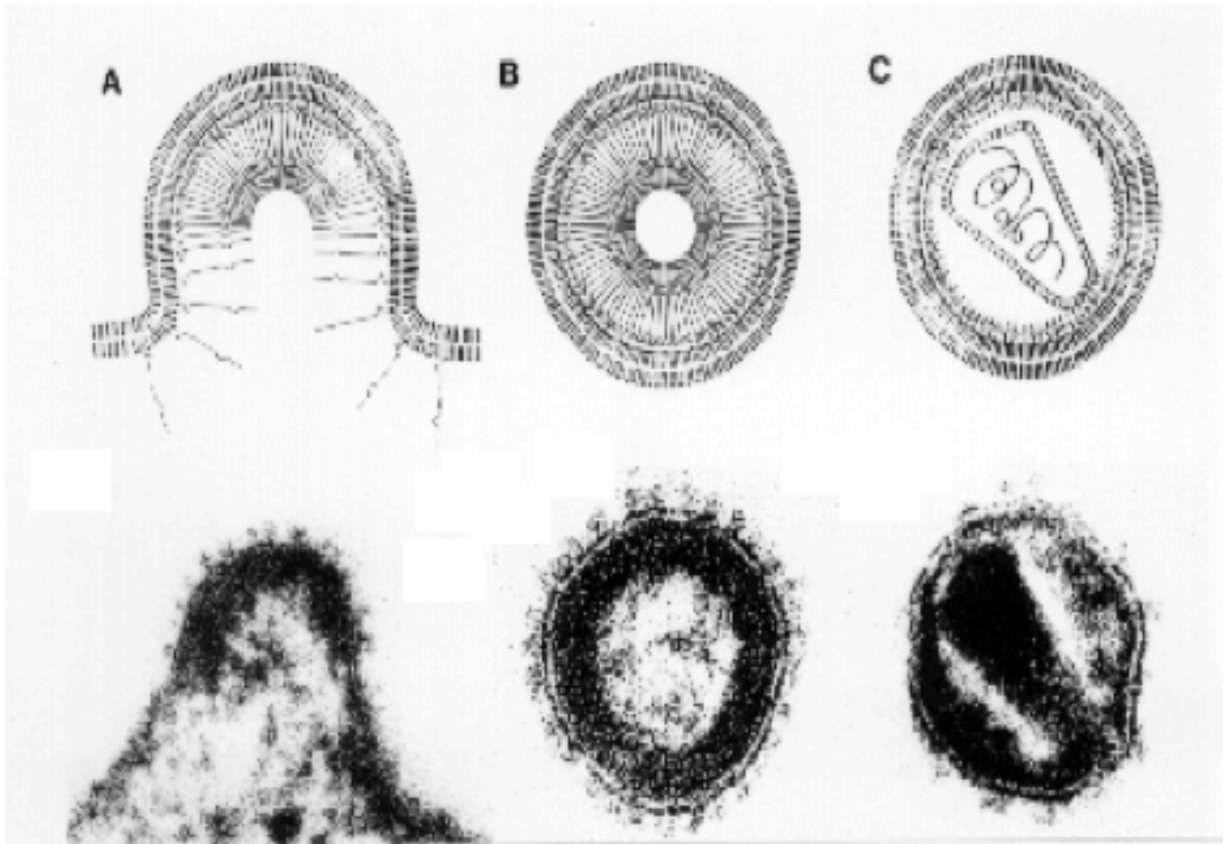


Figure 23. HIV-1 virion maturation. The three different stages in the maturation of HIV-1 are illustrated schematically along with the electron micrographs at each step. In (A), the particle is budding from the cell, In (B), the immature particle that has left the cell is shown, and (C), the mature particle is formed viral protease cleavage of the Gag and Gag-Pro-Pol precursors (Gelderblom, H. R. (1997). *Fine Structure of HIV and SIV. Los Alamos National Laboratory, Los Alamos, NM*).



(1) chemokine receptor antagonists; (2) fusion inhibitors; (3) nucleoside-analog reverse transcriptase inhibitors (NNRTIs); (4) non-nucleoside reverse transcriptase inhibitors (NNRTIs); (5) integrase inhibitors; and (6) protease inhibitors (PIs) (Arts & Hazuda, 2012). I will discuss the different classes of anti-retroviral drugs in the context of the viral replication cycle.

Inhibitors of virus **entry** include the attachment inhibitors, chemokine receptor antagonists, and fusion inhibitors. In the history of entry inhibitor development, investigators have focused on ways to hinder the interaction between viral envelope protein gp120 and cell surface receptor CD4. One inhibitor that targets the entry process is a small-molecule called BMS-378806. It alters the conformation of the envelope protein by binding to a pocket on gp120 that is important for CD4 binding (Lin et al., 2003). Besides these molecules, investigators have developed antibodies to prevent gp120 binding to CD4. TNX-355 is a humanized anti-CD4 monoclonal antibody that binds to CD4 without compromising CD4 function in immunological contexts, thus inhibiting HIV-1 envelope binding (Reimann et al., 1997). However, neither BMS-378806 nor TNX-355 have been approved for clinical application. The step after gp120 binding to CD4 is the gp120 binding to chemokine coreceptor. Two agents have been approved by FDA, which target the interaction between Env and the coreceptor CCR5: fuzeon (T20) and maraviroc. Fuzeon is a peptide-based fusion inhibitor. The rational design of fuzeon is based on the finding that two homologous leucine zipper domains in the viral gp41 protein must interact with each other for fusion. Replacing one of the domains with a heterologous protein, such as alpha-helical peptides, can disrupt the intramolecular interaction of the gp41. Based on the ordered solution structure, investigators ultimately developed fuzeon with potent antiviral activity in 2003. Maraviroc is a small-molecule antagonist of CCR5 that targets a hydrophobic



transmembrane cavity of CCR5. The binding alters the conformation of the second extracellular loop of CCR5 and prevents its interaction with the V3 stem loop of gp120.

**Reverse transcription** has been extensively used as a target for antiretroviral drug development. Two types of reverse transcriptase inhibitors currently exist: 1) the nucleotide reverse transcriptase inhibitors (NRTIs); and 2) the non-nucleotide reverse transcriptase inhibitors (NNRTIs). NRTIs are nucleoside analogues with altered nucleosides lacking 3'-hydroxyl groups. Thus, the incorporation of NRTIs prevents the formation of 3'-5'-phosphodiester bond for the incoming 5'-nucleoside triphosphates, resulting in chain termination. NRTIs are prodrugs that require host cell entry and require phosphorylation by cellular kinases into triphosphates. There are currently seven FDA-approved NRTIs. Zidovudine (AZT) was the first antiretroviral drug approved by the FDA in 1987 for the treatment of HIV-1. Other FDA approved NRTIs are Didanosine (ddI), Stavudine (d4T), Lamivudine (3TC), Abacavir (ABC), Emtricitabine (FTC), and Tenofovir (Viread). The other class of RT inhibitors is the NNRTIs, which inhibit reverse transcription by binding to the hydrophobic pocket proximal to the active site. This binding alters the spatial conformation of the substrate-binding site, thus reducing the polymerase activity. There are currently four FDA-approved NNRTIs: Etravirine, Rilpivirine, Efavirenz, and Nevirapine. Unlike NRTIs, NNRTIs only target HIV-1 (M group) but not the reverse transcriptase of other lentiviruses such as HIV-2 and SIVs. Interestingly, many NNRTI-resistance mutations are found in the wild-type sequences in HIV-1 group O and HIV-2 viruses (Arts & Hazuda, 2012).

The **integrase** catalyzes 3' end processing of viral DNA and strand transfer. All integrase inhibitors (InSTIs) target the strand transfer step by interacting with both the integrase enzyme and its substrate-viral DNA. InSTIs are comprised of two components: a metal-binding

pharmacophore, sequestering the two essential magnesium metal ion cofactors between the integrase active site and the viral DNA, and a hydrophobic group interacting with both the viral DNA and the enzyme in the complex (Grobler et al., 2002). There are currently three FDA approved InSTIs: Raltegravir (RAL), Dolutegravir (DTG), and Elvitegravir (EVG). InSTIs-resistance mutations map to the integrase active site near the amino acids that coordinate the essential magnesium cofactors. Thus, mutant viruses usually have damaged enzymatic function and dampened viral replication (Hazuda et al., 2000; Marinello et al., 2008).

The **protease** is the key enzyme involved in virus maturation to produce infectious virions. Protease inhibitors (PIs) prevent the cleavage of viral Gag and Gag-Pro-Pol precursors by binding to HIV-1 protease binding pocket. There are currently eight PIs approved by FDA: atazanavir (ATV, Reyataz), darunavir (DRV, Prezista), fosamprenavir (Lexiva), indinavir (IDV, Crixivan), nelfinavir (NFV, Viracept), ritonavir (FOS-APV, Norvir), saquinavir (SQV, Fortovase/ Invirase), and tipranavir (TPV, Aptivus). Although the number of FDA approved PIs is impressive, the significant drawback is that all PIs share similar structures and bind to the same pocket of protease. Thus, cross-resistance is a common occurrence (Arts & Hazuda, 2012). Similar to InSTIs, resistant mutants and primary PI-resistant mutations cluster near the active site of the enzyme. Thus, these mutations result in damaged enzymatic function and impaired replication fitness. However, investigators have found that HIV-1 viruses can: 1) undergo secondary/compensatory mutations to repair the enzymatic functions while maintaining the escaping capacity from PIs; and 2) select mutants that have altered cleavage sites of the polyproteins to serve as substrates for the mutated protease. As a result, the dramatic plasticity of HIV-1 genome helps the viruses to escape from drug recognition, while maintaining replication fitness. The HIV-1 life cycle presents many potential opportunities for therapeutic intervention.

Theoretically, any steps of the viral life cycle that can be distinguished from host systems can be exploited for novel therapeutics. Here, I have only discussed the viral replication steps that have yield FDA approved antiretroviral drugs.

Due to the high mutation rate of HIV-1 (approximately one mutation in each viral genome with every replication cycle), current therapy uses a combination or cocktail of different drugs called highly active antiretroviral therapy (HAART) and more recently combined ART (cART). The critical number of CD4<sup>+</sup> cell count for HAART initiation was raised from 350 to 500 cells/mL in the US and from 200 to 350 cells/mL in mid- and low-income countries. Most current HAART regimens consist of three drugs: 2 NRTIs ("backbone")+ a PI/NNRTI/INSTI ("base"). Initial regimens prefer drugs with a high efficacy and low side effect. As of July 2016 (DHHS, 2016), the United States Department of Health and Human Services (US DHHS) recommended the following initial regimens: 1) tenofovir/emtricitabine and raltegravir; 2) tenofovir/emtricitabine and dolutegravir; 3) abacavir/lamivudine (two NRTIs) and dolutegravir for patients who have been tested negative for the HLA-B\*5701 gene allele; 4) tenofovir/emtricitabine, elvitegravir (an integrase inhibitor) and cobicistat (inhibiting metabolism of the former) in patients with good kidney function (gfr > 70); 5) tenofovir/emtricitabine, ritonavir, and darunavir. HAART has dramatically increased the patient outcome of HIV-1-infected individuals. But this requires strict adherence of the drug cocktails, which are very expensive and have significant side effects. Unfortunately, there is currently no solution to eliminate the virus completely from infected patients. Even with proper adherence, reservoirs viruses can slowly cause problems such as HIV-1-associated neurocognitive disorder (HAND).

## **B Host Restriction of HIV-1 replication**

(See Figure 24 for currently known host restriction factors against HIV-1)

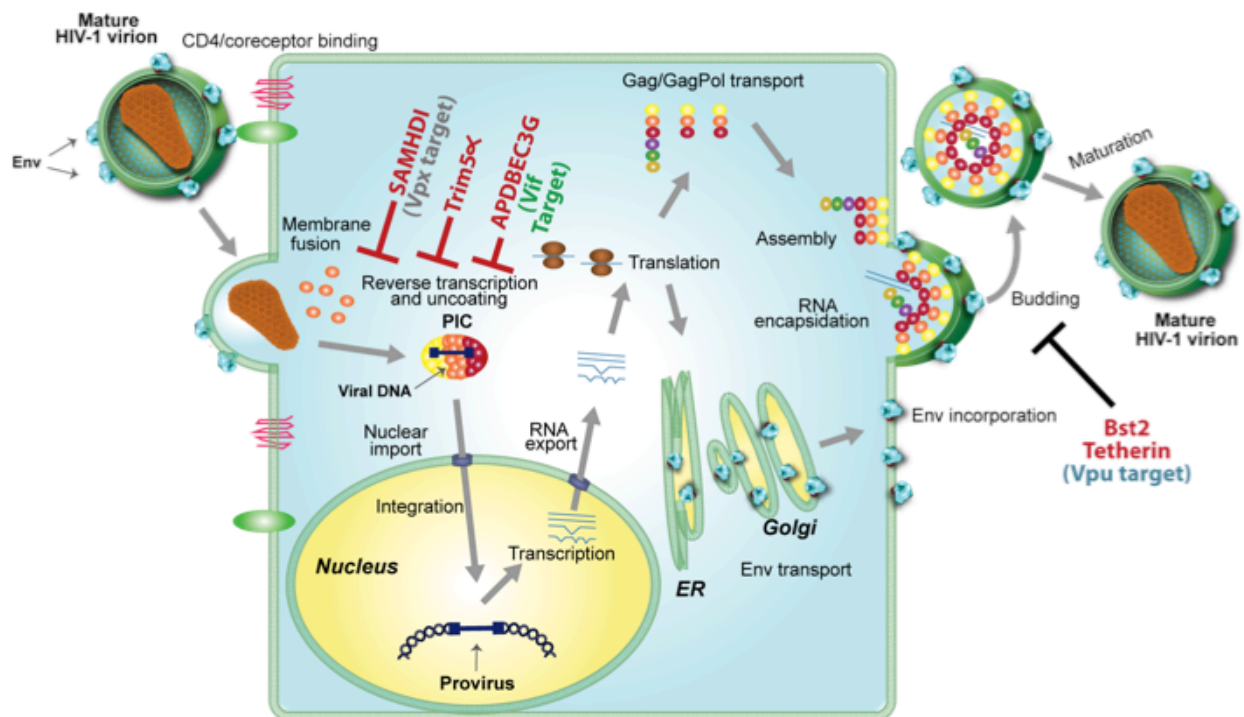
### 1. TRIM5 $\alpha$

TRIM5 $\alpha$  is short for tripartite motif-containing protein 5 alpha isoform, which is also known as RING finger protein 88. It plays an important role in the innate immune defense by mediating species-specific restriction of retroviruses at an early post-entry stage. HIV-1 has a very narrow host range that is limited to humans and chimpanzees. TRIM5 $\alpha$  plays a critical role in restricting HIV-1 replication in Old World monkey cells, such as rhesus and pig-tailed macaques (Shibata, Sakai, Kawamura, Tokunaga, & Adachi, 1995).

TRIM5 $\alpha$  is 493 amino acids in length and has a RING domain, B-box 2 domain, coiled-coil domain, and PRYSPRY domain. The RING and B-box 2 domains are required for efficient restriction activity of TRIM5 $\alpha$ . The RING domain is located at the N-terminus, and contains a cysteine-rich zinc binding sequence. TRIM5 $\alpha$  is able to self-ubiquitinate in a RING domain-dependent manner, and can also be ubiquitinated by another E3 ubiquitin ligase, Ro52 (Yamauchi, Wada, Tanji, Tanaka, & Kamitani, 2008). The ubiquitination of TRIM5 $\alpha$  is essential for HIV-1 restriction. The B-box 2 domain facilitates the cooperative binding to the HIV-1 capsid by mediating higher-order self-association (X. Li & Sodroski, 2008). The coiled-coil domain, composed of multiple  $\alpha$ -helices, promotes protein-protein interactions and is responsible for TRIM5 $\alpha$  oligomerization, which contributes to the binding avidity to the target capsid. The PRYSPRY domain of TRIM5 $\alpha$  forms dimers and directly interacts with various viral capsids to determine species specificity of retroviral restriction (Langelier et al., 2008; Song, 2009).

TRIM5 $\alpha$  is present in the cytoplasm and recognizes motifs on the capsid protein resulting in an accelerated uncoating process (Stremlau et al., 2006). The premature uncoating

Figure 24. Cellular restriction factors. Decades of antiviral research have revealed that several host restriction factors including SAMHD1, Trim5 $\alpha$ , APOBEC3 and BST-2 that target different steps of viral replication. However, the virus has also evolved strategies to counteract these restriction factors. For example Vif protein, short for Viral Infectivity Factor, can target APOBEC3G to facilitate its degradation.



inhibits the initiation of reverse transcription and the transport of the preintegration complex (PIC) to the nucleus. Proteasome activity also contributes to the restriction activity of TRIM5 $\alpha$  (Anderson et al., 2006). Unlike other restriction factors, there is no viral accessory protein to counteract TRIM5 $\alpha$ . The amino acid sequences of the variable region 1 (V1) of TRIM5 $\alpha$  PRYSPRY domain have been shown to determine the aforementioned species-specific restriction of retrovirus infection (Nakayama, Miyoshi, Nagai, & Shioda, 2005). Thus, it is possible to modify the human TRIM5 $\alpha$  gene through mutations in the V1 region or insertion of a CypA gene as found in monkeys to render restriction towards HIV-1 replication (Nakayama & Shioda, 2015). The structure and function of TRIM5 $\alpha$  is shown in Figure 25.

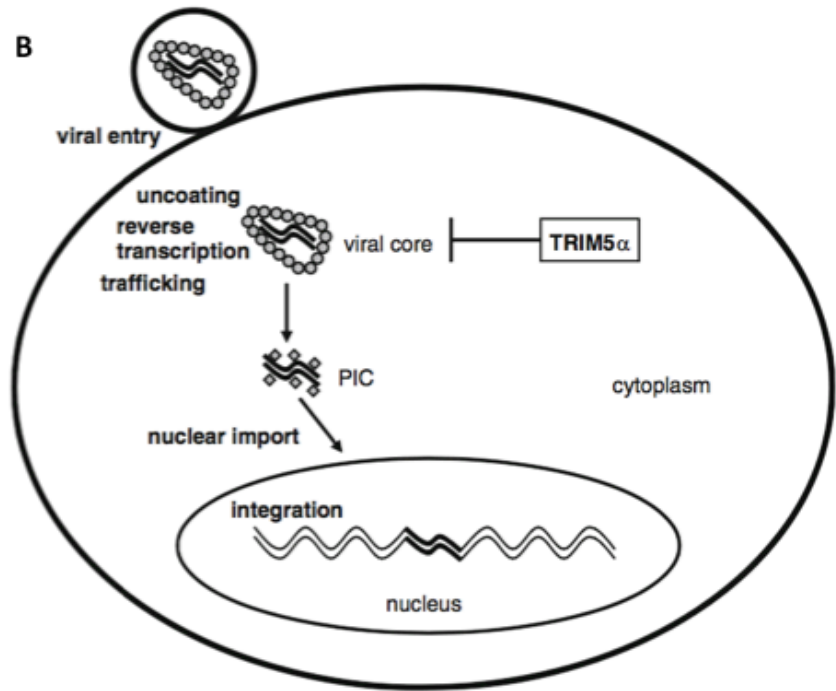
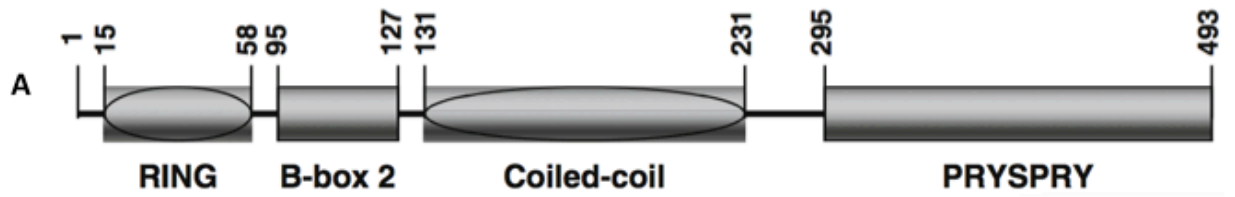
## **2. BST-2 and Vpu**

BST-2 (bone marrow stromal antigen 2), also known as tetherin, CD317, or HM1.24, is a lipid raft associated protein (Ishikawa et al., 1995). In 2008, two independent groups reported that BST-2 or tetherin acts as a host restriction factor that is counteracted by HIV-1 protein Vpu (Neil et al., 2008; Van Damme et al., 2008). In addition, BST-2 has been found to trap the budding of several enveloped viruses including Kaposi's sarcoma-associated herpesvirus (KSHV), Ebola virus, Sendai virus, Chikungunya virus (CHIKV), Herpes simplex virus 1 (HSV-1) by tethering progeny virus like particles (VLPs) to the cell surface (Swiecki, Omattage, & Brett, 2013). Additionally, it can reduce cell-to-cell transmission by inhibiting virological synapse formation. (Giese & Marsh, 2014) Similar to HIV-1, those viruses also evolved viral proteins to counteract the inhibition of BST-2 (Cao et al., 2009). One study has found that the topology of tetherin with two membrane anchors and a flexible ectodomain rather than the specific amino acid sequence is the determinant of preventing virions leaving the cells

(Le Tortorec, Willey, & Neil, 2011).

Figure 25. TRIM5 $\alpha$  structure and function. (A) Schematic representation of the domain structure of human TRIM5 $\alpha$  protein. It has a RING domain, B-box 2 domain, coiled-coil domain, and PRYSPRY domain; (B) Postentry restriction (premature uncoating) by TRIM5 $\alpha$  (Song, B. (2009). TRIM5 $\alpha$ . In *HIV Interactions with Host Cell Proteins* (pp. 47-66). Springer Berlin Heidelberg).





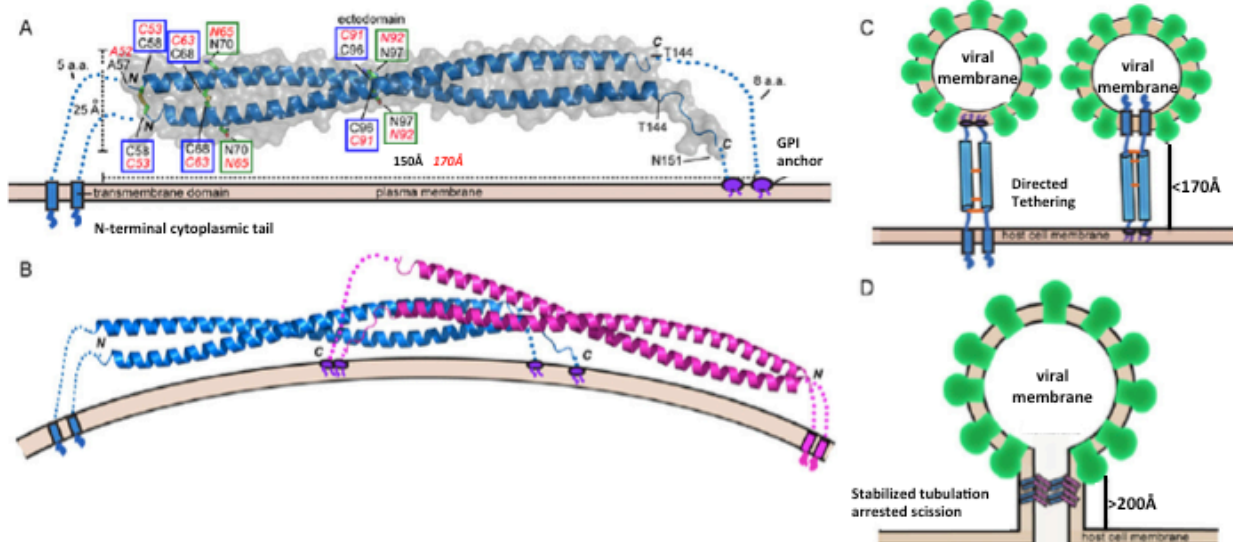
BST-2 is constitutively expressed in only a few cell types including mature B cells, plasma cells and plasmacytoid dendritic cells. In many other cells, the expression level of BST-2 can be dramatically induced by IFN- $\alpha$  and IFN- $\gamma$ . (Van Damme et al., 2008)

BST-2 is a type 2 integral membrane protein, which consists of “a short N-terminal cytoplasmic tail (CT), a single transmembrane region (TM) consisting of a single  $\alpha$ -helix, an ectodomain (ED), a second membrane anchor, and a C-terminal glycosylphosphatidylinositol (GPI)”. (Picture 26 shows the structure of BST-2 and the models of BST-2 antiviral action) (Swiecki et al., 2013). The ectodomain by itself is unstable and denatures at approximately 35°C. Crystal structure studies of the BST-2 ectodomain reveal that it forms a more stable parallel homodimer and serves as an adaptor connecting the host plasma membrane and progeny viruses at a distance of 170Å. (Hinz et al., 2010; Swiecki et al., 2011)

BST-2 seems to be an ideal broad-spectrum inhibitor since it is able to restrict many enveloped virus like-particles tested to date. In addition, it targets a host cell derived component (viral envelope from the host cell plasma membrane) instead of a virus element that is very susceptible to mutations. In many cases, inhibitors targeting a viral component result in corresponding viral mutations that are able to counteract the drug. In contrast, it is way more difficult for viruses to mutate their lipid envelope as it originated from host cells. As such, the envelope is more resistant to mutations than viral components. (Swiecki et al., 2013) However, viruses have evolved mechanisms to counteract the inhibition mediated by BST-2 (Swiecki et al., 2013).

The HIV-1 Vpu protein counteracts the action of BST-2. Vpu down-regulates the BST-2 level by two mechanisms. First, Vpu and BST-2 interact with each other in trans Golgi

Figure 26. Structure of BST-2 and models of BST-2 antiviral action. (A) BST-2 contains a short N-terminal cytoplasmic tail (CT), a single transmembrane region (TM) consisting of a single  $\alpha$ -helix, an ectodomain (ED), a second membrane anchor, and a C-terminal glycosylphosphatidylinositol (GPI); (B) A dimeric assembly based on the mouse BST-2 crystal structure; (C) Direct tethering mode of BST-2-mediated inhibition of viral budding. Parallel BST-2 dimers insert one set of membrane anchors each into the viral membrane and host membrane. (D) BST-2 oligomers stabilize the tubulation and prevent membrane scission (Swiecki, M, et al. (2013). BST-2/tetherin: structural biology, viral antagonism, and immunobiology of a potent host antiviral factor. *Molecular Immunology*, 54(2), 132-139).



network (TGN) and Vpu inhibits the transport of BST-2 to the plasma membrane (A. J. Andrew, Miyagi, & Strebel, 2011; Dube et al., 2009; S. Schmidt, Fritz, Bitzegeio, Fackler, & Keppler, 2011). Second, Vpu induces the ubiquitination and degradation of BST-2 at the cell plasma membrane. The conserved di-serine motif (DSGxxS) in the cytoplasmic part of Vpu recruits the Skp, Cullin, F-box containing complex (SCF) E3 ubiquitin ligase complex via the adaptor protein  $\beta$ -TrCP. The ubiquitinated BST-2 enters the ESCRT-dependent endolysosomal pathway for degradation (Goffinet et al., 2010; Mangeat et al., 2009; Sauter, 2014; Swiecki et al., 2013). HIV-2 uses its Env protein, especially the endocytic motif in gp41, to induce clathrin-dependent internalization and sequestration of BST-2 to TGN (Hauser et al., 2010; Le Tortorec & Neil, 2009). Most SIV Nef proteins interact with the N-terminus of BST-2 and recruits the endocytic adaptor AP-2, resulting in clathrin-dependent intracellular sequestration (Gotz et al., 2012; Serra-Moreno, Jia, Breed, Alvarez, & Evans, 2011; Serra-Moreno, Zimmermann, Stern, & Evans, 2013). Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8) has a protein K5, which is an E3 RING-CH ubiquitin ligase. K5 protein is recruited to BST-2 via the N-terminal cytoplasmic tail (NT) of BST-2 and induces its proteasomal or ESCRT-dependent endo-lysosomal degradation (Bartee, McCormack, & Fruh, 2006; Mansouri et al., 2009; Pardieu et al., 2010). Interestingly, Ebola virus does not inhibit the action of BST-2 by decreasing the surface levels of this restriction factor. (Lopez et al., 2010) The mechanism of how Ebola virus escapes BST-2 restriction is unknown. However, investigators have identified that the glycoprotein (Gp) of Ebola, especially the GP2 subunit, is critical in enhancing virion release (Kuhl et al., 2011; Lopez et al., 2010). Finally, glycoprotein M (gM) of HSV-1 also serves as an antagonist of BST-2 restriction through at least two independent mechanisms: gM-mediated relocalization and a

antagonists of BST-2 is shown in Figure 27.

gM-independent suppression of tetherin mRNA (Y. Liu et al., 2015). A summary of viral

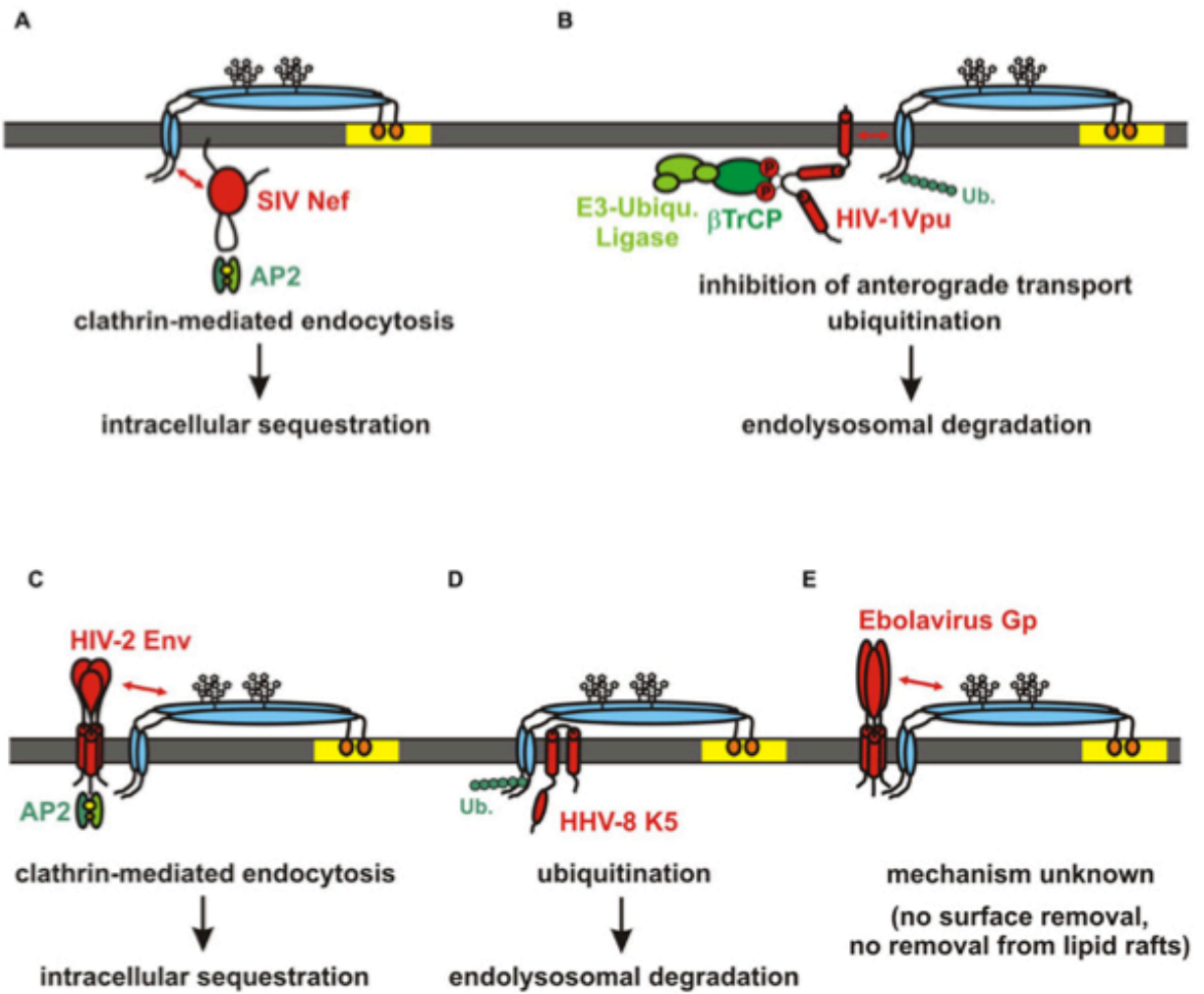
In addition to its antiviral function, BST-2 plays important roles such as: 1) serving as a ligand for the leukocyte inhibitory receptor ILT7 and modulating the IFN responses of the human plasmacytoid dendritic cells (pDCs) in a negative feedback manner (Cao et al., 2009; Gilliet, Cao, & Liu, 2008); 2) promoting metastasis and tumor cell evasion from immune surveillance (Cai et al., 2009; Yi et al., 2013); 3) enhancing viral entry and mediates early detection (Cao et al., 2009; Viswanathan et al., 2011); 4) facilitating monocyte adhesion to endothelial cells (Yoo, Park, Ye, & Kim, 2011); and 5) promoting cellular interactions (Swiecki et al., 2013).

### **3. SAMHD1 and Vpr**

SAMHD1 is short for sterile alpha motif (SAM) and histidine-aspartic domain (HD) containing protein 1. As the most recently identified HIV-1 restriction factor (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 is a host deoxynucleoside triphosphate phosphohydrolase that hydrolyzes deoxynucleoside triphosphates (dNTP) into corresponding deoxynucleosides (dN) and inorganic triphosphates (Goldstone et al., 2011; Powell, Holland, Hollis, & Perrino, 2011). Therefore, it is responsible for depleting the cellular dNTP pool that is required for the reverse transcription, which reduces the replication of HIV-1 in non-dividing cells, such as dendritic cells and macrophages.

SAMHD1 is a tetramer (Yan et al., 2013) and is comprised of three regions: the N-terminus, the catalytic core domain, and the C-terminus. The N-terminus contains a nuclear localization signal at residues 11-14 (Brandariz-Nunez et al., 2012), and a sterile alpha motif (SAM) that is folded into four  $\alpha$ -helices. The catalytic core domain (residues 110–599)

Figure 27. Schematic representation of viral antagonists to BST-2: **(A)** SIV Nef; **(B)** HIV-1 Vpu; **(C)** HIV-2 Env; **(D)** HHV-8 K5; and **(E)** Ebolavirus Gp. Tetherin is shown in blue, the viral antagonists are shown in red (Sauter, D. (2015). Counteraction of the multifunctional restriction factor tetherin. *Manipulation of the Host Cell by Viral Auxiliary Proteins*, 5, 105).





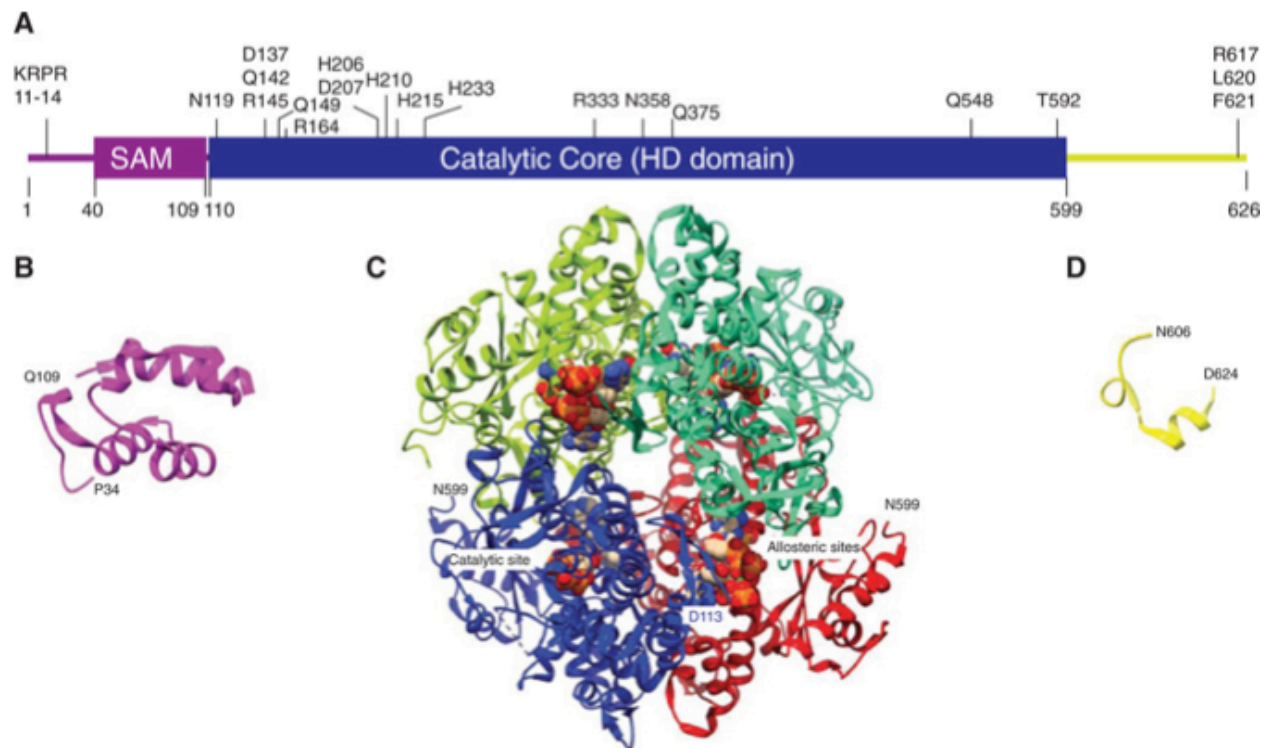
contains the deoxyribonucleoside triphosphate triphosphohydrolase (dNTPase). The stability of the tetramer is closely linked to its antiviral activity (Yan et al., 2013). The initial GTP-binding to the primary sites results in the dimerization of SAMHD1 followed by dNTP binding to the secondary allosteric site to induce the conversion to a catalytically active tetrameric form. In addition to the well-characterized dNTPase activity, the catalytic core domain has a controversial DNA/RNA exonuclease activity (Beloglazova et al., 2013). The C-terminus (residues 600–626) forms a short  $\alpha$ -helical structure with an extended loop (Schwefel et al., 2014). It also contains a conserved di-hydrophobic amino acid motif (Leu620-Phe621) that is recognized by cell cycle regulatory protein, cyclin A2-cyclin dependent kinase (cyclin A2-CDK). Cyclin A2 binds to this motif and allows CDK to mediate a critical post-translational phosphorylation at Thr592, which determines cell type dependent-HIV-1 restriction (Yan et al., 2015). The phosphorylation at Thr592 determines the stability of the SAMHD1 tetramer and hence its antiviral activity. Both phosphorylated and non-phosphorylated SAMHD1 tetramers possess similar levels of catalytically activity (Arnold et al., 2015; Hansen, Seamon, Cravens, & Stivers, 2014). However, after depletion of dNTP pools, the phosphorylated SAMHD1 tetramer rapidly dissociates to an inactive form, while the low levels of cyclin A2-CDK in non-permissive and non-dividing cells allow unmodified SAMHD1 to form a long-lived, stable and catalytically active tetramer. (Arnold et al., 2015; Hansen et al., 2014). SAMHD 1 domain structures are shown in Figure 28.

## **C APOBEC and Vif**

### **1. APOBEC3 family of proteins**

APOBEC3 or A3 is short for “**ap**olipoprotein **B** mRNA editing enzyme, **c**atalytic

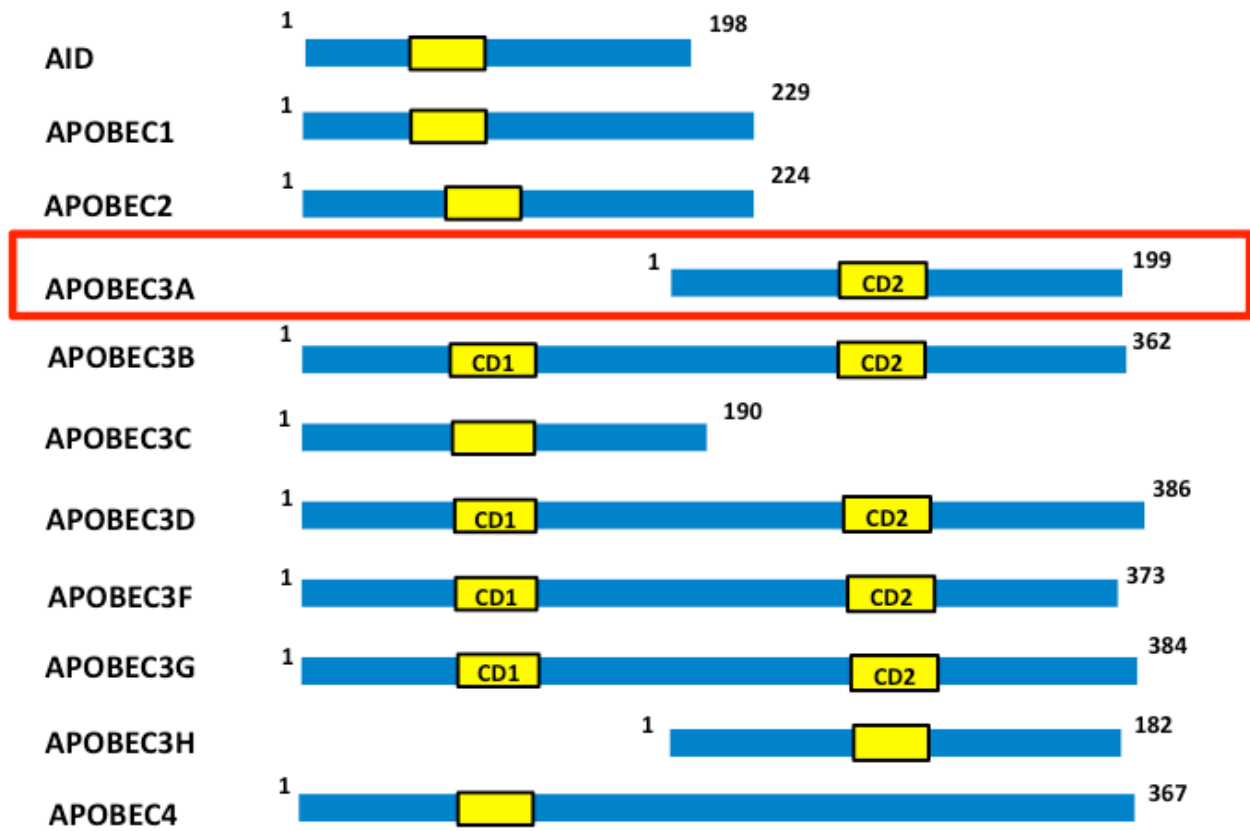
Figure 28. SAMHD1 Domain Structures. (A) A linear diagram of SAMHD1 comprising three domains; the N-terminus: Sterile Alpha Motif (purple), catalytic core: Histidine-Aspartic (HD) domain-containing domain (blue); and the C-terminus (yellow.) The nuclear localization signal (NLS) KRPR resides at amino acids 11–14. (B) The crystal structure of tetrameric human SAMHD1 catalytic core (each monomer is identified with distinct colors) in complex with dGTP (sphere) (Ahn, J. (2016). Functional organization of human SAMHD1 and mechanisms of HIV-1 restriction. *Biological Chemistry*, 397(4), 373-379).



polypeptide-like” proteins 3. APOBEC3 proteins belong to a family of proteins that includes APOBEC1, activation-induced CDA (AID), APOBEC2, and APOBEC4. These proteins mediate the enzymatic conversion of cytosine (C) to uracil (U) on DNA (AID, A3) and/or RNA (A1) molecules, which results in multiple alternations of the nucleotide strand called hypermutation. The A3 family contains seven cytidine deaminases (A3A, A3B, A3C, A3D, A3F, A3G and A3H). Human A3 genes are highly related and are tandemly located in a single cluster on chromosome 22 at q13.2 (Jarmuz et al., 2002). Old World monkeys, such as the rhesus macaque (*Macaca mulatta*), also express seven A3 genes, which are tandemly located on chromosome 10. This family plays an important role as sentinels in innate restriction to a broad numbers of mobile genetic elements (endogenous retroelements and exogenous viruses), including HIV-1, and other viruses such as parvoviruses and papillomavirus (Ahasan et al., 2015; H. Chen et al., 2006; H. C. Smith, Bennett, Kizilyer, McDougall, & Prohaska, 2012). The activity of APOBEC4 on RNA or DNA is currently unknown.

All A3 proteins are characterized by one or two zinc-coordinating domains (Z domains) with the sequence H-x-E-x<sub>23-28</sub>-PC-x<sub>2-4</sub>-C. The histidine and cysteine residues coordinate Zn<sup>2+</sup>, while glutamic acid functions as a proton shuttle during the deaminase reaction (Betts, Xiang, Short, Wolfenden, & Carter, 1994). The Z domains catalyze cytidine deamination by removing the amine group (NH<sub>2</sub>) from cytosines, resulting in uracils on a variety of RNA/DNA targets (Romani, Engelbrecht, & Glashoff, 2009). Three A3 family members (hA3A, hA3C and hA3H) have only one Z domain, while four (A3B, A3D, A3F and A3G) possess two Z domains (shown in Figure 29). Phylogenetic analysis revealed a clustering among A3 zinc-finger motifs, further classified as A3Z1, A3Z2 or A3Z3 (Wang, Schmitt, Guo, Santiago, & Stephens, 2016). For A3 proteins that have two Z-domains, generally only one domain is catalytically

Figure 29. Zinc (Z) coordinating-type domains of human A3 enzymes. The A3 proteins contain either a single cytidine deaminase domain (A3A, A3C, A3H) or duplicated domains (A3B, A3D, A3F, and A3G). Adapted from Feng, Yuqing, et al., *Manipulation of the Host Cell by Viral Auxiliary Proteins* (2015)



active. An exception is A3B, which may have two catalytically active domains (Bogerd, Wiegand, Doehle, & Cullen, 2007).

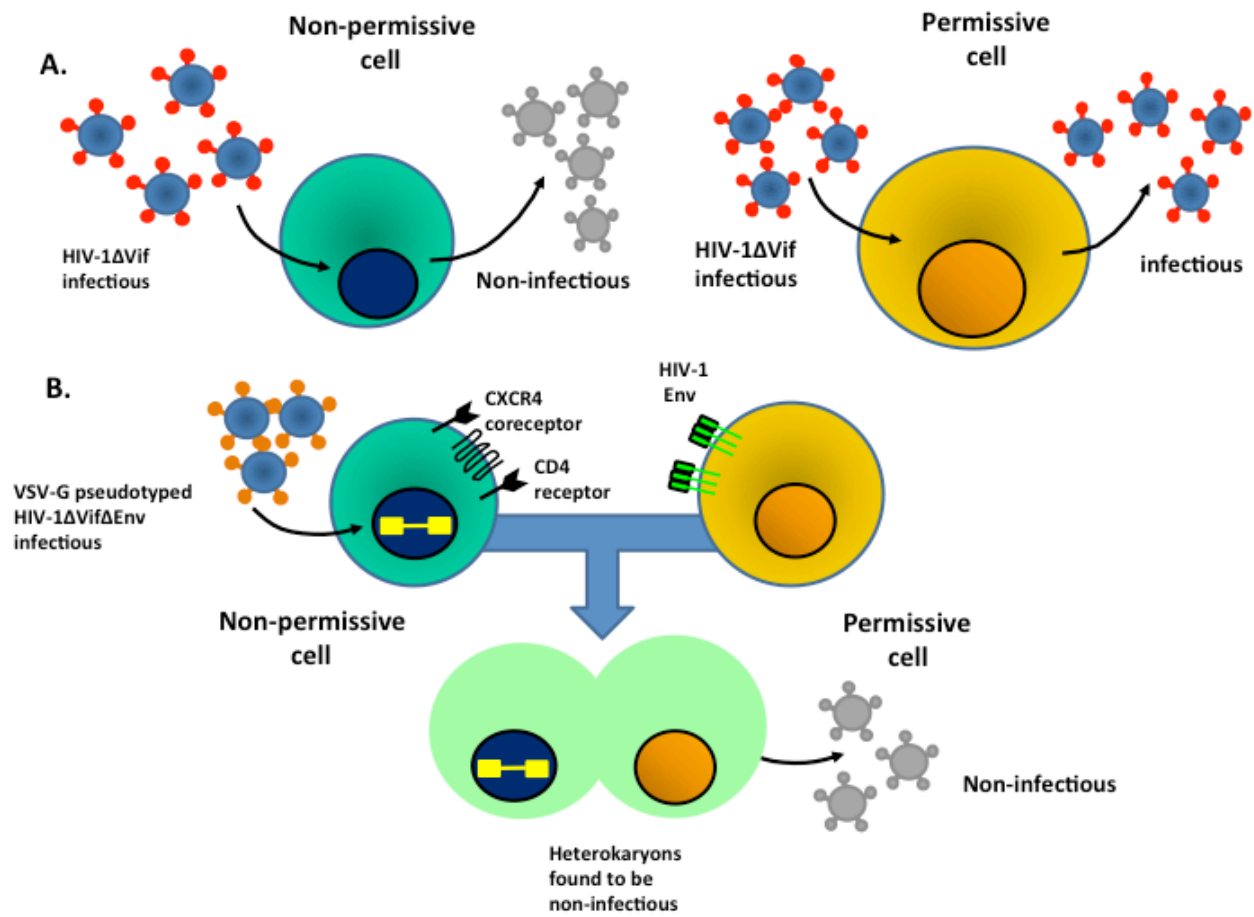
## **2. APOBEC3G and its restriction of HIV-1 (canonical and non-canonical pathway)**

A3G was the first identified A3 member with anti-HIV-1 activity and has been the most intensively characterized member of APOBEC3 family. The initial observation was that HIV-1 lacking *vif* (HIV-1 $\Delta$ *vif*) was severely restricted in the ability to replicate in non-permissive cells, such as primary CD4<sup>+</sup> T cells, macrophages and some T cell lines (e.g. H9, CEM) (Sova & Volsky, 1993). In contrast, HIV-1 $\Delta$ *vif* replicated well in permissive cell lines including HeLa, COS, SupT1, Jurkat, CEM-SS (J. H. Simon, Gaddis, Fouchier, & Malim, 1998; Sova & Volsky, 1993). In order to explain the different phenotypes of non-permissive and permissive cell lines, two hypotheses were proposed. In the first hypothesis, permissive cells expressed additional factors to compensate the absence of Vif. The second hypothesis was that the non-permissive cells possessed inhibitory factors that were counteracted by Vif. To distinguish between these two possibilities, investigators applied an experimental approach by fusing a non-permissive cell to a permissive cell, and found that the transient heterokaryon did not support HIV-1 $\Delta$ *vif* replication. They concluded that the non-permissive cells “have an activity whose expression results in the inhibition of HIV-1 infectivity, and that the function of Vif is to counteract this”. This classic experiment is shown in Figure 30 (J. H. Simon et al., 1998). Later in 2002, these investigators identified that this host restriction factor as CEM15 and later named APOBEC3G, through a complementary DNA subtraction strategy (Sheehy, Gaddis, Choi, & Malim, 2002).

Human A3G possesses two homologous deaminase domains: an inactive N-terminal

Figure 30. Strategy to test the infectivity of HIV-1 from transient non-permissive and permissive heterokaryons. A non-permissive cell and a permissive cell were fused. The transient heterokaryon did not support HIV-1 $\Delta vif$  replication. This classical experiment allows investigators to conclude that the non-permissive cells “have an activity whose expression results in the inhibition of HIV-1 infectivity, and that the function of Vif is to counteract this” (Simon, J. H, et al. (1998) Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nature Medicine*, 4(12), 1397-1400).

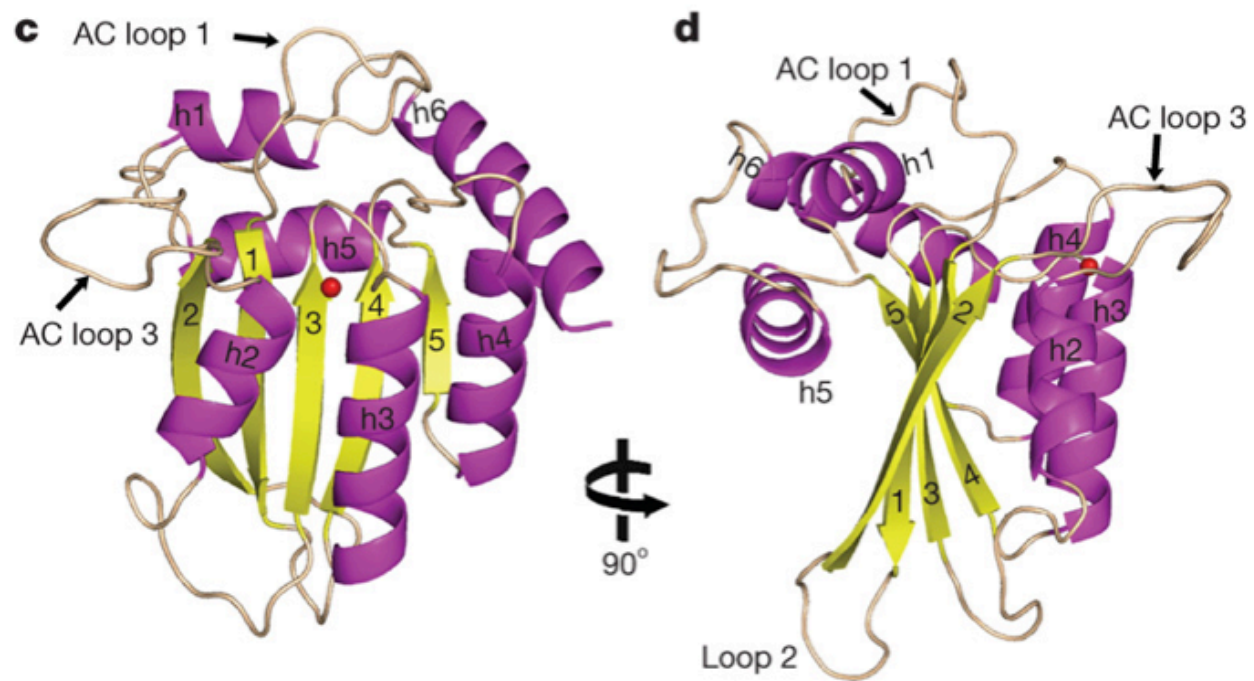




CD1 domain (A3G-CD1) and active C-terminal CD2 domain (A3G-CD2). A3G-CD1 is responsible for substrate binding like DNA and RNA. The incorporation of A3G into virions is essential for the 5'→3' deamination processivity. In addition, it interacted with Vif to result in the recruitment of E3-ubiquitin ligase complex. However, the structure of A3G-CD1 remains elusive due to its poor solubility (Xiao, Li, Yang, & Chen, 2016). In contrast, the NMR and crystal structure of A3G-CD2 (residues 197–380) was successfully resolved in 2008 (K. M. Chen et al., 2008; Holden et al., 2008). The A3G-CD2 has a core  $\beta$ -sheet that is composed of five  $\beta$ -strands surrounded by six  $\alpha$ -helices. Two of the six  $\alpha$ -helices form the zinc-coordinating active site. The structure deduced from X-ray diffraction studies revealed that the AC loops 1 and 3 and the regions near the active site form a deep, spacious groove that runs horizontally across the active center pocket and is involved in substrate recognition & binding (K. M. Chen et al., 2008; Holden et al., 2008). The X-ray structure of A3G-CD2 is shown in Figure 31. The CD2 is catalytically active and critical for deamination and motif specificity. In general, in the absence of Vif, A3G is efficiently incorporated into progeny virions released from producer cells. It restricts further virus replication in target cells through both canonical (deaminase-dependent) and non-canonical (deaminase-independent) pathways.

In the absence of Vif, the newly translated A3G is recruited and packaged into HIV-1 virions by associating with viral RNA. The binding to viral RNA is essential for the packaging of A3G. However, this association also diminishes the enzymatic activity of A3G. Nevertheless, this inactivation is relieved by viral RNase H during the reverse transcription in target cells (Soros, Yonemoto, & Greene, 2007). Studies have shown that N-terminal CD domain constitutes a viral packaging signal in A3G (Ma et al., 2013). More specifically, the tryptophan at position 127 and tyrosine at position 124 are critical since mutation at either site inhibited

Figure 31. Ribbon representation of enzymatically active APOBEC3G-CD2 based on the X-ray structure (two views rotated 90°). It contains five-stranded-sheet core surrounded by six helices. The zinc is represented as a red sphere (Holden, L. G et al., (2008) Crystal structure of the anti-viral APOBEC3G catalytic domain and functional implications. *Nature*, 456(7218), 121-124).



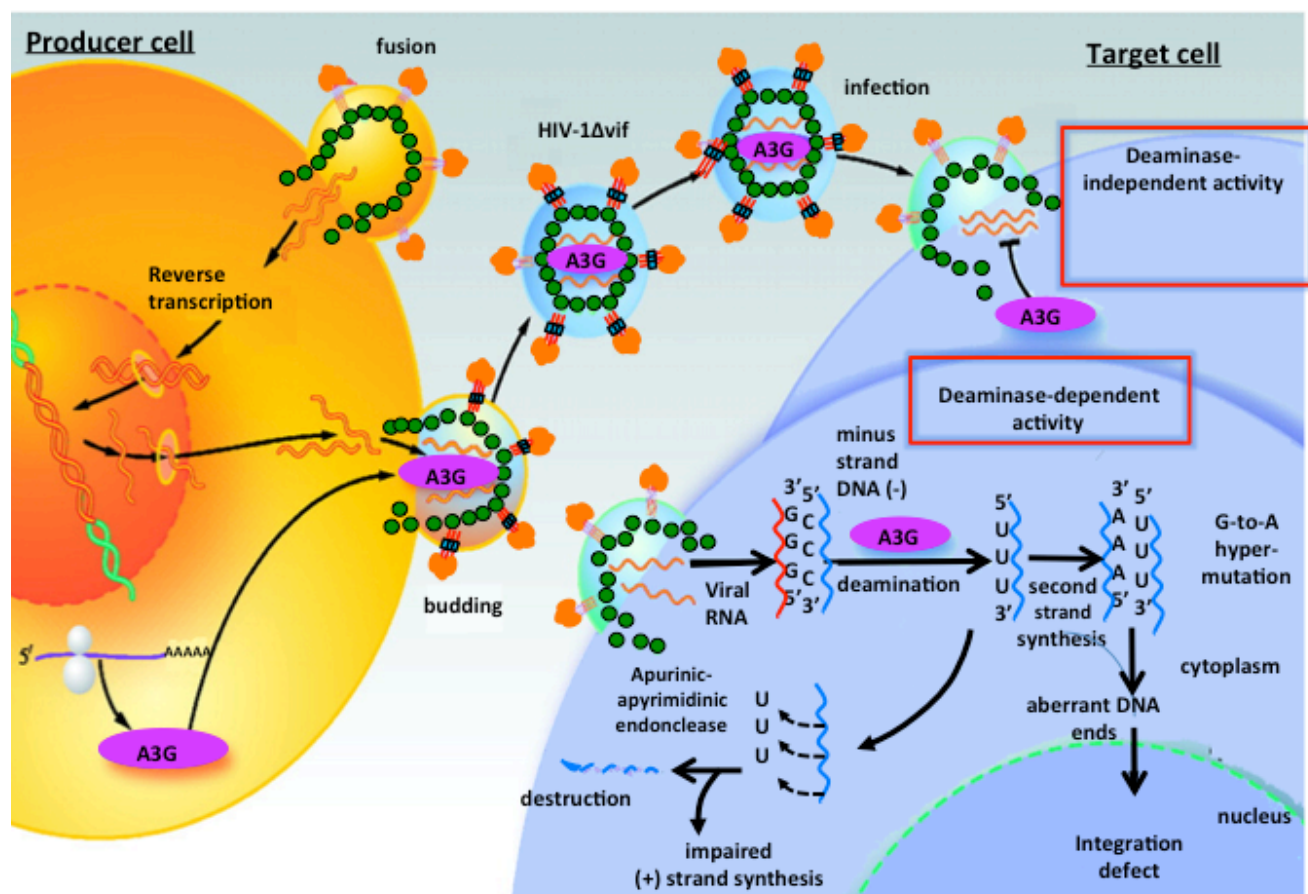
A3G encapsidation (M. A. Khan et al., 2009). In addition, investigators identified that GC-associated nuclear protein (GANP) contributes to A3G encapsidation into HIV-1 virions by revealing a positive correlation between cellular GANP level and A3G encapsidation level (Maeda et al., 2013).

***Canonical pathway:***

In this pathway, as depicted in Figure 32, HIV $\Delta$ vif infects non-permissive producer cells (i.e., expressing A3G) and A3G is incorporated into the virus released from the producer cells. Upon release from the producer cell, virus infects the “target cell” where the restriction occurs. A3G targets the newly made minus-strand DNA during reverse transcription and mediates extensive deamination of dC residues to dU residues, preferentially at the 3'-dC with context 5'-CC dinucleotides (R. S. Harris et al., 2003; H. Zhang et al., 2003). This mutation of dC to dU residues in the nucleotide strand is called hypermutation. The abnormal presence of uracils in DNA strand can be recognized by uracil-DNA glycosylase and subject to excision, leading to abasic sites that could result in DNA degradation in the presence of apurinic–apyrimidinic endonuclease (Yang, Chen, Zhang, Huang, & Zhang, 2007). For the few minus DNA strands that survive this attack, the dU in the minus template strand leads to dA during plus strand synthesis, resulting in the G to A mutation in the viral genome. This alters the viral opening reading frames and ultimately results in the mutation of codons leading to translation termination (R. S. Harris et al., 2003; Lecossier, Bouchonnet, Clavel, & Hance, 2003; Yang et al., 2007; H. Zhang et al., 2003). In addition, A3G restricts the replication of other retroviruses, such as simian immunodeficiency virus (SIV), equine infectious anemia virus (EIA), murine leukemia virus (MLV), human foamy viruses (HFV), human T-lymphotropic virus (HTLV-1), and human hepatitis B virus (HBV) at various levels through its enzymatic deaminase activity

(Y. L. Chiu & Greene, 2009).

Figure 32. In the Absence of Vif- Canonic Pathway of A3G restricting HIV-1. In the absence of Vif, A3G is incorporated into viral particle and upon second round infection, A3G will target minus-strand DNA during reverse transcription and lead to C to U mutation. Then 2 things will happen. . One is with the abnormal presence of U in DNA strand, it will be recognized by uracil-DNA glycosylase and subject to destruction or, second strand will still be synthesized. As A pairs with U, it will end up with a G to A hypermutation in the viral genome ultimately leading to G-to-A hypermutations in the viral genome. Also, A3G can function in deaminase independent manner (Chiu, Y. L et al., (2009). *Philosophical Transactions of the Royal Society of London B: Biological Sciences*).



### ***Deaminase-independent pathway***

Human A3G also functions in deaminase-independent manner by inhibiting reverse transcription and integration. The incorporation of A3G into the HIV-1 $\Delta$ vif virions also reduces the ability of tRNA<sup>Lys3</sup> to prime reverse transcription by over 50 percent (F. Guo, Cen, Niu, Saadatmand, & Kleiman, 2006). A3G also reduces the efficiency and specificity of primer tRNA processing and removal of tRNA<sup>Lys3</sup> during plus-strand DNA transfer, leading to the formation of aberrant viral DNA ends that ultimately hinders the integration of PIC into host chromosome (Mbisa et al., 2007). Finally, the N-terminal linker region of A3G is a docking site for the C-terminal domain of HIV-1 integrase. The association of A3G protein with integrase may prevent the entry of PIC into the nucleus and impair viral DNA integration. (Luo et al., 2007)

### **3. APOBEC3/Vif Interactions**

Human A3G expression in non-permissive cells significantly restricts the replication and spread of HIV-1 $\Delta$ vif. However, it is incapable of restricting wild type HIV-1 replication since the viral protein Vif counteracts the action of human A3G. Vif serves as an adaptor protein in organizing the Vif–CBF $\beta$ –CUL5–ELOB–ELOC–Rbx2 pentameric complex that ultimately results in the polyubiquitylation and degradation of both A3G and Vif (Jager et al., 2011). First, Vif contains a SOCS-box motif to serve as a receptor for a Cullin-RING E3 ubiquitin-ligase complex consisting of Cul5, EloB and EloC, and the RING protein, Rbx2. Vif then specifically binds and recruits the substrate APOBEC3 proteins (A3C, D, F, G, or H) to the E3 ubiquitin ligase for covalent modification with polyubiquitin chains, which act as a target for proteasomal degradation (Salter, Morales, & Smith, 2014). Additionally, two independent groups identified that a cellular chaperone protein, core binding factor  $\beta$  (CBF- $\beta$ ), is required for the stabilization



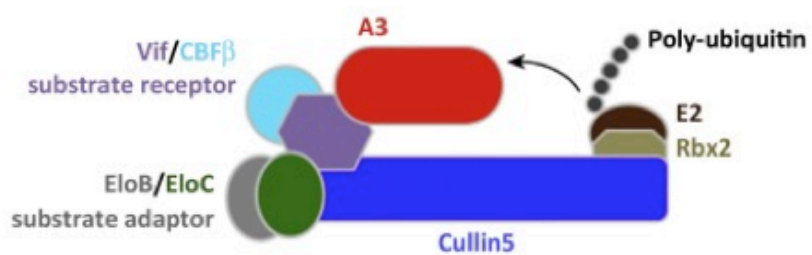
of the interactions among Vif, the E3 ubiquitin-ligase complex, and APOBEC3 (Jager et al., 2011; W. Zhang, Du, Evans, Yu, & Yu, 2011).

Specifically, the N-terminal region of Vif binds to an N-terminal region of A3G (amino acids 54–124); the SLQ(Y/F)LA motif (amino acids 144–150) of Vif mediates binding to the elongin C (EloC) component of the E3 ligase complex; the zinc-binding motif, (HCCH, amino acids 108–139) within Vif, containing two conserved cysteines, mediates a second interaction with the Cullin 5 (Cul5) component; and CBF- $\beta$  forms three critical interactions with the N-terminus and zinc-binding domain of Vif. The complex of Vif- CBF- $\beta$ -EloB-EloC-Cullin5-Rbx2 E3 ubiquitin-ligase complex is depicted in Figure 33 (Y. L. Chiu & Greene, 2009; Conticello, Harris, & Neuberger, 2003; Y. Guo et al., 2014; Marin, Rose, Kozak, & Kabat, 2003; Sheehy, Gaddis, & Malim, 2003).

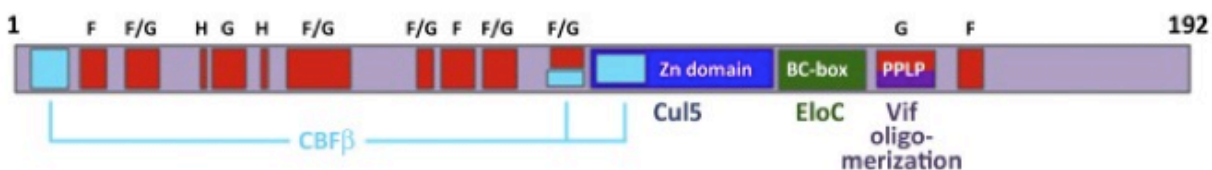
Additionally, Vif is capable of down-regulating A3G expression level by partially impairing the translation of A3G mRNA. Unlike the well-characterized Vif-induced degradation of A3G, the functional role of the inhibition of A3G translation by Vif remained unclear until Santiago and colleagues reported that Vif requires stem-loops 2 and 3 in the 5'UTR of A3G mRNA to inhibit translation (Guerrero et al., 2016). The specific molecular mechanism still remains unknown. However, this down-regulation is possible through direct interaction since Vif binds to a few high-affinity binding sites in this region (Guerrero et al., 2016; Mercenne et al., 2010; Stopak, de Noronha, Yonemoto, & Greene, 2003). Investigators have also found that the proteasomal degradation and translation inhibition of A3G by HIV-1 Vif are two independent pathways since “K26R mutation in Vif abolishes degradation of A3G by the proteasome but has no effects at the translational level” (Guerrero et al., 2016). These two pathways also appear to be redundant since the inhibition of A3G translation alone in the absence of proteasomal

Figure 33. Cartoon depiction of a Vif-CBF- $\beta$ -EloB-EloC-Cullin5-Rbx2 E3 ubiquitin-ligase complex. (A) Schematic depiction of Vif-mediated E3 ubiquitin-ligase complex. A3 proteins (red) are recruited to the N-terminal half of Cul5 (blue) through specific interaction with Vif (purple). EloB/EloC substrate adaptor (grey and green) bound to the Vif facilitates the association between Vif and Cul5. CBF- $\beta$  stabilizes interactions of Vif with Cul5 and A3 substrates. The C-terminal half of Cul5 bound to Rbx2 recruits an E2 ubiquitin conjugation enzyme responsible for ubiquinating A3 proteins. (B) Functional motifs of Vif involved in protein-protein interactions of the E3 ubiquitin ligase complex. Regions of Vif that interact with CBF $\beta$ , Cul5, EloC, are colored cyan, blue, and green respectively. Regions implicated in binding A3 proteins are color highlighted in red. (Salter, J. D, et al. (2014). Structural insights for HIV-1 therapeutic strategies targeting Vif. *Trends in Biochemical Sciences*, 39(9), 373-380).

(A) Cartoon depiction of a Vif-CBF $\beta$ -EloB-EloC-Cullin5-Rbx2 E3 ubiquitin-ligase complex.



(B) Schematic diagram of Vif's interacting motifs.



degradation is sufficient to partially restore viral infectivity (Guerrero et al., 2016).

#### **4. Restriction by other human A3 proteins**

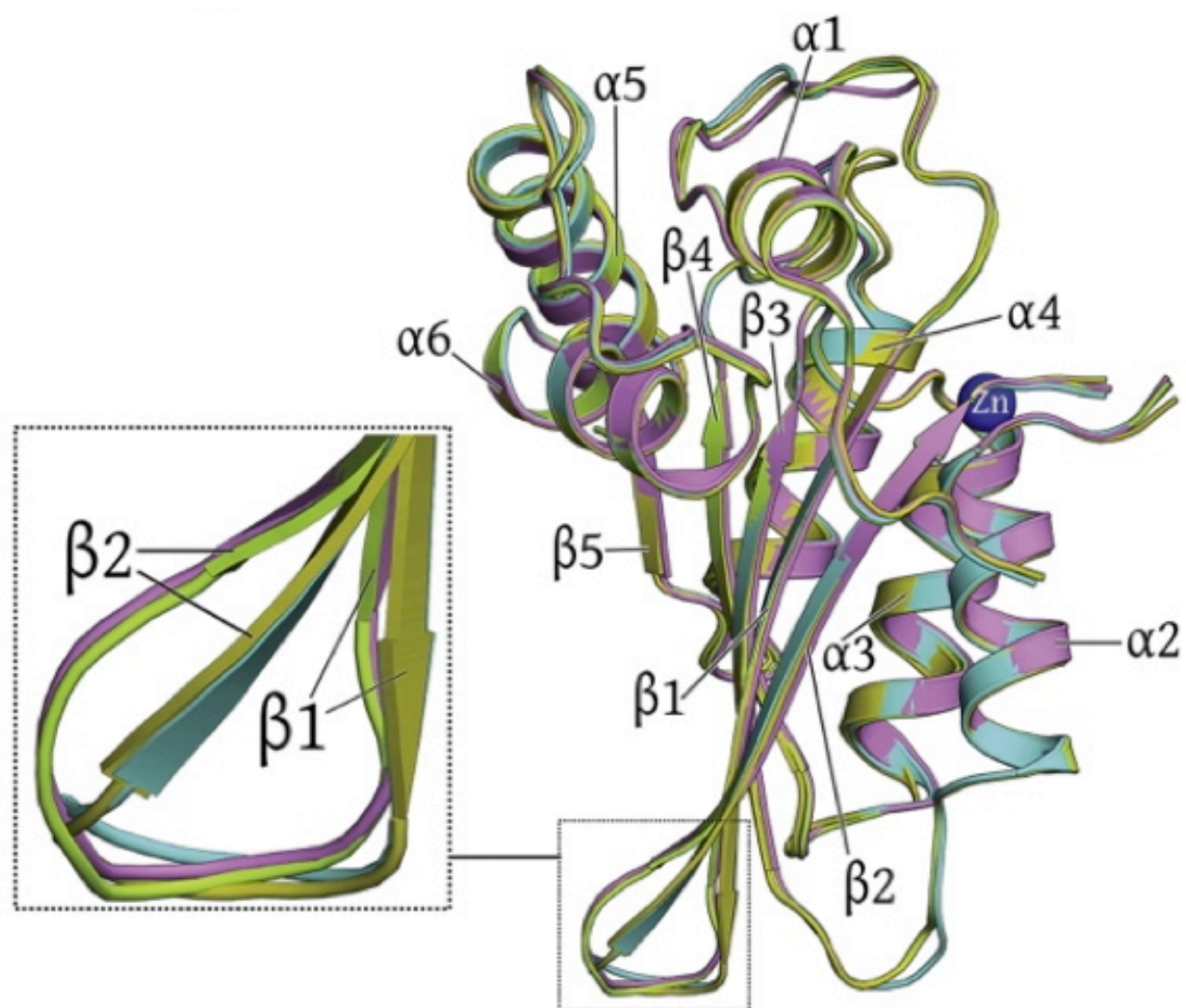
##### ***A3F***

A3F is the most closely related member to A3G in the APOBEC3 family with a sequence identity of approximately 50 %, and most significantly, their N termini (first 60 amino acids) are nearly identical. Although less potent than A3G, A3F shows a similar antiviral activity against HIV-1, which is antagonized by HIV-1 Vif (Bishop, Holmes, Sheehy, Davidson, et al., 2004; Liddament, Brown, Schumacher, & Harris, 2004; Takaori-Kondo, 2006). In addition, it is able to restrict simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), and foamy viruses (FV) replication to equivalent level as A3G (Delebecque et al., 2006; Zielonka et al., 2009).

As mentioned earlier, A3F contains two CD domains. The structure of A3F-CTD was resolved in 2013. It has “a canonical DNA cytosine deaminase fold, composed of five  $\beta$  strands, six  $\alpha$  helices, and the catalytic zinc-binding site” (Bohn et al., 2013). Investigators also identified that “the zinc-coordinating residue (C283) and the catalytic residue E251 are located on helices  $\alpha$ 2 and  $\alpha$ 3 and define the catalytic pocket” (Bohn et al., 2013). The structure of human A3F-CTD is shown in Figure 34.

Similar to A3G, A3F is incorporated into progeny virions and induces G-to-A hypermutation, to a less extent compared to A3G (at least 10 times less than the antiviral activity of A3G) (Bishop, Holmes, Sheehy, Davidson, et al., 2004; Liddament et al., 2004; B. Liu, Sarkis, Luo, Yu, & Yu, 2005). As with A3G, the enzymatic C-terminal CD domain mediates deamination whereas the non-enzymatic N-terminal domain is responsible for efficient

Figure 34. A3F-CTD structure. The A3F-CTD has a canonical cytosine deaminase fold with five  $\beta$  strands and six  $\alpha$  helices and the catalytic zinc-binding site. The  $\beta$ 1- $\beta$ 2 loop region (inset) has conformational plasticity (Bohn, M. F, et al. (2013). Crystal structure of the DNA cytosine deaminase APOBEC3F: the catalytically active and HIV-1 Vif-binding domain. *Structure*, 21(6), 1042-1050).



packaging into virions (R. K. Holmes, Koning, Bishop, & Malim, 2007; Jonsson et al., 2006).

Despite the shared features and sequence homology between A3F and A3G, A3F targets a different consensus dinucleotide 5'-TC (substrate cytidine underlined) for deamination.

Moreover, A3F also appears to be capable of inducing C-to-T mutations in plus-stranded HIV-1 cDNA at low levels (Bishop, Holmes, Sheehy, Davidson, et al., 2004; Bishop, Holmes, Sheehy, & Malim, 2004). Interestingly, the cytidine deaminase activity of A3F is not absolutely required for its antiviral activity (R. K. Holmes et al., 2007). In addition to the canonical hypermutation antiviral strategy, both A3G and A3F have been found to interact with HIV-1 integrase, hence inhibiting proviral DNA formation (Luo et al., 2007).

Although A3F is also targeted by HIV-1 Vif through the recruitment of the Cullin5-elongin B/C-Rbx2 ubiquitin ligase complex for proteasomal degradation similar to A3G, A3F is less efficiently degraded by Vif-activated pathways than A3G (Marin, Golem, Rose, Kozak, & Kabat, 2008). In addition, these two enzymes require distinct determinants for interactions with Vif. Amino acids <sup>40</sup>YRHHY<sup>44</sup> of Vif are essential for A3G binding while <sup>14</sup>DRMR<sup>17</sup> of Vif is critical for A3F binding (Russell & Pathak, 2007; V. Simon et al., 2005; Tian et al., 2006). Substituting the critical binding sites for A3F/A3G in Vif to alanine revealed that A3G exerts a greater restriction effect on HIV-1 than A3F and A3DE in primary CD4<sup>+</sup> T cells and macrophages (Chaipan, Smith, Hu, & Pathak, 2013). Investigators also identified that the <sup>289</sup>EFLARH<sup>294</sup> region, and E<sup>324</sup> of hA3F as critical for interactions with HIV-1 Vif and proteasomal degradation (Albin et al., 2010; J. L. Smith & Pathak, 2010). In addition, substitution of lysine at position 128, a residue critical for A3G and HIV-1 Vif association, does not affect the interaction between A3F and Vif (B. Liu et al., 2005). In addition to their most potent anti-HIV-1 activities among the APOBEC family, A3G and A3F are both expressed in

human CD4<sup>+</sup> T cells, which is the principal target for HIV-1 infection *in vivo*. This further supports the central role of both enzymes as innate host restriction factors that can naturally encounter HIV-1, and the virus evolves different determinants in Vif to evade the anti-viral activities of each (Franca, Spadari, & Maga, 2006; Russell & Pathak, 2007).

### **A3B**

A3B only has a weak anti-HIV-1 activity in cell culture assays. A3B contains two CDA that are both enzymatically active. The inhibition of HIV-1 by A3B protein is at least partly independent of DNA editing since inactivation of both catalytic domains didn't completely abolish its antiviral activity (Bogerd et al., 2007; Doehle, Schafer, & Cullen, 2005; Malim, 2009). Interestingly, this restriction is not relieved by HIV-1 Vif protein. Therefore, A3B is able to restrict wild-type HIV-1 replication to a limited extent (Doehle et al., 2005). Unfortunately, endogenous A3B is barely expressed in primary human lymphoid cells including CD4<sup>+</sup> T cells, thus is unlikely to significantly reduce HIV-1 replication during natural infection. However, this dilemma also raises the possibility of a novel strategy for inhibiting HIV-1 replication *in vivo* through activation of the endogenous A3B gene expression in primary human lymphoid cells (Doehle et al., 2005).

In addition to its anti-HIV-1 activity, A3B has been revealed to restrict other exogenous viruses and endogenous retrotransposons. It is able to inhibit human T-lymphotropic virus type 1 (HTLV-1) in a deaminase-dependent manner (Ooms, Krikoni, Kress, Simon, & Munk, 2012). A3B is capable of suppressing HBV replication in hepatocytes, and has been reported to result in less persistence, and a lowered risk for hepatocellular carcinoma (HCC) development (T. Zhang et al., 2013). A3B restricts HBV by inhibiting a positive regulator of



HBV expression, heterogeneous nuclear ribonucleoprotein K (hnRNP K), thus reducing its transcription. A3B is also a potent inhibitor of core-associated HBV DNA replication (W. Zhang et al., 2008). It has been shown that A3B expression is upregulated by HPV E6/E7 oncoproteins. A cellular zinc-finger protein, ZNF384, plays an important role in the proximal-region-mediated activation of A3B by E6 (Mori, Takeuchi, Ishii, & Kukimoto, 2015). Additionally, investigators found that endogenous A3B appears to restrict Long Interspersed Nuclear Element-1 (LINE-1 or L1) retrotransposition in a broad range of cell types, including pluripotent human embryonic stem cells (hESCs) (Wissing, Montano, Garcia-Perez, Moran, & Greene, 2011).

Moreover, A3B has been revealed to play a role in cancer development (Ooms et al., 2012). A3B is the only family member of APOBEC that has steady-state nuclear localization (Lackey et al., 2012). The presence of A3B in the nucleus provides A3B with the potential to edit cellular DNA. It has been reported that A3B can impair genomic stability by inducing base substitutions in genomic DNA in human cells (Shinohara et al., 2012). The investigators also found that “A3B is highly expressed in several lymphoma cells and somatic mutations occur in some oncogenes of the cells highly expressing A3B” (Shinohara et al., 2012). Additionally, A3B mRNA was found to be overexpressed in tumors and a clinical study showed that A3B high expression status is associated with aggressive phenotype such as lymph node metastasis and pathological nuclear grade in Japanese breast cancers (Tsuboi et al., 2016).

### **A3A**

A3A is generally thought to have no anti-HIV-1 activity (Franca et al., 2006; R. S. Harris & Liddament, 2004; Malim, 2009). However, investigators later found that A3A is critical in monocyte resistance to HIV-1 (Peng et al., 2007). It contains a single CDA domain, is

naturally expressed in immature monocytes, and can be induced by IFN- $\alpha$ . A more detailed discussion of human A3A and its antiviral activity will be discussed later.

### **A3C**

A3C also contains a single CDA domain. It is expressed in CD4<sup>+</sup> T cells, and can act in target cells to introduce limited G-to-A mutations at sub-lethal levels during infection by some HIV-1 strains. Since the A3C induced G-to-A mutation doesn't abolish the viral replication, it may instead contribute to viral fitness and diversity, helping virus to escape from immune surveillance and antiviral drug treatment (Bourara, Liegler, & Grant, 2007; Malim, 2009). A3C is sensitive to Vif-mediated degradation. Specifically, residues Leu72, Phe75, Cys76, Ile79, Leu80, Ser81, Tyr86, Glu106, Phe107 and His111 in A3C are critical for forming interaction with Vif, while Arg122 is important for A3C encapsidation into progeny virions (Kitamura et al., 2012; Stauch et al., 2009). The A3C and Vif interface is marked by a hydrophobic bottom and hydrophilic edges (Kitamura et al., 2012). Investigators found that A3C dimerization is critical for its antiviral activity (Stauch et al., 2009).

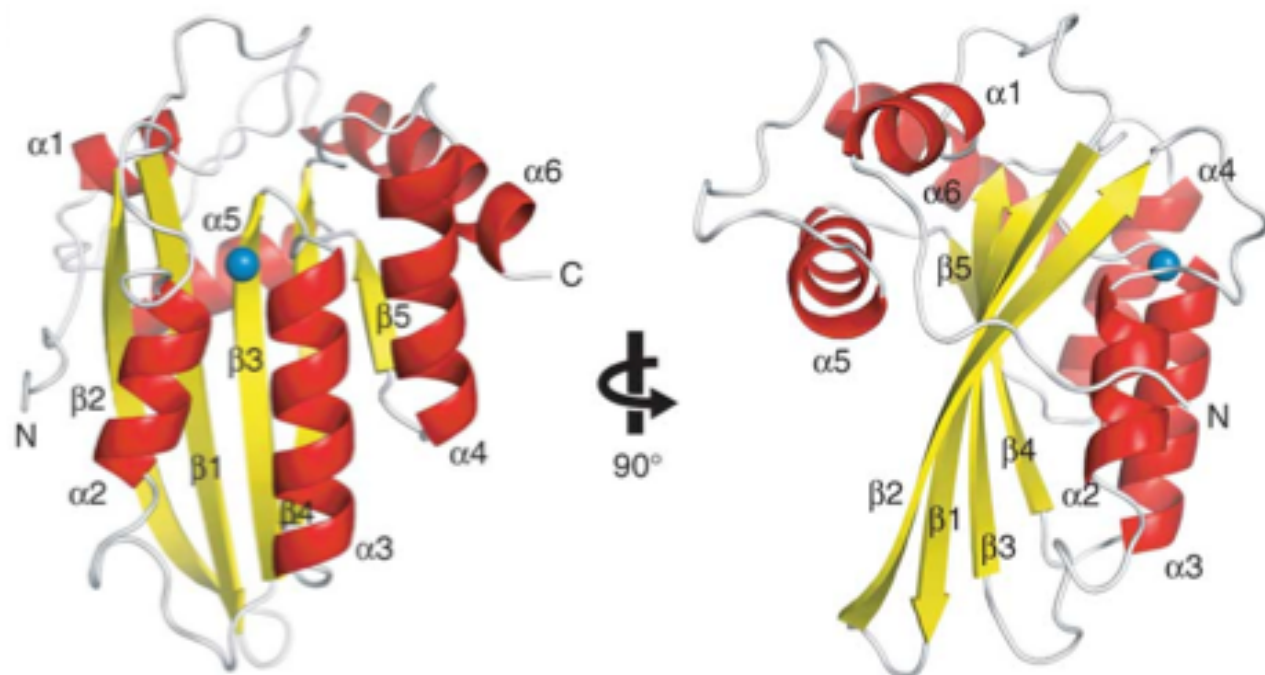
In addition to its weak antiviral activity to HIV, human A3C can effectively restrict SIVmac $\Delta$ Vif and SIVagm $\Delta$ Vif (Q. Yu et al., 2004). Studies have shown that the counteraction of Vif and A3C is species specific that Vif proteins from diverse HIV/SIV lineages have distinct interaction interfaces with A3C (Z. Zhang et al., 2016). It can also inhibit the replication of human papillomavirus (HPV) infectivity (Ahasan et al., 2015). The restriction of HPV is not mediated by hypermutation, but possibly through a direct interaction between A3C and HPV16 pseudovirion capsid protein L1 (Ahasan et al., 2015).

The crystal structure of full-length human A3C has been resolved in 2012. “The A3C structure has a core platform composed of six  $\alpha$ -helices ( $\alpha 1$ – $\alpha 6$ ) and five  $\beta$ -strands ( $\beta 1$ – $\beta 5$ ), with a coordinated zinc ion that is well conserved in the cytidine deaminase superfamily” (Kitamura et al., 2012), as shown in Figure 35. The core structure of A3C and A3G C-terminus domain (A3G CTD) are similar, while their loop structures are distinct. The  $\beta 2$ -strand of A3C exhibits a continuous well-ordered characteristics with high stability, which is remarkably different from that of the A3G CTD (Kitamura et al., 2012).

### **A3D**

A3D is previously referred as A3D/E. A3D can also modestly inhibit HIV-1 infection when overexpressed in virus producing cells. In the absence of Vif, A3D can be packaged into progeny virions and is capable of inducing G-to-A mutations in viral minus strand during reverse transcription at a lower frequency (0.13%) compared to A3G (0.5%). A3D targets a novel dinucleotide site 5'CC3' (substrate cytidine underlined) and is expressed at higher levels than A3F in non-permissive cells including MT4, H9, PM1, Hut78, JM1, and CEM cells and primary peripheral blood lymphocyte (PBL), and is broadly expressed in tissues in a more abundant manner (Dang, Wang, Esselman, & Zheng, 2006). In addition, it forms heteromultimers with A3F or A3G in the cell, which might contribute to the intracellular defense network (Dang et al., 2006; Malim, 2009). A3D is also sensitive to Vif-mediated degradation. Vif mediates A3D polyubiquitylation by Cul5-based E3 ligases, which is further degraded in the 26S proteasome (Dang et al., 2006). Ten residues in A3D (Leu268, Phe271, Cys272, Ile275, Leu276, Ser277, Tyr282, Glu302, Phe303 and His307) are important for Vif interaction (Kitamura et al., 2012).

Figure 35. The X-ray crystal structure of full-length A3C. Two views of the A3C structure, rotated by 90°. A3C contains six  $\alpha$ -helices (red) and five  $\beta$ -strands (yellow). The zinc is represented as a blue sphere (Kitamura, Shingo, et al. "The APOBEC3C crystal structure and the interface for HIV-1 Vif binding." *Nature Structural & Molecular Biology* 19.10 (2012): 1005-1010).



Similar to A3F, <sup>14</sup>DRMR<sup>17</sup> motif in Vif is necessary for the degradation of APOBEC3D (Sato et al., 2014). In addition to its antiviral activity to HIV-1, it also restricts the replication of SIV<sub>agm</sub>, and SIV<sub>mac</sub>, but is not able to inhibit Murine leukemia virus (MLV) (Dang et al., 2006).

A3D, similar as A3G, A3H, and A3F, fundamentally works as restriction factor against HIV-1 by inducing hypermutation in the viral genome. However, at the same time, the mutations may positively contribute to viral diversification and evolution *in vivo*. Investigators have shown that viruses are specifically and significantly diversified in a humanized mouse model (Sato et al., 2014).

Interestingly, it has recently been reported that human A3D is able to antagonize A3F and A3G restriction of HBV replication through direct binding to itself, A3F, and A3G proteins. Thus, A3D suppresses the antiviral activity of A3F and A3G, resulting in an enhanced HBV replication (Bouzidi et al., 2016).

### ***A3H***

Human A3H is the only APOBEC3 protein that contains a single copy of a Z3-type APOBEC3 catalytic domain. Several studies revealed that several stable APOBEC3H haplotypes are present *in vivo* and act as barriers to HIV-1 $\Delta$ vif propagation, and Vif is capable of adapting to these restrictive pressures, reaching for an evolutionary equilibrium (Ooms et al., 2013; Refsland et al., 2014).

There are several single-nucleotide polymorphisms (SNPs) been found in A3H gene, making it as the most polymorphic member of the APOBEC3 family. Seven haplotypes (hap I–VII) and four mRNA splicing variants (SV) of A3H have been identified (Gu et al., 2016). Different haplotypes possess great variability in protein stability, subcellular distribution, and/or

RNA binding and virion packaging (Gu et al., 2016; Zhen, Du, Zhou, Xiong, & Yu, 2012). There are primarily four haplotypes circulating in human population: HapI, 18R/105G/121K/178E; HapII, 18R/105R/121D/E/178D; HapIII, d15N/18R/105R/121D/E/178D; and HapIV, d15N/18L/105R/121D/E/178D (Zhen, Wang, Zhao, Xiong, & Yu, 2010). It has been observed that functional APOBEC3H polymorphisms are associated with the susceptibility to HIV-1 infection and progression to AIDS *in vivo* (Sakurai et al., 2015).

A3H\_HapII and A3H\_HapI differ only three amino acids. However, they share distinct characteristics and antiviral profiles. A3H\_HapII is the most stable and restricts HIV-1 $\Delta$ Vif at the highest level (Harari, Ooms, Mulder, & Simon, 2009). Similar to A3G, A3H\_HapII induces proteasome-mediated degradation in the presence of Vif. The E121 residue in hA3H hapII plays a critical role in determining its sensitivity to HIV-1 Vif derived from the NL4-3 strain. Noteworthy, the interaction between A3H\_HapII and Vif is mediated through unique substrate recognition domains of Vif that are distinct from those for the human A3G and A3F proteins. Although A3H\_HapI is less stable to A3H\_HapII, investigators have found that it is insensitive to Vif-mediated degradation. Further, a single amino acid change in HapI (G105R) greatly stabilized the protein and increased its antiviral activity against wild type HIV-1 (Zhen et al., 2010).

A study comparing primate A3H sequences revealed that a premature termination codon was identified on the fifth exon of the human and chimpanzee A3H genes, leading to a significant decrease in their mRNA and protein expression. Specifically, the investigators found that the truncated protein was as stable as the full-length protein while the truncated mRNA is very unstable. Restoring full length A3H expression by correcting the premature termination codon allows human A3H to reduce HIV-1 infectivity up to 150-fold through a deamination-

independent mechanism. Despite the fact that HIV-1 Vif is capable of to form a direct association with the optimized A3H (A3H helices  $\alpha 3$  and  $\alpha 4$  interact with the Vif  $\beta$ -sheet ( $\beta 2$ - $\beta 5$ ) (Ooms, Letko, & Simon, 2016)), it is unable to counter the restriction carried by A3H (Dang et al., 2008; Malim, 2009; Sakurai et al., 2015). Thus, optimizing the expression of endogenous A3H *in vivo* could be a novel strategy of anti-HIV therapy (Dang et al., 2008). However, this study didn't specify which haplotype of A3H was studied.

A3H\_HapII can be packaged in human T-lymphotropic virus (HTLV-1) particles and has been found to restrict HTLV-1 replication. Interestingly, this restriction is acted in a deaminase-independent manner, thus A3H hapII does not require an intact deaminase domain for function (Ooms et al., 2012).

Noteworthy, A3H\_HapI is thought to contribute to breast and lung cancer mutagenesis. Previously, A3B was shown to play a critical role in causing cytosine mutations within TCA/T motifs. However, it has been observed that A3B-null breast tumors still have this mutational bias. The investigators were surprised to find that a previously deemed unstable and inactive variant - A3H\_HapI showed a statistically significant association with the mutations. They further testified that A3H\_HapI is catalytically active through biochemical and cell-based assays. In addition, A3H\_HapI has increased nuclear localization, providing a mechanism for accessing genomic DNA. (Starrett et al., 2016).

## **D APOBEC3A and HIV-1**

### **1. Human A3A**

Human A3A belongs to the APOBEC family and has only one zinc-coordinating domain (Holden et al., 2008; Kitamura et al., 2012). Human A3A has been shown to play an

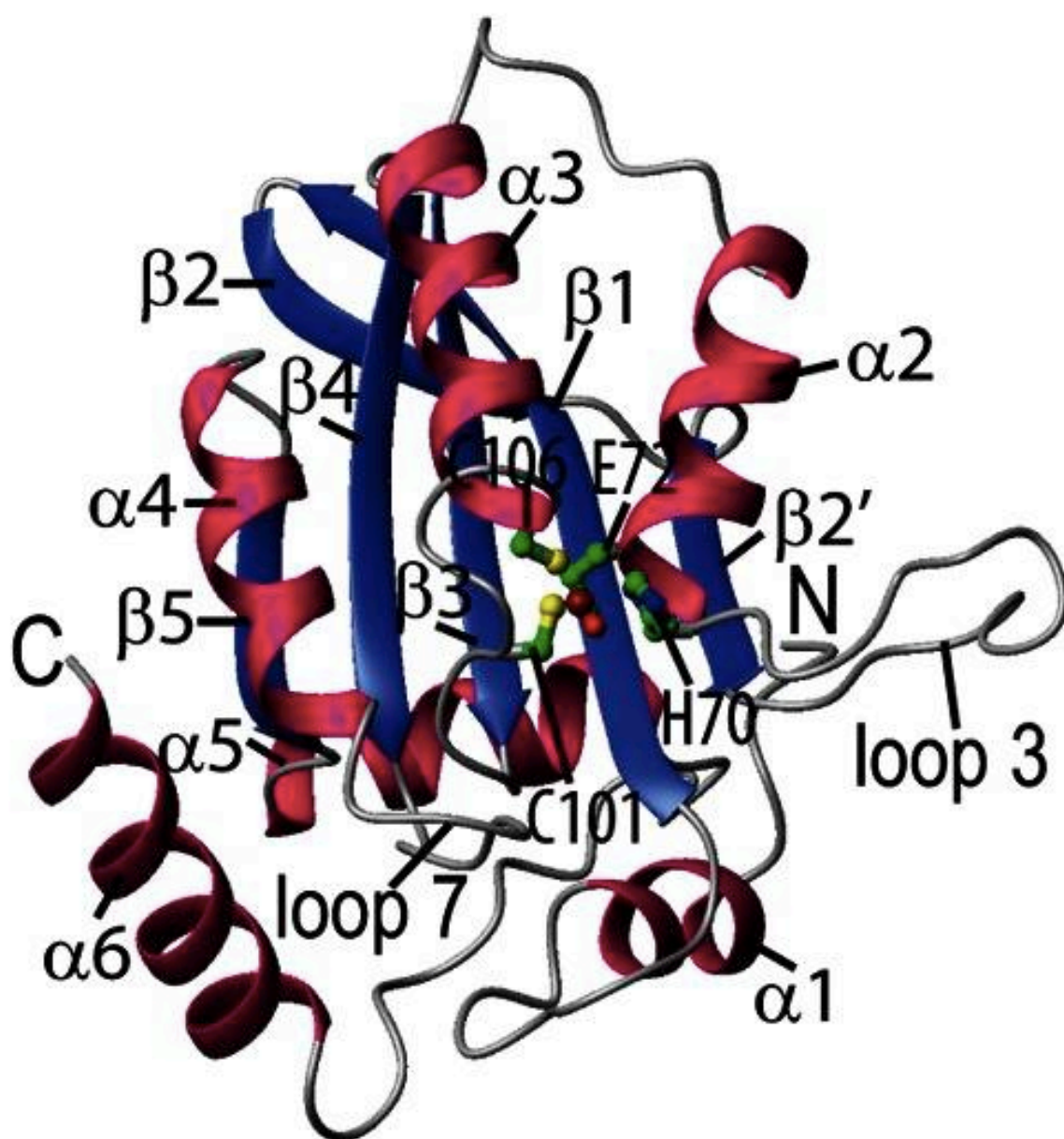


important role in the innate restriction by displaying a broad range of activities against exogenous viral pathogens and endogenous retrotransposons (Y. L. Chiu & Greene, 2008).

The nuclear magnetic resonance (NMR) structure of A3A (residues 10-194 of 199 aa) has been resolved. The structure consists of six helices surrounding a central  $\beta$  sheet of five strands (shown in Figure 36), which is common to all known APOBEC protein structures (Byeon et al., 2013). Specifically, A3A NMR structure is most similar to the X-ray structure of the A3G-CTD (residues 191-384), with some differences in the loop region. An interesting feature of hA3A is the presence of four amino acids between the two cysteine residues of the canonical deaminase domain. The group also shows that A3A binds TTCA- or CCCA-containing single-stranded oligonucleotides ( $\geq 9$  nt). It has been noted that A3A binds to ssDNA much more weakly ( $\sim 1000$ -fold) than A3G (Byeon et al., 2013). Interestingly, the NMR experiment shows that RNA binding is specific, but almost tenfold weaker than binding of the corresponding 9-nt DNA. However, gel shift assays with a 40nt of ssRNA and ssDNA results in a different conclusion: the binding efficiency is three folds higher for ssRNA than for the corresponding 40-nt ssDNA. This observed difference in the binding affinity may attribute to the differences in the EMSA and NMR, as well as the specific and non-specific binding to a long RNA (Byeon et al., 2013; Mitra et al., 2014).

Human A3A was previously thought to exist as monomers. However, a recent salient finding showed that A3A actually forms homodimers and the dimerization creates a highly positive groove that connects the active site of both monomers, allowing the A3A homodimers to recognize substrate cooperatively with high affinity and specificity. The investigators also identified that H11/H56 and H16/K30 are critical for cooperative DNA binding (Bohn et al., 2015).

Figure 36. Ribbon representation of human A3A based on NMR structure. It consists of six helices (hot pink) surrounding a central  $\beta$  sheet of five strands (royal blue) (Byeon, In-Ja L., et al. "NMR structure of human restriction factor APOBEC3A reveals substrate binding and enzyme specificity." *Nature Communications* 4 (2013): 1890).



Human A3A has been involved in regulating the activity of many viruses.

Importantly, human A3A can act as a specific inhibitor of the early phases of HIV-1 infection in myeloid cells such as dendritic cells and macrophages (Berger et al., 2011). Human A3A is expressed at high levels in cells of the myeloid lineage including monocytes and macrophages, and its expression is stimulated in response to interferon-alpha (IFN-  $\alpha$ ) (Koning et al., 2009; Peng et al., 2007; Refsland et al., 2010; Thielen et al., 2010). The high level of A3A expression explains why myeloid cells are generally resist to HIV-1 infection during the early phase of infection. In addition, silencing of A3A in monocytes was associated with increased susceptibility to HIV-1 infection. Finally, A3A-specific editing of HIV-1 minus-strand DNA during reverse transcription was found to occur in infected myeloid cells, suggesting a role for A3A deaminase activity in HIV-1 restriction (Berger et al., 2011; Peng et al., 2007).

In another study, it was shown that A3A, together with A3C and A3H, are expressed in keratinocytes and skin, and is able to edit human papillomavirus (HPV) DNA. As a result, this editing contributes to the initial benign and precancerous lesions, eventually promoting skin cancer development (Vartanian, Guetard, Henry, & Wain-Hobson, 2008). In contrast, another study suggests that human A3A actually exerts a protective effect by significantly suppressing HPV propagation. They first noticed that HPV-positive cervical and head-and-neck cancers exhibited higher rates of APOBEC3 mutation signatures than most HPV-negative cancers. Then they found that hA3A expression levels are highly elevated in HPV-positive keratinocytes and cervical tissues in early stages of cancer progression. Importantly, the team reported that HPV virions assembled in the presence of human A3A have significantly lower infectivity compared to HPV virions assembled in the absence of A3A or with the presence of a catalytically inactive mutant, hA3A/E72Q. However, no evidence of mutations was found (Ahasan et al., 2015).

Additionally, knocking-down A3A in human keratinocytes results in a significant increase in HPV infectivity (Warren et al., 2015). Thus, human A3A may act as a double-edged sword: it serves as restriction factor against HPV infection, while contributing to cancer mutagenesis at a cost.

Investigators also found that the alpha-retrovirus, Rous sarcoma virus (RSV), is susceptible to inhibition by all human APOBEC proteins tested including human A3A. The fact that alpha-retroviruses resides in avian hosts and usually do not have access to human APOBEC3 proteins in their normal life cycle, but are susceptible to restriction of all human APOBEC3 proteins, “suggests that the resistance of mammalian retroviruses to inhibition by the APOBEC3 proteins expressed in their normal host species is likely to have evolved subsequent to the appearance of this family of mammalian antiretroviral proteins some 35 million years ago; i.e., the base state of a naive retrovirus is susceptibility to inhibition.” (Wiegand & Cullen, 2007)

The single-stranded DNA viruses, parvovirus, is also a target of human A3A (H. Chen et al., 2006; Narvaiza et al., 2009). The first report reveals that human A3A potently inhibits parvovirus adeno-associated virus (AAV) replication. Although the conserved amino acids of the hA3A active site are needed, there are no mutations found in the retroelement sequences (H. Chen et al., 2006). In addition, a more recent study confirmed that deaminase activity is not required for the antiviral activity of A3A against parvoviruses. Through generation the chimeric proteins of A3A and A3G, they were able to define that the VS1 region of A3A (residues 60 to 67) contributes to the antiviral activity against parvovirus (Narvaiza et al., 2009).

Human A3A is also able to inhibit human T-lymphotropic virus type 1 (HTLV-1) in a deaminase dependent manner. The investigators showed that human A3A was efficiently

packaged in HTLV-1 virions, and a catalytic-site mutant abolished HTLV-1 restriction (Ooms et al., 2012).

More recently, a study analyzed the restriction of the retrovirus murine leukemia virus in hA3A and hA3G transgenic mice in a mouse A3-knockout background (Stavrou et al., 2014). These investigators showed that hA3G and hA3A restricted murine retroviruses by different mechanisms. They observed that hA3G was packaged into virions and caused extensive deamination of the viral genome, while hA3A was not packaged into virions and restricted infection when expressed in target cells (Stavrou et al., 2014). This study also found that hA3A can restrict murine retroviruses by a deamination-independent mechanism (Wang, Schmitt, et al., 2016).

In addition, human A3A is capable of recognizing and degrading foreign DNA (Stenglein, Burns, Li, Lengyel, & Harris, 2010), inhibiting retrotransposition of LINE-1, *Alu*, and LTR retroelements. Interestingly, the signature mutation of A3A was not detected in LINE-1 and other retrotransposon DNA sequences (Bogerd et al., 2006; Kinomoto et al., 2007; Muckenfuss et al., 2006). Furthermore, it possesses a unique ability of deaminating 5-methylcytosine, a methylated form of the DNA base cytosine that is involved in the regulation of gene transcription, to thymine (T) (Carpenter et al., 2012; Wijesinghe & Bhagwat, 2012). It has been reported that overexpression of A3A in cells results in increased level of DNA breaks and activation of damage responses in a deaminase-dependent manner, leading to cell cycle arrest (Landry, Narvaiza, Linfesty, & Weitzman, 2011). However, those experiments are done using overexpression of exogenous A3A, while the endogenous A3A has been shown to reside primarily in cytoplasm, thus nongenotoxic. It should be noted that the antibody used to detect endogenous hA3A in the study was probably not specific to human A3A. More convincing proof

like identification of a cytoplasmic retention signal similar to that of human A3G is needed for further confirmation (Taggart et al., 2013). A study has shown that cellular genome becomes especially vulnerable to A3A editing during DNA replication when cytoplasmic A3A accesses nuclear DNA (Green et al., 2016). This genomic instability caused by A3A is relieved and balanced by the expression of Tribbles Homolog 3 (TRIB3), a human protein kinase induced by NF- $\kappa$ B. Either overexpression of A3A or knock-down of TRIB3 leads to elevated levels of nuclear DNA editing and cell cycle arrest (Aynaud et al., 2012).

## **2. Non-human primate A3A proteins**

This part is an excerpt from Wang, Y., Schmitt, K., Guo, K., Santiago, M. L., & Stephens, E. B. (2016). Role of the single deaminase domain APOBEC3A in virus restriction, retrotransposition, DNA damage and cancer. *Journal of General Virology*, 97(1), 1-17.

In contrast to hA3A, a previous study in our laboratory about rhesus macaques showed that rhesus macaque A3A (rhA3A) was capable of restricting the replication of Vif-deficient SHIV $\Delta$ vif at similar levels as rhesus macaque A3G (rhA3G) (Schmitt et al., 2011). Although another group observed that rhA3A did not restrict replication (Hultquist et al., 2011), this discrepancy was probably due to the position of the hemagglutinin (HA) tag fused to the rhA3A (Schmitt et al., 2013). Furthermore, hA3A was capable of significantly restricting SHIV $\Delta$ vif although not to the same extent as rhA3G (Schmitt et al., 2011). These results indicate that hA3A is capable of restricting lentiviruses other than HIV-1 $\Delta$ vif. Similar to the conclusions observed above for hA3A (Love, Xu, & Chelico, 2012), we observed that rhA3A was capable of cytidine deamination but not at the same frequency as rhA3G (Schmitt et al., 2011).

Our results with rhA3A raised the question of whether the A3A proteins from other non-human primates restrict HIV-1/SIV/SHIV and the importance of the 3 amino acids deletion in hominid A3A proteins. Recently, we examined the restriction properties of A3A proteins from an additional hominid (Lars gibbon; gibA3A), two additional Old World monkeys [African green monkey (agmA3A) and black and white colobus monkey (colA3A)] and two New World monkeys [squirrel monkey (sqmA3A) and northern owl monkey (nomA3A)] (Schmitt et al., 2013). Similar to hA3A, gibA3A did not restrict HIV-1 $\Delta$ vif or SHIV $\Delta$ vif, indicating that the lack of HIV-1 $\Delta$ vif restriction is probably conserved among hominids. Similar to rhA3A, we found that agmA3A and colA3A restricted the replication of SHIV $\Delta$ vif and that all three Old World monkey A3A proteins (agmA3A, colA3A, and rhA3A) restricted HIV-1 $\Delta$ vif to some extent, with mndA3A being the most effective. Among the New World monkeys, sqmA3A restricted SHIV $\Delta$ vif but not HIV-1 $\Delta$ vif, whereas nomA3A restricted neither SHIV $\Delta$ vif nor HIV-1 $\Delta$ vif (Schmitt et al., 2013). As the A3A proteins from select Old World monkeys could restrict HIV-1 $\Delta$ vif, we investigated the molecular determinants that define restriction. Using the distinct phenotypes of rhA3A (virus restriction) and hA3A (no virus restriction) in epithelial cells, a series of hA3A/rhA3A chimeric proteins were constructed and their restriction properties on HIV-1 $\Delta$ vif were analyzed (Schmitt et al., 2013). Our results showed that a chimeric A3A protein, designated rh<sub>25-33</sub>hA3A, which had the AC loop 1 region of hA3A replaced by the AC loop 1 region of rhA3A, restored the restriction properties of hA3A to a similar level as rhA3A restriction of SHIV $\Delta$ vif (Schmitt et al., 2013). Interestingly, rhA3A amino acids 25–33 map to the AC-loop1, and molecular modeling suggests that the AC-loop1 of rhA3A has a greater molecular surface compared with hA3A. Due to its predicted solvent accessibility, this additional



molecular surface may be an important region for protein–protein interactions that do not occur with hA3A.

The finding that sqmA3A restricted SHIV $\Delta$ *vif* is interesting, as the New and Old World monkeys branched approximately 30–35 million years ago with the separation of South America from Africa. To date, no lentiviruses have been isolated from New World monkeys. This brings up the question of whether some New World monkey A3A proteins may have evolved to restrict other viruses. One potential candidate is the foamy viruses (FVs), which are exogenous, persistent and non-pathogenic retroviruses in the subfamily *Spumaretrovirinae* (Switzer et al., 2005). FVs have been isolated from a broad range of mammals including non-human primates (both New and Old World monkeys), horses, cows and cats (Hussain et al., 2003; Meiering & Linial, 2001; Rethwilm, 2010). Previous studies have shown that the FVs have co-speciated with their mammalian hosts. For example, FVs express the Bet protein, which antagonizes A3 restriction and may play a role in both particle release and virus persistence (Alke et al., 2001; Chareza et al., 2012; Lochelt et al., 2005; Russell et al., 2005; Saib, Koken, van der Spek, Peries, & de The, 1995). A remarkable feature of the FVs is that they have the capacity to cross species barriers and pose a significant risk of interspecies transmission to humans (Heneine, Schweizer, Sandstrom, & Folks, 2003; Switzer et al., 2005). While no disease has been associated with the transmission of FVs to humans, the ease of primate-to-primate transmission implies the co-evolution of host restriction factors, as viral disease mechanisms are not sufficient to prevent cross-species transmission (A. S. Khan, 2009; Leendertz et al., 2008). As the FVs are promising candidates for the development of viral vectors for gene delivery and vaccination, it will be of interest to determine whether New and Old World monkey A3A

proteins are capable of restricting the replication of spumaviruses (Rethwilm, 2010; Schwantes, Truyen, Weikel, Weiss, & Lochelt, 2003; Trobridge, 2009).

## **Chapter II : Identification of three Old World Monkey A3A proteins that were able to inhibit WT HIV-1**

### **Abstract**

Previous studies have shown that human A3A (hA3A) normally does not restrict the replication of HIV-1 $\Delta$ *vif* in CD4<sup>+</sup> T cells. The exception is that, when expressed at high levels in macrophages and monocytes in response to IFN- $\alpha$ , hA3A restricts HIV-1 $\Delta$ *vif* without incorporation. The mechanism of restriction is still unknown (Berger et al., 2011; Bishop, Holmes, Sheehy, & Malim, 2004; Koning, Goujon, Bauby, & Malim, 2011; Thielen et al., 2010). Previous research in our lab revealed that A3A proteins from various hominid and non-human primates possess distinct restriction patterns towards HIV-1/SIV/SHIV. Similar to rhA3A, we found that agmA3A and colA3A restricted the replication of SHIV $\Delta$ *vif* and that all three Old World monkey A3A proteins (agmA3A, colA3A, and rhA3A) restricted HIV-1 $\Delta$ *vif* to some extent, with mndA3A being the most effective. More importantly, three OWM A3A-colA3A, mndA3A and debA3A were not only able to inhibit the replication of HIV-1 $\Delta$ *vif*, but also WT HIV-1 and HIV-1 $\Delta$ *vif*.

### **Introduction**

The inability of hA3A to restrict HIV-1 $\Delta$ *vif* has been linked to its poor incorporation into the nucleoprotein complex (NPC) during maturation. Targeting hA3A to the nucleoprotein complex by either fusion to the N-terminal domain of A3G or fusion to Vpr results in enhanced incorporation and restriction activity (Aguiar, Lovsin, Tanuri, & Peterlin, 2008; Goila-Gaur, Khan, Miyagi, Kao, & Strebel, 2007). In contrast to hA3A, a study in rhesus macaques showed that rhesus macaque APOBEC3A (rhA3A) is capable of restricting both simian–human

immunodeficiency virus (SHIV $\Delta$ vif) and human immunodeficiency virus (HIV-1 $\Delta$ vif) to a greater extent than hA3A. More impressively, previous work in our lab showed that the A3A protein from the black and white colobus monkey (mantled guereza; *Colobus guereza*; colA3A) potently inhibited not only HIV-1 $\Delta$ vif replication but also wild-type HIV-1 in producer cells (Schmitt et al., 2013) Sequence alignment of various A3As revealed an important region - AC loop 1 in determining the restriction pattern against lentiviruses. Thus, we analyzed the ability of A3A proteins from *Mandrillus sphinx* (mndA3A) and *Cercopithecus neglectus* (debA3A), which have similar AC-loop1 region as colA3A, to restrict HIV-1.

## Results

### Cells expressing HA-colA3A inhibit HIV-1 production

293 cells were co-transfected with the vector expressing colA3A and either HIV-1 or HIV $\Delta$ vif. In the presence of colA3A, HIV-1 and HIV $\Delta$ vif released 0.1% p24 (HIV-1: mean of 0.58 ng/ml, range of 0.21 to 0.91 ng/ml; HIV-1 $\Delta$ vif: mean of 0.48 ng/ml, range of 0.18– 0.60 ng/ml) compared to the other A3A proteins (HIV-1: mean of 1110 ng/ml, range of 700–1272 ng/ml; HIV-1 $\Delta$ vif: mean of 2781 ng/ml; range of 1112–4985 ng/ml (Schmitt et al., 2013) (Figure 37).

Similar to the co-transfection experiment in 293 cells, we expressed colA3A expression into a more relevant cell line, TZM-bl cells. TZM-bl cells were transfected with a vector expressing HA-colA3A and selected for G-418 resistance for 2 weeks. The resulting G-418 resistant cells were analyzed for the expression of HA-colA3A by immunoprecipitation using an antibody against the HA-tag. The immunoprecipitation analysis revealed that the TZM-bl cells transfected with the vector expressing HA-colA3A expressed HA-colA3A while the

parental TZM-bl cell line was negative for HA-colA3A expression (Figure 38A.). We used these HA-colA3A expressing cells to determine if they would restrict the replication of HIV-1. TZM-bl cells expressing HA-colA3A were inoculated with HIV-1 at an MOI of 1.0 and at 48 h post-inoculation, the culture supernatants and cell lysates were assessed for Gag p24 release and infectivity test on TZM-bl cells (Figure 38B). Diagram of the restriction assay is shown in Figure 39. The results indicate that HIV-1 production was significantly inhibited in TZM-bl cells expressing HA-colA3A (Katuwal et al., 2014).

### **Comparison of the N-terminal sequences of the several OWM A3As**

N-terminal sequences of the several OWM A3As were aligned together to determine if the A3A sequences from other OWM were similar to colA3A (Figure 40). Our analysis indicates that the A3A proteins from *Mandrillus sphinx* (mndA3A) and *Cercopithecus neglectus* (debA3A) had an AC-loop1 region that was similar to colA3A. We generated constructs in pcDNA3.1(+) vector as we previously described for HA-rhA3A, and HA-colA3A.

### **Other Old World monkey A3A proteins restrict HIV-1 replication similar to colA3A**

Both mndA3A and debA3A were expressed well in 293 cells (Figure 41). 293 cells were transfected with vectors expressing either hA3A, rhA3A, colA3A, debA3A, or mndA3A and plasmids with the genomes of HIV-1. The culture supernatants were harvested and cell lysates prepared at 48 h and assayed for p24 content and infectivity using TZM-bl cells. The experiment was repeated at least four times. As shown in Figure 41B, colA3A, debA3A and mndA3A restricted HIV-1 while hA3A and rhA3A did not restrict HIV-1 replication. Similar to

Figure 37. The colA3A inhibits HIV-1 $\Delta$ vif and HIV replication by a novel mechanism. Panels A-D. The colA3A restricts the synthesis and release of viral p24. 293 cells were transfected with vectors expressing either rhA3G, hA3A, rhA3A, gibA3A, agmA3A, colA3A, nomA3A or sqmA3A and either HIV-1, or HIV-1 $\Delta$ vif genomes. At 48 h, the culture medium was collected and lysates prepared from cells. The levels of p24 in the medium (Panels AB) and cell lysates (Panels CD) were determined using commercial p24 kits. In all panels, error bars correspond to standard deviations from triplicate determinations and statistical differences with the wild-type control were evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.

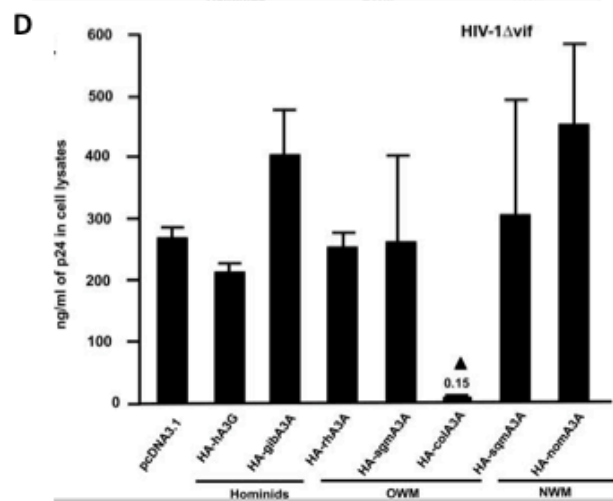
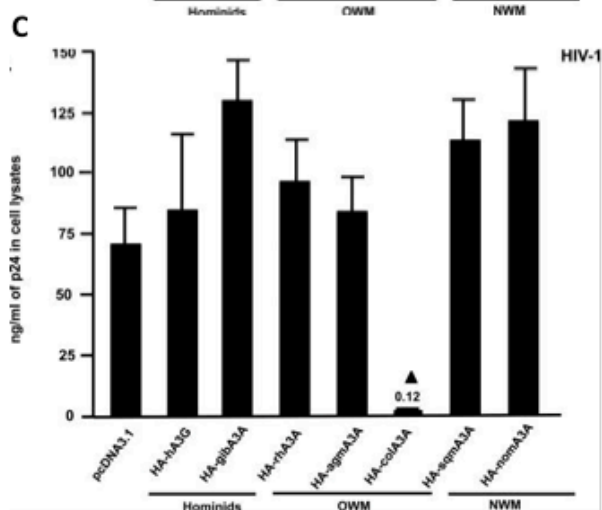
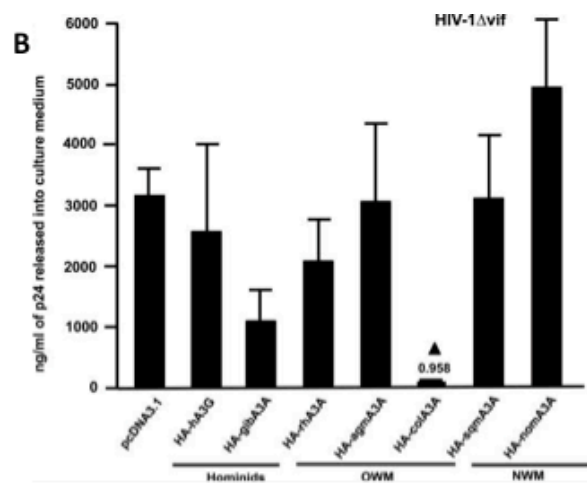
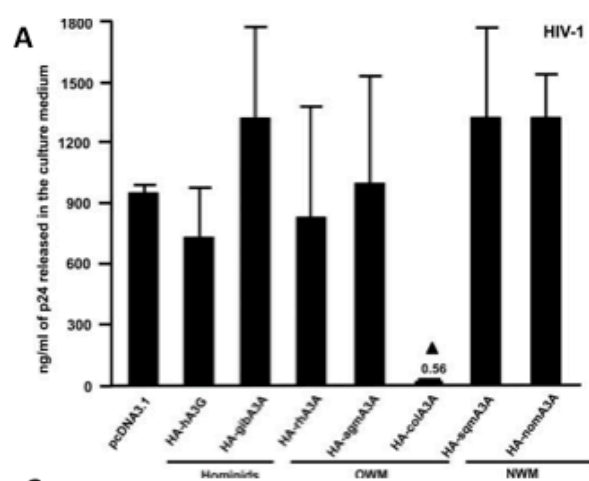


Figure 38. TZM-bl cells expressing HA-colA3A restrict HIV-1 replication. Panel A. TZM-bl cells were transfected with pcDNA3.1(+) expressing HA-colA3A and selected with G-418 for two weeks. These cultures were starved for methionine/cysteine and then radiolabeled with 200  $\mu$ Ci of  $^{35}$ S-methionine/cysteine for 6 h. Cell lysates were prepared and HA-containing proteins immunoprecipitated using an anti-HA antibody. The proteins were separated by SDS-PAGE (12% gel) and visualized by standard autoradiography techniques. Lane 1. HA-containing proteins immunoprecipitated from TZM-bl-colA3A cells using a control rabbit antibody. Lane 2. HA-containing proteins immunoprecipitated from TZM-bl-colA3A cells using a rabbit anti-HA antibody. Panel B. TZM-bl cells expressing HA-colA3A cells restrict HIV-1 replication. TZM-bl and TZM-bl cells expressing HA-colA3A in 6-well plates were inoculated with  $10^3$  TCID<sub>50</sub> of HIV-1 or HIV-1 $\Delta$ vif for 4 h. The inoculum was removed, washed three times and fresh medium added to cultures. At 48 h, the culture medium was harvested and virion infectivity measured using TZM-bl cells. The experiments were performed at least three times. Shown is the mean percentage virion infectivity of virus inoculated onto TZM-bl cells normalized to 100%. Statistical differences with the wild-type control were evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.



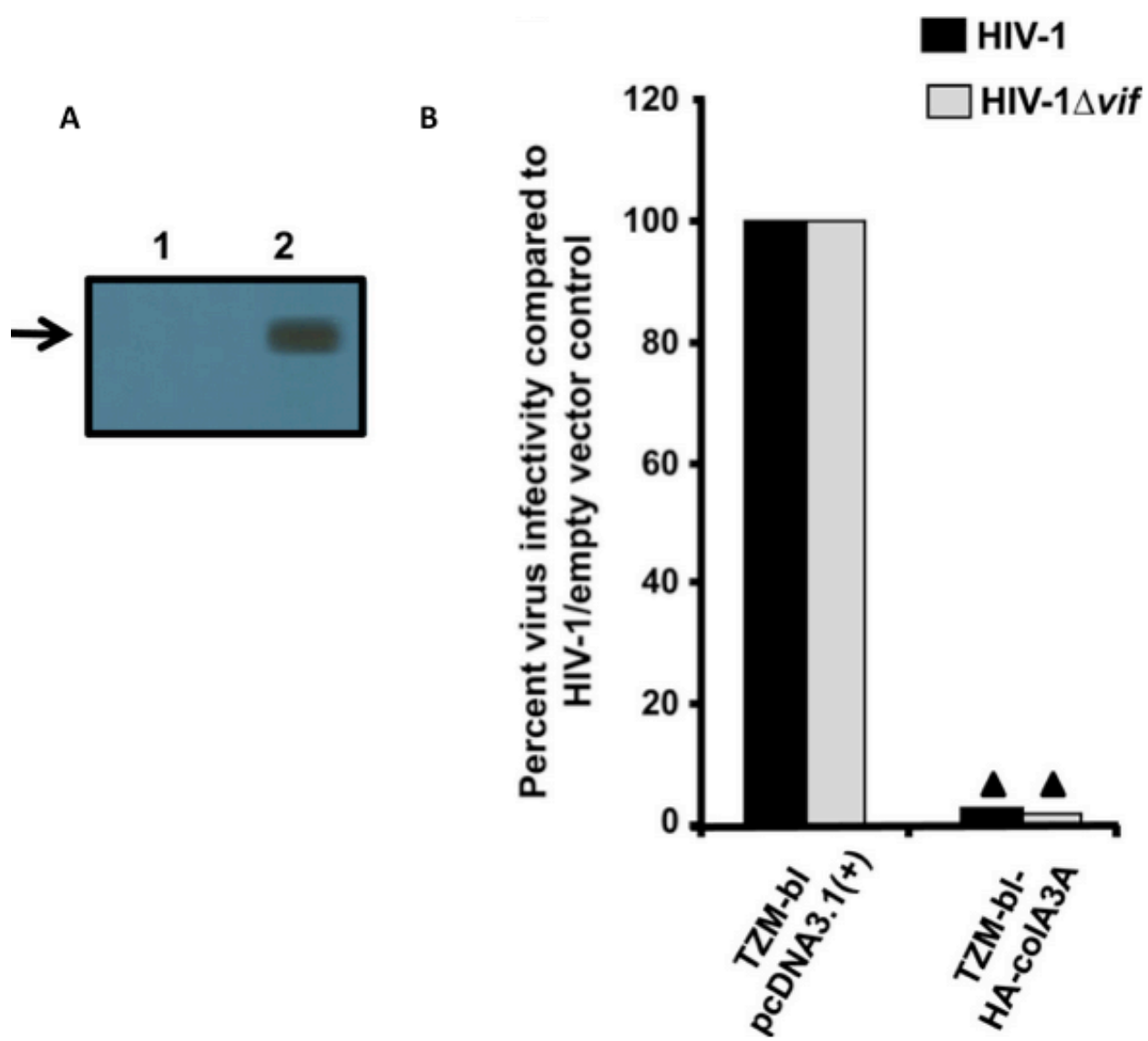
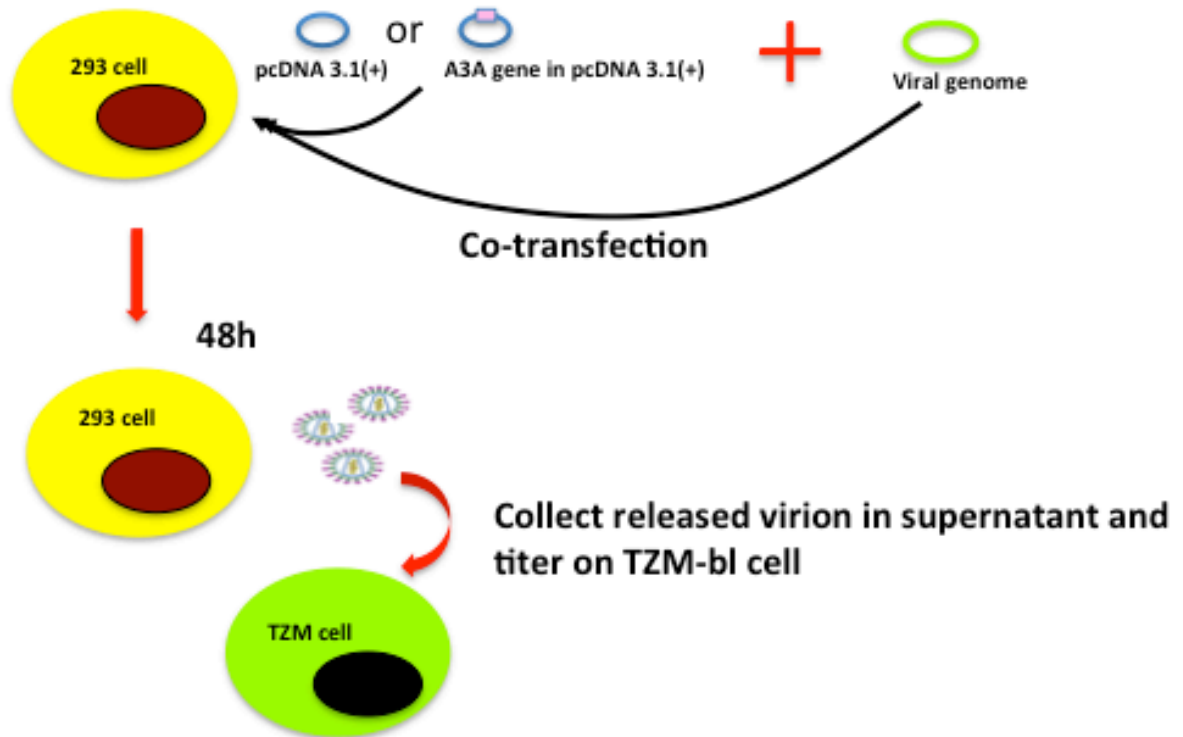


Figure 39. The procedure of the restriction assay. 293 cells were co-transfected with various A3A genome or pcDNA3.1(+) as control, and viral genome. At 48 h post-transfection, culture supernatants were collected and titered on TZM-bl cells. The TZM-bl cell line is an engineered HeLa cell clone that expresses human CD4, CCR5 and CXCR4, which allows virus entry and contains HIV-1 Tat-regulated reporter gene  $\beta$ -galactosidase. At 48 h post-infection, cells are fixed and stained with X-gal and blue reaction indicates the cells were infected, expressed functional Tat and led to transcription of the  $\beta$ -galactosidase gene and protein production.

## Restriction Assay



48h later, Stain with x-gal and count blue reactions

Figure 40. The N-terminal amino acid sequence of the A3A proteins from hominid and several Old World monkey species. The predicted AC-loop 1 region is within the box from each A3A showing that the AC loop 1 region of *Homo sapiens* (human), *Hylobates lar* (lar gibbon), *Macaca mulatta* (rhesus macaque), *Chlorocebus aethiops* (Grivet), *Colobus guereza* (black and white colobus monkey), *Cercopithecus neglectus* (De Brazza's monkey) and *Mandrillus sphinx* (mandrill). The AC loop 1 sequence of A3A proteins from De Brazza's monkey and mandrill are most closely resemble that of colobus monkey.

## 2 OWM A3A Contain a Similar AC loop to colA3A

	AC Loop 1	
<i>Homo sapiens</i>	MEASPASGPRHLMDPHIT	SNFNNGI---GRHKTYLCYEVERLD-NCTSVKMDQHRGFLH
<i>Hylobates lar</i>	M..N...R.....NV..	.....RW.....-..W.....
<i>Macaca mulatta</i>	MDG...R.....NT..F....DLSVR...	Q.....-...W.P..ER...C
<i>Chlorocebus aethiops</i>	MDG...R.....DT..F....DLSIL...	RQ.....-...W.P..ER....
<i>Colobus guereza</i>	MDG...R.G.V...DT..P....KPWVS	QRE.....K...SH-.D.W.PLN.....R
<i>Cercopithecus neglectus</i>	MDG...R.G.V...GT.....KPWVS	QRE.....K...SH-.D.W.LLN.....R
<i>Mandrillus sphinx</i>	MDS...R.....GT.....KLWVS	Q.E.....T...PH-.D.W.LLN.....Q

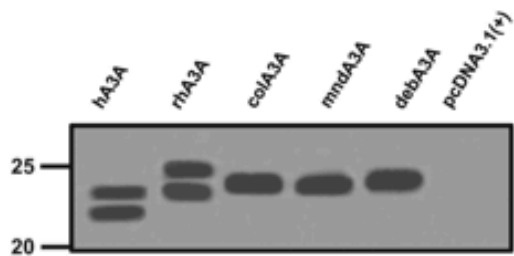
colA3A, the levels of p24 in the culture supernatants and cell lysates prepared from producer cells were significantly reduced compared to empty vector controls or cells transfected with vectors expressing hA3A (Figure 41C and D).

## Discussion

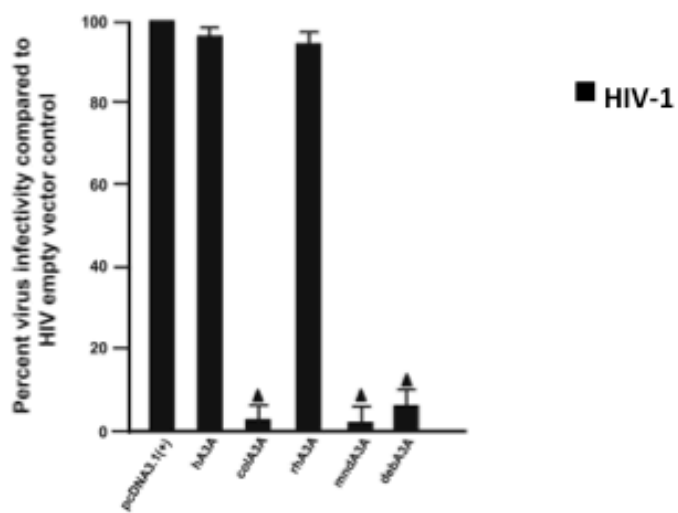
Deciphering the mechanism of colA3A restriction could be instrumental in designing novel antiviral strategies against HIV-1. We show here that TZM-bl cells expressing colA3A (TZM-bl-colA3A) inoculated with either HIV-1 or HIV-1 $\Delta$ vif viruses. Analysis of the AC-Loop-1 regions from hA3A and colA3A revealed significant variability in N-terminal domain sequences. We analyzed the AC loop 1 sequence of the A3A proteins from other OWM and compare them to colA3A. We found that the A3A proteins from both *C. neglectus* (debA3A; <sup>25</sup>KPWVSGQRE<sup>33</sup>) and *M. sphinx* (mndA3A; <sup>25</sup>KLWVSGQHE<sup>33</sup>) were either identical or nearly identical to colA3A in this region. Similar to colA3A, we observed that debA3A and mndA3A restricted HIV-1 with mndA3A being slightly more restrictive than colA3A and debA3A. The Old World monkeys (Family Cercopithecoidea) are composed of two subfamilies (Cercopithecoidea and Colobinae). The Cercopithecoidea are further classified into two tribes, the Cercopithecoini, and the Papionini. The Colobinae are generally classified into the African and Asian colobines. Our findings indicate that restriction of wild type HIV-1 by A3A is represented by species in two tribes of the Cercopithecoidea (*Cercopithecoini*, debA3A; and *Papionini*, mndA3A) and by the African group of the Colobinae. These results also reinforce our hypothesis that the sequence of the AC Loop1 region is critical for restriction of wild type HIV-1 by OWM A3A proteins. Moreover, it will be of interest to determine if the hA3A protein can potentially

Figure 41. The A3A proteins from other Old World monkeys also restrict HIV-1 by a similar mechanism. Panel A. Expression of the wild type and chimeric A3 proteins. 293 cultures were transfected with the empty pcDNA3.1(+) vector or those expressing hA3A, rhA3A colA3A, mndA3A, or debA3A. At 48 h, cells were starved and radiolabeled with  $^{35}\text{S}$ -methionine/cysteine for 6 h. The medium was removed, cell lysates prepared in 1X RIPA buffer and HA-tagged proteins immunoprecipitated with a rabbit antibody against the HA-tag and protein A Sepharose. The proteins were resolved using SDS-PAGE and visualized by standard radiographic techniques. The lanes correspond to the vector used to transfect the 293 cells. Panels B–D. 293 cells were transfected with pcDNA3.1(+)empty vector or vectors expressing either hA3A, colA3A, rhA3A, mndA3A, or debA3A and the vector with the HIV-1 genome. At 48 h, the culture supernatants were collected and assayed for infectious virus using TZM-bl as the indicator cell line or p24 levels in the culture supernatant and in cell lysates. Panel B. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. Panel C. Levels of p24 antigen in the culture supernatants from transfected cells using commercially available p24 antigen capture kits. Panel D. Levels of p24 antigen in the cell lysates from transfected cells using commercially available p24 antigen capture kits.

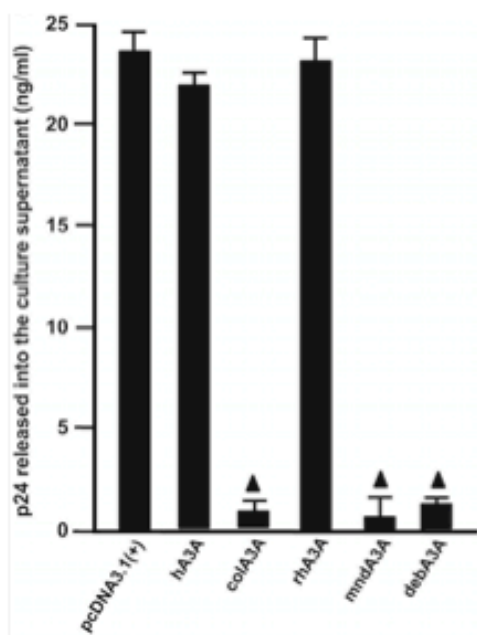
A



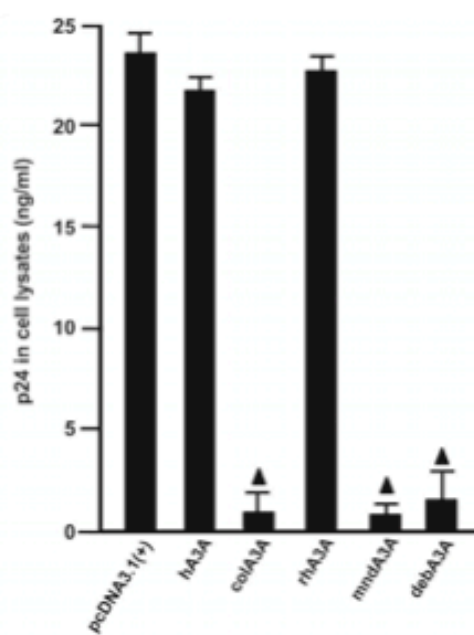
B



C



D





inhibit HIV-1 with a few amino acid substitutions mimicking the three OWM A3A proteins in the AC-loop 1 region.

It should be noted that the A3A sequence we used in these studies for the colobus monkey, Debrazza's monkey and mandrill were from a study that used primers in an exon-by-exon amplification approach (Henry, Terzian, Peeters, Wain-Hobson, & Vartanian, 2012). However, in a recent study, it was shown that the oligonucleotides primers also matched the intronic sequence on either side of exon 5 of A3G. Thus, it is likely that the studies with the colA3A were most likely a colA3A/G chimeric protein (McLaughlin, Gable, Wittkopp, Emerman, & Malik, 2016). Further, these investigators that the actual colA3A of the colobus monkey doesn't contain the WVS motif in the AC loop 1 gene and no restriction of HIV-1 is observed with the correct colA3A sequence. Although the correct A3A sequence for Mandrill and Debra is not examined, it is very possible that the mndA3A and debA3A we used are also mndA3A/G and debA3A/G chimeras. It was frustrating to find that the old world monkey A3A sequences we used were artifacts of the oligonucleotides used in the PCR process, we later appreciate this unexpected "accident" resulted in chimeric APOBEC proteins (colA3A/G, mndA3A/G, and debA3A/G) that are able to restrict wild type HIV-1 and leads to our later finding that the AC loop 1, especially the WVS motif, is critical in restoring hA3A's antiviral activity.

## **Materials and Methods**

### ***Cells and viruses***

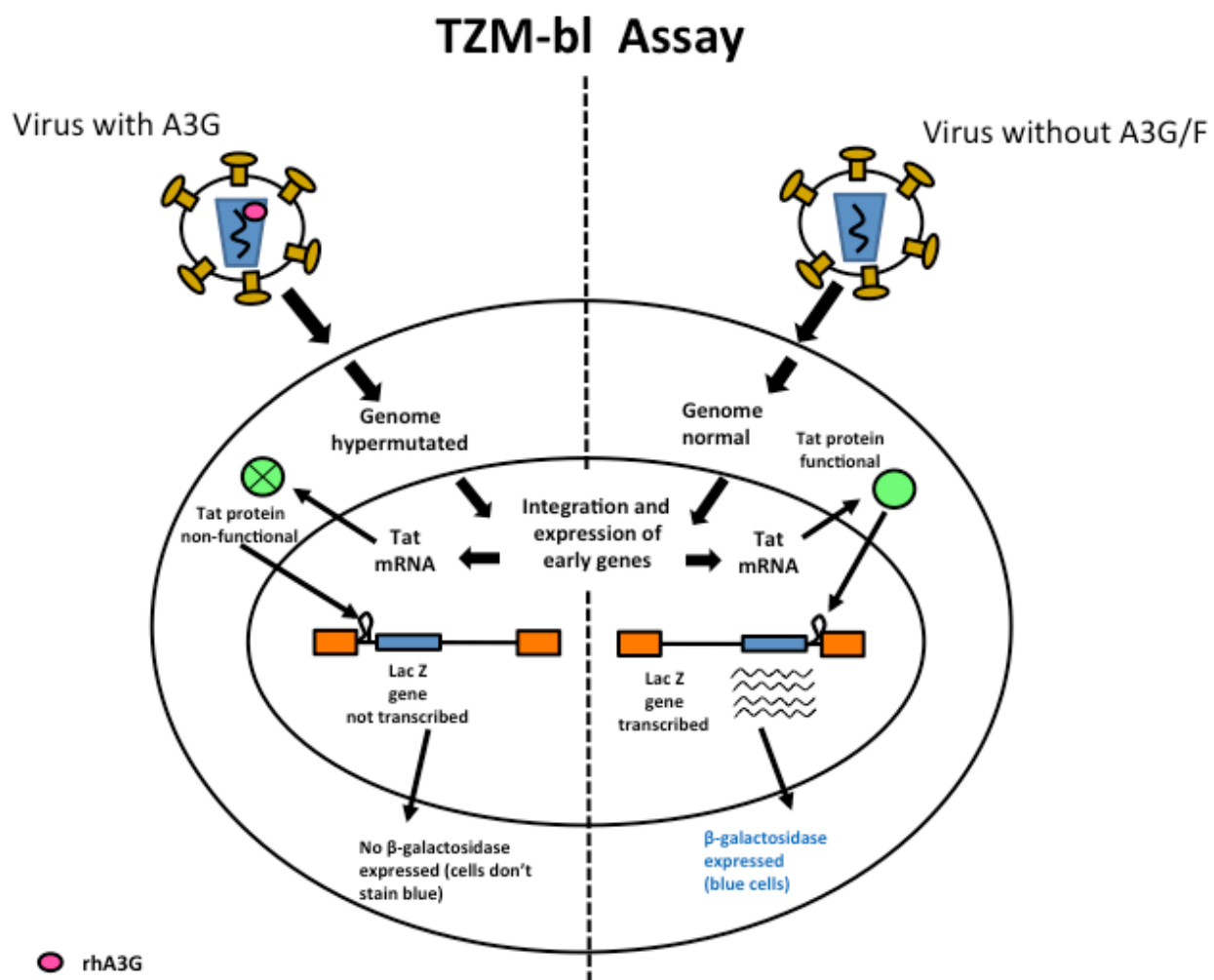
293 cell line is a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. TZM-bl cell line is an engineered HeLa cell clone that expresses

human CD4, CCR5 and CXCR4 and contains HIV-1 Tat-regulated reporter genes for firefly luciferase and  $\beta$ -galactosidase as shown in Figure 42. 293 cells were used for transfection of vectors expressing various A3 proteins, full-length HIV-1 (NL4-3) or HIV-1 $\Delta$ *vif*. The TZM-bl indicator cell line was used to measure the infectivity of viruses. Both cell lines were maintained in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (R10FBS), 10 mM HEPES buffer, pH 7.3, and 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin and 5  $\mu$ g gentamicin. A plasmid with the genome of HIV-1 strain NL4-3 (referred to in the text as HIV-1; NIH AIDS Research and Reference Reagent Program) and a  $\Delta$ *vif* version (referred to in text as HIV-1 $\Delta$ *vif*; Schmitt et al., 2011). To make the HIV-1 $\Delta$ *vif* plasmid, NL4-3 plasmid was digested with PflM1, phenol:chloroform extracted, and ethanol precipitated. The 5'-protruding ends were filled using DNA Polymerase I Large Fragment (Klenow; Promega). The resulting Klenow fragment reaction produced blunt ended fragments that were purified and re-ligated. The resulting plasmid was sequenced and found to contain a deletion in pNL4-3 *vif* between base pairs 5301 to 5308. Therefore, only the first 79 amino acids of pNL4-3 *Vif* could be expressed (Schmitt et al., 2011)

### ***Expression of colA3A in TZM-bl cells***

TZM-bl cells in 6-well plates (approximately 50% confluent) were transfected with pcHA-colA3A (the colA3A gene with a HA-tag at the N-terminus subcloned into the KpnI and EcoRV sites of pcDNA3.1(+)) using ExGen500 reagent according to the manufacturers' instructions. At 48 h, the medium was removed and replaced with growth medium containing 1 mg/ml of G-418. The cells were incubated with the medium replaced every 3 days until non-transfected cells were killed. Cells were then trypsinized, resuspended and diluted such that single cells were

Figure 42. The principle behind titration of infectious virus on TZM-bl cells. The parent TZM-bl cell line was engineered HeLa cell clone that expresses human CD4, CCR5 and CXCR4 and contains HIV-1 Tat-regulated reporter gene for  $\beta$ -galactosidase. On the right, when restriction factors are not present, functional Tat protein is produced and induces the transcription of LacZ gene under the promoter of TAR.  $\beta$ -galactosidase is produced and results in blue color reaction in the presence of X-gal. On the left, when a restriction factor such as A3G is present, a non-functional Tat protein is produced (due to deamination) and the lacZ gene not transcribed and thus cells remain colorless when stained with X-gal.



seeded into each well of 96 well plate. Cells were allowed to grow until they were confluent, trypsinized and seeded into 6 well plates and then into T-25 flasks. Cultures were examined for the expression of HA-colA3A using immuno-precipitation analysis using an antibody against the HA-tag (Santa Cruz Biotechnology). The TZM-bl cells were used for virus infectivity assays.

***Production of HIV-1 $\Delta$ vif and HIV virions in the presence of A3 proteins***

293 cells or TZM-bl cells were co-transfected with HIV-1 or HIV-1 $\Delta$ vif and of HA-tagged A3A proteins. After 48 h, supernatants were collected and the cellular debris was removed by low speed centrifugation. The cells were lysed in 500  $\mu$ l of 1X RIPA (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% deoxycholate, 0.2% SDS, 10 mM EDTA) and the nuclei removed by high speed centrifugation. The amount of Gag p24 present in the supernatants and cell lysates was measured using a commercially available p24 ELISA kit (Zeptometrix). The experiment was run in triplicate and the differences between the mean percentages were calculated using a two-tailed Student's t-test with  $p < 0.05$  considered significant.

**Chapter III : Chimeric and deletion studies show that the AC loop1 region, especially the <sup>27</sup>WVS<sup>29</sup> motif is sufficient to restore anti HIV-1 activity while reducing anti AAV-2 restriction.**

**Abstract**

Old World monkey (OWM) and hominid A3A proteins exhibit differential restriction activities against lentiviruses and DNA viruses. We identified that the N-terminal region of the colA3A, mndA3A, and debA3A contains the critical determinants for restricting HIV-1 production through chimeric and truncation experiments. We further confirmed that the AC Loop 1 region is crucial through constructing a chimeric hA3A/mndA3A in which the AC Loop 1 region of hA3A (<sup>27</sup>GI-GRHK<sup>32</sup>) was exchanged for AC Loop 1 region of mndA3A (<sup>27</sup>KLWVSGQRE<sup>35</sup>). More specifically, we constructed a chimeric hA3A with only three amino acids insertion (hA3A[<sup>27</sup>WVS<sup>29</sup>]) that conferred potent HIV-1 restriction activity. Interestingly, insertion of “WVS” into hA3A modestly reduces restriction of adeno-associated virus 2 (AAV-2) while the insertion of the AC Loop-1 region of the mndA3A/G into hA3A abolished AAV-2 restriction, strengthening the role of this molecular interface in the functional evolution of primate A3A.

**Introduction**

Previous research in our lab showed that rhA3A has stronger restriction towards lentivirus replication than hA3A. In contrast to the weak antiviral activity of hA3A, rhA3A inhibits SHIV $\Delta$ vif infectivity by approximately 20-fold and modestly inhibited HIV $\Delta$ vif infectivity by about 3-fold. Comparison of the amino acid sequences of human and rhesus

macaque A3A reveals a monkey-specific insertion at positions 27–29 (SVR) that is critical for SHIV $\Delta$ *vif* and HIV-1 $\Delta$ *vif* inhibition by rhA3A that was lost in the hominid lineage. Deletion of SVR from rhA3A result in the lost of antiviral activity, while the addition of the SVR motif to hA3A partially restored the inhibition against HIV-1 $\Delta$ *vif*. This suggests the importance of the SVR motif, which located in the AC loop 1 region in determining the antiviral activity.

Additionally, a similar study in TRIM5 $\alpha$  revealed that a single amino acid determines the antiviral potency of human TRIM5 $\alpha$  (Stremlau, Perron, Welikala, & Sodroski, 2005). If a positively charged arginine residue in the C-terminal domain of human TRIM5 $\alpha$  is either deleted or replaced with an uncharged amino acid, human cells gain the ability to inhibit HIV-1 infection (Y. Li, Li, Stremlau, Lee, & Sodroski, 2006; Stremlau, 2007).

Previously, we showed that the A3A proteins from three Old World monkey species, the colobus monkey, DeBrazza's monkey and the mandrill were capable of restricting not only HIV $\Delta$ *vif* but also HIV-1 (Katuwal et al., 2014). Thus, we want to determine the critical restriction motif of colA3A, mndA3A or debA3A, which can be used in designing gene-therapy approaches to restore hA3A antiviral activity. A unique feature of the reported A3A proteins from these Old World monkeys was the presence of an AC-loop 1 region characterized by the sequence (<sup>25</sup>KP/LWVSGQR/HE<sup>33</sup>), which differed from the sequence (<sup>25</sup>DLSV/IRGRH/RQ<sup>33</sup>) found in other Old World Monkeys (Henry et al., 2012). Therefore, it is of great interest to determine the molecular elements that are responsible for the restriction.

## Results

**Chimera studies showing the N-terminal region of the colA3A contains the critical determinants for restricting HIV-1 production.**

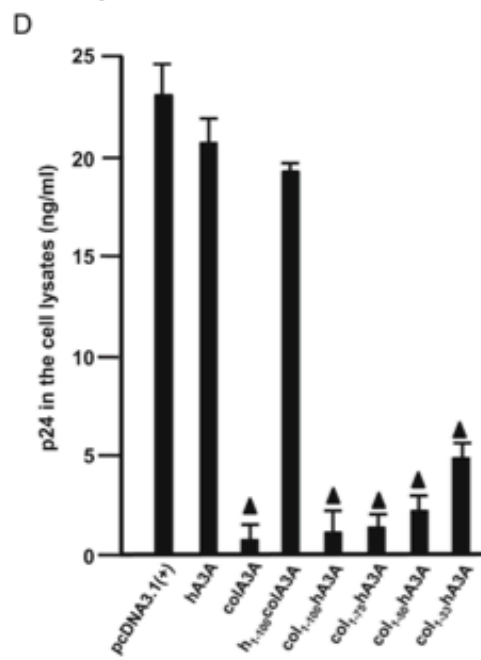
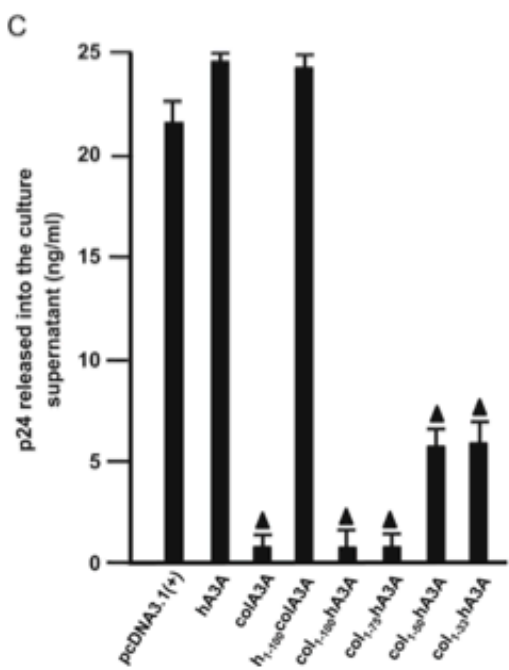
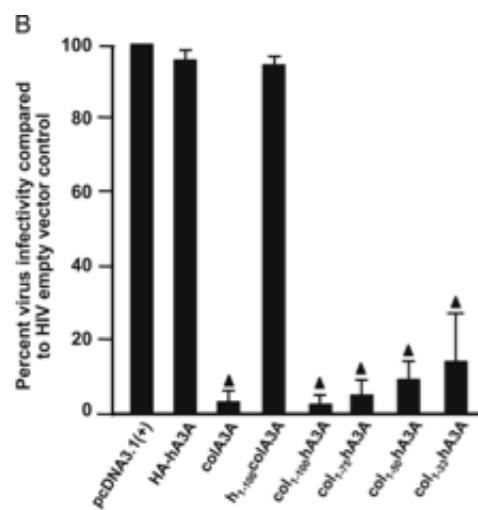
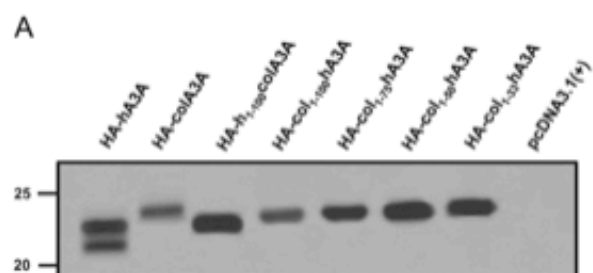
We previously showed that hA3A did not restrict HIV-1 $\Delta$ *vif* while colA3A restricted both HIV-1 and HIV-1 $\Delta$ *vif* (Schmitt et al., 2011; Schmitt et al., 2013). Chimeric colobus/human A3A proteins were constructed to define the determinants of colA3A restriction. All human/colobus chimeric proteins were expressed well in 293 cells (Figure 43A). The first two chimeric colobus/human A3A chimeras tested was col<sub>1-100</sub>hA3A, in which the first 100 amino acids of colA3A were fused to the C-terminal region of hA3A and h<sub>1-100</sub>colA3A, in which with the first 100 amino acids of hA3A fused to the C-terminal region of colA3A. The results of the restriction assays indicated that col<sub>1-100</sub>hA3A but not h<sub>1-100</sub>colA3A restricted HIV-1 infectious virus production as measured using the TZM.bl assay (Figure 43B). We also examined chimeric proteins with the N-terminal 75, 50, 33 amino acids of colA3A fused to hA3A (col<sub>1-75</sub>hA3A, col<sub>1-50</sub>hA3A, col<sub>1-33</sub>hA3A). Restriction assays showed that col<sub>1-75</sub>hA3A, col<sub>1-50</sub>hA3A, col<sub>1-33</sub>hA3A restricted HIV-1 at approximately 5%, 10%, and 15% of the empty vector control, respectively. We also examined the levels of p24 release and obtained similar results (Figure 43C and D). However, if we normalized infectious titer to p24 levels we observed no effect on HIV-1 virion infectivity (Fig). These results indicate that the colA3A N-terminal domain harbor the critical determinants for HIV-1 restriction.

### **Truncated colA3A, mndA3A and debA3A proteins can restrict HIV-1**

As the above results indicated that the first 100 amino acids of colA3A contained the determinants of HIV-1 restriction, we determined if truncated colA3A, mndA3A and debA3A proteins could also restrict HIV-1 replication. Truncated versions of colA3A were constructed



Figure 43. Chimeric colobus/human A3A proteins revealed that the N-terminal region of colA3A has the determinants to restrict HIV-1. Panel A. Expression of the wild type and chimeric A3 proteins. 293 cultures were transfected with vectors expressing each wild type and chimeric protein. At 48 h, cells were starved and radiolabeled with  $^{35}\text{S}$ -methionine/cysteine for 6 h. The medium was removed, cell lysates prepared in 1X RIPA buffer and HA-tagged proteins immunoprecipitated with an antibody against the HA-tag and protein A Sepharose. The proteins were resolved using SDS-PAGE and visualized by standard radiographic techniques. The lanes correspond to the vector used to transfect the 293 cells. Panels B-D. 293 cells were transfected with plasmids expressing hA3A, colA3A, col<sub>1-100</sub>hA3A, h<sub>1-100</sub>colA3A, col<sub>1-75</sub>hA3A, col<sub>1-50</sub>hA3A, or col<sub>1-33</sub>hA3A the plasmid with the HIV-1 genome. At 48 h, the culture supernatants were collected and assayed for infectious virus using the TZM-bl indicator cell line and p24 levels in the culture supernatants and cell lysates. Panel B. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. Panel C. Levels of p24 antigen in the culture supernatants from transfected cells using commercially available p24 antigen capture kits. Panel D. Levels of p24 antigen in the cell lysates from transfected cells using commercially available p24 antigen capture kits. The experiments in Panels B–D were performed at least three times and statistical differences with the pcDNA3.1(+) empty vector/HIV-1 control evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.

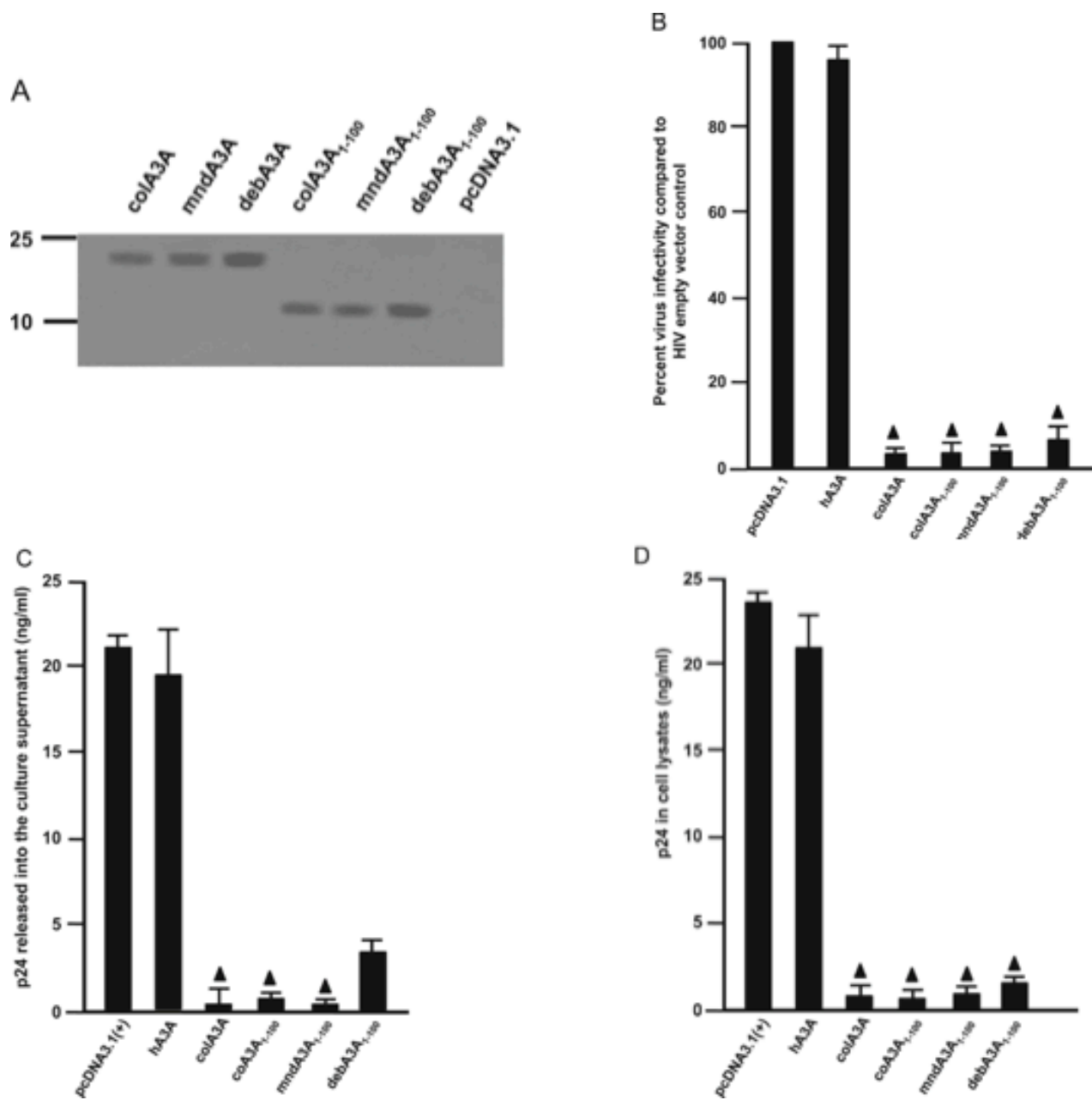


that expressed the N-terminal 100 amino acids (colA3A<sub>1-100</sub>, mndA3A<sub>1-100</sub>, debA3A<sub>1-100</sub>). We next examined the intracellular localization of HA-colA3A and HA-colA3A<sub>1-100</sub> in transfected 293 cells using immunofluorescence and confocal microscopy to determine if the truncated A3A was localized in different compartments than the unmodified A3A protein. 293 cells were transfected with the empty vector or vectors expressing either mndA3A or mndA3A<sub>1-100</sub>. At 48 h cells were examined for the intracellular localization of the A3A proteins using an anti-HA antibody. The results indicate that similar to HA-colA3A, HA-colA3A<sub>1-100</sub> was also localized in both the cytoplasmic and nuclear compartments of the cell. We next examined the restriction activity of these A3A proteins. 293 cells were transfected with plasmids with the HIV-1 genome and 48 h later the culture medium was harvested. The levels of p24 Gag were measured and levels of infectious virus determined using TZM-bl assays. Our results show that the truncated A3A proteins were just as effective as the full-length colA3A at restricting infectious HIV-1 production (Figure 44B). The truncated colA3A, debA3A, and mndA3A also inhibited HIV-1 p24 synthesis in producer cells and release into the culture supernatants, similar to the full length counterparts (Figure 44C and D). As negative controls, rhA3A did not restrict HIV-1 in these experiments.

### **A chimeric hA3A containing the AC Loop1 region of mndA3A restricts HIV-1**

Previously we reported that substitution of the hA3A AC loop 1 region with rhA3A restricted the replication of HIV-1 $\Delta$ vif. We were very interested in the mndA3A as it restricted HIV-1 to a higher level than either colA3A and debA3A. We constructed and expressed a chimeric hA3A/mndA3A in which the AC Loop1 region of hA3A (<sup>27</sup>GI-GRHK<sup>32</sup>) was exchanged for AC Loop 1 region of mndA3A (<sup>27</sup>KLWVSGQRE<sup>35</sup>). This chimera, designated as

Figure 44. Truncated colA3A, mndA3A, and debA3A proteins restrict wild type HIV-1. Panel A. Expression of the wild type and chimeric A3 proteins. 293 cultures were transfected with the empty pcDNA3.1(+) vector or vectors expressing colA3A, mndA3A, debA3A, colA3A<sub>1-100</sub>, mndA3A<sub>1-100</sub>, or debA3A<sub>1-100</sub>. At 48 h, cells were starved and radiolabeled with <sup>35</sup>S-methionine/cysteine for 6 h. The medium was removed, cell lysates prepared in 1×RIPA buffer and HA-tagged proteins immunoprecipitated with an antibody against the HA-tag and protein A Sepharose. The proteins were resolved using SDS-PAGE and visualized by standard radiographic techniques. The lanes correspond to the vector used to transfect 293 cells. Panels B–D. 293 cells were transfected with either the empty vector or plasmids expressing hA3A, colA3A, colA3A<sub>1-100</sub>, mndA3A<sub>1-100</sub>, or debA3A<sub>1-100</sub>. At 24 h post-transfection, cells were transfected with plasmids with the HIV-1 genome. At 72 h, the culture supernatants were collected and assayed for infectious virus using TZM-bl assays and p24 levels in culture supernatants and in cell lysates. Panel B. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. Panel C. Levels of p24 antigen in the culture supernatants from transfected cells using commercially available p24 antigen capture kits. Panel D. Levels of p24 antigen in the cell lysates from transfected cells using commercially available p24 antigen capture kits. The experiments in Panels B–D were performed at least four times and statistical differences with the pcDNA3.1(+)/HIV-1 control evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.



mnd<sub>25-34</sub>hA3A, was used in restriction assays along with hA3A, mndA3A, colA3A and an empty vector control. The results of the restriction assays revealed that the mnd25–34hA3A was nearly as effective at mndA3A at restricting HIV-1 while hA3A did not restrict HIV-1 (Figure 45). These results indicate that the AC Loop1 region of mndA3A was critical to its restriction properties.

### **The introduction of the <sup>27</sup>WVS<sup>29</sup> motif into hA3A is sufficient to restrict HIV-1**

We next analyzed the ability of HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] to restrict HIV-1 and HIV-1Δ*vif* virions. As shown in Figure 46A and we previously reported, neither hA3A nor hA3G restricted HIV-1 infectious titers as measured by the TZM.bl assay. As expected, HA-hA3G but not HA-hA3A restricted HIV-1Δ*vif*. In contrast to HA-hA3A, HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] restricted both HIV-1 and HIV-1Δ*vif* (Figure 46A and B). These results indicated that the insertion of [<sup>27</sup>WVS<sup>29</sup>] into hA3A was sufficient to confer hA3A with the ability to restrict HIV-1 infectious titers. To determine if this effect is due to reduction in virus particle production, we next determined the levels of p24 in the supernatant. Interestingly, p24 released from cells transfected with HA-hA3G, HA-hA3A or HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] were similar to cells transfected with the empty pcDNA3.1(+) vector (Figure 46C). Analysis of the cell viability at the end of the experiment indicated that there was significant difference in cell viability at the end of the assay (48 h) (Figure 46F). Overall, our data indicates that HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] inhibits HIV-1 virion infectivity.

### **Restriction of parvovirus AAV-2 by hA3A[<sup>25</sup>KLWVSGQHE<sup>33</sup>] and hA3A[<sup>27</sup>WVS<sup>29</sup>]**

Figure 45. A chimeric hA3A containing the AC Loop1 region of mndA3A restricts HIV-1. 293 cells were grown to 50% confluency and transfected with an empty vector or vectors expressing hA3A, mndA3A, mnd<sub>25-34</sub>hA3A, or hA3G and a vector containing the HIV-1 genome. At 48 h, the culture supernatants were collected and assayed for infectious virus using the TZM-bl indicator cell line or p24 levels in the culture supernatant and in cell lysates. Panel A. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. Panel B. Levels of p24 antigen in the culture supernatants from transfected cells using commercially available p24 antigen capture kits. Panel C. Levels of p24 antigen in the cell lysates from transfected cells using commercially available p24 antigen capture kits. The experiments in Panels A–C were performed three times and statistical differences with the pcDNA3.1(+)/HIV-1 control evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.

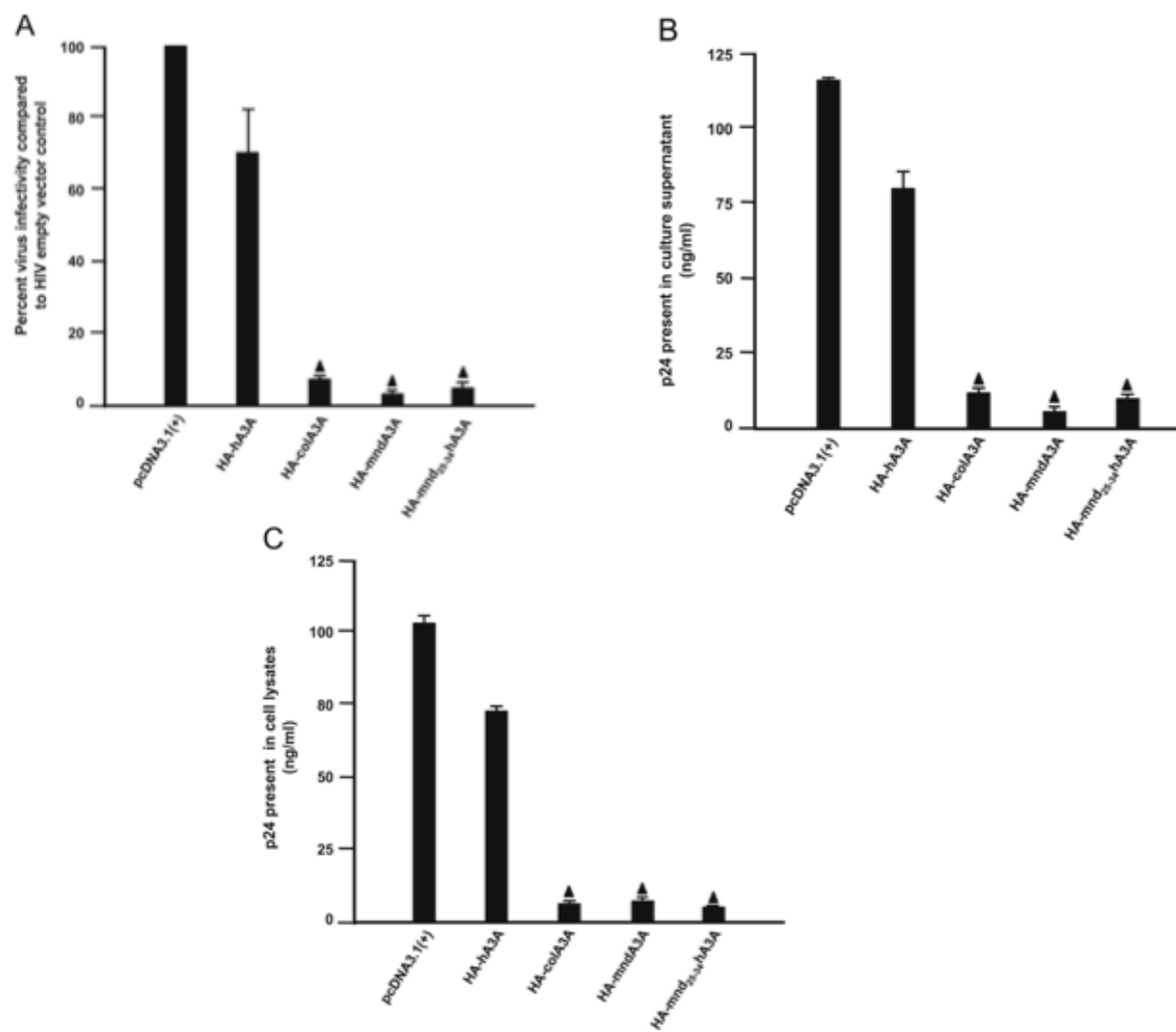
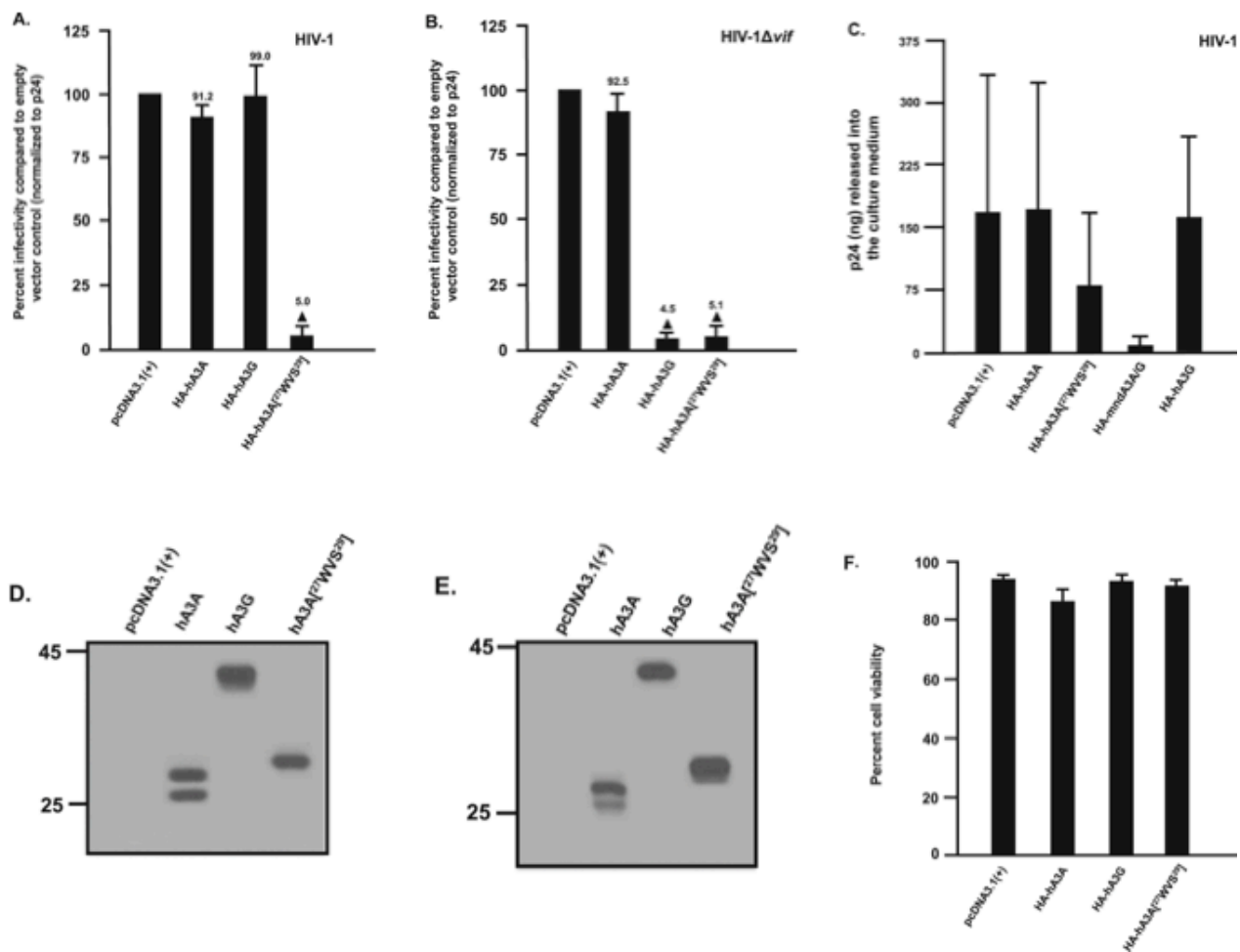


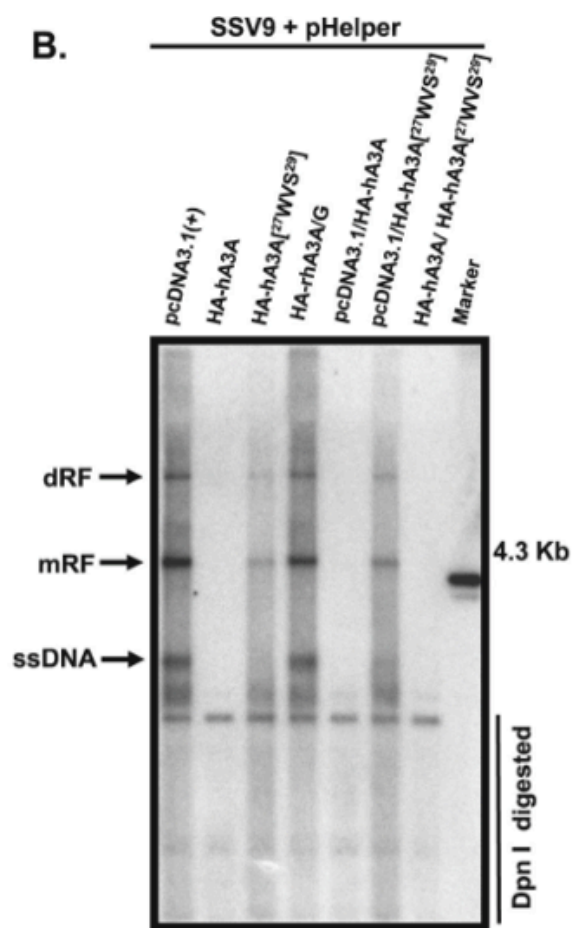
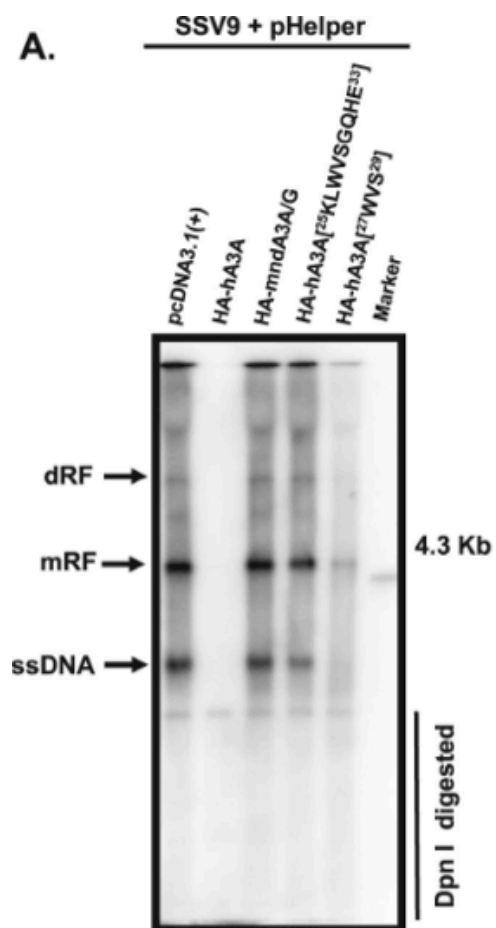


Figure 46. The introduction of the motif WVS into hA3A results the ability of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> to restrict HIV-1. 293 cells were co-transfected with either the empty vector or plasmids expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G and a plasmid with the HIV-1 genome (pNL4-3) or HIV-1 $\Delta$ *vif* (pNL4-3 $\Delta$ *vif*). At 48 h, the culture supernatants were collected and cell lysates prepared. The levels of p24 determined in the culture supernatants was quantified using p24 antigen capture assays and equivalent levels of p24 were used to determine virus infectivity using TZM-bl cell assays. The expression of A3A proteins in cell lysates was determined by Western blot. Panels A and B. The level of infectious virus in the culture supernatants from cells co-transfected with pNL4-3 genome or pNL4-3 $\Delta$ *vif* and plasmids expressing the A3 proteins, respectively. Panel C. The quantity of p24 released from cells from four experiments following transfection with pNL4-3. Panels D and E. Western blots from representative transfections showing expression of HA-tagged A3A proteins from restriction assays. Restriction experiments were performed at least four times and statistical differences with the pcDNA3.1(+)/HIV-1 control evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant. Panel F. The viability of transfected cells from a representative experiment from Panel A was assessed using the Cyto-Tox-Glo Cytotoxicity assay according to the manufacturer's instructions.



Previous studies have shown that hA3A could restrict the replication of adenovirus associated virus 2 (AAV-2) and we showed that rhA3A did not restrict AAV-2 (H. Chen et al., 2006; K. Schmitt et al., 2011). We hypothesized that the AC-Loop1 region may be important in the restriction of AAV-2. We determined if HA-hA3A [<sup>25</sup>KLWVSGQHE<sup>33</sup>] and HA-hA3A [<sup>27</sup>WVS<sup>29</sup>] could restrict AAV-2. 293 cells were transfected with the empty pcDNA3.1(+) vector or pcDNA3.1(+) expressing HA-hA3A (positive control), HA- mndA3A/G, HA-hA3A [<sup>25</sup>KLWVSGQHE<sup>33</sup>], or HA-hA3A [<sup>27</sup>WVS<sup>29</sup>] and plasmids SSV9 (an infectious molecular clone of AAV-2) and pHelper. At 48h, the cells were harvested, the extrachromosomal DNA isolated by Hirt extraction and analyzed using Southern blots for the presence of replicating AAV-2 DNA (mRF and dRF). The results, shown in Figure 47, indicate that HA-hA3A inhibited AAV-2 replication as previously reported while HA-rhA3A did not restrict AAV-2 replication (Narvaiza et al., 2009; Chen et al., 2006; Schmitt et al., 2011). Cells transfected with HA-mndA3A/G and HA-hA3A [<sup>25</sup>KLWVSGQHE<sup>33</sup>] did not significantly restrict AAV-2 while cells transfected with HA-hA3A [<sup>27</sup>WVS<sup>29</sup>] restricted AAV-2 replication at approximately 18% of the pcDNA3.1(+) control (Figure 47A). Similarly, we found that when HA-hA3A and HA-hA3A [<sup>27</sup>WVS<sup>29</sup>] were expressed together, AAV-2 replication was similar in cells expressing hA3A or hA3A and pcDNA3.1(+) (Figure 47 B). A Western blot from an aliquot of the 293 lysates showed that the A3 proteins were similarly expressed. These results indicate that the insertion of the WVS into HA-hA3A modestly reduced hA3A's restriction activity of AAV2 but that substitution of the entire AC loop1 region from mndA3A/G completely abrogated restriction of the parvovirus AAV-2. We also assessed the viability of transfected cells at the end of the experiment. Our results indicate that there was no significant loss of viability when compared to the pcDNA3.1(+) control.

Figure 47. Human A3A<sup>[27WVS<sup>29</sup>]</sup> does not significantly restrict parvovirus AAV-2 DNA replication. Panel A . Low molecular weight DNA extracted from 293 cells co-transfected with recombinant AAV-2 plasmids and either empty vector pcDNA3.1(+) or vectors expressing HA-hA3A, HA-mndA3A/G, HA-hA3A<sup>[25KLWVSGQHE<sup>33</sup>]</sup>, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. A 1% Southern blot was run and hybridized with a <sup>32</sup>P-labeled probe from SSV9 as previously described (Qiu et al., 2002). The following AAV replication products are shown as dRF (double replicative form), mRF (monomeric replicative form), and ssDNA. These experiments were performed two times with the same result. Panel B. Low molecular weight DNA extracted from 293 cells transfected with recombinant AAV-2 plasmids and either empty vector pcDNA3.1(+), or vectors expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, HA-rhA3A, pcDNA3.1(+) and HA-hA3A, pcDNA3.1(+) and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, and HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>.



## Discussion

Determining the sequence requirements that permit hA3A to gain function (i.e., restriction activity), may not only provide clues to the evolutionary and structural biology of these proteins but also inform translational strategies that utilize A3A with enhanced restriction properties.

We previously used a chimeric protein approach to show that substitution of AC loop 1 of hA3A with that of rhA3A restored the ability to hA3A to restrict HIV-1 $\Delta$ vif ((K. Schmitt et al., 2013)). We hypothesized that the three amino acid insertion in AC loop 1 of rhA3A could result in a AC-Loop1 with a greater molecular surface compared to hA3A. Due to its predicted solvent-accessibility, this additional molecular surface may be an important region for protein-protein interactions (K. Schmitt et al., 2013).

Using a similar approach we found that determinants within the N-terminal domain (i.e. the first 100 amino acids) which includes the AC Loop-1 region of colA3A are likely involved in the restriction of HIV-1 as chimeric proteins col<sub>1-100</sub>hA3A restricted at levels similar to full length A3A while col<sub>1-75</sub>hA3A, col<sub>1-50</sub>hA3A, and col<sub>1-33</sub>hA3A showed a gradual reduction in the level of restriction although they were still at statistically significant levels. As our chimeric data showed that the N-terminal region of colA3A had the determinants for HIV-1 restriction and that mndA3A and debA3A also restricted HIV-1, we determined if truncation of these A3A proteins retained the ability to restrict HIV-1. Our data clearly indicated that all three truncated A3A proteins (colA3A<sub>1-100</sub>, mndA3A<sub>1-100</sub>, and debA3A<sub>1-100</sub>) restricted HIV-1. Similar to the full-length A3A proteins, the restriction involved reduced Gag expression in the producer cells.

As our chimeric and truncation experiment indicated that the determinants of restriction were associated with the N-terminal domain, we determined if the AC loop1 region was once again critical to this restriction. As the mndA3A consistently restricted HIV-1 to a higher level of the three Old World monkey A3A proteins tested, we constructed a hA3A contained the mndA3A AC loop 1 region. Our results indicated that the level of virus restriction by HA- hA3A<sup>[25KLWVSGQHE<sup>33</sup>]</sup> was similar to mndA3A indicating that this region was critical for restriction of HIV-1. Recently, it was shown that this region in hA3A was important in controlling substrate specificity (Logue et al., 2014). More support for the importance of this region in restriction has come from a recent study using oligonucleotide substrates with a single deamination site ((Logue et al., 2014). These investigators found that A3A preferentially deaminated at TC followed by an A or G residue. Further, using chimeric hA3A/hA3G proteins these investigators found that amino acids GI25–26 of AC-Loop 1 region of hA3A (referred to as the recognition loop 1; RL1) specified the 5' nucleotide of the recognition motif and that a chimeric A3A/A3G with the RL1 of A3G was more flexible with respect to the 5' nucleotide (AC, CC, GC all used with equal frequency).

Finally, we showed that the introduction of a <sup>27</sup>WVS<sup>29</sup> motif into hA3A (hA3A<sup>[27WVS<sup>29</sup>]</sup>) was sufficient to restrict both HIV-1 $\Delta$ *vif* and HIV-1. While HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> restricted HIV-1, restriction appeared to occur through a different mechanism than with HA- hA3A<sup>[25KLWVSGQHE<sup>33</sup>]</sup> as the latter significantly reduced the levels of viral p24 released from cells compared to cultures transfected with HA-hA3A, HA-hA3G, and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>.

## Materials and methods

### ***Construction of chimeric colobus/human A3A and truncated colA3A proteins***

As the hA3A protein and colA3A were phenotypically different regarding restriction of HIV-1 in producer cells, we adopted to a chimeric approach to determine if specific regions of the colA3A protein were responsible for the inhibition of replication. We generated a series of chimeric colobus/human A3A proteins, which included col<sub>1-100</sub>hA3A (N-terminal 100 amino acids of colA3A substituted for same region of hA3A), h<sub>1-100</sub>colA3A, col<sub>1-75</sub>hA3A, col<sub>1-50</sub>hA3A and col<sub>1-33</sub>hA3A. All genes were synthesized with an HA-tag at the N-terminus (Genscript) and subcloned into the pcDNA3.1(+) vector and sequenced to confirm the integrity of the gene. All lots of A3A plasmids were prepared in *Escherichia coli* grown at 30°C and each lot sequenced to insure no mutations were introduced. The expression of each chimeric protein from each plasmid was confirmed by Western blot analysis before viral replication assays were performed.

For construction of truncated colA3A, mndA3A and debA3A proteins, sited-directed mutagenesis was use to introduce stop codons after the N-terminal 100 amino acids (colA3A mndA3A<sub>1-100</sub>, debA3A<sub>1-100</sub>). All genes were sequenced to insure that only the desired mutations were introduced into the gene.

### ***Analysis of virus replication in the presence of A3A proteins***

To analyze the virus restriction properties of the different A3A proteins, chimeric colobus/human proteins and truncated A3A proteins described above, 293 cells were first transfected with a vector expressing one of the A3A proteins and with a plasmid containing the complete NL4-3 viral genome. At 48 h post-transfection, the culture medium was collected, clarified by low speed centrifugation and analyzed for virus infectivity using TZM.bl cells. The diagram of the restriction assay is shown in Figure 39. The parent TZM-bl cell line is an engineered HeLa cell clone that expresses human CD4, CCR5 and CXCR4 and contains HIV-1



Tat-regulated reporter genes for firefly luciferase and  $\beta$ -galactosidase. The principle of TZM-bl assay is depicted in Figure 42. All assays were performed with hA3A (non-restrictive); and colA3A (restrictive). All assays were performed at least four times and analyzed for statistical significance using two-tiered Student's *t*-test.

### ***Immunofluorescence studies of A3A localization***

To examine the intracellular localization of HA–colA3A and the truncated HA–colA3A<sub>1–100</sub> proteins, 293 cells on cover slips were transfected with empty vector, or vectors expressing HA–colA3A or HA–colA3A<sub>1–100</sub>. At 48 h post-transfection, cells were washed twice in cold PBS, and fixed in 2% paraformaldehyde for 5 min at room temperature. The cells were washed three times in cold PBS, and incubated in a 0.5% Triton X-100 solution in PBS for 5 min. Cells were once again washed with cold PBS and incubated with a rabbit antibody against the HA-tag conjugated with Alexa488 prepared in PBS with 1% BSA for 1 h. The cells were then washed three times with PBS, mounted and examined using a Leica TCS SPE confocal microscope using a 63X objective using filters for Alexa488 and DAPI.

### ***Analysis of AAV-2 replication in the presence of HA-hA3A, HA- mndA3A/G, HA-rhA3A, HA-hA3A<sup>[25]KLWVSGQHE<sup>33</sup>]</sup>, and HA-hA3A<sup>[27]WVS<sup>29</sup>]</sup>***

The ability of hA3A and hA3A<sup>[27]WVS<sup>29</sup>]</sup> to inhibit the replication of AAV-2 was assessed. 293 cells were transfected using LipoD 293 transfection reagent (SigmaGen Labs, Rockville, MD) into 60 mm dishes in duplicate with 1  $\mu$ g SSV9 (an AAV2 infectious clone, psub201) (Oiu and Pintel, 2002), 2 $\mu$ g pHelper (Stratagene, La Jolla, CA) (Xiao et al., 1998), and 1  $\mu$ g of the empty expression vector (pcDNA3.1(+)) or expressing HA-hA3A, HA-mndA3A/G, HA-hA3A<sup>[25]KLWVSGQHE<sup>33</sup>]</sup>, HA-hA3A<sup>[27]WVS<sup>29</sup>]</sup>. At 48h post-transfection, the cells were

harvested and resuspended in PBS. The supernatant was discarded and Hirt DNA was extracted using Hirt solution (10 mM Tris-HCl, pH 7.5; 10 mM EDTA, 0.6% SDS) at room temperature for 10 min followed by 5 M NaCl at 4 °C overnight. The mixture was centrifuged at 14,000 rpm at 4 °C for 20 min. Following centrifugation, the pellet was discarded and the supernatant was treated with proteinase K (50 µg/ml) at 37 °C for 1 h. The low molecular weight (LMW) DNA was extracted twice with saturated phenol and once with chloroform/isoamyl alcohol. The resulting DNA was ethanol precipitated and column purified using the Qiagen gel extraction kit (Qiagen). The LMW DNA was run on a 1% agarose gel, transferred to nitrocellulose and analyzed by Southern hybridization using a <sup>32</sup>P-labeled probe consisting of a fragment representing nucleotides 184–4490 (Xba I digestion) from SSV9 as previously described (Qiu & Pintel, 2002). Densitometry was performed on the MF and RF forms to determine the level of viral DNA replication with pcDNA3.1(+) serving as the control. To ensure that all samples were expressing the desired A3 proteins, aliquots of harvested cells utilized in the Southern blot were lysed in RIPA buffer. The nuclei were removed and the protein was precipitated using methanol, resuspended in 2X sample reducing buffer, and boiled for 5 min. Proteins were separated on a 12% SDS- PAGE gel and probed using a rabbit polyclonal anti-HA antibody (HA-probe, Santa Cruz). The viability of cells transfected with the plasmids as described above was assessed using the Cyto-Tox-Glo Cytotoxicity assay (Promega) according to the manufacturer's instructions.

## Chapter IV : Amino Acid determinants of restriction

### Abstract

Previously, we showed that the insertion of three amino acids “WVS” to hA3A (hA3A<sup>[27WVS<sup>29</sup>]</sup>) is alone and sufficient to restore the antiviral activity against HIV-1. We further interrogated the amino acid requirements at these three positions in HIV-1 restriction through single amino acid substitution and single/double deletion mutants. We found that all single amino acid substitutions retained the ability to restrict HIV-1 at some level. While deletion of one amino acid still resulted in restriction of HIV-1, deletion of two amino acids resulted in restriction that was not statistically significant when compared to hA3A. Taken together, these results indicate that length of this sequence is more important than the charge or hydrophobicity of the amino acids at these positions amino acids accounted for restriction activity.

### Introduction

It is known that the hA3A protein lacks restriction activity against wild type HIV-1. However, our lab identified that the A3A proteins from certain Old World monkeys including colobus monkey A3A (colA3A), mandrill A3A (mndA3A) and De Brazza’s monkey A3A (debA3A) possess significant antiviral activity against wild type HIV-1. Sequence analysis indicates that all hominid species have a three amino acids indel in the AC loop 1 region of this protein, while colA3A, mndA3A and debA3A all have a consistent WVS motif insertion at that spot. This WVS motif appears to play a very important role in restricting HIV-1. We confirmed the importance of this WVS motif by constructing a chimeric hA3A containing the <sup>27</sup>WVS<sup>29</sup> sequence from the Old World Monkey A3As known as hA3A<sup>[27WVS<sup>29</sup>]</sup> and the chimeric hA3A<sup>[27WVS<sup>29</sup>]</sup> retained potent HIV-1 restriction activity as WT mndA3A. It is of great interest

to further define the importance of each amino acids in the <sup>27</sup>WVS<sup>29</sup> motif. Thus, we substituted/deleted each amino acids in the <sup>27</sup>WVS<sup>29</sup> motif to determine the minimal molecular requirement for HIV-1 restriction.

## Results

### **Amino acid substitutions within the <sup>27</sup>WVS<sup>29</sup> with differing charge and hydrophobicity do not affect HIV-1 restriction activity of the chimeric A3A**

We next determined if substitution each amino acid in the <sup>27</sup>WVS<sup>29</sup> motif of HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] with amino acids with either similar or different properties (hydrophobicity, charge) would affect the restriction of HIV-1. For amino acids with similar properties, we constructed HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] mutants with the tryptophan at position 27 substituted with phenylalanine HA-hA3A [<sup>27</sup>YVS<sup>29</sup>], the valine at position 28 with leucine HA-hA3A [<sup>27</sup>WLS<sup>29</sup>] and the serine at position 29 with threonine HA-hA3A [<sup>27</sup>WVT<sup>29</sup>]. These A3A proteins were assayed for the ability to restrict HIV-1. The sequence alignment of the single amino acid mutants and expression in 293 cells are shown in Figure 48 and the structural illustration is depicted in Figure 49. Our results show that HA-hA3A[<sup>27</sup>YVS<sup>29</sup>], HA-hA3A[<sup>27</sup>WLS<sup>29</sup>], and HA-hA3A[<sup>27</sup>WVT<sup>29</sup>] restricted HIV-1 to similar levels as the HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] while negative controls hA3A and hA3G did not restrict HIV-1 (Figure 50). These results show that single conservative amino acid substitutions were tolerated in this motif.

Figure 48. Panel A sequence alignment of hA3A<sup>[27WVS<sup>29</sup>]</sup> single amino acid mutant proteins, for each mutant, the amino acid substitution/insertion is bolded. Panel B The mutant proteins are stably expressed in cells. 293 cells were transfected with vectors expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> or the mutant proteins. At 48 h, cell lysates were prepared, and processed for Western blots. HA-containing proteins detected using an anti-HA antibody. Western blot showing expressing of the single amino acid substitution mutants of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> (upper panel) and GAPDH loading controls (lower panel).

## A

hA3A	MEASPASGPRHLMDPHIFTSNFNNGI --- IGRHKTYLCYEVERLDNGTSVKMDQHRGFLHN	57
hA3A [27WVS <sup>29</sup> ]	.....WVS.....	60
hA3A [27FVS <sup>29</sup> ]	..... <b>F</b> VS.....	60
hA3A [27YVS <sup>29</sup> ]	..... <b>Y</b> VS.....	60
hA3A [27HVS <sup>29</sup> ]	..... <b>H</b> VS.....	60
hA3A [27WLS <sup>29</sup> ]	..... <b>W</b> LS.....	60
hA3A [27WTS <sup>29</sup> ]	..... <b>W</b> TS.....	60
hA3A [27WDS <sup>29</sup> ]	..... <b>W</b> DS.....	60
hA3A [27WVT <sup>29</sup> ]	..... <b>W</b> V <b>T</b> .....	60
hA3A [27WTA <sup>29</sup> ]	..... <b>W</b> V <b>A</b> .....	60
hA3A [27WVD <sup>29</sup> ]	..... <b>W</b> V <b>D</b> .....	60

## B

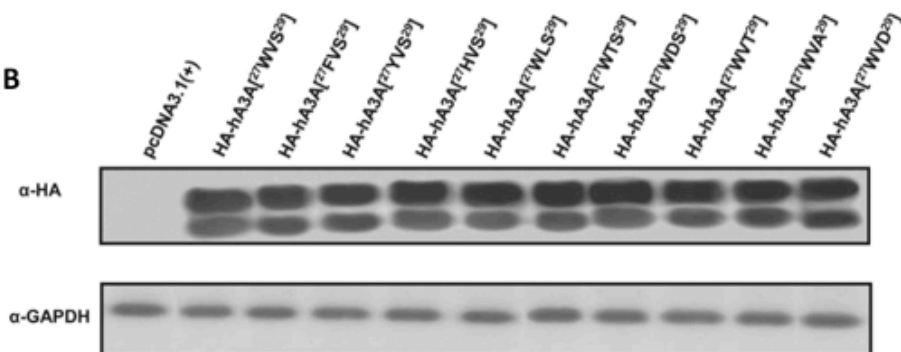


Figure 49. Illustration of the single amino acid substitutions in the hA3A<sup>[27WVS<sup>29</sup>]</sup> protein.

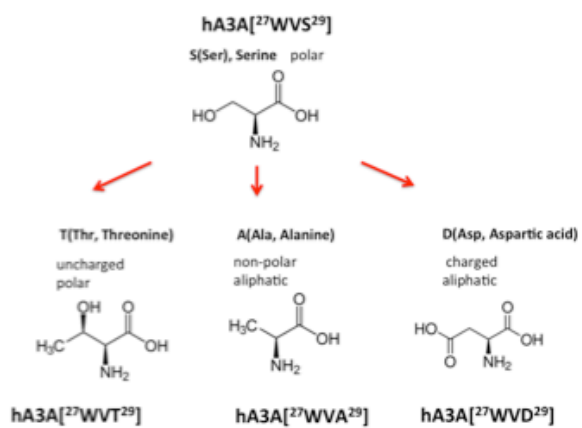
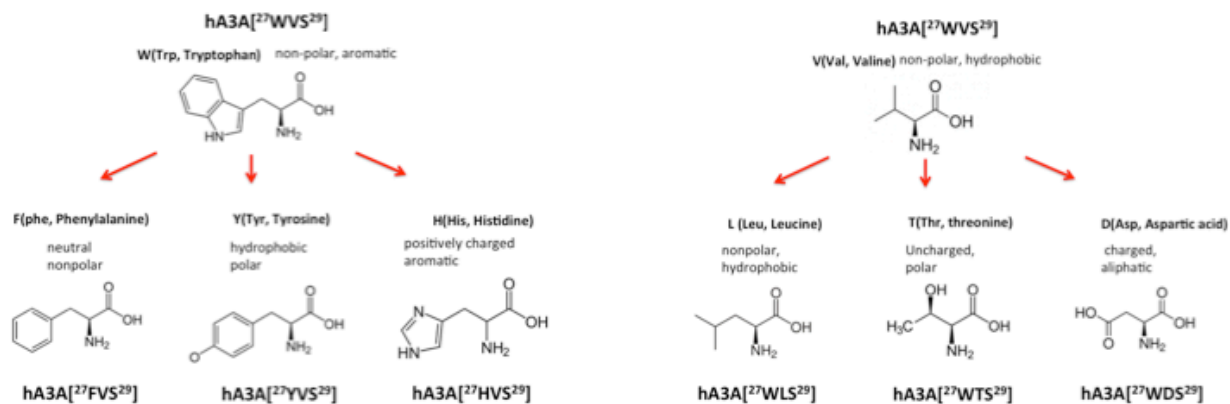
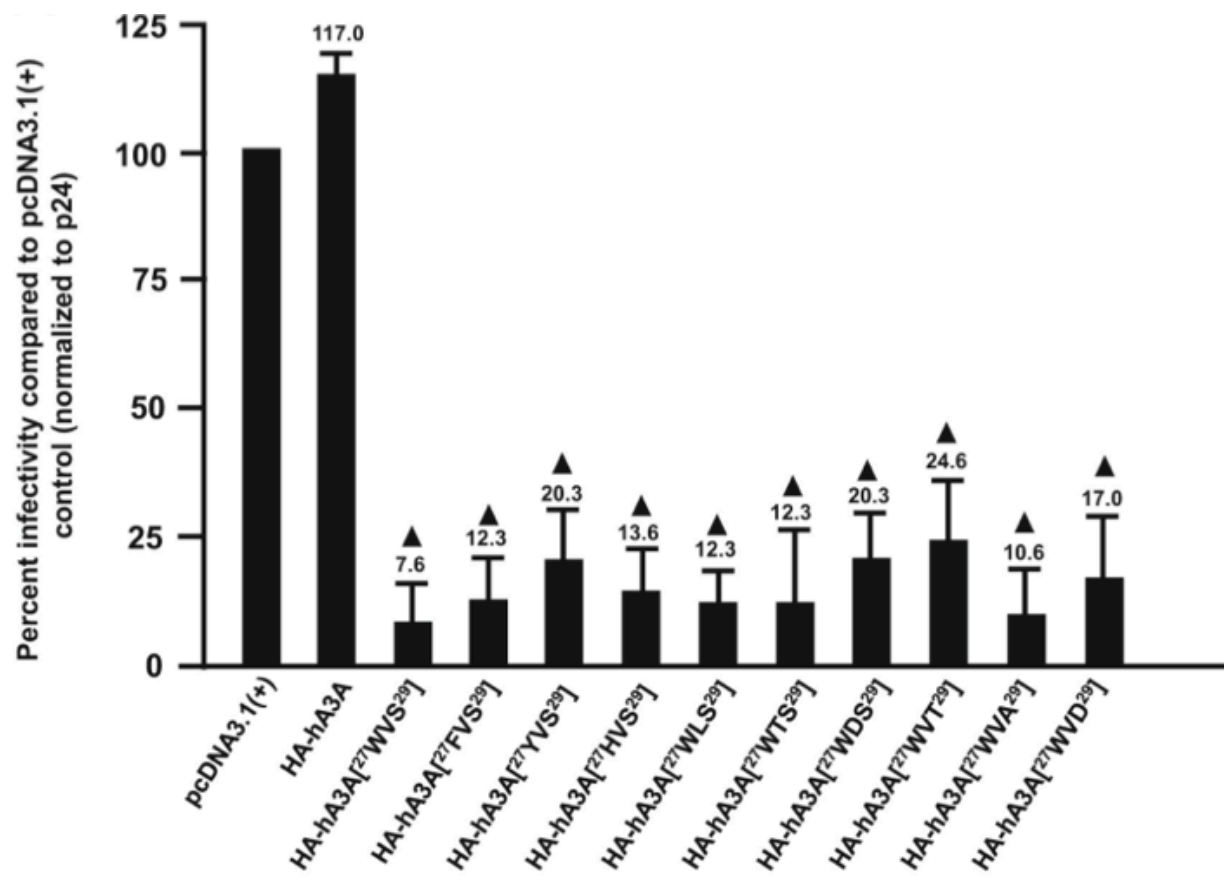




Figure 50. Substitution of <sup>27</sup>WVS<sup>29</sup> with conservative or non-conservative amino acid changes also resulted in restriction of HIV-1. 293 cells were co-transfected with either the empty vector or plasmids expressing HA-tagged hA3A, hA3A[<sup>27</sup>WVS<sup>29</sup>], hA3A [<sup>27</sup>YVS<sup>29</sup>], hA3A[<sup>27</sup>FVS<sup>29</sup>], hA3A[<sup>27</sup>HVS<sup>29</sup>], hA3A[<sup>27</sup>WLS<sup>29</sup>], hA3A[<sup>27</sup>WTS<sup>29</sup>], hA3A [<sup>27</sup>WDS<sup>29</sup>], hA3A[<sup>27</sup>WVT<sup>29</sup>], hA3A[<sup>27</sup>WVA<sup>29</sup>], or hA3A[<sup>27</sup>WVD<sup>29</sup>] and a plasmid with the HIV-1 genome. At 48 h, the culture supernatants were collected. The levels of p24 in the culture supernatants were quantified using p24 antigen capture assays and equivalent levels of p24 were used to determine the levels of virus infectivity using TZM-bl cell assays. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. The experiments were performed at least four times and statistical differences with the pcDNA3.1(+)/HIV-1 control evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.



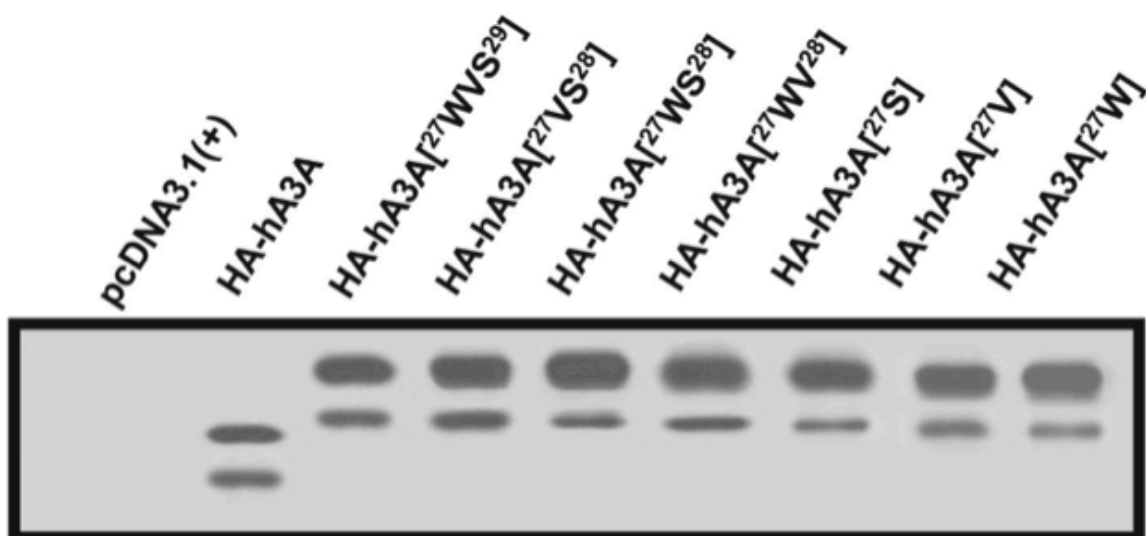
We also determined if substitution of <sup>27</sup>WVS<sup>29</sup> motif of HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] with amino acids differing in polarity or charge would restrict HIV-1. These included the substitution of the tryptophan at position 27 with tyrosine HA-hA3A[<sup>27</sup>YVS<sup>29</sup>] or histidine HA-hA3A[<sup>27</sup>HVS<sup>29</sup>], the valine at position 28 with threonine HA-hA3A[<sup>27</sup>WTS<sup>29</sup>] or aspartic acid HA-hA3A[<sup>27</sup>WDS<sup>29</sup>], and the serine at position 29 with alanine HA-hA3A[<sup>27</sup>WVA<sup>29</sup>] or aspartic acid HA-hA3A[<sup>27</sup>WVD<sup>29</sup>]. The sequence alignment of the single amino acid mutants and expression in 293 cells are shown in Figure 48 and the structural illustration is depicted in Figure 49. Restriction assays with these mutants revealed that all six significantly restricted HIV-1 with most restricting at levels similar to HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] (Figure 50). Combined, our results indicate that the nature of the amino acid was not critical for restriction of HIV-1 by HA-hA3A[<sup>27</sup>WVS<sup>29</sup>].

### **The role of one or two amino acid deletions in the WVS motif of HA-hA3A[<sup>27</sup>WVS<sup>29</sup>]**

We next determined if removal of one or two amino acids from the WVS tripeptide would reduce the ability of the chimeric protein to restrict HIV-1. Five different constructs, HA-hA3A[<sup>27</sup>VS<sup>28</sup>], HA-hA3A[<sup>27</sup>WS<sup>28</sup>], HA-hA3A[<sup>27</sup>WV<sup>28</sup>], HA-hA3A[<sup>27</sup>S], HA-hA3A [<sup>27</sup>V], and HA-hA3A[<sup>27</sup>W] were constructed and analyzed for expression and the ability to restrict HIV-1. The sequence alignment of deletion mutants are shown in Figure 51. All five mutants were expressed well in cells compared with HA-hA3A and HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] as shown in Figure 51. The single amino acid deletion mutants (HA-hA3A[<sup>27</sup>VS<sup>28</sup>], HA-hA3A[<sup>27</sup>WS<sup>28</sup>], and HA-hA3A [<sup>27</sup>WV<sup>28</sup>]) modestly restricted HIV-1, varying from 16 to 26% (p<0.01) compared with 8% for HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] (Fig. 5A). However, deletion of two amino acids from the WVS motif

Figure 51 Panel A. Sequence alignment of hA3A<sup>[27WVS<sup>29</sup>]</sup> deletion mutant proteins Panel B.  
The hA3A<sup>[27WVS<sup>28</sup>]</sup> deletion mutants were stably expressed in cells shown by Western blot.

hA3A	MEASPASGPRHLMDPHIFTSNFNNGI---GRHKTYLCYEVEERLDNGTSVKMDQHRGFLH
hA3A <sup>[27WVS<sup>29</sup>]</sup>	.....WVS.....
hA3A <sup>[28VS<sup>29</sup>]</sup>	.....-VS.....
hA3A <sup>[27WS<sup>29</sup>]</sup>	.....W-S.....
hA3A <sup>[27WV<sup>28</sup>]</sup>	.....WV-.....
hA3A <sup>[W<sup>27</sup>]</sup>	.....W--.....
hA3A <sup>[V<sup>28</sup>]</sup>	.....-V-.....
hA3A <sup>[S<sup>29</sup>]</sup>	.....--S.....



resulted in less restriction, with HA-hA3A<sup>[27S]</sup> and HA-hA3A<sup>[27V]</sup> restriction not being statistically significant ( $p < 0.05$ ) compared to HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> (Figure 52). These results indicate that the presence of two amino acids in this region was critical for restriction of HIV-1.

### **Chimeric HA-hA3A<sup>[27AAA<sup>29</sup>]</sup> and HA-hA3A<sup>[27GGG<sup>29</sup>]</sup> also restrict HIV-1**

As the above results indicated that length and not the nature of the amino acids were critical to the restriction activity, we next tested two additional constructs, one with the “WVS” motif substituted with “AAA” and a second with “GGG.” These constructs, HA-hA3A<sup>[27AAA<sup>29</sup>]</sup> and HA-hA3A<sup>[27GGG<sup>29</sup>]</sup>, were analyzed for restriction of HIV-1 along with HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. Both HA-hA3A<sup>[27AAA<sup>29</sup>]</sup> and HA-hA3A<sup>[27GGG<sup>29</sup>]</sup> along with HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> restricted HIV-1 while HA-hA3A did not restrict HIV-1 (Figure 53). Thus, these results confirm that the amino acid length of this region was most important for activity.

## **Discussion**

Previously, we analyzed the A3A proteins from several Old World monkey species and showed that they could restrict SHIV $\Delta$ *vif*. Significantly, we showed that the A3A protein from the black and white colobus monkey, the mandrill and DeBrazza's monkey not only restricted HIV-1 $\Delta$ *vif* but also HIV-1. We further showed that the introduction of a <sup>27</sup>WVS<sup>29</sup> motif into hA3A (hA3A<sup>[27WVS<sup>29</sup>]</sup>) was sufficient to restrict both HIV-1 $\Delta$ *vif* and HIV-1 (Katuwal et al., 2014; K. Schmitt et al., 2013). Our results indicate that substitution of one amino acid of the WVS motif with either a conservative, non-conservative or charged amino acid was

Figure 52. Deletion of one or two amino acids from the WVS motif of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> chimeric protein reduces the ability of hA3A<sup>[27WVS<sup>29</sup>]</sup> to restrict HIV-1. 293 cells were co-transfected with either the empty vector or plasmids expressing HA-tagged hA3A, hA3A<sup>[27WVS<sup>29</sup>]</sup>, hA3A<sup>[27VS<sup>28</sup>]</sup>, hA3A<sup>[27WS<sup>28</sup>]</sup>, hA3A<sup>[27WV<sup>28</sup>]</sup>, or hA3A<sup>[27S]</sup> and a plasmid with the HIV-1 genome. At 48 h, the culture supernatants were collected and cell lysates prepared. The levels of p24 were quantified in the culture supernatants using p24 antigen capture assays and equivalent levels of p24 were used to determine the levels of virus infectivity using TZM-bl cell assays. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. The experiments were performed at least four times and statistical differences with the pcDNA3.1(+)/HIV-1 control evaluated using a two-tailed Student's *t*-test, ▲ with  $p < 0.05$  ( ) considered significant.

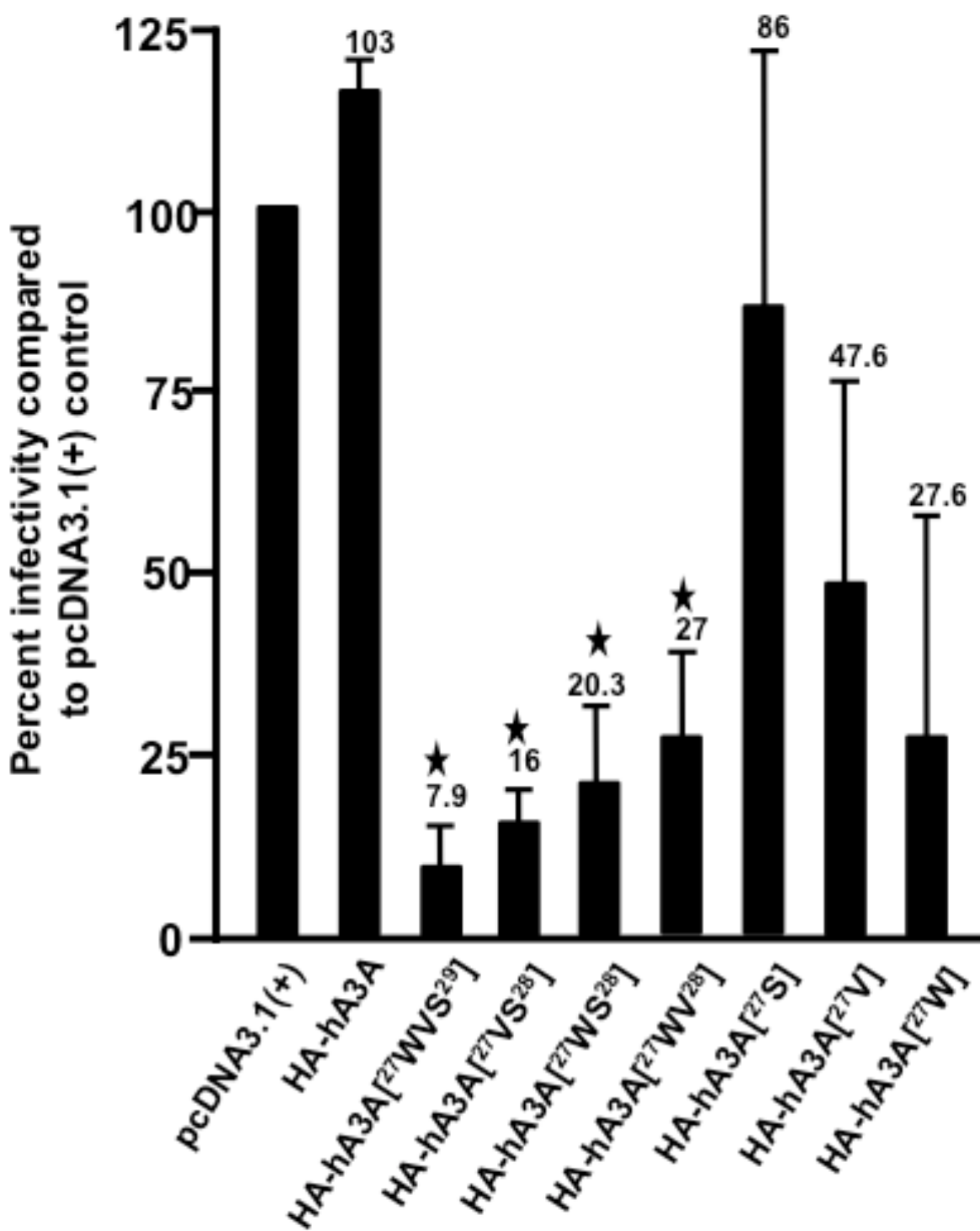
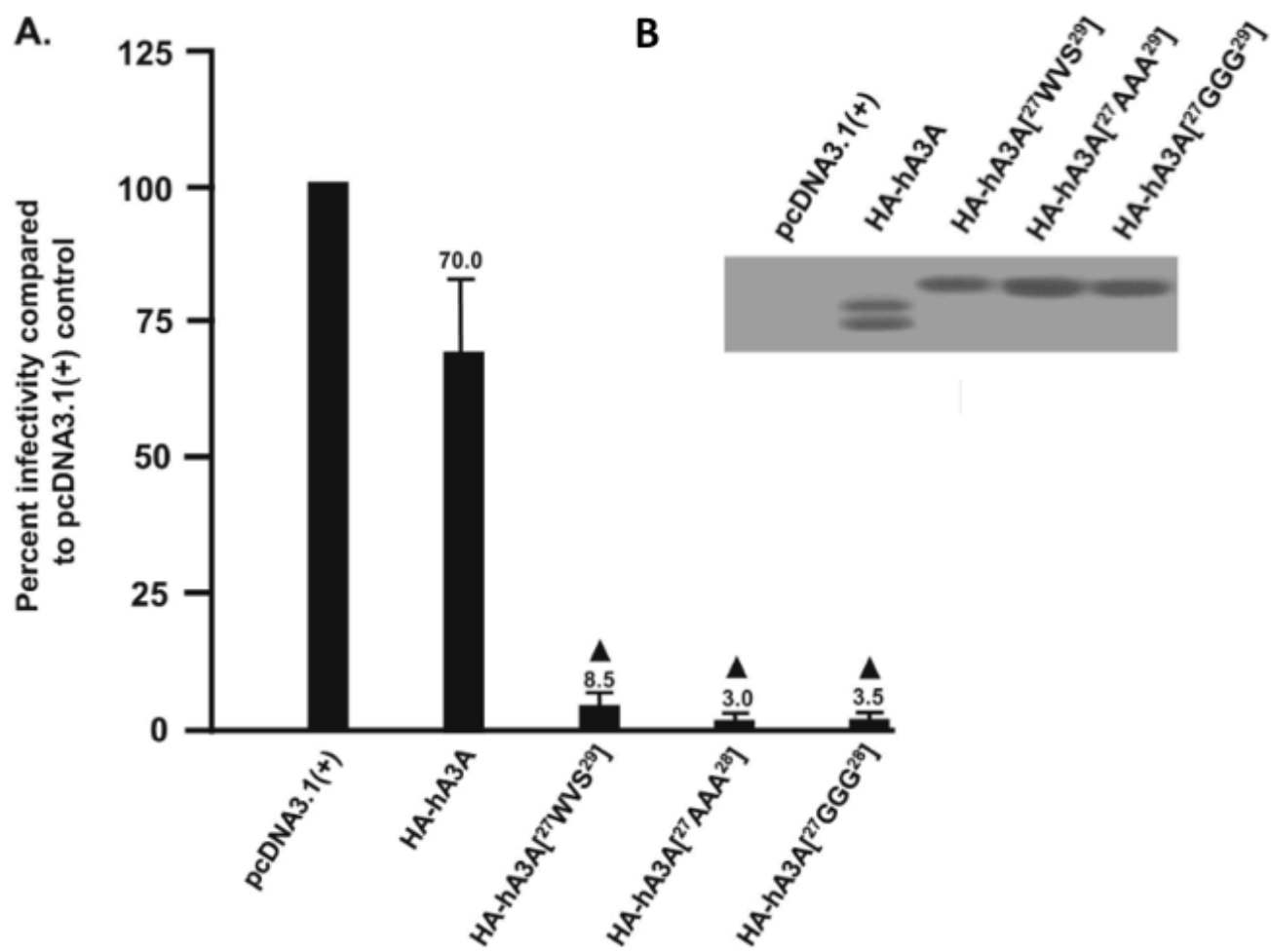




Figure 53. Chimeric HA-hA3A<sup>[27 AAA<sup>29</sup>]</sup> and HA-hA3A<sup>[27 GGG<sup>29</sup>]</sup> also restrict HIV-1. 293 cells were transfected with either the empty vector or plasmids expressing HA-tagged hA3A, hA3A<sup>[27 WVS<sup>29</sup>]</sup>, hA3A<sup>[27 AAA<sup>29</sup>]</sup>, or hA3A<sup>[27 GGG<sup>28</sup>]</sup> and a plasmid with the HIV-1 genome. At 48 h, the culture supernatants were collected and cell lysates prepared. The levels of p24 were quantified in the culture supernatants using p24 antigen capture assays and equivalent levels of p24 were used to determine the levels of virus infectivity using TZM-bl cell assays. The expression of A3A proteins in cell lysates determined by Western blot. Panel A. The level of infectious virus in the culture supernatants from transfected cells using TZM-bl assays. Panel B. Western blot from a representative transfection showing expression of HA-tagged A3A proteins. The experiments were performed at least three times and statistical differences with the pcDNA3.1(+)/HIV-1 control evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  ( ) ▲ considered significant.



inconsequential in the restriction of HIV-1. In fact, replacement of this tripeptide region with alanines or glycines had no effect on HIV-1 restriction. These results indicate that the amino acids at these positions can have different properties (polar, non-polar, or charged) and retain restriction activity. While the nature of the amino acids in this region did not seem to be critical for HIV-1 restriction, our results indicate that deletion of one amino acid resulted in less restriction than HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> and deletion of two amino acids resulted in even less restriction. These results suggest that the presence of the three amino insertion results in optimal restriction of HIV-1.

## **Materials and Methods**

### ***Site-directed mutagenesis of A3A genes***

The hA3A in these studies was constructed with an HA-tag at the N-terminus (HA-hA3A) and expressed using the pcDNA3.1(+) vector (pHA-hA3A). The introduction of mutations resulting in amino acid substitutions or deletions was accomplished using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. All plasmid inserts were sequenced to ensure the validity of the mutations and that no other mutations were introduced during the mutagenesis or cloning processes.

### ***Detection of A3A proteins***

For analysis of A3A protein expression in cells, 293 cells in 6-well plates were transfected with either pcDNA3.1(+) or pcDNA3.1(+) expressing each of the A3A proteins and incubated at 37°C. At 48 h, the medium was removed, cells lysed in 1X

radioimmunoprecipitation assay buffer (RIPA: 50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.5% deoxycholate; 0.2% SDS; 10 mM EDTA). The nuclei and other cell debris removed by centrifugation in a mi- crocentrifuge for 10 min and the supernatant collected in a fresh tube. Equal aliquots of cell lysates were boiled in sample reducing buffer, separated by SDS-PAGE, and proteins transferred to membranes HA-containing proteins were detected by Western blot using a rabbit anti-HA antibody (Santa Cruz Biotechnology Y-11 (sc805), a secondary goat anti-rabbit IgG-alkaline phosphatase antibody (Sigma, #A3687) and Novex AP chemiluminescent substrate (CDP-Star; InVitrogen, #WP20002). Proteins were detected using a Fuji Phosphoimager (LAS-4000).

#### ***Analysis of virus replication in the presence of A3A proteins***

To analyze the virus restriction properties of the mutant A3A proteins, 293 cells were first transfected with either the empty pcDNA3.1(+) or a vector expressing one of the A3A proteins and with a plasmid containing either the HIV or HIV-1 $\Delta$ vif viral genome. At 48h post-transfection, the culture medium was collected, clarified by low speed centrifugation and the supernatant analyzed for p24 using antigen capture assays. Equivalent levels of p24 were used to titrate virus infectivity using TZM.bl cell assays. All assays were performed with the empty pcDNA3.1(+) vector (non-restrictive for HIV or HIV-1 $\Delta$ vif), hA3AG (restrictive for HIV-1 $\Delta$ vif; nonrestrictive for HIV-1). All assays were performed at least four times and analyzed for statistical significance using two-tiered Student's t-test.

## Chapter V : Mechanism of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> inhibiting HIV-1

### Abstract

Initially, we found that hA3A<sup>[27WVS<sup>29</sup>]</sup> expressing producer cells release similar amount of virions through p24 ELISA and pulse-chase experiments, while results in lower levels of HIV-1 protein synthesis in target cells, indicating that the restriction happens in the next round of replication in the target cells. Next we analyzed that viral RNA is packaged at similar level in the presence or absence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. Then it is revealed that the differences in the retroviral restriction properties by HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> don't come from the differences in the subcellular localization since both proteins were observed in both the cytoplasm and nucleus in the producer cell. Further we excluded the canonical mechanism that the hA3A<sup>[27WVS<sup>29</sup>]</sup> restrict HIV-1 through cytidine deamination of the viral genome. Furthermore, we showed that hA3A<sup>[27WVS<sup>29</sup>]</sup> is package into progeny virions and it resulted in lower levels of HIV-1 transcripts in target cells. In addition, The presence of hA3A<sup>[27WVS<sup>29</sup>]</sup> results in decreased proviral integration in target cells. Finally, we confirmed that hA3A<sup>[27WVS<sup>29</sup>]</sup> restricts HIV-1 at the level of reverse transcription in target cells. However, colorimetric Reverse Transcriptase Assay revealed that hA3A<sup>[27WVS<sup>29</sup>]</sup> didn't impair exogenous reverse transcriptase enzyme activity *in vitro*.

### Introduction

Previously, we showed that the insertion of WVS motif is alone and sufficient to restore the antiviral activity towards HIV-1. Single amino acid substitution and single/double deletion mutants revealed that the length and not necessarily the nature of the amino acid is most important for defining HIV-1 restriction. However, the mechanism of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>

restricting HIV-1 remains unknown. Initial experiments showed that in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>, the virions can successfully be released but have impaired replication for the next round of infection. Thus we will follow the replication map (figure 54) to explore the possible inhibition mechanism step by step.

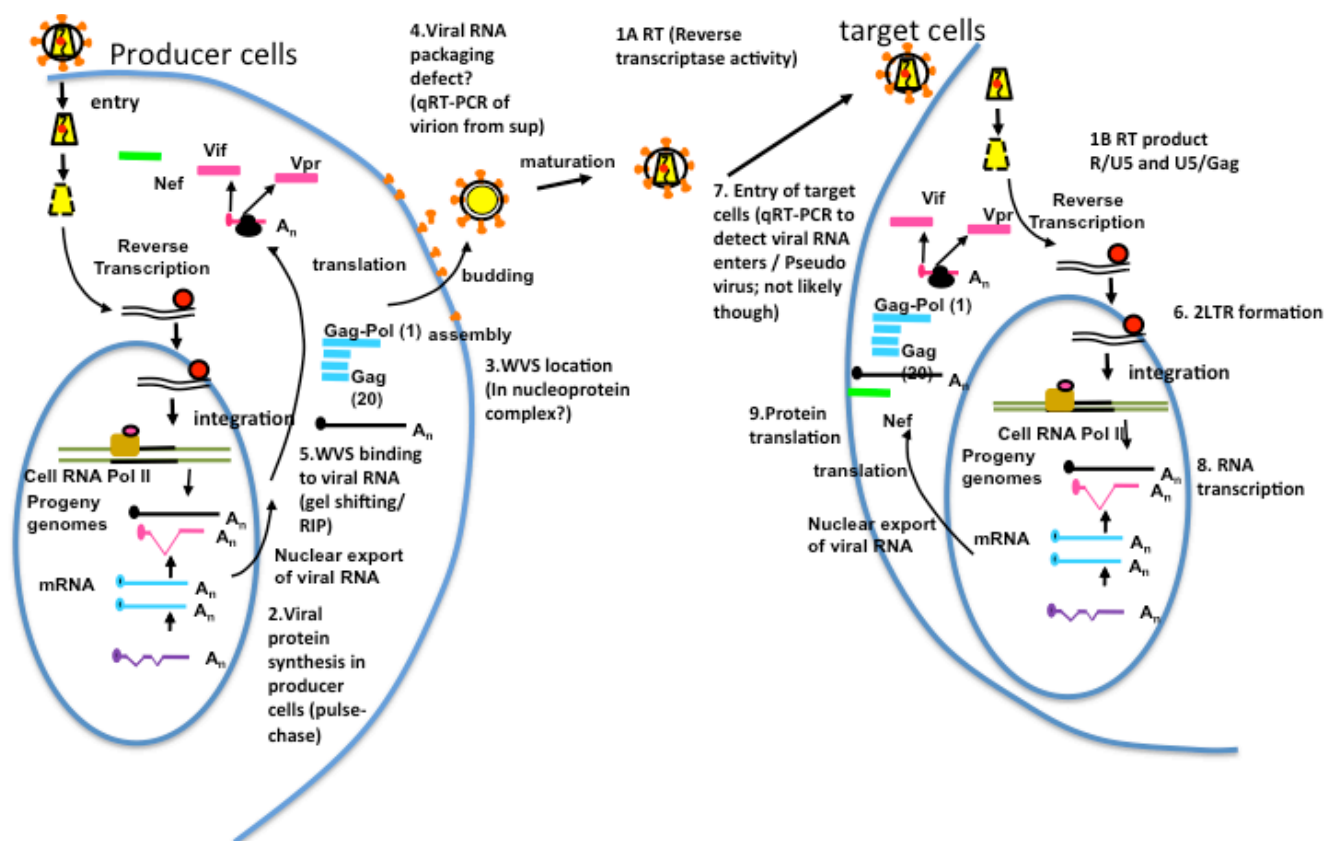
## Results

### **Viral proteins are synthesized and processed similarly in the presence of hA3A or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>**

Previously, we shown that the infectivity of virions produced from hA3A<sup>[27WVS<sup>29</sup>]</sup> expressing cells are down by TZM-bl titrating. To determine if there are less virions released or further infection/replication is impaired, we determined the level of p24 (HIV-1 capsid protein) released from producer cells using HIV-1 p24 Antigen ELISA 2.0 kit from Zeptometrix. In detail, 293 cells were co-transfected with APOBEC plasmids and pNL4-3 (widely used lab-adapted HIV-1 strain). The supernatant, containing released virions was collected 48h post-transfection and subjected to p24 ELISA. It was shown that the p24 level is not significantly lower than control. That means in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>, the virions can successfully be released but have impaired replication for the next round of infection.

Then we confirmed that by showing that the biosynthesis and processing of viral proteins are same in the presence of the empty vector (pcDNA3.1(+), or in vectors expressing HA-hA3A or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> using pulse-chase. We performed pulse-chase analysis using 293 cells transfected with either the empty vector pcDNA3.1(+), or vectors expressing HA-hA3A or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> and a plasmid with the HIV-1 genome. At 48h post-transfection,

Figure 54. Possible mechanism through which hA3A<sup>[27WVS<sup>28</sup>]</sup> could restrict HIV-1.





cells were starved and radiolabeled with <sup>35</sup>S-methionine/cysteine followed by removal and chase in cold excess methionine/cysteine for 0, 3, and 6 h. Viral proteins were immunoprecipitated from the cell lysates and culture supernatants. Our results indicate that the synthesis and processing of the Env (gp160) and Gag (p55) precursors in cells and the release of gp120 and p24 into the culture supernatants were similar for cultures co-transfected with pcDNA3.1(+), HA-A3A, and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> (Figure 55). These results confirm that HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> expressed similar levels of viral proteins in producer cells.

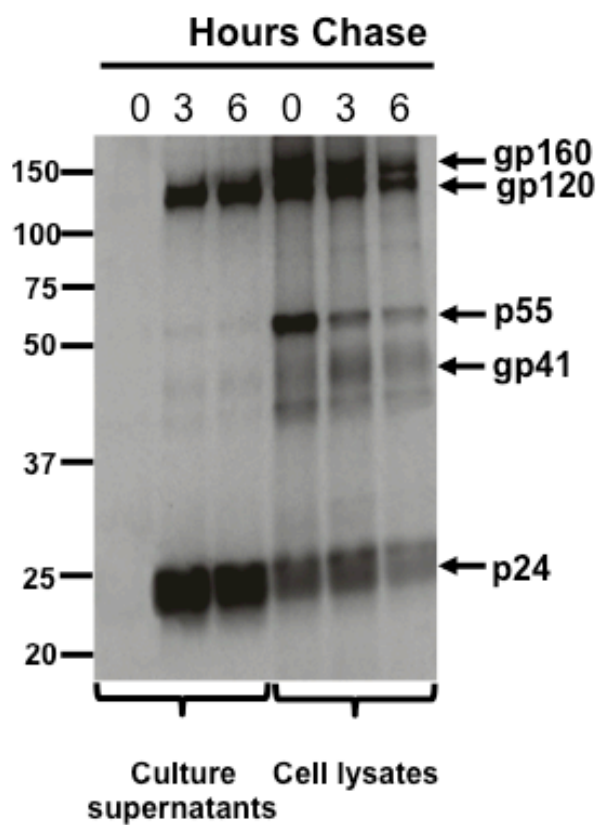
### **Viral RNA package in progeny virions in the presence of HA- hA3A<sup>[27WVS<sup>29</sup>]</sup>**

From previous experiments we knew that similar level of virions were released from producer cells, but we didn't know if they were intact virions or "empty viruses" missing viral RNA. To examine that, we first collected viruses as previous described. Next, we subjected same amount of viruses (calculation is based on p24 ELISA data) to ultracentrifugation through at 20% sucrose cushion to concentrate viruses. The viral RNA was extracted, reverse transcribed and quantified using qPCR. qPCR with HIV-1 Gag indicates that viral RNA is packaged at similar level either with or without the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup> as shown in Figure 56.

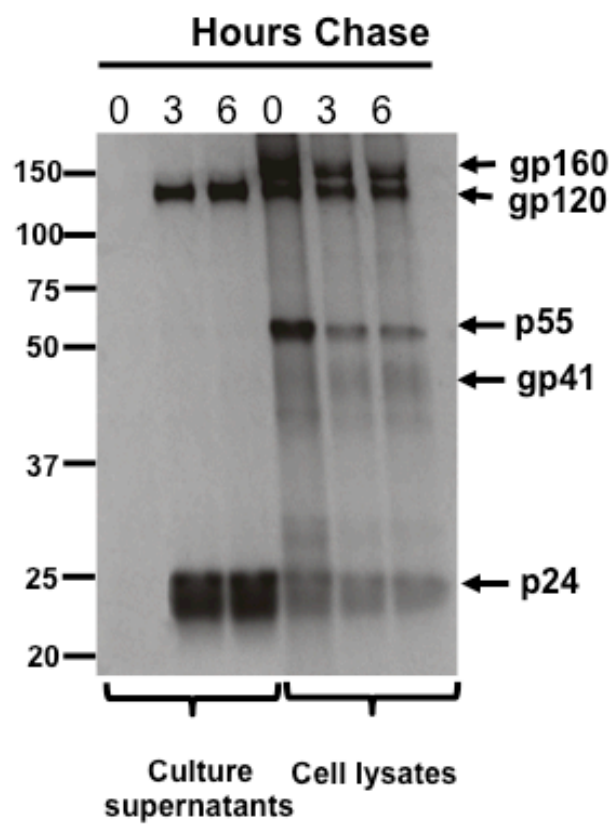
### **hA3A<sup>[27WVS<sup>29</sup>]</sup> results in lower levels of HIV-1 protein synthesis in target cells**

After transcription, both spliced and unspliced (full-length) RNA act as template for translation to produce various viral proteins. They will be assembled, packaged together with the full-length virus genome into progeny virions. The progeny virions will be released and undergo maturation after budding. To examine the viral protein synthesis in target cells, we infect TZM cells with viruses that produce with/without the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>. Then we trace the

Figure 55. Pulse-chase analysis of viral proteins synthesized in the presence of empty pcDNA3.1(+) vector, HA-hA3A, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. 293 cells were transfected with a plasmid with HIV-1 NL4-3 genome (pNL4-3) and either pcDNA3.1(+) or pcDNA3.1(+) expressing HA-A3A, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. At 48 h post-transfection, cells were washed and starved for methionine/cysteine for 2 h. Cells were radiolabeled with 500 µCi of <sup>35</sup>S-methionine/cysteine was added for 30 min, the medium with radiolabel removed and cells incubated in medium containing cold excess methionine/cysteine for 0, 3, or 6 h. HIV proteins were immunoprecipitated from cell lysates using plasma pooled from several pig-tailed monkeys infected previously with SHIV and a monoclonal antibody directed against HIV-1 p24 as described in the Material and Methods. Uninfected 293 cells, radiolabeled and chased for 6 h served as a negative control. All immunoprecipitates were collected on protein A Sepharose, the beads washed with RIPA buffer, and the samples resuspended in sample reducing buffer. Samples were boiled and the SHIV specific proteins analyzed by SDS-PAGE (10% gel). Proteins were then visualized by standard autoradiographic techniques. Panel A. Proteins immunoprecipitated from culture supernatants and cell lysates from cells co-transfected with pcDNA3.1(+) and pNL4-3. Panel B. Proteins immunoprecipitated from culture supernatants and cell lysates from cells co-transfected with vectors expressing HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> and pNL4-3.

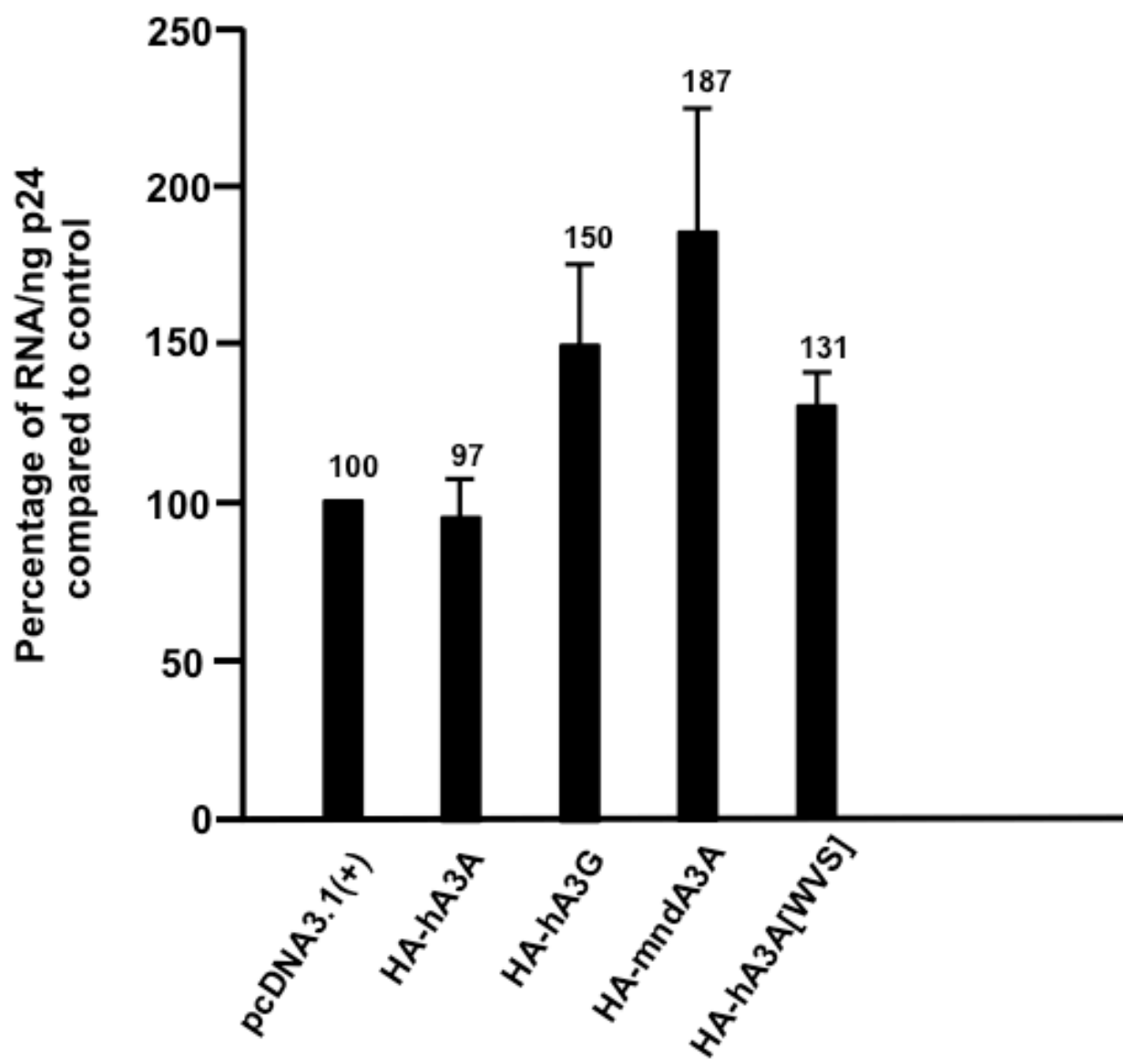


pcDNA3.1(+)/NL4-3



pHA-hA3A<sup>[27WVS<sup>29</sup>]</sup>/NL4-3

Figure 56. Viral RNA is packaged in progeny virions at similar level in the absence and presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>. The viruses were produced by co-transfecting 293 as previously described. Next, the same amount of viruses were subjected (calculation is based on p24 ELISA data) to ultracentrifugation through at 20% sucrose cushion to concentrate viruses. The viral RNA was extracted, reverse transcribed and quantified using qPCR. qPCR with HIV-1 Gag indicates that viral RNA is packaged at similar level either with or without hA3A<sup>[27WVS<sup>29</sup>]</sup>.



biosynthesis of viral protein by pulse-chase label. The viral protein are immunoprecipitated and run on gel for radioautograph. The results indicate that that the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup> led to a decrease in viral protein biosynthesis and subsequent release of viral proteins from target cells (Figure 57).

### **hA3A<sup>[27WVS<sup>29</sup>]</sup> and hA3A are localized to the intracellular compartments**

Previous studies have shown that the hA3G is localized in the cytoplasm of the cell while hA3A and rhA3A are distributed in both the cytoplasm and nucleus (K. Schmitt et al., 2011; Wichroski, Ichiyama, & Rana, 2005). We determined if HIV-1 restriction by HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> could be due to its intracellular localization. 293 cells were transfected with either the empty vector or one expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> or HA-hA3AG. At 48h, cells were fixed and immunostained using an antibody to the HA-tag. Using confocal microscopy, we found that both HA-hA3A and hA3A<sup>[27WVS<sup>29</sup>]</sup> were observed in both the cytoplasm and nucleus (Figure 58) while HA-hA3G was detected in the cytoplasm of cells. Thus, the differences in the retroviral restriction properties by HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> could not be explained by differences in the subcellular localization.

### **hA3A<sup>[27WVS<sup>29</sup>]</sup> is incorporated into virus particles similar to hA3A**

Previous studies have shown that hA3A is incorporated in both HIV-1 and HIV-1 $\Delta$ *vif* virus particles (Goila-Gaur et al., 2007). We determined if HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> was incorporated into virus particles at higher levels than the unmodified hA3A. 293 cells were co-transfected with the empty pcDNA3.1(+) vector or vectors expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G and the plasmid with the HIV-1 $\Delta$ *vif* or HIV-1. At 48h the culture

Figure 57. Lower Level of Virions were Produced with hA3A<sup>[27WVS<sup>29</sup>]</sup> in target Cells. To assess/visualize the production and maturation of virus in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>, we used <sup>35</sup>S-methionine/cysteine to radiolabel all proteins and immunoprecipitate viral proteins using antibody cocktail including anti-SHIV serum (which has antibodies against the Env protein), an anti-p24 monoclonal antibody, and an anti-Env monoclonal antibody. 293 cells were co-transfected with APOBEC3 plasmids and pNL4-3. At 48 h post-transfection, the culture medium containing virus was collected and incubated with 20% polyethylene glycol (PEG) overnight at 4°C. The next day, the mixture was spun at 18,000 x g for 20 min and virus was re-suspended in FBS free medium. The concentrated virus was used to infect target cells for 48 h. Then the medium was removed and target cells were starved in methionine/cysteine-free medium for 2 h. The cells were then radiolabeled with 500 µCi of <sup>35</sup>S-methionine/cysteine for 16 h. The viral proteins in both supernatant and cell lysate were immunoprecipitated using anti-SHIV, anti-p24 and anti-Env antibodies for 18 h. The beads were washed three times and resuspended in same amount of sample reducing buffer. The samples were run on SDS-PAGE and visualized using standard autoradiographic techniques. The gel indicated that that the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup> led to a decrease in viral protein biosynthesis and subsequent release of viral proteins from target cells.

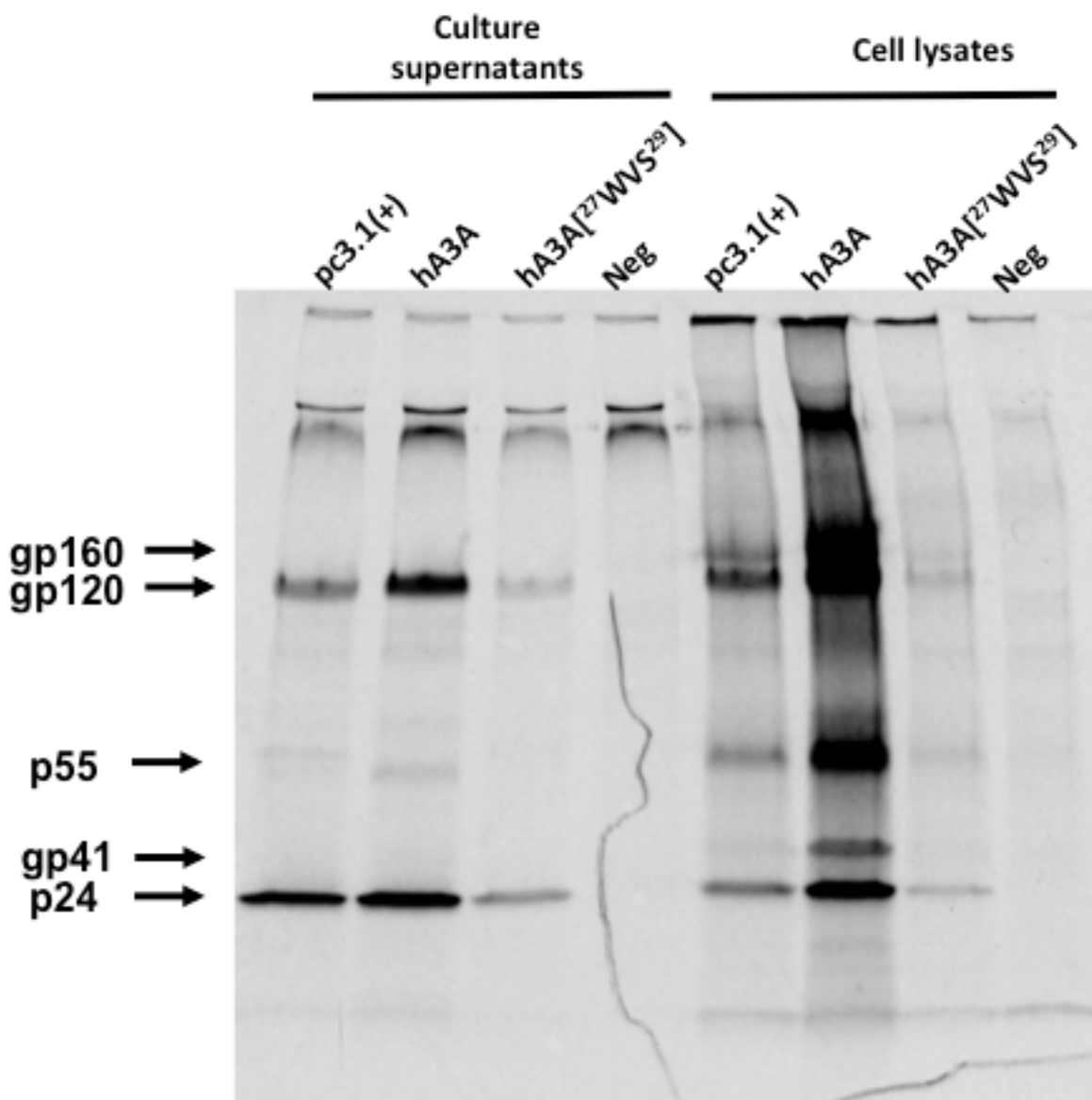
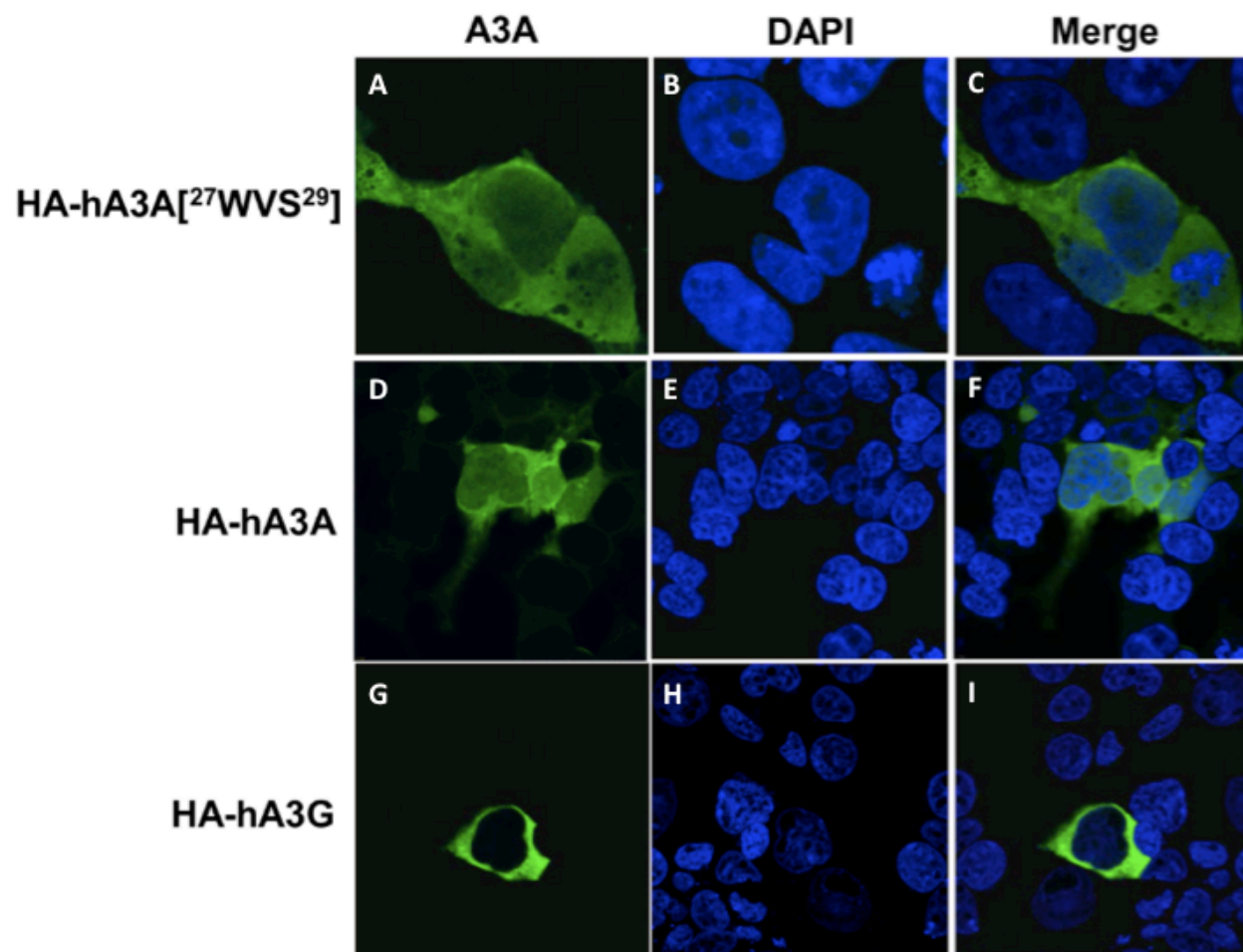




Figure 58. Both hA3A and hA3A<sup>[27WVS<sup>29</sup>]</sup> are localized to the same intracellular compartments of the cell. Panel A-I. 293 cells were transfected with 1.5 µg HA-tagged vectors expressing HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> (Upper three panels), HA-hA3A (Middle three panels), or HA-hA3G (Lower three panels). At 48 h, cells were fixed and stained with an anti-HA-FITC antibody for 1h, washed and stained with DAPI for 5 min. The cover slips were mounted and examined using a Leica TCS SPE Confocal Microscope confocal microscope using a 63× objective with a 2× digital zoom using the Leica Application Suite X (LAS X, LASX) software package as described in Section 4. A 488 nm filter was used to visualize the FITC and a 405 filter used to visualize the DAPI staining. Shown are images for A3A (Left three panels), DAPI staining of nuclei (Middle three panels) and merged GFP and DAPI images (Right three panels).



supernatant was harvested and subjected to ultracentrifugation through a 20% sucrose cushion. The virus was then analyzed on Western blots for the presence of HA containing proteins. As shown in Figure 59, the cells 293 cells transfected with vectors expressing HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> were incorporated into HIV-1 and HIV-1 $\Delta$ vif particles at similar levels based on equivalent levels of p24 Gag). As expected, the levels of HA-hA3G was significantly decreased in HIV-1 virus particles.

### **hA3A<sup>[27WVS<sup>29</sup>]</sup> does not restrict through cytidine deamination**

We examined whether cytidine deamination was the mechanism through which hA3A<sup>[27WVS<sup>29</sup>]</sup> restricted the HIV-1. We first examined whether HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G caused cytidine deamination of the HIV-1 $\Delta$ vif genome. 293 cells were transfected with pcDNA3.1(+) empty vector or one expressing HA-hA3A, HA-hA3G or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> and a plasmid containing the HIV-1 $\Delta$ vif genome. At 48h, the culture supernatant was harvested, DNase-I treated and the virus containing supernatant were used to infect TZM-bl cells for 16h. Total DNA was extracted and the nef gene was amplified, cloned and sequenced. The results of the analysis is presented in Figure 60 and Table 2 . As expected, cells transfected with vectors expressing HA-hA3G and HIV-1 $\Delta$ vif showed many G-to-A mutations (155 mutations/4850 base sequenced or 3.2%) in the nef gene, most of which were in the 5'-GG-3'(81.2%) and 5'-GA-3'(17.4%) contexts. Cells transfected with the empty vector or one expressing HA-hA3A or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> did not exhibit cytidine deamination of the nef gene product. Cells transfected with vectors expressing HA-hA3G, HA-hA3A, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> and HIV-1 resulted in G-to-A mutation levels comparable to the empty pcDNA3.1(+) vector (data not shown). Taken together these results indicate that the HA-

Figure 59. Analysis of the incorporation of hA3A, hA3G and hA3A<sup>[27WVS<sup>29</sup>]</sup> into virus particles. 293 cells were transfected with empty vector pcDNA3.1(+) or with pcDNA3.1(+) expressing HA-hA3A, HA-A3G, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> and a plasmid with the HIV-1 genome. At 36 h, the cells were starved for methionine/cysteine and radiolabeled with <sup>35</sup>S-methionine/cysteine for 18 h. The culture supernatants were collected and virus semi-purified as described in the text. Viral p24 was immunoprecipitated from the viral pellets using an anti-p24 antibody and immunoprecipitates collected on protein A Sepharose as described in the text. The levels of p24 were normalized and the levels of HA-A3 proteins immunoprecipitated using an anti-HA antibody, boiled in sample reducing buffer and separated by SDS-PAGE and visualized by standard autoradiography. Shown are the A3 proteins in virus (panel A), and viral p24 (Panel B) proteins using appropriate antibodies.

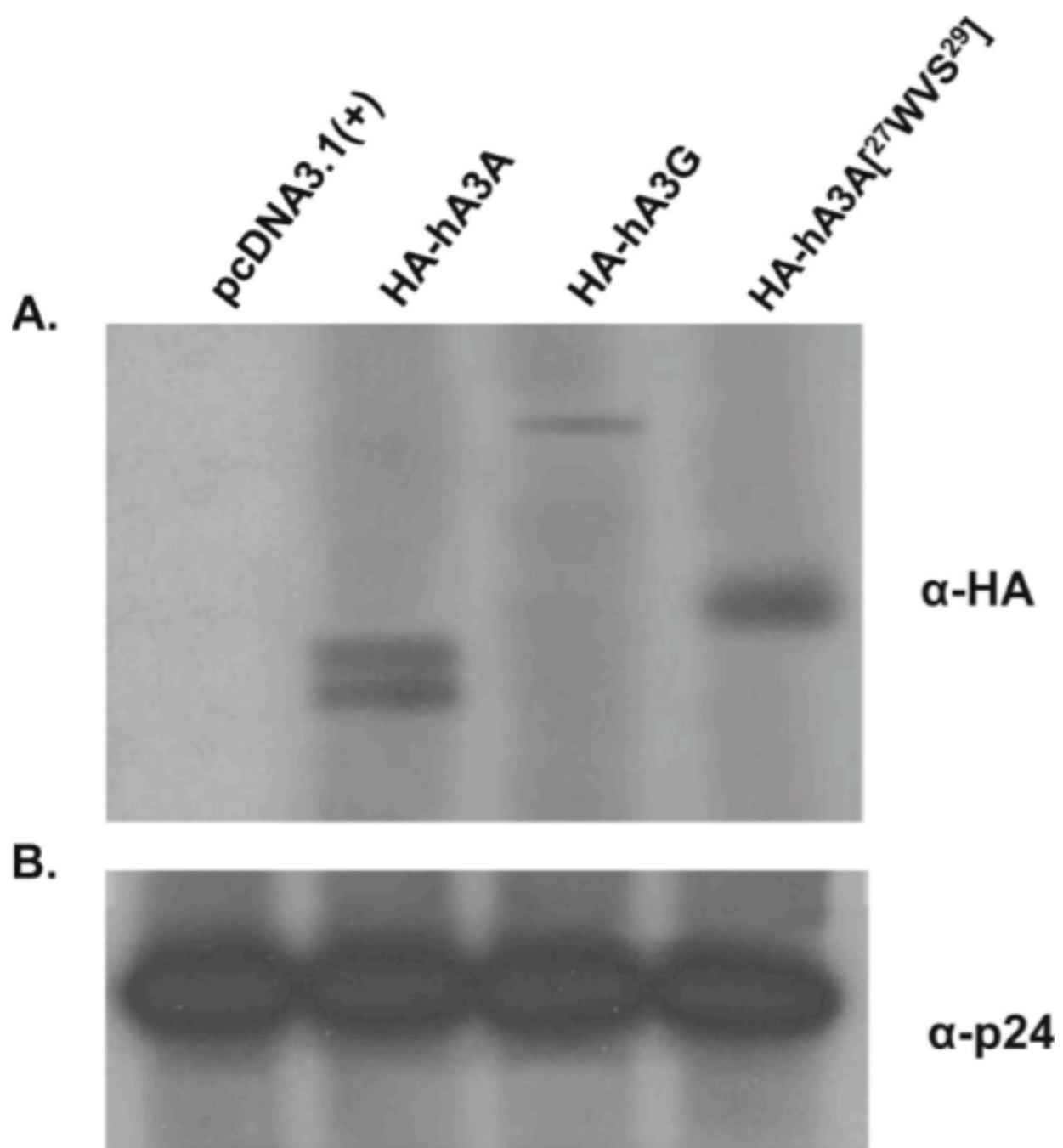


Figure 60. hA3A<sup>[27WVS<sup>29</sup>]</sup> does not restrict HIV-1 through cytidine deamination. 293 cells were transfected with pcDNA3.1(+), or one expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G and a plasmid with the HIV-1 $\Delta$ *vif* genome (strain NL4-3). At 48 h post-transfection the culture medium was harvested, DNase I treated, and used to infect TZM-bl cells. At 16 h following inoculation of TZM-bl cells, the cells harvested and total DNA extracted. A region of *nef* spanning the polypurine tract was amplified by PCR, cloned into pGEM-TEasy vector and 10 clones sequenced. The number of G-to-A changes are presented as ticks on the region sequenced. Panel A. G-to-A mutations from cells transfected with HA-hA3G/HIV-1 $\Delta$ *vif*. Panel B. G-to-A mutations from cells transfected with HA-hA3A/HIV-1 $\Delta$ *vif*. Panel C. G-to-A mutations from cells transfected with HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>/HIV-1 $\Delta$ *vif*. Panel D. G-to-A mutations from cells transfected with pcDNA3.1(+)/HIV-1 $\Delta$ *vif*.

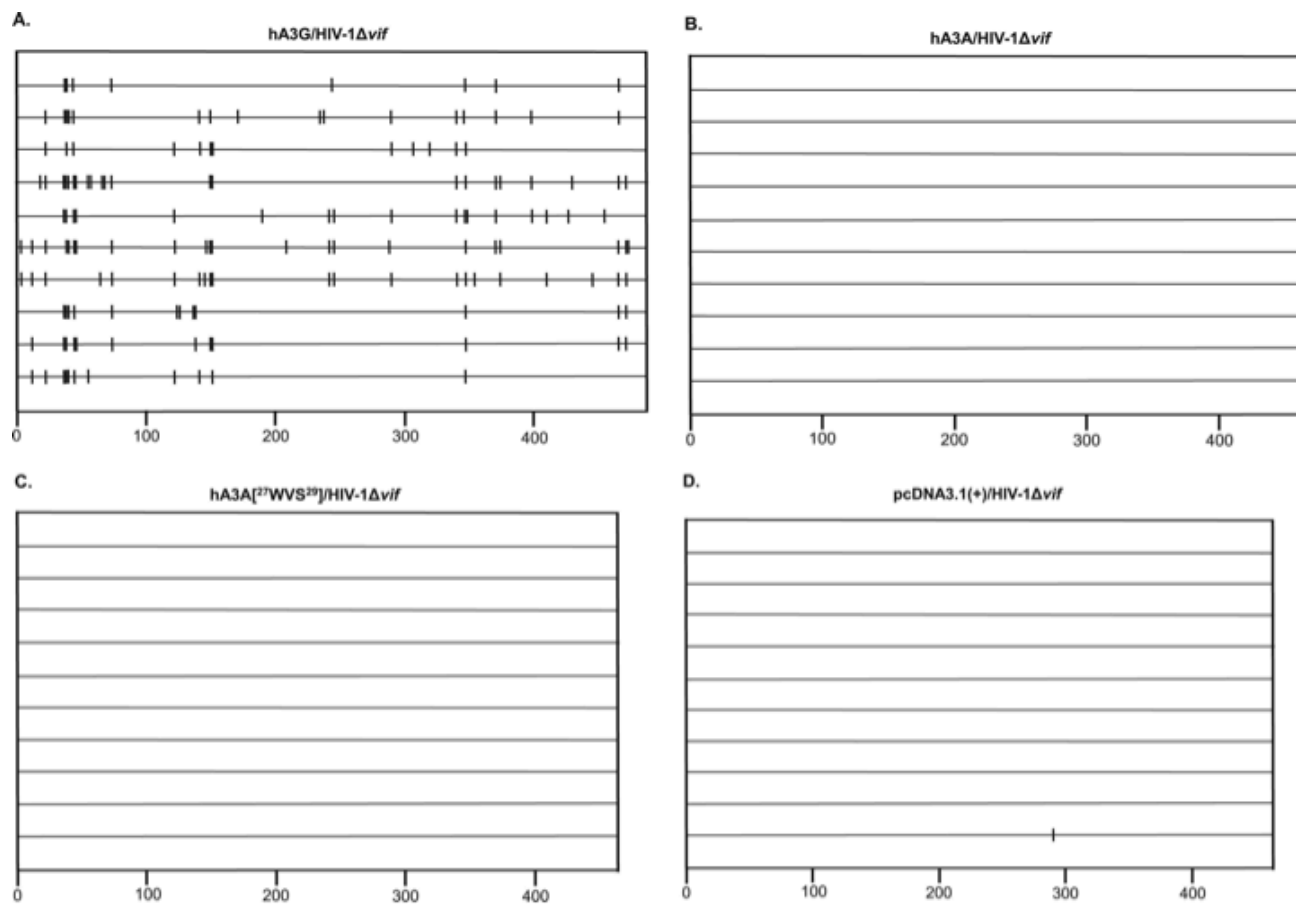


Table 2

**Hypermutation assay of HIV-1 $\Delta$ vif grown in the presence of hA3G, hA3A, hA3A[WVS] or empty plasmid.**

	Number of bases sequenced	Number G-to- A mutations (% mutations)	Number G-to-A mutations in 5'-GG-3' context (% of G-to-A mutations)	Number G-to-A mutations in 5'-GA-3' context (% of G-to-A mutations)
Vector				
hA3G	4,850	155 (3.2)	126 (81.2)	27 (17.4)
hA3A	4,850	0 (0)	0 (0)	0 (0)
hA3A <sup>[27WVS29]</sup>	4,850	0 (0)	0 (0)	0 (0)
pcDNA3.1(+)	4,850	1 (0.02)	1 (0.02)	0 (0)



hA3A<sup>[27WVS<sup>29</sup>]</sup> did not restrict HIV-1 through cytidine deamination of the viral genome.

### **hA3A<sup>[27WVS<sup>29</sup>]</sup> leads to less viral transcripts in target cells**

Transcription process initially results in the early synthesis of regulatory HIV-1 proteins such as Tat and Rev. Tat will bind to the Transactivation Response element and stimulates the transcription of longer RNA transcripts. Rev also helps with this process, and ensures the export of the correctly processed messenger (such as Gag) and genomic RNA from the nucleus to the cytoplasm. Thus, we will quantify the amount of early transcript (Rev) and later transcript (Gag) in target cells. 293 cells were co-transfected with either the empty pcDNA3.1 (+) vector or hA3A, hA3A<sup>[27WVS<sup>29</sup>]</sup> and pNL4-3 (a plasmid containing the intact HIV-1 genome). After 48 h, the culture supernatant was harvested and the virus containing supernatant was used to infect TZM-bl cells for 48 h. Total RNA was extracted and subjected to qRT-PCR with either Rev or Gag primers and the probe. My results indicate that the RNA level of Rev and Gag transcripts were significantly decreased in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup> as shown in figure 61.

### **The presence of hA3A<sup>[27WVS<sup>29</sup>]</sup> results in decreased proviral integration in target cells.**

After reverse transcription, the newly formed HIV c-DNA will be accompanied by integrase to form preintegration complex (PIC). The PIC enters nucleus and the double stranded linear template inserted into host chromosome at various sites with a preference on genes possessing high numbers of introns. To determine whether it function at integration step, We first examined the amount of 2-LTR in target cell post infection. Since TZM-bl cells were engineered to contain HIV-1 Long terminal repeats (LTRs) consisting U3, R, and U5 region, we used HIV pseudotyped with VSV-G to infect 293 cells (pseudoviruses are produced as shown in figure 62). The qPCR revealed that the 2LTR is not significantly different with/without the presence of

Figure 61. hA3A<sup>[27WVS<sup>29</sup>]</sup> results in less early transcription product (Rev) and less late transcription product (Gag). 293 cells were co-transfected with various A3A protein and HIV-1 genome. 48h post transfection, supernatant were collected and used to infect TZM-bl cells for 48h. Total RNA was extracted and subjected *in vitro* reverse transcription and the level of early transcript (Rev) and late transcript (Gag) are quantified though qPCR and normalized by  $\beta$ -actin.

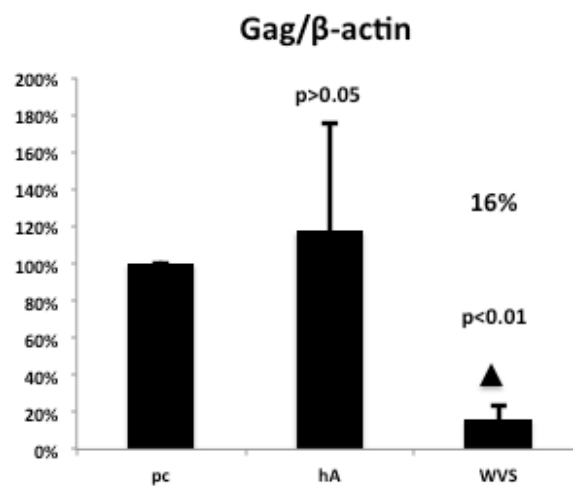
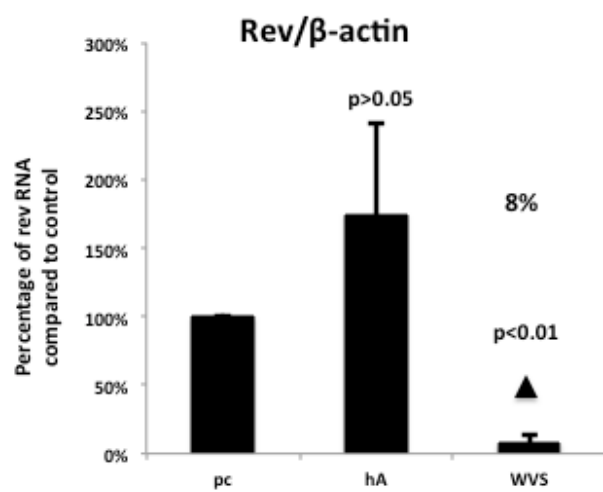
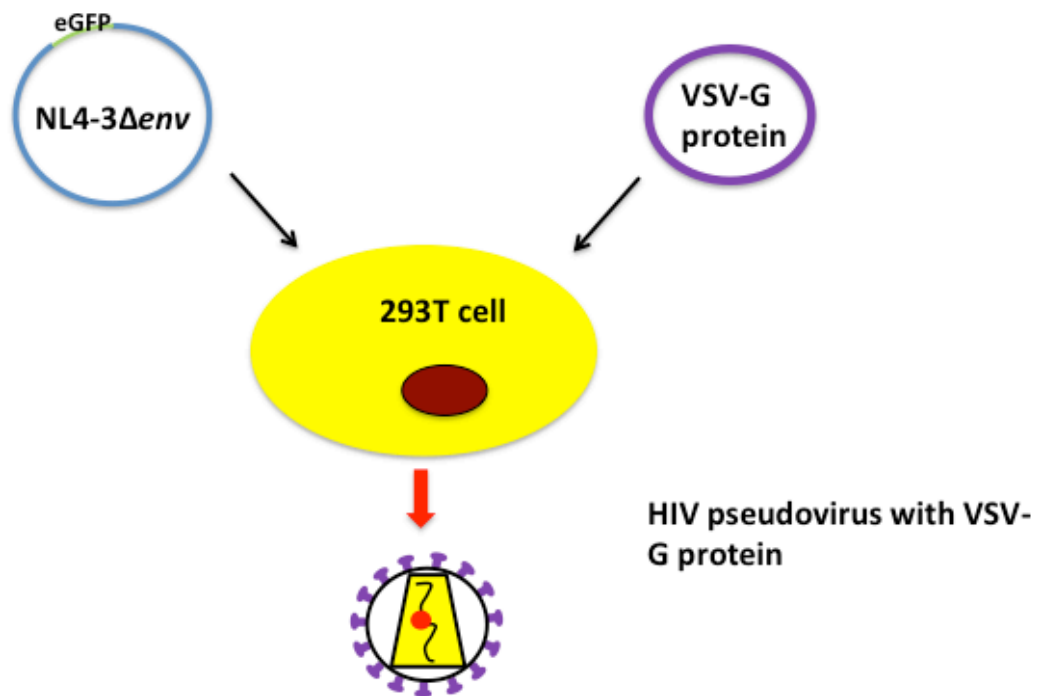


Figure 62. Diagram of VSV-G pseudotyped HIV-1 virion.

## Pseudovirus



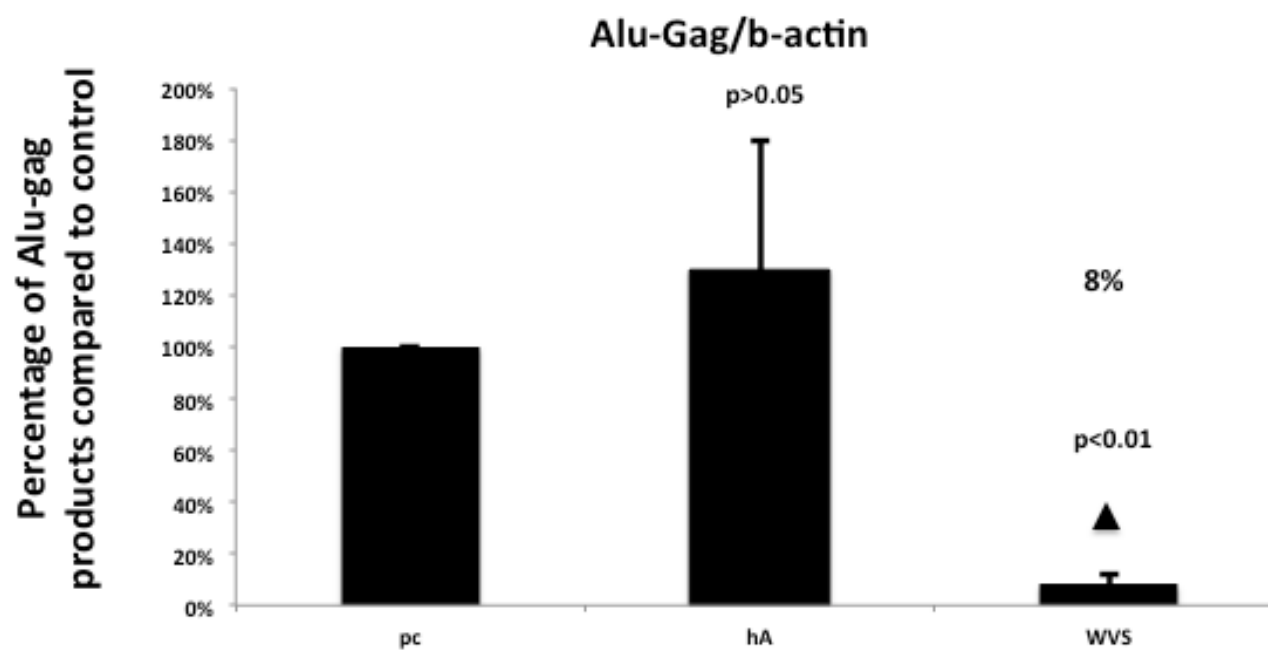
hA3A<sup>[27WVS<sup>29</sup>]</sup>. That means either hA3A<sup>[27WVS<sup>29</sup>]</sup> doesn't inhibit integration step or the effect inefficient integration and low level of double strand viral DNA counteract each other.

To distinguish these two possibilities, we checked the level of Alu-gag, which is the direct indicator of viral integration level. Alu is a family of repetitive transposable elements in the human genome. Each provirus has a unique distance to the nearest Alu sequence. Thus, with a primer specific to the Alu sequence and a primer specific for the inside of provirus sequence, we could amplify the junction part of the integration. However, since the distance between each provirus and the closest Alu sequence varies, it is difficult to produce a standard curve for quantification. Alternatively, we applied a two-step amplification by first performing a regular amplification using Alu-Gag primers followed by amplification of 1 ul of the sample from first round PCR to quantitative real-time PCR with R/U5 primers and a probe. The two-step amplification confirmed that in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>, the integration level was significantly diminished as shown in figure 63.

### **hA3A<sup>[27WVS<sup>29</sup>]</sup> does not interfere with virion reverse transcriptase but does decrease reverse transcription in target cell**

We next examined the reverse transcriptase activity of virus grown in the presence of empty vector pcDNA3.1(+), or vectors expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G. 293 cells were transfected with each pcDNA3.1(+) vector and a plasmid with the NL4-3 genome. At 48 h, the culture medium was collected, centrifuged at low speed to remove cellular debris, and the virus pelleted through a 20% sucrose cushion. The p24 levels were determined and equivalent levels of virus (based on p24 content) were used in reverse transcriptase assays with an exogenous template according to the manufacturer's instructions. The results indicate

Figure 63. hA3A<sup>[27WVS<sup>29</sup>]</sup> Results in less Integration (Alu-Gag). 293 cells were co-transfected with various A3A protein or pcDNA3.1(+) as control, and HIV-1 $\Delta env$  and VSV-G. 48h post transfection, supernatant are collected and used to infect 293 cells for 48h. Total DNA is extracted and the Alu-Gag level is quantified through a two step nested qPCR.





that reverse transcriptase activity of the viruses grown in the presence of the three A3A proteins was similar, indicating that the presence of the A3A proteins did not block reverse transcriptase activity (Figure 64). Controls in which AZT (100 nM) was added to the reactions abolish reverse transcriptase activity.

We also analyzed reverse transcription in target cells. For these experiments, 293 cells were transfected with plasmid with the pNL4-3 genome and either the empty pcDNA3.1(+) vector or vectors expressing HA-hA3A, HA-hA3G, or HA-A3A<sup>[27WVS<sup>29</sup>]</sup>. At 48 h the virus stocks were harvested and the p24 levels in stocks determined. Using equivalent levels of p24, viruses were used to inoculate C8166 cells for 24 h. Total cellular DNA was isolated and the early R/U5 and late U5/Gag reverse transcription products quantified using real DNA PCR as previously described (Suzuki et al., 2003). Our results indicate that the empty vector, pcDNA3.1(+), and vectors expressing HA-hA3A and HA-hA3G had similar levels of R-U5 and U5-gag products while HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> had a reduction in the levels of R-U5 and U5-gag products compared to the pcDNA3.1(+) control as shown in figure 65. These results indicate that HA-hA3A <sup>[27WVS<sup>29</sup>]</sup> reduced the levels of reverse transcription products in target cells.

## Discussion

Our results presented here indicate that viral protein synthesis and processing was similar in cells transfected with vectors expressing HA-hA3A or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. We first exclude the possibility that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> inhibits HIV-1 replicating by causing cytidine hypermutation of the HIV-1 genome. We further showed that HA-hA3A and HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> both had a nucleocytoplasmic localization in producer cells. We also analyzed virus produced in the presence of A3 proteins for reverse transcriptase (RT) activity using an

Figure 64. The presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> does not decrease virion reverse transcriptase activity. Reverse transcriptase (RT) activity of virus grown in the presence of HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, and HA-hA3G. 293 cells were co-transfected with a plasmid with HIV-1 NL4-3 genome and either pcDNA3.1(+) or expressing HA-A3A, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G. At 48 h, the culture supernatants were collected and partially purified as described in the text. The levels of p24 were quantified and equivalent levels of p24 were used to determine RT activity using a colorimetric Roche RT kit. Activity is plotted as percent RT activity with pcDNA3.1(+)/pNL4-3 normalized to 100%. AZT was used to inhibit reverse transcriptase activity

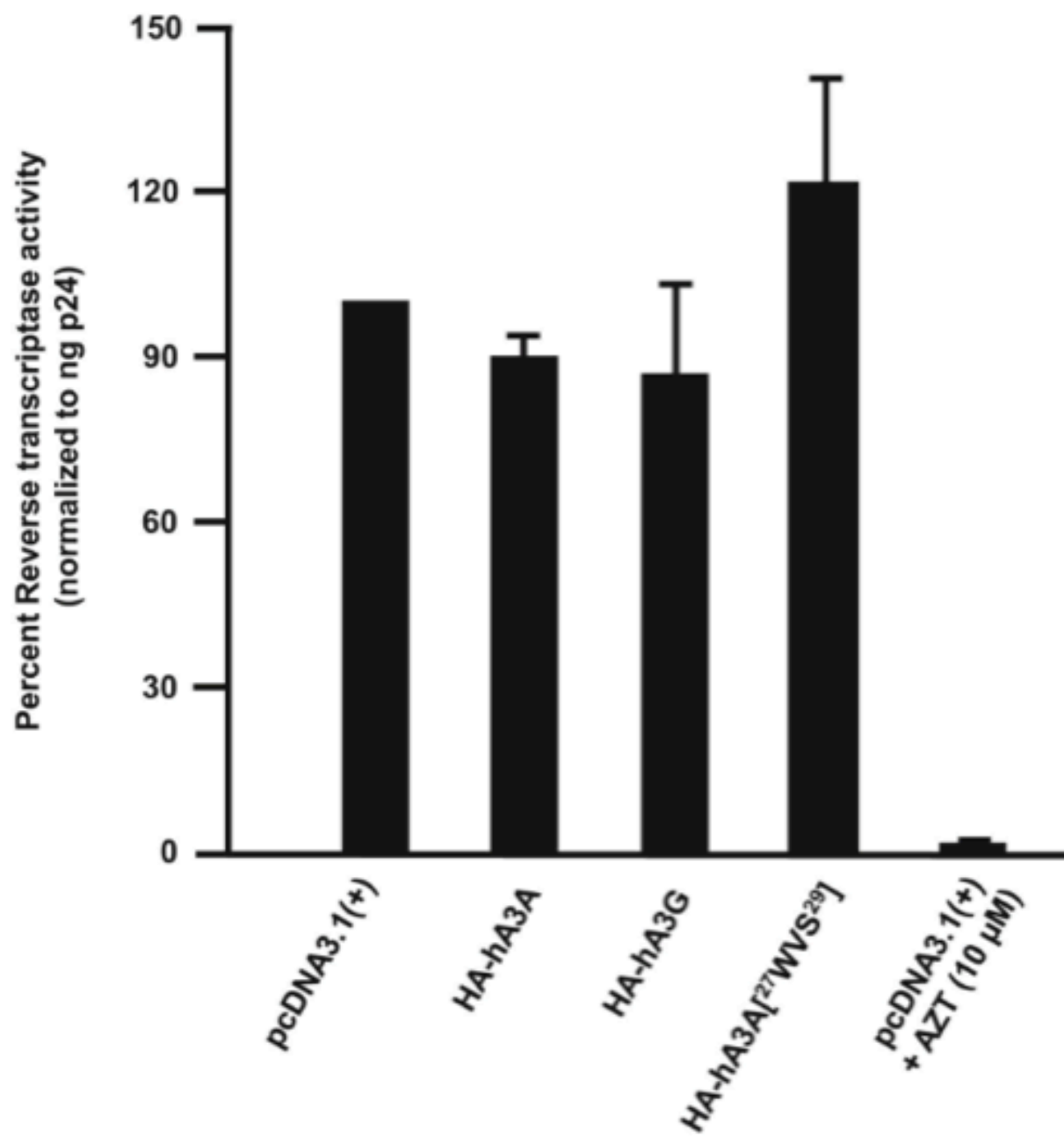
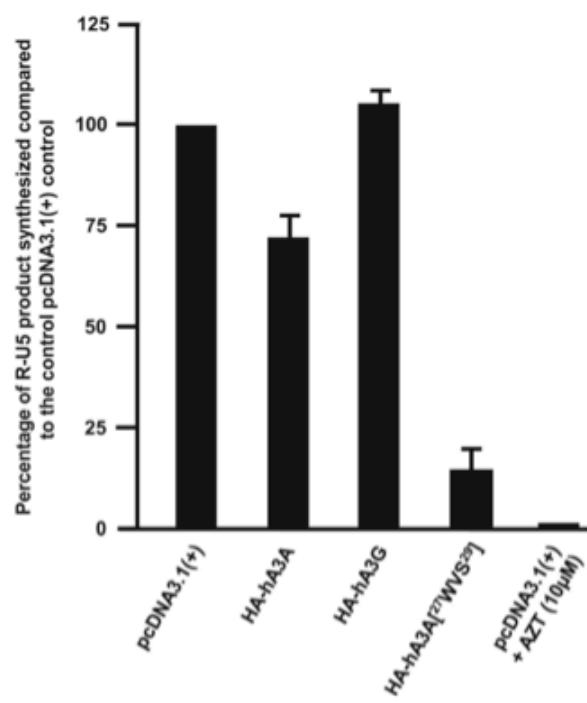
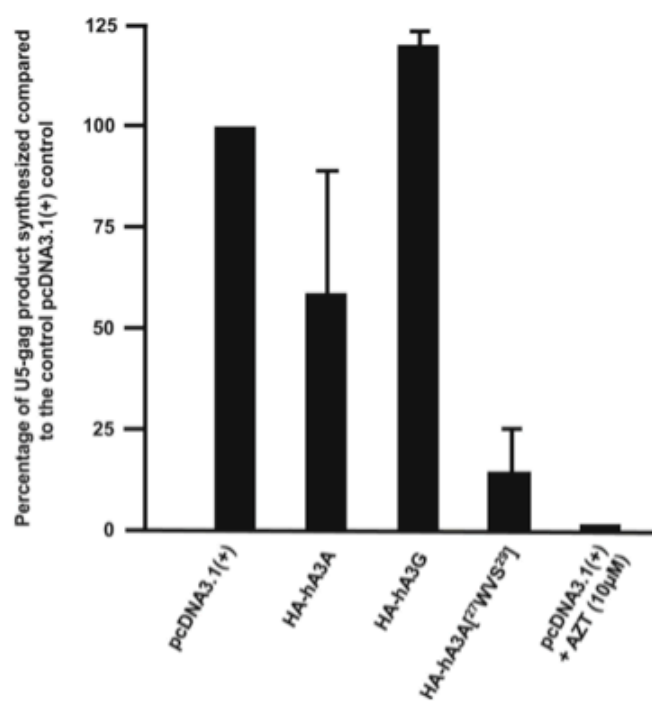


Figure 65. The presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> decreases reverse transcription in target cells. 293 cells were co-transfected with a plasmid pNL4-3 (containing the NL4-3 genome) and either empty vector pcDNA3.1(+) or one expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G. Virus was harvested, p24 levels determined, and equivalent levels of p24 were used to inoculate C8166 cells. At 24 h post-inoculation, the cells were washed three times, total DNA extracted, and the levels of early RT product R-U5 (Panel A) and late RT product U5-*gag* (Panel B) transcripts quantified using real time DNA PCR as described in the text. The values obtained are presented as percent product compared to the pcDNA3.1(+)/pNL4-3 control. AZT was used to inhibit reverse transcriptase activity. The experiments were performed three times and statistical differences with the pcDNA3.1(+)/pNL4-3 control were evaluated using a two-tailed Student's *t*-test, with  $p < 0.05$  (▲) considered significant.

**A****B**

exogenous template. Our results indicate that RT activity was similar for HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, and HA-hA3G. These results suggest that incorporated HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> did not directly interact with reverse transcriptase to block its activity. Alternatively, it is also possible that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> may interact weakly with the reverse transcriptase but that the conditions of the reverse transcriptase disrupted this interaction. However, when virus grown in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> was used to inoculate target cells, we observed a significant decrease in the levels of early (R-U5) and late (U5-gag) reverse transcriptase products. Since the RT activity of virus grown in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> was not significantly decreased, it suggests that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> may interact with the viral RNA to decrease levels of early and late RT products. This would explain the deaminase-independent restriction by HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. Previous studies have shown that hA3A binds to longer RNA templates (40 nucleotides) at a slightly higher binding affinity than single stranded DNA (Byeon et al., 2013; Mitra et al., 2014) and it is possible that the insertion into HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> may slightly alter its structure to permit better binding to regions of the viral RNA.

## **Materials and Methods**

### ***Pulse-chase analysis of viral proteins in the presence of hA3 proteins***

293 cells were transfected with a plasmid containing the HIV-1 NL4-3 genome (pNL4-3) and empty pcDNA3.1(+) vector or expressing either HA-hA3A or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. At 48h post-transfection, the medium was removed and cells were incubated in methionine/cysteine-free medium for 2h. The cells were then radiolabeled with 500 mCi of <sup>35</sup>S-Translabel (methionine and cysteine, MP Biomedical) for 30 min. The radiolabel was chased in DMEM containing 100X unlabeled methionine/cysteine medium for 0, 3 and 6 h. HIV-

1 proteins were immunoprecipitated using a monkey anti-SHIV antibody and a monoclonal antibody against p24 (#24-4 from the NIH AIDS Reagent Program; Fouchier et al., 1997; Simon et al., 1997) and immunoprecipitates collected on protein A-Sepharose beads. The beads were washed three times with 1X radioimmunoprecipitation buffer (RIPA: (50 mM TrisHCl, pH 7.5; 50 mM NaCl; 0.5% deoxycholate; 0.2% SDS; 10 mM EDTA), and the samples resuspended in sample reducing buffer. The samples were boiled, proteins separated by SDS-PAGE (12% gel), and proteins visualized using standard autoradiographic techniques.

### ***Subcellular localization of hA3 proteins***

For intracellular localization of A3A proteins, 293 cells were plated onto 13 mm cover slips in a 6 well plate and transiently co-transfected with vectors expressing HA-A3A, hA3A<sup>[27WVS<sup>29</sup>]</sup> or hA3G using a polyethylenimine (PEI) transfection reagent (ExGen<sup>TM</sup> 500, MBI Fermentas) according to the manufacturer's instructions. Cultures were maintained for 48h, the medium removed, washed in PBS (pH 7.4) and fixed in 4% paraformaldehyde (prepared in PBS) for 15 min. Cover slips were washed in PBS, permeabilized in 2% Triton-X-100, and incubated in PBS containing 5% BSA. The coverslips were washed and incubated with a mouse monoclonal antibody against the HA-tag conjugated to FITC. (Abcam AB1208) for 1 h. The cover slips were counterstained with DAPI (1 µg/ml) for 5 min, washed and mounted in glycerol containing mounting media (Slowfade antifade solution A, Invitrogen). The cover slips were examined using a Leica TCS SPE Confocal Microscope confocal microscope using a 63 objective with a 2 digital zoom using the Leica Application Suite X (LAS X, LASX) software package. A 488 nm filter was used to visualize the FITC and a 405 filter used to visualize the DAPI staining.

### ***Detection of hA3A<sup>[27WVS<sup>29</sup>]</sup> in virus particles***

To determine if hA3A<sup>[27WVS<sup>29</sup>]</sup> was incorporated into virus particles at higher levels than hA3A, 293 cells were transfected with a plasmid with the HIV-1 NL4-3 genome or one lacking a functional Vif (HIV-1 $\Delta$ vif) and either the pcDNA3.1(+) as the empty vector control or pcDNA3.1(+) expressing HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, or HA-hA3G. At 36h, the cells were starved for methionine/cystine for 2h and radiolabeled with 35S-methionine/ cystine for 16h. At 48h post-transfection, the culture medium was harvested, cell debris removed by low speed centrifugation at 1,200 rpm for 10 min. The virus containing supernatant was layered onto a 20% sucrose cushion and subjected to ultracentrifugation at 30,000 rpm (SW55Ti rotor, 109,000 g) for 2 h. The virus pellet was resuspended in PBS, pH 7.4 and viral p24 was immunoprecipitated from the viral pellets using an anti-p24 antibody and immunoprecipitates collected on protein A Sepharose. The levels of p24 were normalized and the levels of HA-A3 proteins immunoprecipitated using an anti-HA antibody, boiled in sample reducing buffer and separated by SDS-PAGE and visualized by standard autoradiography.

### ***Analysis of cytidine deamination by A3 proteins***

Previous studies have shown that hA3A did not significantly introduce G-to-A mutations into HIV-1 $\Delta$ vif. We determined if hA3A<sup>[27WVS<sup>29</sup>]</sup> could introduce G-to-A mutations into the HIV- 1 $\Delta$ vif genome. For hypermutation analysis, 293 cells were transfected with empty pcDNA3.1(+) vector or pcDNA3.1(+) expressing HA-hA3A, HA-hA3G or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, and a vector with the HIV-1 or HIV-1 $\Delta$ vif genome. After 48h, the supernatant containing virus was subjected to low speed centrifugation. The resulting supernatant was DNase I-treated (Fermentas) at 37<sup>0</sup>C for 30 min to eliminate any trace of plasmid carry-over from the in-



itial transfection. The DNase-I-treated supernatant was titrated on TZM-bl cells to assess both infectivity and hypermutation. Twenty-four hours post-infection, total DNA cellular DNA was harvested and extracted using the DNeasy kit and the manufacturer's instructions (Qiagen). The DNA was used in a DNA polymerase chain reaction to amplify a 480 base pair fragment of nef. The PCR reaction was carried out using rTaq, the manufacturer's instructions (Takara). The PCR reactions were performed using an Applied Systems 7500 Thermal Cycler with the following thermal profile: 95 °C for 2 min, 1 cycle; 95 °C for 30 s, 48 °C for 30 s, 65 °C for 2 min, 35 cycles; 65 °C for 7 min. The PCR products were separated on a 1% agarose gel, isolated from the gel and the products from five reactions were pooled. The purified products were cloned into pGEM-T Easy vector and the inserts sequenced. The frequency of mutations were determined in the negative controls (pcDNA3.1(+) vector plus pNL4-3 and pcDNA3.1(+) vector plus pNL4-3 $\Delta$ vif) and positive controls (HA-hA3G plus pNL4-3 $\Delta$ vif) and compared with hA3A and hA3A<sup>[27WVS<sup>29</sup>]</sup>. The flow path of hypermutation assay is shown in figure 66.

### ***Assessment of HIV-1 protein synthesis in target cells***

To assess/visualize the production and maturation of the progeny virions in the presence of hA3A<sup>[27WVS<sup>29</sup>]</sup>, we used 35S-methionine/cysteine to radioactively label all proteins and immunoprecipitate viral proteins using an antiviral protein antibody cocktail that includes anti-SHIV serum, an anti-p24 monoclonal antibody, and an anti-env monoclonal antibody. 293 cells were co-transfected with various APOBEC3 plasmids and pNL4-3. At 48h post-transfection, the culture medium containing viruses was collected and incubated with 20% polyethylene glycol (PEG) overnight at 4°C. The next day, the mixture was spun at 18,000Xg for 20 min, the viruses were re-suspended in FBS-free media. The concentrated virus preparation

was used to infect target cells for 48 h. Then, the media were removed, and target cells were starved in methionine/cysteine-free media for 2 h. The cells were then radiolabeled with 500  $\mu$ Ci of  $^{35}$ S-methionine/cysteine for 16 h. Viral proteins in both the supernatant and cell lysate were immunoprecipitated using the antiviral protein antibody cocktail for 18 h. The beads were washed three times and resuspended in same amount of the reducing sample buffer. The samples were run on SDS-PAGE and visualized autoradiographically.

***Assessment of viral transcripts in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>***

As shown in figure 67, to detect the viral transcripts level in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, we will quantify the amount of early transcript (Rev) and later transcript (Gag) in target cells. 293 cells were co-transfected with either the empty pcDNA3.1 (+) vector or hA3A, hA3A<sup>[27WVS<sup>29</sup>]</sup> and pNL4-3 (a plasmid containing the intact HIV-1 genome). At 48 h, the culture supernatant was harvested and the virus containing supernatant was used to infect TZM-bl cells for 48 h. Total RNA was extracted and subjected to qRT-PCR with either Rev or Gag primers and probe.

***Assessment of integration in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>***

To determine if HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> interfered with viral genome integration, we employed two different assays. The first assay detected the presence of 2-LTR circles, which occurs at a higher frequency when integration is unsuccessful (Butler et al., 2001). To quantify the levels of 2-LTR circles, 293 cells were transfected with either the empty vector (pcDNA3.1(+)) or the vector expressing HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. At 24 h, equal infectious units of HIV-1 $\Delta$ E-/VSV-G virus were then used to infect 293 cells for 48 h. Total cellular DNA was

Figure 66. APOBEC3 hypermutation assay. 293 were co-transfected with the pNL4-3Δ *vif* genome and with the empty pcDNA3.1(+) vector or one expressing the APOBEC protein. Virus in the culture medium was collected and used to inoculate susceptible target cells. At 48 h, the DNA was extracted and PCR performed using *nef* primers. A3G, which is known to cause hypermutation, was used as positive control

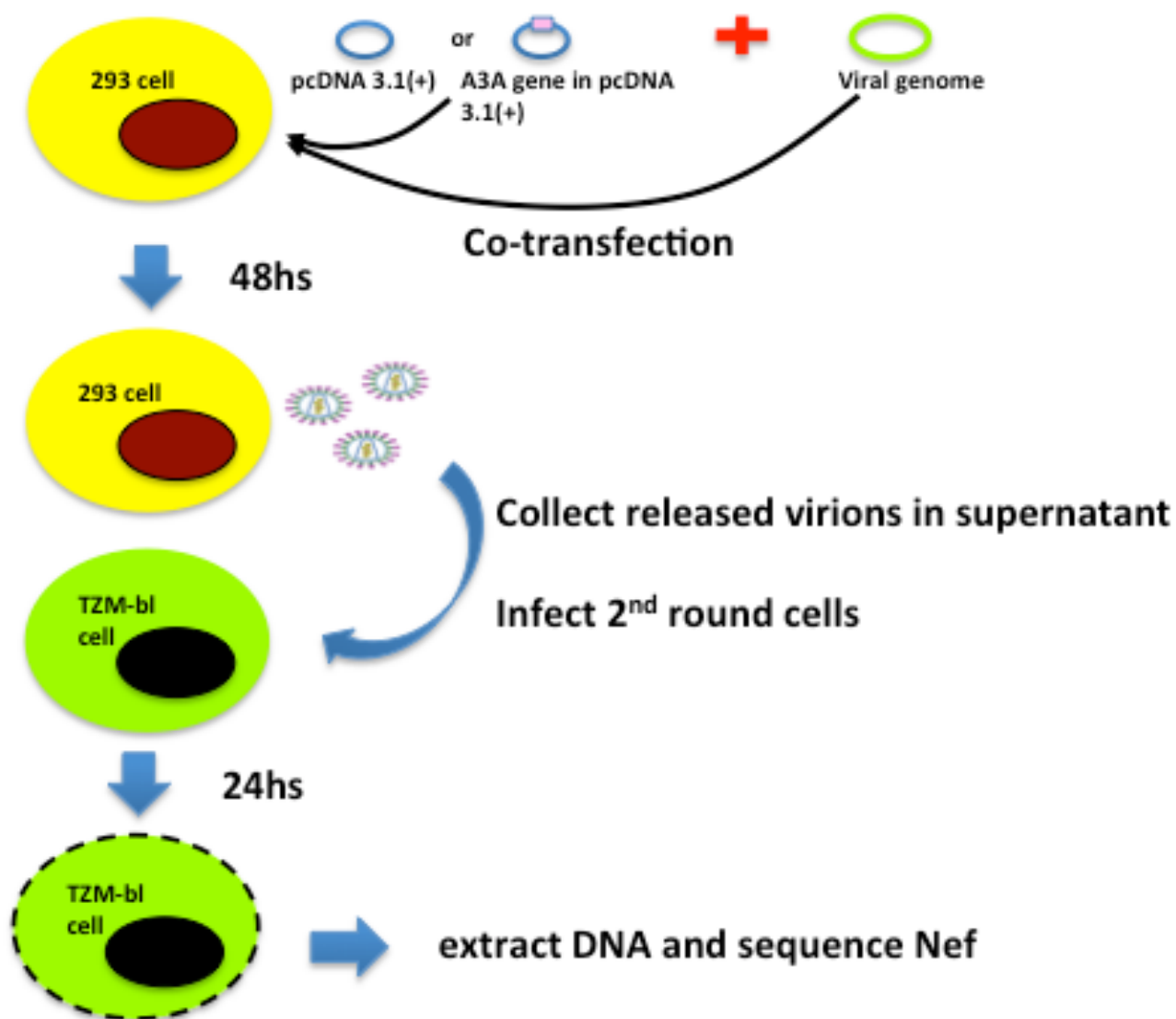
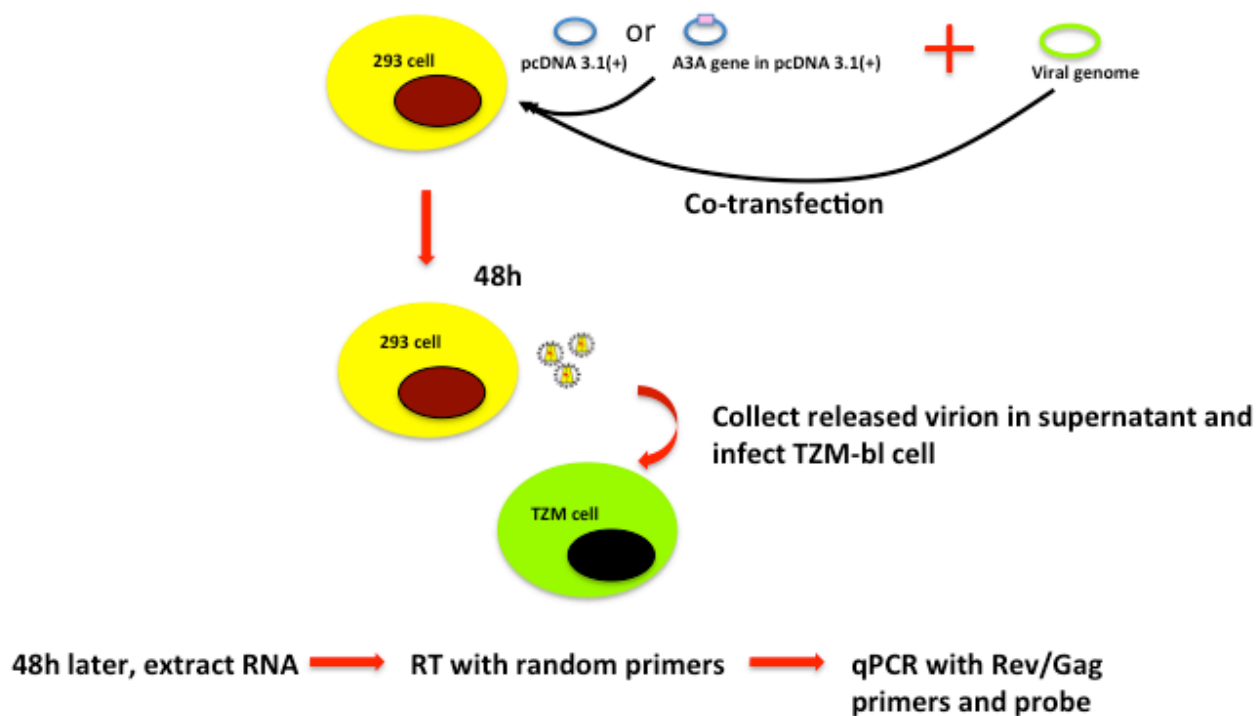


Figure 67. The flowchart for detection of RNA transcripts in target cells. 293 cells were co-transfected with the empty pcDNA3.1(+) vector or expressing various A3A protein, and HIV-1. At 48 h post-transfection, supernatants were collected and used to infect TZM-bl cells for 48 h. Total RNA was extracted and level of early transcript (Rev) and late transcript (Gag) are quantified by RT-qPCR.

## Transcription in target cell



purified using DNeasy Blood & Tissue Kit from Qiagen. The 2- LTR circle levels were detected as previously described (Butler, Hansen, & Bushman, 2001). Real-time PCR was performed using the following oligonucleotide primers: 2-LTR circle forward, MH535: 5'-AAC-TAGGGAACCCACTGCTTAAG-3'; 2-LTR circle reverse, ES2448 (based on MH536): 5' -TCCACAGATCAAGGATCTCTTGTC-3'; and 2-LTR probe, MH603: 5'-(FAM)-ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAM)-3'. A standard curve of the amplification being measured was run ranging from 10 to  $1 \times 10^{11}$  copies. Reactions containing 1X premix (Takara), 0.5  $\mu$ M forward primer, 0.5  $\mu$ M reverse primer, 0.25  $\mu$ M probe and 1X RoxDyeII in a 20ul volume. The thermal cycle is 95  $^{\circ}$ C for 30 s, followed by 40 cycles of amplification at 5s at 95  $^{\circ}$ C and 34s at 60  $^{\circ}$ C.

In addition, we assessed the level of integration was by amplification Alu repeat/gag sequences. The oligonucleotides used for detecting Alu-gag products were: (a) Alu-gag forward oligonucleotide primer, MH535 (above); (b) Alu-gag reverse oligonucleotide primer: SB704: 5'-TGCTGGGATTACAGGCGTGAG-3'. One microlitre of sample from first round regular PCR and subject to real-time PCR with R/U5 primers (forward primer: M667-5'-GGCTAACTAGGGAACCCACTGC-3'; reverse primer: AA55-5'-CTGCTAGAGATT TCCACACTGAC-3') and probe (HIV FAM-5'-TAGTGTGTGCCCGTCTGTTGTGTGAC-3'-TAM) (Suzuki et al., 2003). With each experiment, a standard curve of the amplicon being measured was run in duplicate ranging from 10 to  $1 \times 10^{11}$  copies plus a no-template control. Reactions contained 1 premix (Takara), 0.5  $\mu$ M forward primer, 0.5  $\mu$ M reverse primer, 0.25  $\mu$ M probe primer and 100– 500 ng of template DNA and 1 RoxDyeII in a 20  $\mu$ l volume. After initial incubations of 95  $^{\circ}$ C for 30 s, 40 cycles of amplification were carried out at 5 s at 95  $^{\circ}$ C, 34 s at 60  $^{\circ}$ C. Reactions were analyzed using the ABI Prism 7500 sequence detection system (PE-

Applied Biosystems, Foster City, California). The procedure of integration assay is shown in figure 68 and the two step PCR for quantification of Alu-gag is shown in figure 69 .

***Reverse transcriptase (RT) activity in viruses was measured by a reverse transcriptase colorimetric kit***

A reverse transcriptase colorimetric kit (Roche, USA) was used to measure the RT enzyme activity of progeny viruses from producer cells. Briefly, 293 cells were cotransfected with a plasmid with the NL4-3 genome (pNL4-3) and either the empty pcDNA3.1(+) vector or vectors expressing either HA-hA3A, HA-hA3G, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. At 48 h, the viral culture medium was collected, centrifuged at low speed to removed cellular debris and the virus containing supernatants layered onto a 20% sucrose cushion. The virus was pelleted for 2h at 35,000 rpm (SW41 rotor, Beckman). The virus pellets were resuspended in PBS (pH 7.4) and p24 levels determined and equivalent levels of p24 were used in RT assays according to manufacturer's instructions. The relative RT activity was plotted with the pcDNA3.1(+) control set at 100% activity. The Test principle of *in vitro* RT activity is shown in figure 70 and the procedure is described in figure 71.

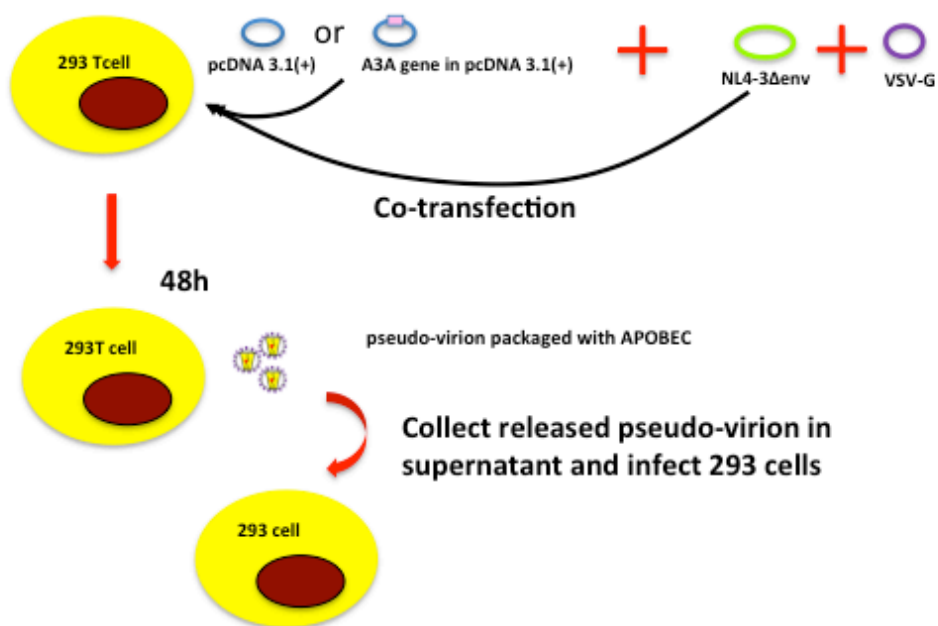
***Assessment of reverse transcription in the presence of HA-A3 proteins***

In order to assess whether incorporation of HA-hA3A <sup>[27WVS<sup>29</sup>]</sup> would block virion reverse transcriptase, 293 cells in 60 mm dishes were co-transfected with vector pNL4-3 and the empty pcDNA3.1(+) vector or one expressing either HA-hA3A, HA-hA3G, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. The virus containing culture medium was harvested at 48 h post-transfection, the medium centrifuged at low speed to remove cellular debris and then pelleted through a 20%



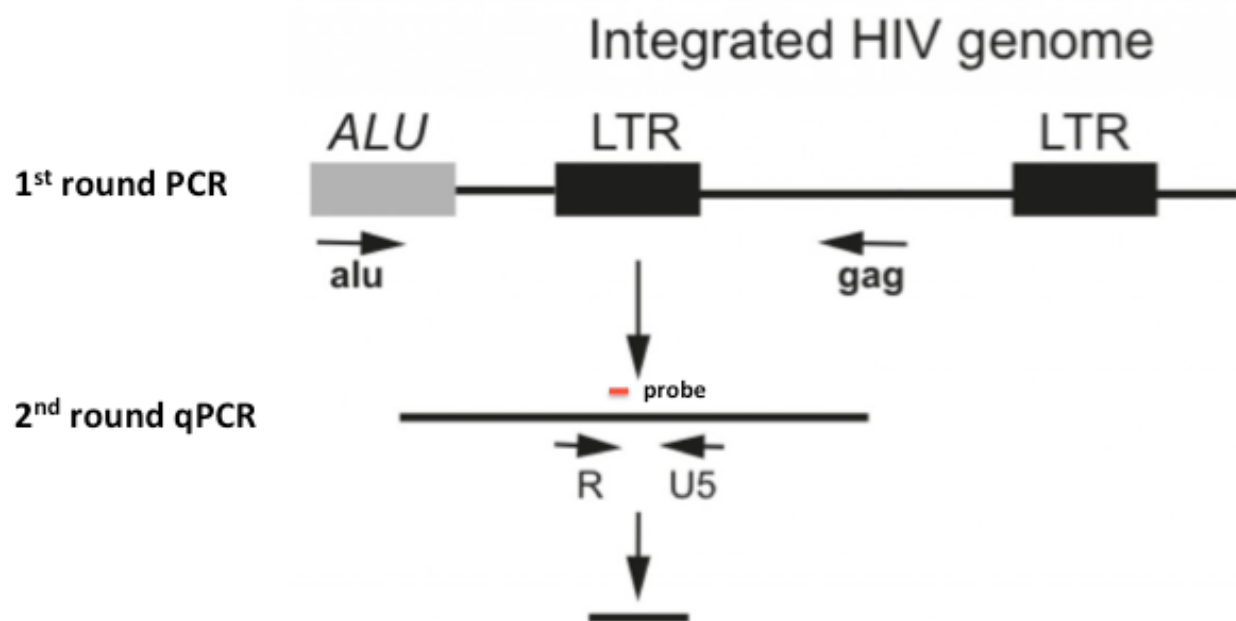
Figure 68. Flowchat for detection of viral integration level in target cells. 293 cells were co-transfected with various A3A protein or pcDNA3.1(+) as control, and HIV-1 $\Delta env$  and VSV-G. At 48 h post-transfection, supernatants were collected and used to inoculate 293 cells for 48h. Total DNA was extracted and the Alu-Gag level was quantified through a two step nested qPCR.

## Integration in Target Cell



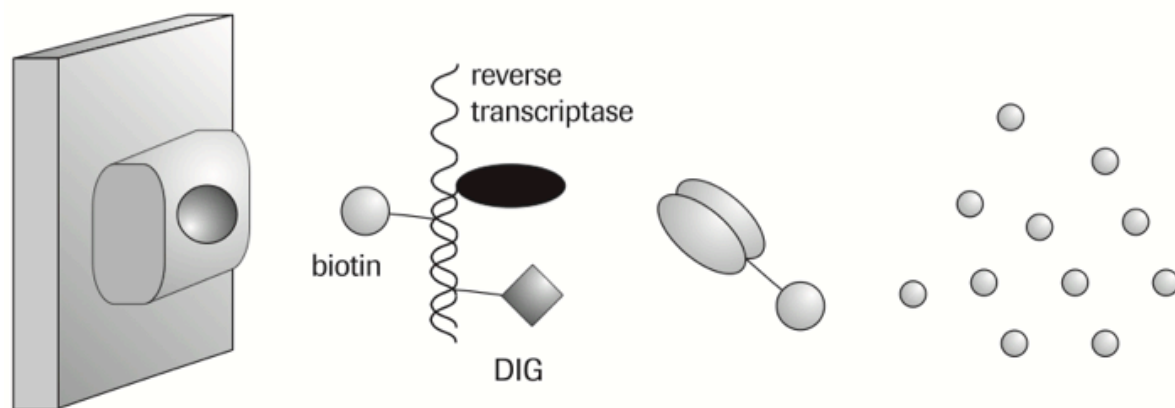
48h later, extract DNA → PCR with Alu-Gag primer → qPCR with R-U5 primer

Figure 69. Alu-gag nested PCR coupled with quantitative real time PCR for Quantitation. In the first step, PCR is performed with primers sets that flank the Alu sequence and the HIV structural gene gag to amplify the integrated form of HIV-1 gene sequence. In the second round of qPCR, the copy number of integrated HIV-1 is quantified by primer sets R-U5, which flank viral LTR region.



sucrose cushion at 35,000 rpm (Beckman SW41 rotor, 210,000 g) for 2 h. The virus containing pellet was resuspended in PBS (pH 7.4) and the p24 levels determined. Using equivalent amounts of p24, reverse transcriptase activity was determined using the Roche colorimetric Reverse Transcriptase Assay kit according to the manufacturer's instructions. The reverse transcriptase activity was plotted with the RT activity of the pcDNA3.1(+)/NL4-3 virus set at 100%. For these experiments, 293 cells were transfected with plasmid with the pNL4-3 genome and either the empty pcDNA3.1(+) vector or vectors expressing HA-hA3A, HA-hA3G, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. At 48 h the virus stocks were harvested and the p24 levels in stocks determined. Using equivalent levels of p24, viruses were used to inoculate C8166 cells for 24 h. Total cellular DNA was purified using DNeasy Blood & Tissue Kit from Qiagen. The R/U5 and U5/Gag levels were detected as previously described (Suzuki et al., 2003). Real-time PCR was performed using the following oligonucleotide primers: for R/U5, forward primer: M667-5'-GGCTAACTAGGGAACCCACTGC-3'; reverse primer: AA55-5'-CTGCTAGAGATTTTCCACACTGAC-3') and probe (HIV FAM-5'-TAGTGTGTGCCCGTCTGTTGTGTGAC-TAM; for U5/gag, forward primer: LG564-5'CCGTCTGTTGTGTGACTCTGGT-3' reverse primer: LG699-5'GAGTCCTGCGTCGAGAGATCT-3' and probe: LG-FAM-5'-(FAM)-CCCGAACAGGGACTTGAAAGCGAA-(TAMRA)-3'. For  $\beta$ -actin, forward primer: ES2474-5'CTGGCATCGTGATGGACTCC-3'; reverse primer: ES2476-5'GGAGCTGGAAGCAGCCG-3' and  $\beta$ -actin probe: 5' (6FAM)GATCCTCACCGAGCGC-(TAMRA)-3'. A standard curve of the amplification being measured was run ranging from 1 to 1  $10^{11}$  copies. Reactions containing 1x premix (Takara), 0.5  $\mu$ M forward primer, 0.5 $\mu$ M reverse primer, 250 nM probe and 1X RoxDyeII in a 20  $\mu$ l volume. Thermal cycle is 95 °C for 30 s, followed by 40 cycles of

Figure 70. The principle behind detection of *in vitro* RT activity. The microplate is precoated with streptavidin. The biotin-labeled DNA then is bound to the streptavidin on the plate. Next, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), is bound to the digoxigenin-labeled DNA. Finally, the peroxidase substrate ABTS is added and catalyzes the cleavage of the substrate, resulting in a colored reaction. The absorbance of the reaction is determined by a ELISA reader and calculated based on standard curve.



streptavidin-coated  
MTP

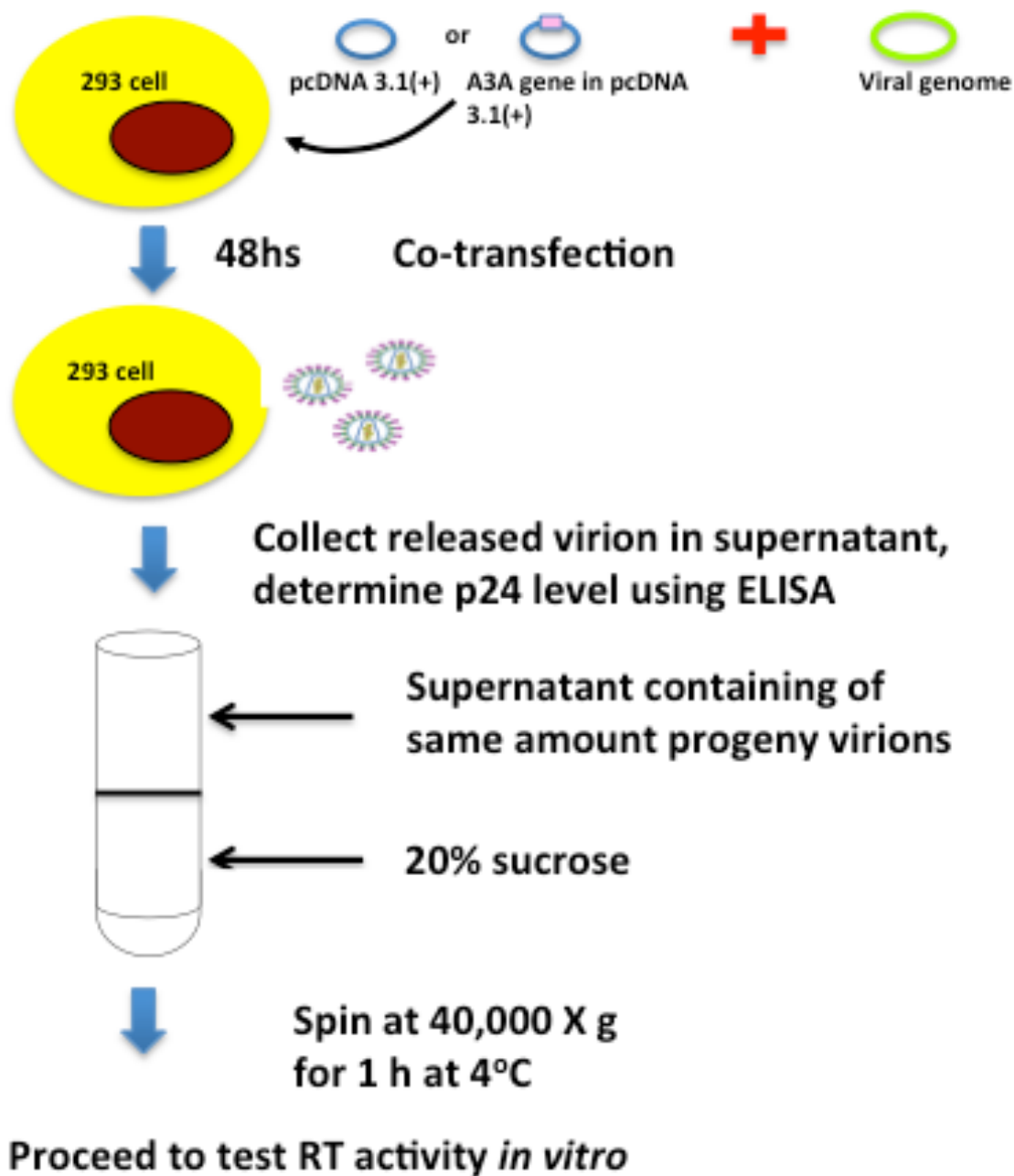
DNA/RNA-hybrid,  
biotin- and DIG-  
labeled by  
RT activity

anti-DIG-  
POD  
Fab-fragment

POD substrate  
(ABTS)

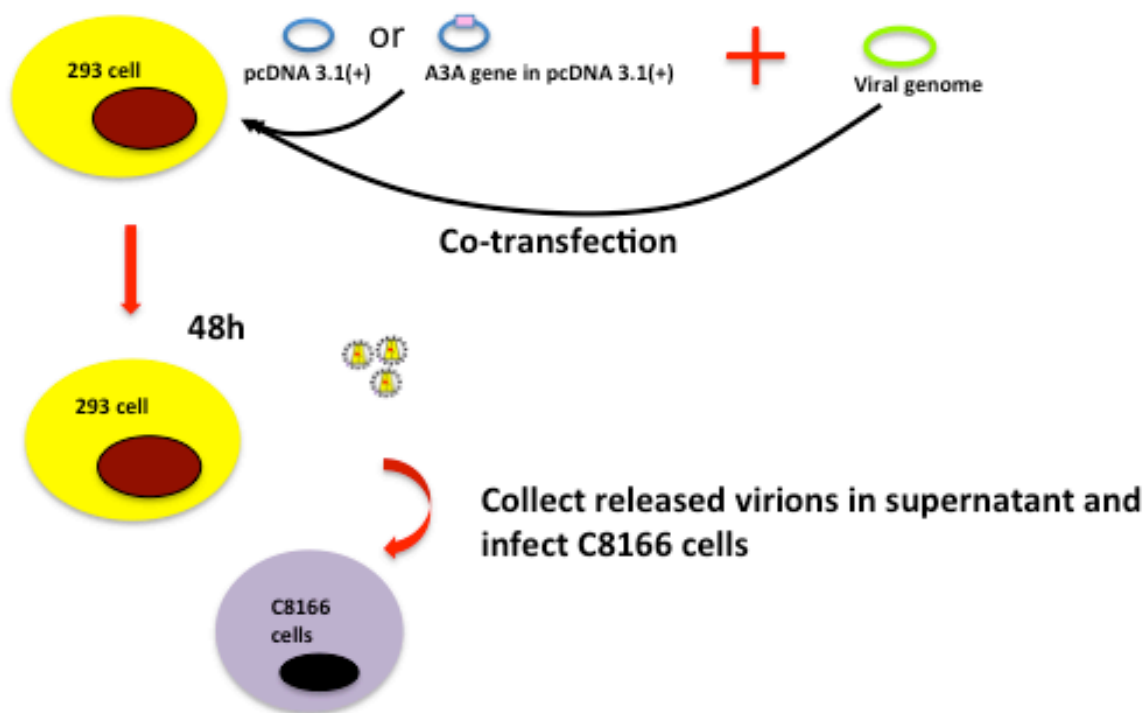
Figure 71. Procedure for preparation of virus for *in vitro* RT assay. 293 cells were co-transfected with a plasmid with the HIV-1 (pNL4–3) and either the empty pcDNA3.1(+) vector or vectors expressing either HA-hA3A, HA-hA3G, or HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. At 48 h, the viral culture medium was collected, centrifuged at low speed to removed cellular debris and the virus containing supernatants layered onto a 20% sucrose cushion. The virus was pelleted for 2 h at 35,000 rpm (SW41 rotor, Beckman). The virus pellets were resuspended in PBS (pH 7.4) and p24 levels determined and equivalent levels of p24 were used in RT assays, which is described in Figure 70.





amplification at 5 s at 95 °C and 34 s at 60 °C. Reactions were analyzed using the 7500 Real Time PCR System (ABI). The experiment was repeated three times, the mean determined, and the results plotted as the percent product with the pcDNA3.1(+)/NL4- 3 control set at 100%. The procedure of RT in target cells is depicted in figure 72.

Figure 72. Reverse transcription in target cell. 293 cells were transfected with plasmid with the pNL4-3 genome and either the empty pcDNA3.1(+) vector or vectors expressing HA-hA3A, HA-hA3G, or HA-hA3A [<sup>27</sup>WVS<sup>29</sup>]. At 48 h the virus stocks were harvested and the p24 levels in stocks determined. Using equivalent levels of p24, viruses were used to inoculate C8166 cells for 24 h. Total cellular DNA was purified using DNeasy Blood & Tissue Kit from Qiagen. The R/U5 and U5/Gag levels were detected.



24h later, extract DNA, and qPCR with R-U5 and U5-Gag primers

## Chapter VI : Conclusions

HIV-1 is the causative agent of AIDS, which causes depletion of CD4<sup>+</sup> T cells and profound immunodeficiency in infected humans. Current therapy focuses on drugs in HAART (highly active antiretroviral therapy) or cART (combined antiretroviral therapy), which was introduced in 1996. The introduction of HAART therapy has changed HIV-1 infection from a fatal disease into a chronic disease. However, current therapies focusing on antiretroviral drugs suffers from the rapid emergence of resistant viral strains resulting from high mutation rate during reverse transcription and the fact that these drugs do not eradicate viral reservoirs from the body. Thus, novel treatment strategies are needed to eliminate these viral reservoirs and eventually eliminate AIDS.

Recent efforts aimed at enhancing the activity of innate host factors are promising. Currently known host restriction factors against HIV-1 include tripartite motif-containing protein 5 alpha isoform (**TRIM5 $\alpha$** ), bone marrow stromal antigen 2 (**BST-2**), sterile alpha motif (SAM) and histidine-aspartic domain (HD) containing protein 1 (**SAMHD1**), and apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (**APOBEC3**). These restriction factors have been shown to exhibit a positive selection signature (Laguetta et al., 2012; Ortiz et al., 2009). The human APOBEC3 family is comprised of seven members that includes hA3A, hA3B, hA3C, hA3D, hA3F, hA3G, hA3H that are tandemly arranged on chromosome 22 (Jarmuz et al., 2002). They are cytidine deaminases that restrict various retroviruses, other viruses and the transposition of endogenous retroelements. Human A3G was the first identified hA3 protein that restricted HIV-1 $\Delta$ vif by both deaminase-dependent and independent mechanisms (Chiu & Greene, 2009). However, HIV-1 circumvents the restriction of A3

proteins by encoding for a Vif protein that binds to certain A3 proteins and shunts them to the proteasome for degradation. Human A3A (hA3A) is generally expressed in myeloid cells such as monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC). The expression of A3A in these cells is boosted in response to type I interferon, which suggests that hA3A might be an important player in the antiviral response (Berger et al., 2011). While hA3A has little activity against HIV-1, it can restrict many other viruses including parvoviruses, adeno-associated virus (AAV), minute virus of mice (MVM), papillomavirus, human T-lymphotropic virus type I (HTLV-1) and some endogenous retroelements (H. Chen et al., 2006; Kinomoto et al., 2007; Kondo et al., 2016; Narvaiza et al., 2009; Ooms et al., 2012). Select A3 genes (A3B, A3D, A3F, A3G, and A3H) have also undergone positive selection (Cagliani et al., 2011; Ortiz et al., 2009; Sawyer, Emerman, & Malik, 2004). In contrast, A3A was shown to be under purifying selection, consistent with the notion that it may not have played a significant role in retrovirus restriction (Sawyer et al., 2004). However, our group and in a recent study have indicated that A3A genes have evolved rapidly under diversifying selection in primates. (McLaughlin et al., 2016; Wang, Schmitt, et al., 2016; Wang, Wang, et al., 2016). The Malik/ Emerman groups found that this rapid evolution has not been driven by long interspersed elements-1 (LINE-1) but rather through the gain of antiviral specificity against other pathogens. We concur with this conclusion as our follow-up experiments on LINE-1 (Schmitt et al., 2011) did not reveal a substantial difference (2-fold or less) in the anti-LINE-1 restriction activity of rhesus macaque A3A versus human A3A.

While HIV-1 infection of humans leads to massive CD4<sup>+</sup> T cell loss and collapse of immune system, many Old World monkeys are naturally infected with simian immunodeficiency viruses and maintained high plasma viremia but do not develop disease. The interaction between host proteins and pathogens may leave positive-selection signature on host proteins. Previous work from our laboratory revealed the A3A protein from rhesus macaques restricted SHIV $\Delta$ *vif* (Schmitt et al., 2011). The purpose of my research was to determine why rhA3A could restrict SHIV $\Delta$ *vif* and why the hA3A could not restrict HIV $\Delta$ *vif*.

The sequence of human A3A (hA3A) and rhesus macaque A3A (rhA3A) have approximately 81% identity at the amino acid level. Studies have shown that human A3A (hA3A) lacks activity against HIV-1 $\Delta$ *vif* activity. However, we previously showed that rhA3A potently restricted the replication of SHIV $\Delta$ *vif* and HIV-1 $\Delta$ *vif* to a much greater extent than hA3A (hA3A generally doesn't restriction HIV-1 or HIV-1 $\Delta$ *vif*) (Schmitt et al., 2011). Since the rhA3A had a different restriction pattern than hA3A, it raised the possibility that A3A from other Old World monkeys might also restrict primate lentiviruses. Additional experiments with two other Old World monkey (OWM) A3A proteins from the African Green monkey A3A (agmA3A), colobus monkey A3A (colA3A), and another hominid A3A (gibA3A) confirmed that OWM A3A proteins restricted SHIV  $\Delta$ *vif* and HIV-1  $\Delta$ *vif* more potently than hominid A3A proteins. Notably, colA3A not only restrict SHIV $\Delta$ *vif* and HIV $\Delta$ *vif*, but also restricted wild type HIV-1 (expressing intact Vif proteins). We also determined if the A3A proteins from other OWM species also restricted HIV-1 in producer cells and if we could modify the hA3A protein with a few amino acid substitutions mimicking to potently inhibit HIV-1.

Analysis of amino acids sequence from hA3A and colA3A revealed significant variability in N-terminal domain sequences, especially within the AC (Active Center) loop 1 region located near the N-terminus. AC loop 1 region is responsible for substrate binding (AC loop 1 is also known as recognition loop 1, or RL1). Additional support for the importance of this region in restriction has come from a recent study using oligonucleotide substrates with a single deamination site (Logue et al., 2014). These investigators found that A3A preferentially deaminated at TC followed by an A or G residue. Further, using chimeric hA3A/hA3G proteins these investigators found that amino acids GI25-26 of AC-Loop 1 region of hA3A (referred to as the recognition loop 1; RL1) specified the 5' nucleotide of the recognition motif and that a chimeric A3A/A3G with the RL1 of A3G was more flexible with respect to the 5' nucleotide (AC, CC, GC all used with equal frequency) (Logue et al., 2014). To test the hypothesis that AC loop 1 region was important in retrovirus restriction, we analyzed the A3A sequences of other OWM with AC Loop1 sequences similar to colA3A (<sup>25</sup>KPWVSGQRE<sup>33</sup>). We found that the A3A proteins from both *Cercopithecus neglectus* (debA3A; <sup>25</sup>KPWVSGQRE<sup>33</sup>) and *Mandrillus sphinx* (mndA3A; <sup>25</sup>KLWVSGQHE<sup>33</sup>) were identical or nearly identical to colA3A in this region. We observed that both mndA3A and debA3A not only restrict the replication of HIV-1 $\Delta$ vif but also intact HIV-1, with mndA3A consistently being more restrictive than colA3A and debA3A. The restriction of HIV-1 by these A3A proteins appears to be specific since mndA3A did not restrict Friend MuLV, gammaretrovirus. The Old World monkeys (Family Cercopithecidae) are composed of two subfamilies (Cercopithecinae and Colobinae). The Cercopithecinae are further classified into two tribes, the Cercopithecini, and the Papionini. The Colobinae



are generally classified into the African and Asian colobines. Our findings indicate that restriction of wild type HIV-1 by A3A is represented by species in two tribes of the Cercopithecinae (Cercopithecini, debA3A; and Papionini, mndA3A) and by the African group of the Colobinae. These results also reinforced our previous findings that the sequence of the AC Loop1 region was critical for restriction of wild type HIV-1 by OWM A3A proteins. Notably, while these three monkey species express an A3A that restricts HIV-1, all three monkeys are known to harbor endemic SIV infections (SIVcol, SIVmnd, and SIVdeb) (Courgnaud et al., 2001; Souquiere et al., 2001; Bibollet-Ruche et al., 2004).

In a recent study, investigators found that the sequences of the colobus monkey, mandrill and DeBrazza's monkey were most likely artifacts of the oligonucleotides used in the PCR process and likely an exon of colA3G (the oligonucleotides primers also matched the intronic sequence on either side of exon 5 of A3G (Henry et al., 2012). Thus, it is likely that the studies with the colA3A were most likely a colA3A/G chimeric protein (McLaughlin et al., 2016). The actual colA3A of the colobus monkey doesn't contain the WVS motif in the AC loop 1 gene. Although the correct A3A sequence for mandrill and De Brazza's monkey were not examined, it is very possible that the mndA3A and debA3A we used are also mndA3A/G and debA3A/G chimeras. Investigators have used the correct sequence of colA3A and observed no restriction of HIV-1. While unexpected, this error provided clues to the amino acid determinants necessary for restriction of HIV-1. Moreover, it led us to identify two additional monkey A3A and A3G chimeras (mndA3A/G, and debA3A/G) that possessed similar restriction capacities towards HIV-1. It is amazing that this unexpected artifact

led to a unique finding that the AC loop 1, especially the WVS motif, was critical in restoring hA3A's antiviral activity.

We previously used a chimeric protein approach to show that substitution of AC loop 1 of hA3A with that of rhA3A restored the ability to hA3A to restrict HIV-1 $\Delta$ vif (Schmitt et al., 2013). We hypothesized that the three amino acid insertion in AC loop 1 of rhA3A could result in a AC-Loop1 with a greater molecular surface compared to hA3A. Due to its predicted solvent-accessibility, this additional molecular surface may be an important region for protein–protein interactions (Schmitt et al., 2013). Next, we took advantage of chimeric protein approach and hypothesized that the amino acids within the N-terminal domain (i.e. the first 100 amino acids) which include the AC Loop-1 region of colA3A/G were likely involved in the restriction of HIV-1.

We first asked whether the N-terminal, C-terminal or both were essential for retaining the restriction. Both hA3A and colA3A/G contains approximately 200 amino acids in length. Thus, we constructed chimeras that either have the first 100 amino acids of hA3A and the last 100 amino acids of colA3A ( $h_{1-100}colA3A$ ), or the first 100 amino acids of colA3A and the last 100 amino acids of hA3A ( $col_{1-100}hA3A$ ). By comparing the restrictive abilities of  $h_{1-100}colA3A$  and  $col_{1-100}hA3A$  with wild type hA3A and colA3A, we could speculate the relative importance of the N-terminal and C-terminal. If the restriction determinants were in the N-terminal region, then the  $h_{1-100}colA3A$  should behave similarly to hA3A (no restriction towards HIV-1), and  $col_{1-100}hA3A$  should act like colA3A/G (strong restriction towards HIV-1). Conversely, if the restriction determinant lies completely in the C-terminal region, then the  $h_{1-100}colA3A$  should behave similarly to colA3A (strong restriction towards HIV-1), and  $col_{1-100}hA3A$  should

act like hA3A (no restriction towards HIV-1). If both N-terminal and C-terminal of A3A proteins contributed to restriction of HIV-1, then both chimeric proteins ( $h_{1-100}colA3A$  and  $col_{1-100}hA3A$ ) should restrict HIV-1 at levels higher than hA3A and lower than colA3A/G. The result of our restriction assays showed that  $col_{1-100}hA3A$  restricted at levels similar to full length colA3A/G while  $h_{1-100}colA3A$  did not restrict HIV-1. This revealed that the first 100 amino acids in the N-terminal contain the key determinants for HIV-1 restriction. In addition, to further confirm this conclusion, we truncated the C-terminal off from all three OWM A3As that possess anti-HIV-1 activity. All three truncated A3A proteins ( $colA3A_{1-100}$ ,  $mndA3A_{1-100}$ , and  $debA3A_{1-100}$ ) retained the restrictive ability similar to the full-length A3A proteins. These results represent the first demonstration that a single deaminase A3 protein could be significantly truncated (i.e., 50%) without loss of restriction activity and further support the idea that N-terminal region of colA3A, mndA3A and debA3A had the determinants for HIV-1 restriction (Katuwal et al., 2014).

Although we determined that the first half (N-terminal) was the key to HIV-1 restriction, we want to further determine if we could narrow down the amino acids in colA3A/G that were required for restriction. Next, we constructed chimeras with the first 75, 50, 33 amino acids from colA3A/G fused to hA3A resulting in three additional chimeric proteins:  $col_{1-75}hA3A$ ,  $col_{1-50}hA3A$ ,  $col_{1-33}hA3A$ . Our results indicated that  $col_{1-75}hA3A$ ,  $col_{1-50}hA3A$ ,  $col_{1-33}hA3A$  restricted HIV-1, although not at the same level as  $col_{1-100}hA3A$ . Additionally, we showed that the introduction of the AC-Loop1 region from mndA3A into hA3A (a total of six amino acid substitutions/insertions; now

designated as HA-hA3A [<sup>25</sup>KLWVSGQHE<sup>33</sup>]) resulted in a protein gained the ability to restrict HIV-1 (Katuwal et al., 2014).

This confirmed our hypothesis that AC loop 1 region in the N-terminal of A3A was the key determinants of HIV-1 restriction. Since the OWM A3A that restricted HIV-1 (colA3A/G, mndA3A/G, and debA3A/G) contained WVS at this site, we introduced <sup>27</sup>WVS<sup>29</sup> motif into hA3A (hA3A[<sup>27</sup>WVS<sup>29</sup>]). By insertion of these three amino acids, we were able to enable hA3A to restrict both HIV-1 $\Delta$ *vif* and HIV-1. While HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] restricted HIV-1, restriction appeared to occur through a different mechanism than with HA-hA3A[<sup>25</sup>KLWVSGQHE<sup>33</sup>] as the latter significantly reduced the levels of viral p24 released from cells compared to cultures transfected with HA-hA3A, HA-hA3G, and HA-hA3A[<sup>27</sup>WVS<sup>29</sup>].

We next explored the importance of each of the three amino acids in the <sup>27</sup>WVS<sup>29</sup> motif in HIV-1 restriction. We first substituted each amino acid in the WVS motif with the ones owing differing charge and polarity, resulting in hA3A[<sup>27</sup>FVS<sup>29</sup>], hA3A[<sup>27</sup>YVS<sup>29</sup>], hA3A[<sup>27</sup>HVS<sup>29</sup>], hA3A[<sup>27</sup>WLS<sup>29</sup>] hA3A[<sup>27</sup>WTS<sup>29</sup>], hA3A[<sup>27</sup>WDS<sup>29</sup>], hA3A[<sup>27</sup>WVT<sup>29</sup>], hA3A[<sup>27</sup>WVA<sup>29</sup>], and hA3A[<sup>27</sup>WVD<sup>29</sup>]. We found that all single amino acid substitutions retained the ability to restrict HIV-1 to some level. The results indicated that the nature of the amino acids in the <sup>27</sup>WVS<sup>29</sup> motif did not appear to be important. To investigate the role of the length of the motif, we deleted either one or two of the amino acids in the WVS motif, resulting in hA3A[<sup>28</sup>VS<sup>29</sup>], hA3A[<sup>27</sup>WS<sup>29</sup>], hA3A[<sup>27</sup>WV<sup>28</sup>], hA3A[W<sup>27</sup>], hA3A[V<sup>28</sup>], and hA3A[S<sup>29</sup>]. While deletion of one amino acid still resulted in restriction of HIV-1, deletion of two amino acids resulted in restriction that was not statistically significant when compared to hA3A. Taken together,

these results indicate that the length and not necessarily the nature of the amino acid was most important for defining HIV-1 restriction. Our results indicated that substitution of one amino acid of the WVS motif with either a conservative, non-conservative or charged amino acid was inconsequential in the restriction of HIV-1. In the final two constructs, we substituted the <sup>27</sup>WVS<sup>29</sup> with <sup>27</sup>AAA<sup>29</sup> or <sup>27</sup>GGG<sup>29</sup>. Similar to our other amino acid substitutions, these constructs also restricted HIV-1. These results confirmed that the nature of the amino acids in this region did not seem to be critical for HIV-1 restriction.

Next, we explored the mechanism through which hA3A[<sup>27</sup>WVS<sup>29</sup>] restricted HIV-1. Deciphering the mechanism of hA3A[<sup>27</sup>WVS<sup>29</sup>] restriction could be instrumental in designing novel antiviral strategies against HIV-1. As our restriction assays showed that the infectivity of virions produced from hA3A[<sup>27</sup>WVS<sup>29</sup>] transfected cells were decreased as evidenced titers in TZM.b1 cells, the level of Gag released from cells were not significantly lowered using ELISA p24 antigen capture/WB. Thus, in the presence of hA3A[<sup>27</sup>WVS<sup>29</sup>], the virions were successfully released but were impaired for replication in the next round of infection. This suggested that the site of restriction was likely in the target cells. Additional evidence comes from our results from pulse-chase experiments that indicated that viral protein synthesis and processing was similar in producer cells transfected with vectors expressing HA-hA3A or HA-hA3A[<sup>27</sup>WVS<sup>29</sup>]. We further showed that HA-hA3A and HA-hA3A[<sup>27</sup>WVS<sup>29</sup>] both had a nucleocytoplasmic localization in producer cells. Therefore, the cellular localization wasn't responsible for the divergence in restriction capacities of HA-hA3A and HA-hA3A[<sup>27</sup>WVS<sup>29</sup>].

We also examined several parameters on the virus released from the producer cells. We determined if the RNA packaged into virions was decreased. Our results

indicate the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> did not affect RNA packaging. We also analyzed the activity of reverse transcriptase (RT) in the progeny virions using an exogenous template. Our results indicate that RT activity was similar for HA-hA3A, HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>, and HA-hA3G. These results suggest that incorporated HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> did not directly interact with reverse transcriptase to block its activity. Alternatively, it is also possible that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> may interact weakly with the reverse transcriptase but that the conditions of the reverse transcriptase assay disrupted this interaction.

We next focused on target cells to explore the inhibition mechanism involved. First we excluded the possibility of hA3A<sup>[27WVS<sup>29</sup>]</sup> restricts viral replication through canonical hypermutation pathway. As the truncation disrupted the Z domain of this protein, it indicated that deamination is not required for colA3A or mndA3A restriction of HIV-1 (Katuwal et al., 2014). Furthermore, our hypermutation experiment confirmed that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> did not cause cytidine hypermutation of the HIV-1 nef gene.

However, when virus grown in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> was used to inoculate target cells, we observed a significant decrease in the levels of early (R-U5) and late (U5-gag) reverse transcriptase products. Since the RT activity of virus grown in the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> was not significantly decreased, it suggests that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> may interact with the viral RNA to decrease levels of early and late RT products. This would explain the deaminase-independent restriction by HA-hA3A<sup>[27WVS<sup>29</sup>]</sup>. As a result of decreased reverse transcription in target cells, we also observed a subsequent decrease in integration, transcription, virion production and release from target cells.

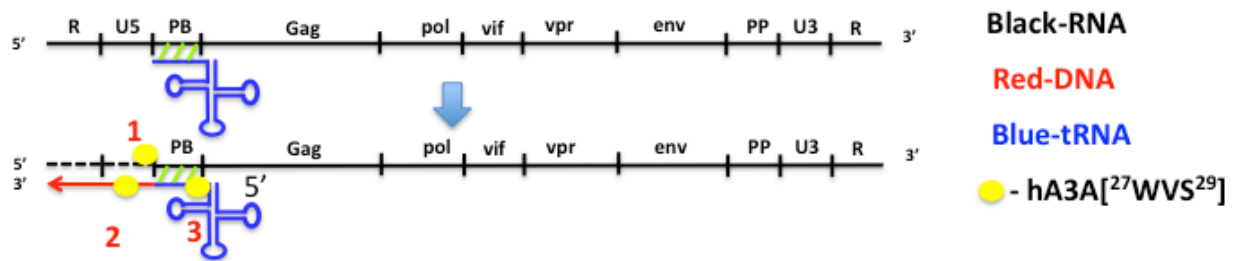
In summary, our results suggested that hA3A<sup>[27WVS<sup>29</sup>]</sup> restricted viral replication at the level of reverse transcription in target cells. As we previously showed that the reverse transcriptase activity of budding virions from producer cells was not inhibited, it is possible that hA3A<sup>[27WVS<sup>29</sup>]</sup> may directly interfere with the interaction with viral RNA/DNA or tRNA<sup>lys3</sup> in the target cells. Although we have not provided direct evidence disclosing the exact viral RNA/DNA sequence that is targeted by hA3A<sup>[27WVS<sup>29</sup>]</sup>, we have previous structural and experimental data to support this hypothesis. Both the NMR and crystal structures of hA3A have been solved (Byeon et al., 2013; Bohn et al., 2015). The crystal structure of A3A-E72A-C171A, which was solved at 2.85 Å resolution, showed that hA3A exists as a homo-dimer (Bohn et al., 2015). These investigators found that A3A dimerization formed a highly positive groove that connects the active site of both monomers. These investigators identified two sets of residues that might contribute to dimerization, H11/H56 and H16/K30. Substitution of these residues showed that both sets of interactions were critical to cooperative DNA binding. Our results could have implications for the structure of A3A proteins from Old World monkey species as K30 is part of the AC Loop 1 region and that the insertion of <sup>27</sup>WVS<sup>29</sup> could possibly alter the interface and perhaps dimer formation and substrate binding. In a previous study, the binding efficiency of hA3A to a 40 nucleotide ssRNA was reported to be approximately 3-fold higher than for the corresponding 40 nt ssDNA (Byeon et al., 2013). More recently, it was shown that hA3A binds single stranded RNA (ssRNA) but no deamination of ssRNA occurs (Mitra et al., 2014). Thus, it is possible that hA3A<sup>[27WVS<sup>29</sup>]</sup> with its different AC-loop-1 region may have a slight change in its structure to permit altered binding to regions of the viral RNA or DNA, therefore

inhibiting virus replication by interfering in the initiation of reverse transcription and elongation. The specific sequence of RNA was not determined but could be possibly located in the 5' end of viral RNA or the newly synthesized DNA fragment (R-U5) as the presence of HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> inhibits reverse transcription at the very early stage. Another possibility is that HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> might have altered binding affinity with the primer tRNA<sup>lys3</sup> as it is a key player in initiating reverse transcription. The possible model of restriction is summarized in Figure 73.

In addition, human A3A has been reported to restrict autonomous parvoviruses and adeno-associated virus type 2 (AAV-2), papillomaviruses, and HTLV-I (Ahasan et al., 2015; Bulliard et al., 2011; Chen et al., 2006; Muckenfuss et al., 2006; Narvaiza et al., 2009; Ooms et al., 2012; Vartanian et al., 2008; Warren et al., 2015). Human A3A restricts AAV-2 DNA replication and was initially thought to be due to cytidine deamination since an E72A mutant of hA3A did not restrict AAV-2 replication (Chen et al., 2006). However, a subsequent study found that the mechanism of restriction was likely deamination-independent (Narvaiza et al., 2009). More recently, we found that unlike hA3A, rhA3A did not restrict AAV-2 (Schmitt et al., 2011). In a previous study, several residues in hA3A were substituted to assess the effect on retro- transposition and restriction of AAV-2 (Bulliard et al., 2011). These investigators showed that substitution of residues such as N57, Y130, and D133 abolished AAV-2 restriction while substitution of residues R28, H29, or K30F had modest effects (50% to 85% restriction compared to unmodified hA3A) on AAV-2 restriction (Bulliard et al., 2011). These results are similar to our observations with HA-hA3A<sup>[27WVS<sup>29</sup>]</sup> (Wang et al., 2016), which restricted AAV-2 replication at approximately 80% of the unmodified hA3A. In contrast,



Figure 73. Proposed model of restriction by hA3A<sup>[27WVS<sup>29</sup>]</sup>. 1.hA3A<sup>[27WVS<sup>29</sup>]</sup> might have altered binding affinity for viral RNA template that prevents the binding of primer tRNA or initiation of RT; 2.hA3A<sup>[27WVS<sup>29</sup>]</sup> might interact with newly synthesized DNA and prevents the initiation & elongation of RT; 3.hA3A<sup>[27WVS<sup>29</sup>]</sup> might binds to the primer tRNA and inhibits the initiation of RT.



1. hA3A<sup>[27WVS<sup>29</sup>]</sup> might have altered binding affinity for viral RNA template that prevents the binding of primer tRNA or initiation of RT;
2. hA3A<sup>[27WVS<sup>29</sup>]</sup> might interact with newly synthesized DNA and prevents the initiation & elongation of RT;
3. hA3A<sup>[27WVS<sup>29</sup>]</sup> might bind to the primer tRNA and inhibits the initiation of RT.

replacement of the entire AC Loop 1 region with the analogous region from mndA3A/G essentially abolished restriction (Wang et al., 2016). These results shown for the first time that the sequence (and structure) of the AC Loop1 region is crucial to hA3A-mediated parvovirus restriction. It also suggests that hA3A with its three amino acid indel (and possibly A3A proteins from other primates) may have been under positive selection to combat parvoviruses and perhaps DNA viruses in general. Since the available data indicates that hA3A does not restrict parvovirus or papillomavirus replication by cytidine deamination, perhaps it can interact with the single stranded DNA of these viruses or interact with proteins involved in DNA replication such as the NS-1 protein of AAV-2. It will be of interest to determine if this region is important for the restriction of human autonomous parvoviruses such as B-19 virus and human bocavirus (HBoV).

In summary, inserting three amino acids in AC loop 1 region can either restore the anti-HIV-1 activity of hA3A or make it lost the ability to restrict AAV-2. These direct evidences confirmed that the key determinants of hA3A for retroviruses and parvoviruses lie in the AC loop 1 region.

As we identified the minimal modification that are required for HIV-1 restriction and constructed a powerful chimeric protein, hA3A<sup>[27WVS<sup>29</sup>]</sup>, that is able to restrict HIV-1 replication though interfering with reverse transcription, it would be interesting to investigate generating a non-immunogenic hA3A (with domains involved in possible cytidine deamination deleted) for potential gene therapy in HIV-1 patients in the future studies. Current HAART therapy based on anti-retroviral drug cocktail improved the life expectancy of HIV-1 patients by converting AIDS from a deadly disease into a manageable chronic infection. However, HAART is not without its side

effects and can result in drug resistance in many cases. Novel therapeutic approaches taking advantage of natural anti-HIV restriction factors inhibiting HIV might offer some effective alternative and should be a booster when combined into current regimen. A distinguished advantage of our chimera protein hA3A<sup>[27WVS<sup>29</sup>]</sup> is that it prevents the initiation and completion of reverse transcription possibly through altered binding to viral RNA/DNA or to other essential players in the reverse transcription such as tRNA, but don't directly interfere with reverse transcriptase enzymatic activity. This is very important because current reverse transcription drugs are either nucleoside analog reverse-transcriptase inhibitors (NRTIs), nucleotide analog reverse-transcriptase inhibitors (NtRTIs) that can prevent the growing of viral DNA chain resulting in chain termination, or Non-nucleoside reverse-transcriptase inhibitors (NNRTIs) that binds to the catalytic groove of reverse transcriptase. The differences in inhibition mechanisms of hA3A<sup>[27WVS<sup>29</sup>]</sup> and current RT drugs indicate that the possibility of resistance is minimized if we introduced this novel hA3A<sup>[27WVS<sup>29</sup>]</sup> into current drug cocktail. Moreover, gene therapy is known for precision and direct therapeutic effects on its targets. However, cell toxicity, immunogenicity, insertional mutagenesis needs to be tested before introducing hA3A<sup>[27WVS<sup>29</sup>]</sup> into infected individuals. Currently, effects on modifying effector cells to specifically kill HIV-1 infected cells has been evaluated in phase I and II clinical trials (Deeks et al., 2002; Mitsuyasu et al., 2000). Other promising attempts have also been made to altering susceptible cells to be resistant to HIV-1 entry and integration (Holt et al., 2010; Maier et al., 2013). These preliminary results from clinical trials and previous experiments have provided feasibility of engineering cells *in vivo* based on safety and efficacy data. It would be very encouraging if we could express

our chimeric protein hA3A<sup>[27WVS<sup>29</sup>]</sup> into a HIV-1 susceptible primary cells using lentiviral vectors and determine how it behaves *in vivo*.

## Chapter VII : Reference

- Abraham, L., & Fackler, O. T. (2012). HIV-1 Nef: a multifaceted modulator of T cell receptor signaling. *Cell Communication and Signaling*, *10*(1), 39. doi: 10.1186/1478-811x-10-39
- Acharya, P., Lusvardi, S., Bewley, C. A., & Kwong, P. D. (2015). HIV-1 gp120 as a therapeutic target: navigating a moving labyrinth. *Expert Opinion on Therapeutic Targets*, *19*(6), 765-783.
- Agosto, L. M., Uchil, P. D., & Mothes, W. (2015). HIV cell-to-cell transmission: effects on pathogenesis and antiretroviral therapy. *Trends in Microbiology*, *23*(5), 289-295. doi: 10.1016/j.tim.2015.02.003
- Aguiar, R. S., Lovsin, N., Tanuri, A., & Peterlin, B. M. (2008). Vpr.A3A chimera inhibits HIV replication. *Journal of Biological Chemistry*, *283*(5), 2518-2525. doi: 10.1074/jbc.M706436200
- Ahasan, M. M., Wakae, K., Wang, Z., Kitamura, K., Liu, G., Koura, M., . . . Muramatsu, M. (2015). APOBEC3A and 3C decrease human papillomavirus 16 pseudovirion infectivity. *Biochem Biophys Res Commun*, *457*(3), 295-299. doi: 10.1016/j.bbrc.2014.12.103
- Aida, Y., & Matsuda, G. (2009). Role of Vpr in HIV-1 nuclear import: therapeutic implications. *Current Hiv Research*, *7*(2), 136-143.
- Albin, J. S., LaRue, R. S., Weaver, J. A., Brown, W. L., Shindo, K., Harjes, E., . . . Harris, R. S. (2010). A single amino acid in human APOBEC3F alters susceptibility to HIV-1 Vif. *Journal of Biological Chemistry*, *285*(52), 40785-40792. doi: 10.1074/jbc.M110.173161
- Alfadhi, A., Barklis, R. L., & Barklis, E. (2009). HIV-1 matrix organizes as a hexamer of trimers on membranes containing phosphatidylinositol-(4,5)-bisphosphate. *Virology*, *387*(2), 466-472. doi: 10.1016/j.virol.2009.02.048
- Alfadhli, A., Huseby, D., Kapit, E., Colman, D., & Barklis, E. (2007). Human immunodeficiency virus type 1 matrix protein assembles on membranes as a hexamer. *Journal of Virology*, *81*(3), 1472-1478. doi: 10.1128/JVI.02122-06
- Alke, A., Schwantes, A., Kido, K., Flotenmeyer, M., Flugel, R. M., & Lochelt, M. (2001). The bet gene of feline foamy virus is required for virus replication. *Virology*, *287*(2), 310-320. doi: 10.1006/viro.2001.1065
- Ambrose, Z., & Aiken, C. (2014). HIV-1 uncoating: connection to nuclear entry and regulation by host proteins. *Virology*, *454-455*, 371-379. doi: 10.1016/j.virol.2014.02.004
- Andersen, J. L., & Planelles, V. (2005). The role of Vpr in HIV-1 pathogenesis. *Current Hiv Research*, *3*(1), 43-51.
- Anderson, J. L., Campbell, E. M., Wu, X. L., Vandegraaff, N., Engelman, A., & Hope, T. J. (2006). Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *Journal of Virology*, *80*(19), 9754-9760.
- Andrew, A., & Strebel, K. (2010). HIV-1 Vpu targets cell surface markers CD4 and BST-2 through distinct mechanisms. *Molecular Aspects of Medicine*, *31*(5), 407-417. doi: 10.1016/j.mam.2010.08.002
- Andrew, A. J., Miyagi, E., & Strebel, K. (2011). Differential effects of human immunodeficiency virus type 1 Vpu on the stability of BST-2/tetherin. *Journal of Virology*, *85*(6), 2611-2619. doi: 10.1128/JVI.02080-10
- Araujo, L. A. L., & Almeida, S. E. M. (2013). HIV-1 Diversity in the Envelope Glycoproteins:

- Implications for Viral Entry Inhibition. *Viruses-Basel*, 5(2), 595-604.
- Arien, K. K., & Verhasselt, B. (2008). HIV Nef: role in pathogenesis and viral fitness. *Current Hiv Research*, 6(3), 200-208.
- Arnold, L. H., Groom, H. C., Kunzelmann, S., Schwefel, D., Caswell, S. J., Ordonez, P., . . . Bishop, K. N. (2015). Phospho-dependent Regulation of SAMHD1 Oligomerisation Couples Catalysis and Restriction. *Plos Pathogens*, 11(10), e1005194. doi: 10.1371/journal.ppat.1005194
- Arthos, J., Cicala, C., Martinelli, E., Macleod, K., Van Ryk, D., Wei, D., . . . Fauci, A. S. (2008). HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nature Immunology*, 9(3), 301-309. doi: 10.1038/ni1566
- Arts, E. J., & Hazuda, D. J. (2012). HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harb Perspect Med*, 2(4).
- Asante-Appiah, E., & Skalka, A. M. (1999). HIV-1 integrase: Structural organization, conformational changes, and catalysis. *Advances in Virus Research*, Vol 52, 52, 351-369. doi: Doi 10.1016/S0065-3527(08)60306-1
- Aynaud, M. M., Suspene, R., Vidalain, P. O., Mussil, B., Guetard, D., Tangy, F., . . . Vartanian, J. P. (2012). Human Tribbles 3 protects nuclear DNA from cytidine deamination by APOBEC3A. *Journal of Biological Chemistry*, 287(46), 39182-39192. doi: 10.1074/jbc.M112.372722
- Barboric, M., Yik, J. H., Czudnochowski, N., Yang, Z., Chen, R., Contreras, X., . . . Zhou, Q. (2007). Tat competes with HEXIM1 to increase the active pool of P-TEFb for HIV-1 transcription. *Nucleic Acids Research*, 35(6), 2003-2012. doi: 10.1093/nar/gkm063
- Bartee, E., McCormack, A., & Fruh, K. (2006). Quantitative membrane proteomics reveals new cellular targets of viral immune modulators. *Plos Pathogens*, 2(10), e107. doi: 10.1371/journal.ppat.0020107
- Basmaciogullari, S., & Pizzato, M. (2014). The activity of Nef on HIV-1 infectivity. *Front Microbiol*, 5, 232. doi: 10.3389/fmicb.2014.00232
- Baur, A. (2004). Functions of the HIV-1 Nef protein. *Curr Drug Targets Immune Endocr Metabol Disord*, 4(4), 309-313.
- Beilhartz, G. L., & Gotte, M. (2010). HIV-1 Ribonuclease H: Structure, Catalytic Mechanism and Inhibitors. *Viruses-Basel*, 2(4), 900-926. doi: 10.3390/v2040900
- Bell, N. M., & Lever, A. M. L. (2013). HIV Gag polyprotein: processing and early viral particle assembly. *Trends in Microbiology*, 21(3), 136-144.
- Beloglazova, N., Flick, R., Tchigvintsev, A., Brown, G., Popovic, A., Nocek, B., & Yakunin, A. F. (2013). Nuclease activity of the human SAMHD1 protein implicated in the Aicardi-Goutieres syndrome and HIV-1 restriction. *Journal of Biological Chemistry*, 288(12), 8101-8110. doi: 10.1074/jbc.M112.431148
- Berger, G., Durand, S., Fargier, G., Nguyen, X. N., Cordeil, S., Bouaziz, S., . . . Cimarelli, A. (2011). APOBEC3A is a specific inhibitor of the early phases of HIV-1 infection in myeloid cells. *Plos Pathogens*, 7(9), e1002221. doi: 10.1371/journal.ppat.1002221
- Betts, L., Xiang, S., Short, S. A., Wolfenden, R., & Carter, C. W., Jr. (1994). Cytidine deaminase. The 2.3 Å crystal structure of an enzyme: transition-state analog complex. *Journal of Molecular Biology*, 235(2), 635-656. doi: 10.1006/jmbi.1994.1018
- Binette, J., & Cohen, E. A. (2004). Recent advances in the understanding of HIV-1 Vpu accessory protein functions. *Curr Drug Targets Immune Endocr Metabol Disord*, 4(4),

- 297-307.
- Bishop, K. N., Holmes, R. K., Sheehy, A. M., Davidson, N. O., Cho, S. J., & Malim, M. H. (2004). Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Current Biology*, *14*(15), 1392-1396. doi: 10.1016/j.cub.2004.06.057
- Bishop, K. N., Holmes, R. K., Sheehy, A. M., & Malim, M. H. (2004). APOBEC-mediated editing of viral RNA. *Science*, *305*(5684), 645. doi: 10.1126/science.1100658
- Blundell, T. L., Lapatto, R., Wilderspin, A. F., Hemmings, A. M., Hobart, P. M., Danley, D. E., & Whittle, P. J. (1990). The 3-D Structure of Hiv-1 Proteinase and the Design of Antiviral Agents for the Treatment of Aids. *Trends in Biochemical Sciences*, *15*(11), 425-430.
- Bogerd, H. P., Wiegand, H. L., Doehle, B. P., & Cullen, B. R. (2007). The intrinsic antiretroviral factor APOBEC3B contains two enzymatically active cytidine deaminase domains. *Virology*, *364*(2), 486-493. doi: 10.1016/j.virol.2007.03.019
- Bogerd, H. P., Wiegand, H. L., Hulme, A. E., Garcia-Perez, J. L., O'Shea, K. S., Moran, J. V., & Cullen, B. R. (2006). Cellular inhibitors of long interspersed element 1 and Alu retrotransposition. *Proc Natl Acad Sci U S A*, *103*(23), 8780-8785. doi: 10.1073/pnas.0603313103
- Bohn, M. F., Shandilya, S. M., Albin, J. S., Kouno, T., Anderson, B. D., McDougle, R. M., . . . Schiffer, C. A. (2013). Crystal structure of the DNA cytosine deaminase APOBEC3F: the catalytically active and HIV-1 Vif-binding domain. *Structure*, *21*(6), 1042-1050. doi: 10.1016/j.str.2013.04.010
- Bohn, M. F., Shandilya, S. M., Silvas, T. V., Nalivaika, E. A., Kouno, T., Kelch, B. A., . . . Schiffer, C. A. (2015). The ssDNA Mutator APOBEC3A Is Regulated by Cooperative Dimerization. *Structure*, *23*(5), 903-911. doi: 10.1016/j.str.2015.03.016
- Bour, S., & Strebel, K. (2003). The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release. *Microbes and Infection*, *5*(11), 1029-1039.
- Bourara, K., Liegler, T. J., & Grant, R. M. (2007). Target cell APOBEC3C can induce limited G-to-A mutation in HIV-1. *Plos Pathogens*, *3*(10), 1477-1485. doi: 10.1371/journal.ppat.0030153
- Bouzidi, M. S., Caval, V., Suspene, R., Hallez, C., Pineau, P., Wain-Hobson, S., & Vartanian, J. P. (2016). APOBEC3DE Antagonizes Hepatitis B Virus Restriction Factors APOBEC3F and APOBEC3G. *Journal of Molecular Biology*, *428*(17), 3514-3528. doi: 10.1016/j.jmb.2016.05.022
- Brandariz-Nunez, A., Valle-Casuso, J. C., White, T. E., Laguette, N., Benkirane, M., Brojatsch, J., & Diaz-Griffero, F. (2012). Role of SAMHD1 nuclear localization in restriction of HIV-1 and SIVmac. *Retrovirology*, *9*, 49. doi: 10.1186/1742-4690-9-49
- Brandenberg, O. F., Magnus, C., Regoes, R. R., & Trkola, A. (2015). The HIV-1 Entry Process: A Stoichiometric View. *Trends in Microbiology*, *23*(12), 763-774. doi: 10.1016/j.tim.2015.09.003
- Brigati, C., Giacca, M., Noonan, D. M., & Albin, A. (2003). HIV Tat, its TARgets and the control of viral gene expression. *Fems Microbiology Letters*, *220*(1), 57-65.
- Briggs, J. A. G., & Krausslich, H. G. (2011). The Molecular Architecture of HIV. *Journal of Molecular Biology*, *410*(4), 491-500. doi: 10.1016/j.jmb.2011.04.021
- Briggs, J. A. G., Riches, J. D., Glass, B., Bartonova, V., Zanetti, G., & Krausslich, H. G. (2009). Structure and assembly of immature HIV. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(27), 11090-11095. doi: 10.1073/pnas.0903535106



- Burniston, M. T., Cimarelli, A., Colgan, J., Curtis, S. P., & Luban, J. (1999). Human immunodeficiency virus type 1 Gag polyprotein multimerization requires the nucleocapsid domain and RNA and is promoted by the capsid-dimer interface and the basic region of matrix protein. *Journal of Virology*, *73*(10), 8527-8540.
- Burton, D. R., & Mascola, J. R. (2015). Antibody responses to envelope glycoproteins in HIV-1 infection. *Nature Immunology*, *16*(6), 571-576.
- Butler, S. L., Hansen, M. S., & Bushman, F. D. (2001). A quantitative assay for HIV DNA integration in vivo. *Nat Med*, *7*(5), 631-634. doi: 10.1038/87979
- Byeon, I. J., Ahn, J., Mitra, M., Byeon, C. H., Hercik, K., Hritz, J., . . . Gronenborn, A. M. (2013). NMR structure of human restriction factor APOBEC3A reveals substrate binding and enzyme specificity. *Nature Communications*, *4*, 1890. doi: 10.1038/ncomms2883
- Cagliani, R., Riva, S., Fumagalli, M., Biasin, M., Caputo, S. L., Mazzotta, F., . . . Sironi, M. (2011). A positively selected APOBEC3H haplotype is associated with natural resistance to HIV-1 infection. *Evolution*, *65*(11), 3311-3322. doi: 10.1111/j.1558-5646.2011.01368.x
- Cai, D., Cao, J., Li, Z., Zheng, X., Yao, Y., Li, W., & Yuan, Z. (2009). Up-regulation of bone marrow stromal protein 2 (BST2) in breast cancer with bone metastasis. *BMC Cancer*, *9*, 102. doi: 10.1186/1471-2407-9-102
- Cameron, P. U., Freudenthal, P. S., Barker, J. M., Gezelter, S., Inaba, K., & Steinman, R. M. (1992). Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. *Science*, *257*(5068), 383-387.
- Campbell, E. M., & Hope, T. J. (2015). HIV-1 capsid: the multifaceted key player in HIV-1 infection. *Nature Reviews Microbiology*, *13*(8), 471-483. doi: 10.1038/nrmicro3503
- Cao, W., Bover, L., Cho, M., Wen, X., Hanabuchi, S., Bao, M., . . . Liu, Y. J. (2009). Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *Journal of Experimental Medicine*, *206*(7), 1603-1614. doi: 10.1084/jem.20090547
- Carlton, J. G., & Martin-Serrano, J. (2009). The ESCRT machinery: new functions in viral and cellular biology. *Biochemical Society Transactions*, *37*, 195-199.
- Carpenter, M. A., Li, M., Rathore, A., Lackey, L., Law, E. K., Land, A. M., . . . Harris, R. S. (2012). Methylcytosine and normal cytosine deamination by the foreign DNA restriction enzyme APOBEC3A. *Journal of Biological Chemistry*, *287*(41), 34801-34808. doi: 10.1074/jbc.M112.385161
- Carter, G. C., Bernstone, L., Baskaran, D., & James, W. (2011). HIV-1 infects macrophages by exploiting an endocytic route dependent on dynamin, Rac1 and Pak1. *Virology*, *409*(2), 234-250. doi: 10.1016/j.virol.2010.10.018
- Casartelli, N. (2016). HIV-1 Cell-to-Cell Transmission and Antiviral Strategies: An Overview. *Current Drug Targets*, *17*(1), 65-75.
- CDC. (2016). Today's HIV/AIDS epidemic.
- Ceccherini-Silberstein, F., Malet, I., D'Arrigo, R., Antinori, A., Marcelin, A. G., & Perno, C. F. (2009). Characterization and Structural Analysis of HIV-1 Integrase Conservation. *Aids Reviews*, *11*(1), 17-29.
- Chaipan, C., Smith, J. L., Hu, W. S., & Pathak, V. K. (2013). APOBEC3G restricts HIV-1 to a greater extent than APOBEC3F and APOBEC3DE in human primary CD4+ T cells and macrophages. *Journal of Virology*, *87*(1), 444-453. doi: 10.1128/JVI.00676-12
- Chan, D. C., Fass, D., Berger, J. M., & Kim, P. S. (1997). Core structure of gp41 from the HIV

- envelope glycoprotein. *Cell*, 89(2), 263-273.
- Chareza, S., Slavkovic Lukic, D., Liu, Y., Rathe, A. M., Munk, C., Zabogli, E., . . . Lochelt, M. (2012). Molecular and functional interactions of cat APOBEC3 and feline foamy and immunodeficiency virus proteins: different ways to counteract host-encoded restriction. *Virology*, 424(2), 138-146. doi: 10.1016/j.virol.2011.12.017
- Chauhan, A., & Khandkar, M. (2015). Endocytosis of human immunodeficiency virus 1 (HIV-1) in astrocytes: a fiery path to its destination. *Microb Pathog*, 78, 1-6. doi: 10.1016/j.micpath.2014.11.003
- Chauhan, A., Tikoo, A., Patel, J., & Abdullah, A. M. (2014). HIV-1 endocytosis in astrocytes: a kiss of death or survival of the fittest? *Neurosci Res*, 88, 16-22. doi: 10.1016/j.neures.2014.08.013
- Checkley, M. A., Luttgge, B. G., & Freed, E. O. (2011). HIV-1 Envelope Glycoprotein Biosynthesis, Trafficking, and Incorporation. *Journal of Molecular Biology*, 410(4), 582-608.
- Chen, H., Lilley, C. E., Yu, Q., Lee, D. V., Chou, J., Narvaiza, I., . . . Weitzman, M. D. (2006). APOBEC3A is a potent inhibitor of adeno-associated virus and retrotransposons. *Current Biology*, 16(5), 480-485. doi: 10.1016/j.cub.2006.01.031
- Chen, K. M., Harjes, E., Gross, P. J., Fahmy, A., Lu, Y., Shindo, K., . . . Matsuo, H. (2008). Structure of the DNA deaminase domain of the HIV-1 restriction factor APOBEC3G. *Nature*, 452(7183), 116-119. doi: 10.1038/nature06638
- Chiozzini, C., & Toschi, E. (2016). HIV-1 Tat and Immune Dysregulation in AIDS Pathogenesis: A Therapeutic Target. *Current Drug Targets*, 17(1), 33-45.
- Chiu, T. K., & Davies, D. R. (2004). Structure and function of HIV-1 integrase. *Current Topics in Medicinal Chemistry*, 4(9), 965-977. doi: Doi 10.2174/1568026043388547
- Chiu, Y. L., & Greene, W. C. (2008). The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annu Rev Immunol*, 26, 317-353. doi: 10.1146/annurev.immunol.26.021607.090350
- Chiu, Y. L., & Greene, W. C. (2009). APOBEC3G: an intracellular centurion. *Philos Trans R Soc Lond B Biol Sci*, 364(1517), 689-703. doi: 10.1098/rstb.2008.0193
- Chukkapalli, V., & Ono, A. (2011). Molecular determinants that regulate plasma membrane association of HIV-1 Gag. *Journal of Molecular Biology*, 410(4), 512-524. doi: 10.1016/j.jmb.2011.04.015
- Conticello, S. G., Harris, R. S., & Neuberger, M. S. (2003). The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. *Current Biology*, 13(22), 2009-2013.
- Costiniuk, C. T., & Jenabian, M. A. (2014). Cell-to-cell transfer of HIV infection: implications for HIV viral persistence. *Journal of General Virology*, 95(Pt 11), 2346-2355. doi: 10.1099/vir.0.069641-0
- Cox, A. L., & Siliciano, R. F. (2014). HIV Not-so-innocent bystanders. *Nature*, 505(7484), 492-493.
- Craigie, R. (2001). HIV integrase, a brief overview from chemistry to therapeutics. *Journal of Biological Chemistry*, 276(26), 23213-23216. doi: Doi 10.1074/Jbc.R100027200
- Craigie, R., & Bushman, F. D. (2012). HIV DNA integration. *Cold Spring Harb Perspect Med*, 2(7), a006890. doi: 10.1101/cshperspect.a006890
- Cullen, B. R. (1986). Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. *Cell*, 46(7), 973-982.

- d'Angelo, J., Mouscadet, J. F., Desmaele, D., Zouhiri, F., & Leh, H. (2001). HIV-1 integrase: the next target for AIDS therapy? *Pathologie Biologie*, *49*(3), 237-246. doi: Doi 10.1016/S0369-8114(01)00135-3
- Daecke, J., Fackler, O. T., Dittmar, M. T., & Krausslich, H. G. (2005). Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. *Journal of Virology*, *79*(3), 1581-1594. doi: 10.1128/JVI.79.3.1581-1594.2005
- Dahiya, S., Nonnemacher, M. R., & Wigdahl, B. (2012). Deployment of the human immunodeficiency virus type 1 protein arsenal: combating the host to enhance viral transcription and providing targets for therapeutic development. *Journal of General Virology*, *93*(Pt 6), 1151-1172. doi: 10.1099/vir.0.041186-0
- Dang, Y., Siew, L. M., Wang, X., Han, Y., Lampen, R., & Zheng, Y. H. (2008). Human cytidine deaminase APOBEC3H restricts HIV-1 replication. *Journal of Biological Chemistry*, *283*(17), 11606-11614. doi: 10.1074/jbc.M707586200
- Dang, Y., Wang, X., Esselman, W. J., & Zheng, Y. H. (2006). Identification of APOBEC3DE as another antiretroviral factor from the human APOBEC family. *Journal of Virology*, *80*(21), 10522-10533. doi: 10.1128/jvi.01123-06
- Das, S. R., & Jameel, S. (2005). Biology of the HIV Nef protein. *Indian Journal of Medical Research*, *121*(4), 315-332.
- Datta, S. A. K., Curtis, J. E., Ratcliff, W., Clark, P. K., Crist, R. M., Lebowitz, J., . . . Rein, A. (2007). Conformation of the HIV-1 Gag protein in solution. *Journal of Molecular Biology*, *365*(3), 812-824.
- Davies, D. R. (1990). The Structure and Function of the Aspartic Proteinases. *Annual Review of Biophysics and Biophysical Chemistry*, *19*, 189-215.
- de la Vega, M., Marin, M., Kondo, N., Miyauchi, K., Kim, Y., Epand, R. F., . . . Melikyan, G. B. (2011). Inhibition of HIV-1 endocytosis allows lipid mixing at the plasma membrane, but not complete fusion. *Retrovirology*, *8*, 99. doi: 10.1186/1742-4690-8-99
- de Rocquigny, H., El Meshri, S. E., Richert, L., Didier, P., Darlix, J. L., & Mely, Y. (2014). Role of the nucleocapsid region in HIV-1 Gag assembly as investigated by quantitative fluorescence-based microscopy. *Virus Research*, *193*, 78-88.
- Deeks, S. G., Wagner, B., Anton, P. A., Mitsuyasu, R. T., Scadden, D. T., Huang, C., . . . Hege, K. M. (2002). A phase II randomized study of HIV-specific T-cell gene therapy in subjects with undetectable plasma viremia on combination antiretroviral therapy. *Molecular Therapy*, *5*(6), 788-797. doi: 10.1006/mthe.2002.0611
- Delebecque, F., Suspene, R., Calattini, S., Casartelli, N., Saib, A., Froment, A., . . . Schwartz, O. (2006). Restriction of foamy viruses by APOBEC cytidine deaminases. *Journal of Virology*, *80*(2), 605-614. doi: 10.1128/JVI.80.2.605-614.2006
- Demirov, D. G., Ono, A., Orenstein, J. M., & Freed, E. O. (2002). Overexpression of the N-terminal domain of TSG101 inhibits HIV-1 budding by blocking late domain function. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(2), 955-960. doi: Doi 10.1073/Pnas.032511899
- DHHS, U. (2016). Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents
- Doehle, B. P., Schafer, A., & Cullen, B. R. (2005). Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif. *Virology*, *339*(2), 281-288. doi: 10.1016/j.virol.2005.06.005
- Domingo, E., & Holland, J. J. (1997). RNA virus mutations and fitness for survival. *Annual*

- Review of Microbiology*, 51, 151-178. doi: Doi 10.1146/Annurev.Micro.51.1.151
- Douek, D. C., Roederer, M., & Koup, R. A. (2009). Emerging concepts in the immunopathogenesis of AIDS. *Annu Rev Med*, 60, 471-484. doi: 10.1146/annurev.med.60.041807.123549
- Dube, M., Bego, M. G., Paquay, C., & Cohen, E. A. (2010). Modulation of HIV-1-host interaction: role of the Vpu accessory protein. *Retrovirology*, 7, 114. doi: 10.1186/1742-4690-7-114
- Dube, M., Roy, B. B., Guiot-Guillain, P., Mercier, J., Binette, J., Leung, G., & Cohen, E. A. (2009). Suppression of Tetherin-restricting activity upon human immunodeficiency virus type 1 particle release correlates with localization of Vpu in the trans-Golgi network. *Journal of Virology*, 83(9), 4574-4590. doi: 10.1128/JVI.01800-08
- Dussupt, V., Javid, M. P., Abou-Jaoude, G., Jadwin, J. A., de La Cruz, J., Nagashima, K., & Bouamr, F. (2009). The Nucleocapsid Region of HIV-1 Gag Cooperates with the PTAP and LYPXnL Late Domains to Recruit the Cellular Machinery Necessary for Viral Budding. *Plos Pathogens*, 5(3). doi: ARTN e1000339  
10.1371/journal.ppat.1000339
- Engelman, A., & Cherepanov, P. (2012). The structural biology of HIV-1: mechanistic and therapeutic insights. *Nature Reviews Microbiology*, 10(4), 279-290. doi: 10.1038/nrmicro2747
- Engelman, A., & Cherepanov, P. (2014). Retroviral Integrase Structure and DNA Recombination Mechanism. *Microbiol Spectr*, 2(6). doi: 10.1128/microbiolspec.MDNA3-0024-2014
- Ewart, G. D., Sutherland, T., Gage, P. W., & Cox, G. B. (1996). The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. *Journal of Virology*, 70(10), 7108-7115.
- Feng, L., Larue, R. C., Slaughter, A., Kessl, J. J., & Kvaratskhelia, M. (2015). HIV-1 Integrase Multimerization as a Therapeutic Target. *Future of Hiv-1 Therapeutics: Resistance Is Futile?*, 389, 93-119.
- Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., . . . Kupfer, A. (1995). Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat Med*, 1(2), 129-134.
- Fitzgerald, P. M. D., & Springer, J. P. (1991). Structure and Function of Retroviral Proteases. *Annual Review of Biophysics and Biophysical Chemistry*, 20, 299-320.
- Foster, J. L., Denial, S. J., Temple, B. R., & Garcia, J. V. (2011). Mechanisms of HIV-1 Nef function and intracellular signaling. *J Neuroimmune Pharmacol*, 6(2), 230-246. doi: 10.1007/s11481-011-9262-y
- Foster, J. L., & Garcia, J. V. (2007). Role of Nef in HIV-1 replication and pathogenesis. *Adv Pharmacol*, 55, 389-409. doi: 10.1016/s1054-3589(07)55011-8
- Foster, J. L., & Garcia, J. V. (2008). HIV-1 Nef: at the crossroads. *Retrovirology*, 5, 84. doi: 10.1186/1742-4690-5-84
- Franca, R., Spadari, S., & Maga, G. (2006). APOBEC deaminases as cellular antiviral factors: a novel natural host defense mechanism. *Med Sci Monit*, 12(5), Ra92-98.
- Frank, G. A., Narayan, K., Bess, J. W., Del Prete, G. Q., Wu, X. W., Moran, A., . . . Subramaniam, S. (2015). Maturation of the HIV-1 core by a non-diffusional phase transition. *Nature Communications*, 6.
- Freed, E. O. (2015). HIV-1 assembly, release and maturation. *Nature Reviews Microbiology*, 13(8), 484-496. doi: 10.1038/nrmicro3490

- Freed, E. O., Myers, D. J., & Risser, R. (1989). Mutational Analysis of the Cleavage Sequence of the Human Immunodeficiency Virus Type-1 Envelope Glycoprotein Precursor-Gp160. *Journal of Virology*, *63*(11), 4670-4675.
- Ganser-Pornillos, B. K., Yeager, M., & Sundquist, W. I. (2008). The structural biology of HIV assembly. *Current Opinion in Structural Biology*, *18*(2), 203-217. doi: 10.1016/j.sbi.2008.02.001
- Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E., & Jones, K. A. (2000). CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol Cell Biol*, *20*(18), 6958-6969.
- Garg, H., Mohl, J., & Joshi, A. (2012). HIV-1 induced bystander apoptosis. *Viruses*, *4*(11), 3020-3043. doi: 10.3390/v4113020
- Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., . . . Sundquist, W. I. (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell*, *107*(1), 55-65. doi: Doi 10.1016/S0092-8674(01)00506-2
- Gatignol, A. (2007). Transcription of HIV: Tat and cellular chromatin. *Adv Pharmacol*, *55*, 137-159. doi: 10.1016/s1054-3589(07)55004-0
- Geyer, M., Fackler, O. T., & Peterlin, B. M. (2001). Structure--function relationships in HIV-1 Nef. *Embo Reports*, *2*(7), 580-585. doi: 10.1093/embo-reports/kve141
- Ghanam, R. H., Samal, A. B., Fernandez, T. F., & Saad, J. S. (2012). Role of the HIV-1 Matrix Protein in Gag Intracellular Trafficking and Targeting to the Plasma Membrane for Virus Assembly. *Front Microbiol*, *3*, 55. doi: 10.3389/fmicb.2012.00055
- Gibellini, D., Vitone, F., Schiavone, P., & Re, M. C. (2005). HIV-1 Tat protein and cell proliferation and survival: a brief review. *New Microbiologica*, *28*(2), 95-109.
- Giese, S., & Marsh, M. (2014). Tetherin can restrict cell-free and cell-cell transmission of HIV from primary macrophages to T cells. *Plos Pathogens*, *10*(7), e1004189. doi: 10.1371/journal.ppat.1004189
- Gilbert, P. B., McKeague, I. W., Eisen, G., Mullins, C., Gueye, N. A., Mboup, S., & Kanki, P. J. (2003). Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal. *Stat Med*, *22*(4), 573-593. doi: 10.1002/sim.1342
- Gilliet, M., Cao, W., & Liu, Y. J. (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nature Reviews Immunology*, *8*(8), 594-606. doi: 10.1038/nri2358
- Gobeil, L. A., Lodge, R., & Tremblay, M. J. (2013). Macropinocytosis-like HIV-1 internalization in macrophages is CCR5 dependent and leads to efficient but delayed degradation in endosomal compartments. *Journal of Virology*, *87*(2), 735-745. doi: 10.1128/JVI.01802-12
- Godet, J., Boudier, C., Humbert, N., Ivanyi-Nagy, R., Darlix, J. L., & Mely, Y. (2012). Comparative nucleic acid chaperone properties of the nucleocapsid protein NCp7 and Tat protein of HIV-1. *Virus Research*, *169*(2), 349-360. doi: 10.1016/j.virusres.2012.06.021
- Goffinet, C., Homann, S., Ambiel, I., Tibroni, N., Rupp, D., Keppler, O. T., & Fackler, O. T. (2010). Antagonism of CD317 restriction of human immunodeficiency virus type 1 (HIV-1) particle release and depletion of CD317 are separable activities of HIV-1 Vpu. *Journal of Virology*, *84*(8), 4089-4094. doi: 10.1128/JVI.01549-09
- Goila-Gaur, R., Khan, M. A., Miyagi, E., Kao, S., & Strebel, K. (2007). Targeting APOBEC3A to

- the viral nucleoprotein complex confers antiviral activity. *Retrovirology*, 4, 61. doi: 10.1186/1742-4690-4-61
- Goila-Gaur, R., & Strebel, K. (2008). HIV-1 Vif, APOBEC, and intrinsic immunity. *Retrovirology*, 5, 51. doi: 10.1186/1742-4690-5-51
- Goldstone, D. C., Ennis-Adeniran, V., Hedden, J. J., Groom, H. C., Rice, G. I., Christodoulou, E., . . . Webb, M. (2011). HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature*, 480(7377), 379-382. doi: 10.1038/nature10623
- Gonzalez, M. E. (2015). Vpu Protein: The Viroporin Encoded by HIV-1. *Viruses*, 7(8), 4352-4368. doi: 10.3390/v7082824
- Gotz, N., Sauter, D., Usmani, S. M., Fritz, J. V., Goffinet, C., Heigele, A., . . . Kirchhoff, F. (2012). Reacquisition of Nef-mediated tetherin antagonism in a single in vivo passage of HIV-1 through its original chimpanzee host. *Cell Host & Microbe*, 12(3), 373-380. doi: 10.1016/j.chom.2012.07.008
- Grandgenett, D. P., Pandey, K. K., Bera, S., & Aihara, H. (2015). Multifunctional facets of retrovirus integrase. *World J Biol Chem*, 6(3), 83-94. doi: 10.4331/wjbc.v6.i3.83
- Green, A. M., Landry, S., Budagyan, K., Avgousti, D. C., Shalhout, S., Bhagwat, A. S., & Weitzman, M. D. (2016). APOBEC3A damages the cellular genome during DNA replication. *Cell Cycle*, 15(7), 998-1008. doi: 10.1080/15384101.2016.1152426
- Grobler, J. A., Stillmock, K., Hu, B., Witmer, M., Felock, P., Espeseth, A. S., . . . Hazuda, D. J. (2002). Diketo acid inhibitor mechanism and HIV-1 integrase: implications for metal binding in the active site of phosphotransferase enzymes. *Proc Natl Acad Sci U S A*, 99(10), 6661-6666. doi: 10.1073/pnas.092056199
- Groot, F., Welsch, S., & Sattentau, Q. J. (2008). Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses. *Blood*, 111(9), 4660-4663. doi: 10.1182/blood-2007-12-130070
- Gu, J., Chen, Q., Xiao, X., Ito, F., Wolfe, A., & Chen, X. S. (2016). Biochemical Characterization of APOBEC3H Variants: Implications for Their HIV-1 Restriction Activity and mC Modification. *Journal of Molecular Biology*, 428(23), 4626-4638. doi: 10.1016/j.jmb.2016.08.012
- Guatelli, J. C. (2009). Interactions of viral protein U (Vpu) with cellular factors. *Curr Top Microbiol Immunol*, 339, 27-45. doi: 10.1007/978-3-642-02175-6\_2
- Guenzel, C. A., Herate, C., & Benichou, S. (2014). HIV-1 Vpr-a still "enigmatic multitasker". *Journal of Neurovirology*, 5, 127. doi: 10.1007/s13365-016-0436-5  
10.3389/fmicb.2014.00127
- Guerrero, S., Libre, C., Batisse, J., Mercenne, G., Richer, D., Laumond, G., . . . Paillart, J. C. (2016). Translational regulation of APOBEC3G mRNA by Vif requires its 5'UTR and contributes to restoring HIV-1 infectivity. *Sci Rep*, 6, 39507. doi: 10.1038/srep39507
- Guo, F., Cen, S., Niu, M., Saadatmand, J., & Kleiman, L. (2006). Inhibition of tRNA(3)(Lys)-primed reverse transcription by human APOBEC3G during human immunodeficiency virus type 1 replication. *Journal of Virology*, 80(23), 11710-11722. doi: 10.1128/JVI.01038-06
- Guo, Y., Dong, L., Qiu, X., Wang, Y., Zhang, B., Liu, H., . . . Huang, Z. (2014). Structural basis for hijacking CBF-beta and CUL5 E3 ligase complex by HIV-1 Vif. *Nature*, 505(7482), 229-233. doi: 10.1038/nature12884
- Hamamoto, I., & Yamamoto, N. (2010). [Progress of basic research on HIV/AIDS: HIV entry mechanism: comparison with influenza virus]. *Nihon Rinsho*, 68(3), 383-388.

- Hammarskjold, M. L., Heimer, J., Hammarskjold, B., Sangwan, I., Albert, L., & Rekosh, D. (1989). Regulation of Human Immunodeficiency Virus Env Expression by the Rev Gene-Product. *Journal of Virology*, *63*(5), 1959-1966.
- Hansen, E. C., Seamon, K. J., Cravens, S. L., & Stivers, J. T. (2014). GTP activator and dNTP substrates of HIV-1 restriction factor SAMHD1 generate a long-lived activated state. *Proc Natl Acad Sci U S A*, *111*(18), E1843-1851. doi: 10.1073/pnas.1401706111
- Hanson, P. I., Roth, R., Lin, Y., & Heuser, J. E. (2008). Plasma membrane deformation by circular arrays of ESCRT-III protein filaments. *Journal of Cell Biology*, *180*(2), 389-402. doi: Doi 10.1083/Jcb.200707031
- Harari, A., Ooms, M., Mulder, L. C., & Simon, V. (2009). Polymorphisms and splice variants influence the antiretroviral activity of human APOBEC3H. *Journal of Virology*, *83*(1), 295-303. doi: 10.1128/JVI.01665-08
- Harris, M. (1999). HIV: a new role for Nef in the spread of HIV. *Current Biology*, *9*(12), R459-461.
- Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., . . . Malim, M. H. (2003). DNA deamination mediates innate immunity to retroviral infection. *Cell*, *113*(6), 803-809.
- Harris, R. S., & Liddament, M. T. (2004). Retroviral restriction by APOBEC proteins. *Nature Reviews Immunology*, *4*(11), 868-877. doi: 10.1038/nri1489
- Hauser, H., Lopez, L. A., Yang, S. J., Oldenburg, J. E., Exline, C. M., Guatelli, J. C., & Cannon, P. M. (2010). HIV-1 Vpu and HIV-2 Env counteract BST-2/tetherin by sequestration in a perinuclear compartment. *Retrovirology*, *7*, 51. doi: 10.1186/1742-4690-7-51
- Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., . . . Miller, M. D. (2000). Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science*, *287*(5453), 646-650.
- Heneine, W., Schweizer, M., Sandstrom, P., & Folks, T. (2003). Human infection with foamy viruses. *Curr Top Microbiol Immunol*, *277*, 181-196.
- Henry, M., Terzian, C., Peeters, M., Wain-Hobson, S., & Vartanian, J. P. (2012). Evolution of the primate APOBEC3A cytidine deaminase gene and identification of related coding regions. *Plos One*, *7*(1), e30036. doi: 10.1371/journal.pone.0030036
- Hetzer, C., Dormeyer, W., Schnolzer, M., & Ott, M. (2005). Decoding Tat: the biology of HIV Tat posttranslational modifications. *Microbes and Infection*, *7*(13), 1364-1369.
- Hill, M., Tachedjian, G., & Mak, J. (2005). The packaging and maturation of the HIV-1 pol proteins. *Current Hiv Research*, *3*(1), 73-85. doi: Doi 10.2174/1570162052772942
- Hinz, A., Miguet, N., Natrajan, G., Usami, Y., Yamanaka, H., Renesto, P., . . . Weissenhorn, W. (2010). Structural basis of HIV-1 tethering to membranes by the BST-2/tetherin ectodomain. *Cell Host & Microbe*, *7*(4), 314-323. doi: 10.1016/j.chom.2010.03.005
- Hiscott, J., Kwon, H., & Genin, P. (2001). Hostile takeovers: viral appropriation of the NF-kappaB pathway. *Journal of Clinical Investigation*, *107*(2), 143-151. doi: 10.1172/JCI11918
- Holden, L. G., Prochnow, C., Chang, Y. P., Bransteitter, R., Chelico, L., Sen, U., . . . Chen, X. S. (2008). Crystal structure of the anti-viral APOBEC3G catalytic domain and functional implications. *Nature*, *456*(7218), 121-124. doi: 10.1038/nature07357
- Holmes, E. C. (2003). Error thresholds and the constraints to RNA virus evolution. *Trends in Microbiology*, *11*(12), 543-546. doi: 10.1016/j.tim.2003.10.006
- Holmes, R. K., Koning, F. A., Bishop, K. N., & Malim, M. H. (2007). APOBEC3F can inhibit

- the accumulation of HIV-1 reverse transcription products in the absence of hypermutation. Comparisons with APOBEC3G. *Journal of Biological Chemistry*, 282(4), 2587-2595. doi: 10.1074/jbc.M607298200
- Holt, N., Wang, J., Kim, K., Friedman, G., Wang, X., Taupin, V., . . . Cannon, P. M. (2010). Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. *Nat Biotechnol*, 28(8), 839-847. doi: 10.1038/nbt.1663
- Hope, T. J. (1999). The ins and outs of HIV Rev. *Archives of Biochemistry and Biophysics*, 365(2), 186-191.
- Hout, D. R., Mulcahy, E. R., Pacyniak, E., Gomez, L. M., Gomez, M. L., & Stephens, E. B. (2004). Vpu: a multifunctional protein that enhances the pathogenesis of human immunodeficiency virus type 1. *Current Hiv Research*, 2(3), 255-270.
- Howcroft, T. K., Strebel, K., Martin, M. A., & Singer, D. S. (1993). Repression of MHC class I gene promoter activity by two-exon Tat of HIV. *Science*, 260(5112), 1320-1322.
- Hrecka, K., Hao, C., Gierszewska, M., Swanson, S. K., Kesik-Brodacka, M., Srivastava, S., . . . Skowronski, J. (2011). Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature*, 474(7353), 658-661. doi: 10.1038/nature10195
- Hsu, K., Seharaseyon, J., Dong, P. H., Bour, S., & Marban, E. (2004). Mutual functional destruction of HIV-1 Vpu and host TASK-1 channel. *Molecular Cell*, 14(2), 259-267. doi: Doi 10.1016/S1097-2765(04)00183-2
- Huang, X., Britto, M. D., Kear-Scott, J. L., Boone, C. D., Rocca, J. R., Simmerling, C., . . . Fanucci, G. E. (2014). The Role of Select Subtype Polymorphisms on HIV-1 Protease Conformational Sampling and Dynamics. *Journal of Biological Chemistry*, 289(24), 17203-17214.
- Hultquist, J. F., Lengyel, J. A., Refsland, E. W., LaRue, R. S., Lackey, L., Brown, W. L., & Harris, R. S. (2011). Human and rhesus APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H demonstrate a conserved capacity to restrict Vif-deficient HIV-1. *Journal of Virology*, 85(21), 11220-11234. doi: 10.1128/JVI.05238-11
- Hurley, J. H. (2010). The ESCRT complexes. *Critical Reviews in Biochemistry and Molecular Biology*, 45(6), 463-487.
- Hurley, J. H., Boura, E., Carlson, L. A., & Rozycki, B. (2010). Membrane Budding. *Cell*, 143(6), 875-887.
- Hurley, J. H., & Hanson, P. I. (2010). Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nature Reviews Molecular Cell Biology*, 11(8), 556-566.
- Hussain, A. I., Shanmugam, V., Bhullar, V. B., Beer, B. E., Vallet, D., Gautier-Hion, A., . . . Switzer, W. M. (2003). Screening for simian foamy virus infection by using a combined antigen Western blot assay: evidence for a wide distribution among Old World primates and identification of four new divergent viruses. *Virology*, 309(2), 248-257.
- Ishikawa, J., Kaisho, T., Tomizawa, H., Lee, B. O., Kobune, Y., Inazawa, J., . . . et al. (1995). Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. *Genomics*, 26(3), 527-534.
- Jacobomolina, A., Ding, J. P., Nanni, R. G., Clark, A. D., Lu, X. D., Tantillo, C., . . . Arnold, E. (1993). Crystal-Structure of Human-Immunodeficiency-Virus Type-1 Reverse-Transcriptase Complexed with Double-Stranded DNA at 3.0 Angstrom Resolution Shows Bent DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 90(13), 6320-6324.



- Jager, S., Kim, D. Y., Hultquist, J. F., Shindo, K., LaRue, R. S., Kwon, E., . . . Krogan, N. J. (2011). Vif hijacks CBF-beta to degrade APOBEC3G and promote HIV-1 infection. *Nature*, *481*(7381), 371-375. doi: 10.1038/nature10693
- James, T., Nonnemacher, M. R., Wigdahl, B., & Krebs, F. C. (2016). Defining the roles for Vpr in HIV-1-associated neuropathogenesis. *22*(4), 403-415. doi: 10.1007/s13365-016-0436-5
- Jarmuz, A., Chester, A., Bayliss, J., Gisbourne, J., Dunham, I., Scott, J., & Navaratnam, N. (2002). An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics*, *79*(3), 285-296. doi: 10.1006/geno.2002.6718
- Jere, A., Fujita, M., Adachi, A., & Nomaguchi, M. (2010). Role of HIV-1 Nef protein for virus replication in vitro. *Microbes and Infection*, *12*(1), 65-70. doi: 10.1016/j.micinf.2009.09.009
- Johri, M. K., Mishra, R., Chhatbar, C., Unni, S. K., & Singh, S. K. (2011). Tits and bits of HIV Tat protein. *Expert Opinion on Biological Therapy*, *11*(3), 269-283.
- Jolly, C., Kashefi, K., Hollinshead, M., & Sattentau, Q. J. (2004a). HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *Journal of Experimental Medicine*, *199*(2), 283-293. doi: 10.1084/jem.20030648
- Jolly, C., Kashefi, K., Hollinshead, M., & Sattentau, Q. J. (2004b). HIV-1 cell to cell transfer across an env-induced, actin-dependent synapse. *Journal of Experimental Medicine*, *199*(2), 283-293.
- Jolly, C., Mitar, I., & Sattentau, Q. J. (2007). Adhesion molecule interactions facilitate human immunodeficiency virus type 1-induced virological synapse formation between T cells. *Journal of Virology*, *81*(24), 13916-13921.
- Jolly, C., & Sattentau, Q. J. (2005). Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity. *Journal of Virology*, *79*(18), 12088-12094. doi: 10.1128/JVI.79.18.12088-12094.2005
- Jolly, C., Welsch, S., Michor, S., & Sattentau, Q. J. (2011a). The Regulated Secretory Pathway in CD4(+) T cells Contributes to Human Immunodeficiency Virus Type-1 Cell-to-Cell Spread at the Virological Synapse. *Plos Pathogens*, *7*(9).
- Jolly, C., Welsch, S., Michor, S., & Sattentau, Q. J. (2011b). The regulated secretory pathway in CD4(+) T cells contributes to human immunodeficiency virus type-1 cell-to-cell spread at the virological synapse. *Plos Pathogens*, *7*(9), e1002226. doi: 10.1371/journal.ppat.1002226
- Jonsson, S. R., Hache, G., Stenglein, M. D., Fahrenkrug, S. C., Andresdottir, V., & Harris, R. S. (2006). Evolutionarily conserved and non-conserved retrovirus restriction activities of artiodactyl APOBEC3F proteins. *Nucleic Acids Research*, *34*(19), 5683-5694. doi: 10.1093/nar/gkl721
- Joseph, A. M., Kumar, M., & Mitra, D. (2005). Nef: "necessary and enforcing factor" in HIV infection. *Current Hiv Research*, *3*(1), 87-94.
- Karn, J., & Stoltzfus, C. M. (2012). Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harb Perspect Med*, *2*(2), a006916. doi: 10.1101/cshperspect.a006916
- Katuwal, M., Wang, Y., Schmitt, K., Guo, K., Halemano, K., Santiago, M. L., & Stephens, E. B. (2014). Cellular HIV-1 inhibition by truncated old world primate APOBEC3A proteins lacking a complete deaminase domain. *Virology*, *468-470*, 532-544. doi: 10.1016/j.virol.2014.09.001
- Keller, P. W., Huang, R. K., England, M. R., Waki, K., Cheng, N. Q., Heymann, J. B., . . . Steven,

- A. C. (2013). A Two-Pronged Structural Analysis of Retroviral Maturation Indicates that Core Formation Proceeds by a Disassembly-Reassembly Pathway Rather than a Displacive Transition. *Journal of Virology*, 87(24), 13655-13664.
- Khan, A. S. (2009). Simian foamy virus infection in humans: prevalence and management. *Expert Rev Anti Infect Ther*, 7(5), 569-580. doi: 10.1586/eri.09.39
- Khan, M. A., Goila-Gaur, R., Kao, S., Miyagi, E., Walker, R. C., Jr., & Strebel, K. (2009). Encapsidation of APOBEC3G into HIV-1 virions involves lipid raft association and does not correlate with APOBEC3G oligomerization. *Retrovirology*, 6, 99. doi: 10.1186/1742-4690-6-99
- Kim, D. Y. (2015). The assembly of Vif ubiquitin E3 ligase for APOBEC3 degradation. *Arch Pharm Res*, 38(4), 435-445. doi: 10.1007/s12272-014-0519-x
- King, J. E., Eugenin, E. A., Buckner, C. M., & Berman, J. W. (2006). HIV tat and neurotoxicity. *Microbes and Infection*, 8(5), 1347-1357.
- Kinomoto, M., Kanno, T., Shimura, M., Ishizaka, Y., Kojima, A., Kurata, T., . . . Tokunaga, K. (2007). All APOBEC3 family proteins differentially inhibit LINE-1 retrotransposition. *Nucleic Acids Research*, 35(9), 2955-2964. doi: 10.1093/nar/gkm181
- Kitamura, S., Ode, H., Nakashima, M., Imahashi, M., Naganawa, Y., Kurosawa, T., . . . Iwatani, Y. (2012). The APOBEC3C crystal structure and the interface for HIV-1 Vif binding. *Nature Structural & Molecular Biology*, 19(10), 1005-1010. doi: 10.1038/nsmb.2378
- Kjems, J., Calnan, B. J., Frankel, A. D., & Sharp, P. A. (1992). Specific Binding of a Basic Peptide from Hiv-1 Rev. *Embo Journal*, 11(3), 1119-1129.
- Klasse, P. J. (2012). The molecular basis of HIV entry. *Cellular Microbiology*, 14(8), 1183-1192.
- Klein, J. S., & Bjorkman, P. J. (2010). Few and Far Between: How HIV May Be Evading Antibody Avidity. *Plos Pathogens*, 6(5).
- Kogan, M., & Rappaport, J. (2011). HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention. *Retrovirology*, 8, 25. doi: 10.1097/COH.0b013e32835049e0  
10.1186/1742-4690-8-25
- Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., & Steitz, T. A. (1992). Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science*, 256(5065), 1783-1790.
- Kondo, S., Wakae, K., Wakisaka, N., Nakanishi, Y., Ishikawa, K., Komori, T., . . . Yoshizaki, T. (2016). APOBEC3A associates with human papillomavirus genome integration in oropharyngeal cancers. *Oncogene*. doi: 10.1038/onc.2016.335
- Koning, F. A., Goujon, C., Bauby, H., & Malim, M. H. (2011). Target cell-mediated editing of HIV-1 cDNA by APOBEC3 proteins in human macrophages. *Journal of Virology*, 85(24), 13448-13452. doi: 10.1128/JVI.00775-11
- Koning, F. A., Newman, E. N., Kim, E. Y., Kunstman, K. J., Wolinsky, S. M., & Malim, M. H. (2009). Defining APOBEC3 expression patterns in human tissues and hematopoietic cell subsets. *Journal of Virology*, 83(18), 9474-9485. doi: 10.1128/JVI.01089-09
- Konvalinka, J., Krausslich, H. G., & Muller, B. (2015). Retroviral proteases and their roles in virion maturation. *Virology*, 479, 403-417.
- Kremer, M., & Schnierle, B. S. (2005). HIV-1 Vif: HIV's weapon against the cellular defense factor APOBEC3G. *Current Hiv Research*, 3(4), 339-344.
- Krueger, B. J., Jeronimo, C., Roy, B. B., Bouchard, A., Barrandon, C., Byers, S. A., . . . Price, D. H. (2008). LARP7 is a stable component of the 7SK snRNP while P-TEFb, HEXIM1 and

- hnRNP A1 are reversibly associated. *Nucleic Acids Research*, 36(7), 2219-2229. doi: 10.1093/nar/gkn061
- Kuhl, A., Banning, C., Marzi, A., Votteler, J., Steffen, I., Bertram, S., . . . Pohlmann, S. (2011). The Ebola virus glycoprotein and HIV-1 Vpu employ different strategies to counteract the antiviral factor tetherin. *Journal of Infectious Diseases*, 204 Suppl 3, S850-860. doi: 10.1093/infdis/jir378
- Kumar, V., Abbas, A. K., Aster, J. C., & Robbins, S. L. (2013). *Robbins basic pathology* (9th ed.). Philadelphia, PA: Elsevier/Saunders.
- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., & Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature*, 393(6686), 648-659.
- Lackey, L., Demorest, Z. L., Land, A. M., Hultquist, J. F., Brown, W. L., & Harris, R. S. (2012). APOBEC3B and AID have similar nuclear import mechanisms. *Journal of Molecular Biology*, 419(5), 301-314. doi: 10.1016/j.jmb.2012.03.011
- Laguet, N., Sobhian, B., Casartelli, N., Ringard, M., Chable-Bessia, C., Segal, E., . . . Benkirane, M. (2011). SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature*, 474(7353), 654-657. doi: 10.1038/nature10117
- Lake, J. A., Carr, J., Feng, F., Mundy, L., Burrell, C., & Li, P. (2003). The role of Vif during HIV-1 infection: interaction with novel host cellular factors. *J Clin Virol*, 26(2), 143-152.
- Landry, S., Narvaiza, I., Lindesty, D. C., & Weitzman, M. D. (2011). APOBEC3A can activate the DNA damage response and cause cell-cycle arrest. *Embo Reports*, 12(5), 444-450. doi: 10.1038/embor.2011.46
- Langelier, C. R., Sandrin, V., Eckert, D. M., Christensen, D. E., Chandrasekaran, V., Alam, S. L., . . . Sundquist, W. I. (2008). Biochemical Characterization of a Recombinant TRIM5 alpha Protein That Restricts Human Immunodeficiency Virus Type 1 Replication. *Journal of Virology*, 82(23), 11682-11694.
- Lassen, K. G., Bailey, J. R., & Siliciano, R. F. (2004). Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells in vivo. *Journal of Virology*, 78(17), 9105-9114. doi: 10.1128/JVI.78.17.9105-9114.2004
- Le Rouzic, E., & Benichou, S. (2005). The Vpr protein from HIV-1: distinct roles along the viral life cycle. *Retrovirology*, 2, 11. doi: 10.1186/1742-4690-2-11
- Le Tortorec, A., & Neil, S. J. (2009). Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. *Journal of Virology*, 83(22), 11966-11978. doi: 10.1128/JVI.01515-09
- Le Tortorec, A., Willey, S., & Neil, S. J. (2011). Antiviral inhibition of enveloped virus release by tetherin/BST-2: action and counteraction. *Viruses*, 3(5), 520-540. doi: 10.3390/v3050520
- Lecossier, D., Bouchonnet, F., Clavel, F., & Hance, A. J. (2003). Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science*, 300(5622), 1112. doi: 10.1126/science.1083338
- Leendertz, F. H., Zirkel, F., Couacy-Hymann, E., Ellerbrok, H., Morozov, V. A., Pauli, G., . . . Junglen, S. (2008). Interspecies transmission of simian foamy virus in a natural predator-prey system. *Journal of Virology*, 82(15), 7741-7744. doi: 10.1128/JVI.00549-08
- Lenburg, M. E., & Landau, N. R. (1993). Vpu-induced degradation of CD4: requirement for specific amino acid residues in the cytoplasmic domain of CD4. *Journal of Virology*, 67(12), 7238-7245.
- Lever, A. M. (2007). HIV-1 RNA packaging. *Adv Pharmacol*, 55, 1-32. doi: 10.1016/s1054-

- 3589(07)55001-5
- Levin, J. G., Guo, J. H., Rouzina, I., & Musier-Forsyth, K. (2005). Nucleic acid chaperone activity of HIV-1 nucleocapsid protein: Critical role in reverse transcription and molecular mechanism. *Progress in Nucleic Acid Research and Molecular Biology, Vol 80, 80*, 217-286. doi: 10.1016/S0079-6603(05)80006-6
- Li, G., Bukrinsky, M., & Zhao, R. Y. (2009). HIV-1 viral protein R (Vpr) and its interactions with host cell. *Current Hiv Research, 7*(2), 178-183.
- Li, H., Dou, J., Ding, L., & Spearman, P. (2007). Myristoylation is required for human immunodeficiency virus type 1 Gag-Gag multimerization in mammalian cells. *Journal of Virology, 81*(23), 12899-12910. doi: 10.1128/jvi.01280-07
- Li, H. L., Chien, P. C., Tuen, M., Visciano, M. L., Cohen, S., Blais, S., . . . Hioe, C. E. (2008). Identification of an N-linked glycosylation in the C4 region of HIV-1 envelope gp120 that is critical for recognition of neighboring CD4 T cell epitopes. *Journal of Immunology, 180*(6), 4011-4021.
- Li, W. X., Li, G. H., Steiner, J., & Nath, A. (2009). Role of Tat Protein in HIV Neuropathogenesis. *Neurotoxicity Research, 16*(3), 205-220.
- Li, X., & Sodroski, J. (2008). The TRIM5 alpha B-Box 2 Domain Promotes Cooperative Binding to the Retroviral Capsid by Mediating Higher-Order Self-Association. *Journal of Virology, 82*(23), 11495-11502.
- Li, Y., Li, X., Stremlau, M., Lee, M., & Sodroski, J. (2006). Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *Journal of Virology, 80*(14), 6738-6744. doi: 10.1128/JVI.00270-06
- Liddament, M. T., Brown, W. L., Schumacher, A. J., & Harris, R. S. (2004). APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. *Current Biology, 14*(15), 1385-1391. doi: 10.1016/j.cub.2004.06.050
- Lin, P. F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N. N., . . . Colonna, R. (2003). A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proceedings of the National Academy of Sciences of the United States of America, 100*(19), 11013-11018. doi: 10.1073/pnas.1832214100
- Lindwasser, O. W., Chaudhuri, R., & Bonifacino, J. S. (2007). Mechanisms of CD4 downregulation by the Nef and Vpu proteins of primate immunodeficiency viruses. *Curr Mol Med, 7*(2), 171-184.
- Lingappa, J. R., Doohar, J. E., Newman, M. A., Kiser, P. K., & Klein, K. C. (2006). Basic residues in the nucleocapsid domain of Gag are required for interaction of HIV-1 gag with ABCE1 (HP68), a cellular protein important for HIV-1 capsid assembly. *Journal of Biological Chemistry, 281*(7), 3773-3784. doi: 10.1074/jbc.M507255200
- Liu, B., Sarkis, P. T., Luo, K., Yu, Y., & Yu, X. F. (2005). Regulation of Apobec3F and human immunodeficiency virus type 1 Vif by Vif-Cul5-ElonB/C E3 ubiquitin ligase. *Journal of Virology, 79*(15), 9579-9587. doi: 10.1128/JVI.79.15.9579-9587.2005
- Liu, Y., Luo, S., He, S., Zhang, M., Wang, P., Li, C., . . . Hu, Q. (2015). Tetherin restricts HSV-2 release and is counteracted by multiple viral glycoproteins. *Virology, 475*, 96-109. doi: 10.1016/j.virol.2014.11.005
- Llewellyn, G. N., Hogue, I. B., Grover, J. R., & Ono, A. (2010). Nucleocapsid Promotes Localization of HIV-1 Gag to Uropods That Participate in Virological Synapses between T Cells. *Plos Pathogens, 6*(10). doi: ARTN e1001167  
10.1371/journal.ppat.1001167

- Lochelt, M., Romen, F., Bastone, P., Muckenfuss, H., Kirchner, N., Kim, Y. B., . . . Munk, C. (2005). The antiretroviral activity of APOBEC3 is inhibited by the foamy virus accessory Bet protein. *Proc Natl Acad Sci U S A*, *102*(22), 7982-7987. doi: 10.1073/pnas.0501445102
- Logue, E. C., Bloch, N., Dhuey, E., Zhang, R., Cao, P., Herate, C., . . . Landau, N. R. (2014). A DNA sequence recognition loop on APOBEC3A controls substrate specificity. *Plos One*, *9*(5), e97062. doi: 10.1371/journal.pone.0097062
- Lopez, L. A., Yang, S. J., Hauser, H., Exline, C. M., Haworth, K. G., Oldenburg, J., & Cannon, P. M. (2010). Ebola virus glycoprotein counteracts BST-2/Tetherin restriction in a sequence-independent manner that does not require tetherin surface removal. *Journal of Virology*, *84*(14), 7243-7255. doi: 10.1128/JVI.02636-09
- Louis, J. M., Ishima, R., Torchia, D. A., & Weber, I. T. (2007). HIV-1 protease: structure, dynamics, and inhibition. *Adv Pharmacol*, *55*, 261-298. doi: 10.1016/s1054-3589(07)55008-8
- Love, R. P., Xu, H., & Chelico, L. (2012). Biochemical analysis of hypermutation by the deoxycytidine deaminase APOBEC3A. *Journal of Biological Chemistry*, *287*(36), 30812-30822. doi: 10.1074/jbc.M112.393181
- Lu, K., Heng, X., & Summers, M. F. (2011). Structural Determinants and Mechanism of HIV-1 Genome Packaging. *Journal of Molecular Biology*, *410*(4), 609-633. doi: 10.1016/j.jmb.2011.04.029
- Lu, M., Blacklow, S. C., & Kim, P. S. (1995). A Trimeric Structural Domain of the Hiv-1 Transmembrane Glycoprotein. *Nature Structural Biology*, *2*(12), 1075-1082.
- Luo, K., Wang, T., Liu, B., Tian, C., Xiao, Z., Kappes, J., & Yu, X. F. (2007). Cytidine deaminases APOBEC3G and APOBEC3F interact with human immunodeficiency virus type 1 integrase and inhibit proviral DNA formation. *Journal of Virology*, *81*(13), 7238-7248. doi: 10.1128/JVI.02584-06
- Ma, J., Li, X., Xu, J., Zhang, Q., Liu, Z., Jia, P., . . . Cen, S. (2013). The roles of APOBEC3G complexes in the incorporation of APOBEC3G into HIV-1. *Plos One*, *8*(10), e74892. doi: 10.1371/journal.pone.0074892
- Maeda, K., Almofty, S. A., Singh, S. K., Eid, M. M., Shimoda, M., Ikeda, T., . . . Sakaguchi, N. (2013). GANP interacts with APOBEC3G and facilitates its encapsidation into the virions to reduce HIV-1 infectivity. *Journal of Immunology*, *191*(12), 6030-6039. doi: 10.4049/jimmunol.1302057
- Maier, D. A., Brennan, A. L., Jiang, S., Binder-Scholl, G. K., Lee, G., Plesa, G., . . . Levine, B. L. (2013). Efficient clinical scale gene modification via zinc finger nuclease-targeted disruption of the HIV co-receptor CCR5. *Hum Gene Ther*, *24*(3), 245-258. doi: 10.1089/hum.2012.172
- Malim, M. H. (2009). APOBEC proteins and intrinsic resistance to HIV-1 infection. *Philos Trans R Soc Lond B Biol Sci*, *364*(1517), 675-687. doi: 10.1098/rstb.2008.0185
- Malim, M. H., Mccarn, D. F., Tiley, L. S., & Cullen, B. R. (1991). Mutational Definition of the Human-Immunodeficiency-Virus Type-1 Rev Activation Domain. *Journal of Virology*, *65*(8), 4248-4254.
- Mangeat, B., Gers-Huber, G., Lehmann, M., Zufferey, M., Luban, J., & Piguet, V. (2009). HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *Plos Pathogens*, *5*(9), e1000574. doi: 10.1371/journal.ppat.1000574

- Mansouri, M., Viswanathan, K., Douglas, J. L., Hines, J., Gustin, J., Moses, A. V., & Fruh, K. (2009). Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. *Journal of Virology*, *83*(19), 9672-9681. doi: 10.1128/JVI.00597-09
- Marin, M., Golem, S., Rose, K. M., Kozak, S. L., & Kabat, D. (2008). Human immunodeficiency virus type 1 Vif functionally interacts with diverse APOBEC3 cytidine deaminases and moves with them between cytoplasmic sites of mRNA metabolism. *Journal of Virology*, *82*(2), 987-998. doi: 10.1128/JVI.01078-07
- Marin, M., Rose, K. M., Kozak, S. L., & Kabat, D. (2003). HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med*, *9*(11), 1398-1403. doi: 10.1038/nm946
- Marinello, J., Marchand, C., Mott, B. T., Bain, A., Thomas, C. J., & Pommier, Y. (2008). Comparison of raltegravir and elvitegravir on HIV-1 integrase catalytic reactions and on a series of drug-resistant integrase mutants. *Biochemistry*, *47*(36), 9345-9354. doi: 10.1021/bi800791q
- Marini, B., Kertesz-Farkas, A., Ali, H., Lucic, B., Lisek, K., Manganaro, L., . . . Lusic, M. (2015). Nuclear architecture dictates HIV-1 integration site selection. *Nature*, *521*(7551), 227-231. doi: 10.1038/nature14226
- Martin-Serrano, J., Zang, T., & Bieniasz, P. D. (2001). HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. *Nat Med*, *7*(12), 1313-1319. doi: Doi 10.1038/Nm1201-1313
- Massiah, M. A., Starich, M. R., Paschall, C., Summers, M. F., Christensen, A. M., & Sundquist, W. I. (1994). Three-dimensional structure of the human immunodeficiency virus type 1 matrix protein. *Journal of Molecular Biology*, *244*(2), 198-223. doi: 10.1006/jmbi.1994.1719
- Mateu, M. G. (2009). The capsid protein of human immunodeficiency virus: intersubunit interactions during virus assembly. *Febs Journal*, *276*(21), 6098-6109. doi: 10.1111/j.1742-4658.2009.07313.x
- Mateu, M. G. (2013). Assembly, stability and dynamics of virus capsids. *Archives of Biochemistry and Biophysics*, *531*(1-2), 65-79. doi: 10.1016/j.abb.2012.10.015
- Matsui, M., Warburton, R. J., Cogswell, P. C., Baldwin, A. S., Jr., & Frelinger, J. A. (1996). Effects of HIV-1 Tat on expression of HLA class I molecules. *J Acquir Immune Defic Syndr Hum Retrovirol*, *11*(3), 233-240.
- Mayol, K., Munier, S., Beck, A., Verrier, B., & Guillon, C. (2007). Design and characterization of an HIV-1 Tat mutant: inactivation of viral and cellular functions but not antigenicity. *Vaccine*, *25*(32), 6047-6060. doi: 10.1016/j.vaccine.2007.05.048
- Mbisa, J. L., Barr, R., Thomas, J. A., Vandegraaff, N., Dorweiler, I. J., Svarovskaia, E. S., . . . Pathak, V. K. (2007). Human immunodeficiency virus type 1 cDNAs produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration. *Journal of Virology*, *81*(13), 7099-7110. doi: 10.1128/JVI.00272-07
- Mccune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R., & Weissman, I. L. (1988). Endoproteolytic Cleavage of Gp160 Is Required for the Activation of Human Immunodeficiency Virus. *Cell*, *53*(1), 55-67.
- McDonald, D., Wu, L., Bohks, S. M., KewalRamani, V. N., Unutmaz, D., & Hope, T. J. (2003). Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science*, *300*(5623), 1295-1297. doi: 10.1126/science.1084238

- McLaughlin, R. N., Jr., Gable, J. T., Wittkopp, C. J., Emerman, M., & Malik, H. S. (2016). Conservation and Innovation of APOBEC3A Restriction Functions during Primate Evolution. *Molecular Biology and Evolution*, 33(8), 1889-1901. doi: 10.1093/molbev/msw070
- Meiering, C. D., & Linial, M. L. (2001). Historical perspective of foamy virus epidemiology and infection. *Clin Microbiol Rev*, 14(1), 165-176. doi: 10.1128/CMR.14.1.165-176.2001
- Melikyan, G. B. (2014). HIV entry: a game of hide-and-fuse? *Curr Opin Virol*, 4, 1-7. doi: 10.1016/j.coviro.2013.09.004
- Meng, B., & Lever, A. M. L. (2013). Wrapping up the bad news - HIV assembly and release. *Retrovirology*, 10.
- Meng, X., Zhao, G. P., Yufenyuy, E., Ke, D. X., Ning, J. Y., DeLucia, M., . . . Zhang, P. J. (2012). Protease Cleavage Leads to Formation of Mature Trimer Interface in HIV-1 Capsid. *Plos Pathogens*, 8(8). doi: ARTN e1002886  
10.1371/journal.ppat.1002886
- Mercenne, G., Bernacchi, S., Richer, D., Bec, G., Henriot, S., Paillart, J. C., & Marquet, R. (2010). HIV-1 Vif binds to APOBEC3G mRNA and inhibits its translation. *Nucleic Acids Research*, 38(2), 633-646. doi: 10.1093/nar/gkp1009
- Merk, A., & Subramaniam, S. (2013). HIV-1 envelope glycoprotein structure. *Current Opinion in Structural Biology*, 23(2), 268-276.
- Micewicz, E. D., & Ruchala, P. (2013). Inhibitors of HIV-1 entry. *Curr Pharm Des*, 19(10), 1784-1799.
- Miller, J. H., Presnyak, V., & Smith, H. C. (2007). The dimerization domain of HIV-1 viral infectivity factor Vif is required to block virion incorporation of APOBEC3G. *Retrovirology*, 4, 81. doi: 10.1186/1742-4690-4-81
- Mitra, M., Hercik, K., Byeon, I. J., Ahn, J., Hill, S., Hinchee-Rodriguez, K., . . . Levin, J. G. (2014). Structural determinants of human APOBEC3A enzymatic and nucleic acid binding properties. *Nucleic Acids Research*, 42(2), 1095-1110. doi: 10.1093/nar/gkt945
- Mitsuyasu, R. T., Anton, P. A., Deeks, S. G., Scadden, D. T., Connick, E., Downs, M. T., . . . Hege, K. M. (2000). Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood*, 96(3), 785-793.
- Miyauchi, K., Kim, Y., Latinovic, O., Morozov, V., & Melikyan, G. B. (2009). HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell*, 137(3), 433-444. doi: 10.1016/j.cell.2009.02.046
- Miyauchi, K., Marin, M., & Melikyan, G. B. (2011). Visualization of retrovirus uptake and delivery into acidic endosomes. *Biochemical Journal*, 434(3), 559-569. doi: 10.1042/BJ20101588
- Moll, M., Andersson, S. K., Smed-Sorensen, A., & Sandberg, J. K. (2010). Inhibition of lipid antigen presentation in dendritic cells by HIV-1 Vpu interference with CD1d recycling from endosomal compartments. *Blood*, 116(11), 1876-1884. doi: 10.1182/blood-2009-09-243667
- Montal, M. (2003). Structure-function correlates of Vpu, a membrane protein of HIV-1. *Febs Letters*, 552(1), 47-53.
- Montefiori, D. C., Robinson, W. E., & Mitchell, W. M. (1988). Role of Protein N-Glycosylation in Pathogenesis of Human Immunodeficiency Virus Type-1. *Proceedings of the National Academy of Sciences of the United States of America*, 85(23), 9248-9252.

- Mori, S., Takeuchi, T., Ishii, Y., & Kukimoto, I. (2015). Identification of APOBEC3B promoter elements responsible for activation by human papillomavirus type 16 E6. *Biochem Biophys Res Commun*, *460*(3), 555-560. doi: 10.1016/j.bbrc.2015.03.068
- Morikawa, Y., Hockley, D. J., Nermut, M. V., & Jones, I. M. (2000). Roles of matrix, p2, and N-terminal myristoylation in human immunodeficiency virus type 1 Gag assembly. *Journal of Virology*, *74*(1), 16-23.
- Mourez, T., Simon, F., & Plantier, J. C. (2013). Non-M variants of human immunodeficiency virus type 1. *Clin Microbiol Rev*, *26*(3), 448-461. doi: 10.1128/CMR.00012-13
- Mousseau, G., Mediouni, S., & Valente, S. T. (2015). Targeting HIV transcription: the quest for a functional cure. *Curr Top Microbiol Immunol*, *389*, 121-145. doi: 10.1007/82\_2015\_435
- Muckenfuss, H., Hamdorf, M., Held, U., Perkovic, M., Lower, J., Cichutek, K., . . . Munk, C. (2006). APOBEC3 proteins inhibit human LINE-1 retrotransposition. *Journal of Biological Chemistry*, *281*(31), 22161-22172. doi: 10.1074/jbc.M601716200
- Muriaux, D., & Darlix, J. L. (2010). Properties and functions of the nucleocapsid protein in virus assembly. *RNA Biol*, *7*(6), 744-753.
- Nair, V. (2002). HIV integrase as a target for antiviral chemotherapy. *Reviews in Medical Virology*, *12*(3), 179-193. doi: 10.1002/rmv.350
- Nakayama, E. E., Miyoshi, H., Nagai, Y., & Shioda, T. (2005). A specific region of 37 amino acid residues in the SPRY (B30.2) domain of African green monkey TRIM5alpha determines species-specific restriction of simian immunodeficiency virus SIVmac infection. *Journal of Virology*, *79*(14), 8870-8877. doi: 10.1128/JVI.79.14.8870-8877.2005
- Nakayama, E. E., & Shioda, T. (2015). Impact of TRIM5alpha in vivo. *Aids*, *29*(14), 1733-1743. doi: 10.1097/qad.0000000000000812
- Narvaiza, I., Linfesty, D. C., Greener, B. N., Hakata, Y., Pintel, D. J., Logue, E., . . . Weitzman, M. D. (2009). Deaminase-independent inhibition of parvoviruses by the APOBEC3A cytidine deaminase. *Plos Pathogens*, *5*(5), e1000439. doi: 10.1371/journal.ppat.1000439
- Neil, S. J., Zang, T., & Bieniasz, P. D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature*, *451*(7177), 425-430. doi: 10.1038/nature06553
- News, A. P. C. (2009). New HIV strain discovered. *08-03*.
- O'connell, R. J., Kim, J. H., & Excler, J. L. (2014). The HIV-1 gp120 V1V2 loop: structure, function and importance for vaccine development. *Expert Review of Vaccines*, *13*(12), 1489-1500.
- Olivetta, E., Arenaccio, C., Manfredi, F., Anticoli, S., & Federico, M. (2016). The Contribution of Extracellular Nef to HIV-Induced Pathogenesis. *Current Drug Targets*, *17*(1), 46-53.
- Ono, A. (2009). HIV-1 Assembly at the Plasma Membrane: Gag Trafficking and Localization. *Future Virology*, *4*(3), 241-257. doi: 10.2217/fvl.09.4
- Ono, A., Ablan, S. D., Lockett, S. J., Nagashima, K., & Freed, E. O. (2004). Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 gag targeting to the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14889-14894. doi: 10.1073/pnas.0405596101
- Ooms, M., Brayton, B., Letko, M., Maio, S. M., Pilcher, C. D., Hecht, F. M., . . . Simon, V. (2013). HIV-1 Vif adaptation to human APOBEC3H haplotypes. *Cell Host & Microbe*, *14*(4), 411-421. doi: 10.1016/j.chom.2013.09.006
- Ooms, M., Krikoni, A., Kress, A. K., Simon, V., & Munk, C. (2012). APOBEC3A, APOBEC3B, and APOBEC3H haplotype 2 restrict human T-lymphotropic virus type 1. *Journal of*



- Virology*, 86(11), 6097-6108. doi: 10.1128/jvi.06570-11
- Ooms, M., Letko, M., & Simon, V. (2016). The structural interface between HIV-1 Vif and human APOBEC3H. *Journal of Virology*. doi: 10.1128/jvi.02289-16
- Ortiz, M., Guex, N., Patin, E., Martin, O., Xenarios, I., Ciuffi, A., . . . Telenti, A. (2009). Evolutionary trajectories of primate genes involved in HIV pathogenesis. *Molecular Biology and Evolution*, 26(12), 2865-2875. doi: 10.1093/molbev/msp197
- Ott, M., Geyer, M., & Zhou, Q. (2011). The control of HIV transcription: keeping RNA polymerase II on track. *Cell Host & Microbe*, 10(5), 426-435. doi: 10.1016/j.chom.2011.11.002
- Overbaugh, J., & Morris, L. (2012). The Antibody Response against HIV-1. *Cold Spring Harbor Perspect Med*, 2(1), a007039. doi: 10.1101/cshperspect.a007039
- Pandey, R. C., Datta, D., Mukerjee, R., Srinivasan, A., Mahalingam, S., & Sawaya, B. E. (2009). HIV-1 Vpr: a closer look at the multifunctional protein from the structural perspective. *Current Hiv Research*, 7(2), 114-128.
- Pardieu, C., Vigan, R., Wilson, S. J., Calvi, A., Zang, T., Bieniasz, P., . . . Neil, S. J. (2010). The RING-CH ligase K5 antagonizes restriction of KSHV and HIV-1 particle release by mediating ubiquitin-dependent endosomal degradation of tetherin. *Plos Pathogens*, 6(4), e1000843. doi: 10.1371/journal.ppat.1000843
- Park, S. H., De Angelis, A. A., Nevzorov, A. A., Wu, C. H., & Opella, S. J. (2006). Three-dimensional structure of the transmembrane domain of Vpu from HIV-1 in aligned phospholipid bicelles. *Biophysical Journal*, 91(8), 3032-3042. doi: 10.1529/biophysj.106.087106
- Pawlak, E. N., & Dikeakos, J. D. (2015). HIV-1 Nef: a master manipulator of the membrane trafficking machinery mediating immune evasion. *Biochim Biophys Acta*, 1850(4), 733-741. doi: 10.1016/j.bbagen.2015.01.003
- Peeters, M., Gueye, A., Mboup, S., Bibollet-Ruche, F., Ekaza, E., Mulanga, C., . . . Delaporte, E. (1997). Geographical distribution of HIV-1 group O viruses in Africa. *Aids*, 11(4), 493-498.
- Peng, G., Greenwell-Wild, T., Nares, S., Jin, W., Lei, K. J., Rangel, Z. G., . . . Wahl, S. M. (2007). Myeloid differentiation and susceptibility to HIV-1 are linked to APOBEC3 expression. *Blood*, 110(1), 393-400. doi: 10.1182/blood-2006-10-051763
- Percario, Z. A., Ali, M., Mangino, G., & Affabris, E. (2015). Nef, the shuttling molecular adaptor of HIV, influences the cytokine network. *Cytokine Growth Factor Rev*, 26(2), 159-173. doi: 10.1016/j.cytogfr.2014.11.010
- Pereira, E. A., & daSilva, L. L. (2016). HIV-1 Nef: Taking Control of Protein Trafficking. *Traffic*, 17(9), 976-996. doi: 10.1111/tra.12412
- Permanyer, M., Ballana, E., & Este, J. A. (2010). Endocytosis of HIV: anything goes. *Trends in Microbiology*, 18(12), 543-551. doi: 10.1016/j.tim.2010.09.003
- Pohlmann, S., Baribaud, F., Lee, B., Leslie, G. J., Sanchez, M. D., Hiebenthal-Millow, K., . . . Doms, R. W. (2001). DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus. *Journal of Virology*, 75(10), 4664-4672. doi: 10.1128/JVI.75.10.4664-4672.2001
- Pollard, V. W., & Malim, M. H. (1998). The HIV-1 Rev protein. *Annual Review of Microbiology*, 52, 491-532.
- Pommier, Y., Pilon, A. A., Bajaj, K., Mazumder, A., & Neamati, N. (1997). HIV-1 integrase as a target for antiviral drugs. *Antiviral Chemistry & Chemotherapy*, 8(6), 463-483.

- Pornillos, O., Ganser-Pornillos, B. K., Kelly, B. N., Hua, Y. Z., Whitby, F. G., Stout, C. D., . . . Yeager, M. (2009). X-Ray Structures of the Hexameric Building Block of the HIV Capsid. *Cell*, *137*(7), 1282-1292. doi: 10.1016/j.cell.2009.04.0634
- Pornillos, O., Ganser-Pornillos, B. K., & Yeager, M. (2011). Atomic-level modelling of the HIV capsid. *Nature*, *469*(7330), 424+. doi: 10.1038/nature09640
- Postler, T. S., & Desrosiers, R. C. (2013). The Tale of the Long Tail: the Cytoplasmic Domain of HIV-1 gp41. *Journal of Virology*, *87*(1), 2-15.
- Powell, R. D., Holland, P. J., Hollis, T., & Perrino, F. W. (2011). Aicardi-Goutieres syndrome gene and HIV-1 restriction factor SAMHD1 is a dGTP-regulated deoxynucleotide triphosphohydrolase. *Journal of Biological Chemistry*, *286*(51), 43596-43600. doi: 10.1074/jbc.C111.317628
- Qiu, J., & Pintel, D. J. (2002). The adeno-associated virus type 2 Rep protein regulates RNA processing via interaction with the transcription template. *Mol Cell Biol*, *22*(11), 3639-3652.
- Quaranta, M. G., Mattioli, B., Giordani, L., & Viora, M. (2009). Immunoregulatory effects of HIV-1 Nef protein. *Biofactors*, *35*(2), 169-174. doi: 10.1002/biof.28
- Rambaut, A., Posada, D., Crandall, K. A., & Holmes, E. C. (2004). The causes and consequences of HIV evolution. *Nature Reviews Genetics*, *5*(1), 52-61. doi: 10.1038/nrg1246
- Raska, M., & Novak, J. (2010). Involvement of Envelope-Glycoprotein Glycans in HIV-1 Biology and Infection. *Archivum Immunologiae Et Therapiae Experimentalis*, *58*(3), 191-208.
- Rausch, J. W., & Le Grice, S. F. J. (2015). HIV Rev Assembly on the Rev Response Element (RRE): A Structural Perspective. *Viruses-Basel*, *7*(6), 3053-3075.
- Refsland, E. W., Hultquist, J. F., Luengas, E. M., Ikeda, T., Shaban, N. M., Law, E. K., . . . Harris, R. S. (2014). Natural polymorphisms in human APOBEC3H and HIV-1 Vif combine in primary T lymphocytes to affect viral G-to-A mutation levels and infectivity. *PLoS Genet*, *10*(11), e1004761. doi: 10.1371/journal.pgen.1004761
- Refsland, E. W., Stenglein, M. D., Shindo, K., Albin, J. S., Brown, W. L., & Harris, R. S. (2010). Quantitative profiling of the full APOBEC3 mRNA repertoire in lymphocytes and tissues: implications for HIV-1 restriction. *Nucleic Acids Research*, *38*(13), 4274-4284. doi: 10.1093/nar/gkq174
- Reimann, K. A., Lin, W., Bixler, S., Browning, B., Ehrenfels, B. N., Lucci, J., . . . Burkly, L. C. (1997). A humanized form of a CD4-specific monoclonal antibody exhibits decreased antigenicity and prolonged plasma half-life in rhesus monkeys while retaining its unique biological and antiviral properties. *AIDS Res Hum Retroviruses*, *13*(11), 933-943. doi: 10.1089/aid.1997.13.933
- Rethwilm, A. (2010). Molecular biology of foamy viruses. *Med Microbiol Immunol*, *199*(3), 197-207. doi: 10.1007/s00430-010-0158-x
- Roberts, J. D., Bebenek, K., & Kunkel, T. A. (1988). The Accuracy of Reverse-Transcriptase from Hiv-1. *Science*, *242*(4882), 1171-1173. doi: Doi 10.1126/Science.2460925
- Rodriguez-Plata, M. T., Puigdomenech, I., Izquierdo-Useros, N., Puertas, M. C., Carrillo, J., Erkizia, I., . . . Martinez-Picado, J. (2013). The infectious synapse formed between mature dendritic cells and CD4(+) T cells is independent of the presence of the HIV-1 envelope glycoprotein. *Retrovirology*, *10*, 42. doi: 10.1186/1742-4690-10-42
- Roeth, J. F., & Collins, K. L. (2006). Human immunodeficiency virus type 1 Nef: adapting to intracellular trafficking pathways. *Microbiol Mol Biol Rev*, *70*(2), 548-563. doi:

- 10.1128/membr.00042-05
- Romani, B., & Engelbrecht, S. (2009). Human immunodeficiency virus type 1 Vpr: functions and molecular interactions. *Journal of General Virology*, *90*(Pt 8), 1795-1805. doi: 10.1099/vir.0.011726-0
- Romani, B., Engelbrecht, S., & Glashoff, R. H. (2009). Antiviral roles of APOBEC proteins against HIV-1 and suppression by Vif. *Archives of Virology*, *154*(10), 1579-1588. doi: 10.1007/s00705-009-0481-y
- Romani, B., Engelbrecht, S., & Glashoff, R. H. (2010). Functions of Tat: the versatile protein of human immunodeficiency virus type 1. *Journal of General Virology*, *91*, 1-12.
- Rose, K. M., Marin, M., Kozak, S. L., & Kabat, D. (2004). The viral infectivity factor (Vif) of HIV-1 unveiled. *Trends Mol Med*, *10*(6), 291-297. doi: 10.1016/j.molmed.2004.04.008
- Roshal, M., Zhu, Y., & Planelles, V. (2001). Apoptosis in AIDS. *Apoptosis*, *6*(1-2), 103-116.
- Rudnicka, D., Feldmann, J., Porrot, F., Wietgreffe, S., Guadagnini, S., Prevost, M. C., . . . Schwartz, O. (2009). Simultaneous Cell-to-Cell Transmission of Human Immunodeficiency Virus to Multiple Targets through Polysynapses. *Journal of Virology*, *83*(12), 6234-6246.
- Ruiz, A., Guatelli, J. C., & Stephens, E. B. (2010). The Vpu protein: new concepts in virus release and CD4 down-modulation. *Current HIV Research*, *8*(3), 240-252.
- Russell, R. A., & Pathak, V. K. (2007). Identification of two distinct human immunodeficiency virus type 1 Vif determinants critical for interactions with human APOBEC3G and APOBEC3F. *Journal of Virology*, *81*(15), 8201-8210. doi: 10.1128/JVI.00395-07
- Russell, R. A., Wiegand, H. L., Moore, M. D., Schafer, A., McClure, M. O., & Cullen, B. R. (2005). Foamy virus Bet proteins function as novel inhibitors of the APOBEC3 family of innate antiretroviral defense factors. *Journal of Virology*, *79*(14), 8724-8731. doi: 10.1128/JVI.79.14.8724-8731.2005
- Ryzhova, E., Whitbeck, J. C., Canziani, G., Westmoreland, S. V., Cohen, G. H., Eisenberg, R. J., . . . Gonzalez-Scarano, F. (2002). Rapid progression to simian AIDS can be accompanied by selection of CD4-independent gp120 variants with impaired ability to bind CD4. *Journal of Virology*, *76*(15), 7903-7909.
- Saad, J. S., Miller, J., Tai, J., Kim, A., Ghanam, R. H., & Summers, M. F. (2006). Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(30), 11364-11369. doi: 10.1073/pnas.0602818103
- Saib, A., Koken, M. H., van der Spek, P., Peries, J., & de The, H. (1995). Involvement of a spliced and defective human foamy virus in the establishment of chronic infection. *Journal of Virology*, *69*(9), 5261-5268.
- Sakurai, D., Iwatani, Y., Ohtani, H., Naruse, T. K., Terunuma, H., Sugiura, W., & Kimura, A. (2015). APOBEC3H polymorphisms associated with the susceptibility to HIV-1 infection and AIDS progression in Japanese. *Immunogenetics*, *67*(4), 253-257. doi: 10.1007/s00251-015-0829-2
- Salter, J. D., Morales, G. A., & Smith, H. C. (2014). Structural insights for HIV-1 therapeutic strategies targeting Vif. *Trends in Biochemical Sciences*, *39*(9), 373-380. doi: 10.1016/j.tibs.2014.07.001
- Sarafianos, S. G., Marchand, B., Das, K., Himmel, D. M., Parniak, M. A., Hughes, S. H., & Arnold, E. (2009). Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. *Journal of Molecular Biology*, *385*(3),

- 693-713. doi: 10.1016/j.jmb.2008.10.071
- Sato, K., Takeuchi, J. S., Misawa, N., Izumi, T., Kobayashi, T., Kimura, Y., . . . Koyanagi, Y. (2014). APOBEC3D and APOBEC3F potently promote HIV-1 diversification and evolution in humanized mouse model. *Plos Pathogens*, *10*(10), e1004453. doi: 10.1371/journal.ppat.1004453
- Sauter, D. (2014). Counteraction of the multifunctional restriction factor tetherin. *Front Microbiol*, *5*, 163. doi: 10.3389/fmicb.2014.00163
- Sawyer, S. L., Emerman, M., & Malik, H. S. (2004). Ancient adaptive evolution of the primate antiviral DNA-editing enzyme APOBEC3G. *Plos Biology*, *2*(9), E275. doi: 10.1371/journal.pbio.0020275
- Scharpe, S., Demeester, I., Hendriks, D., Vanhoof, G., Vansande, M., & Vriend, G. (1991). Proteases and Their Inhibitors - Today and Tomorrow. *Biochimie*, *73*(1), 121-126.
- Schiffner, T., Sattentau, Q. J., & Duncan, C. J. A. (2013). Cell-to-cell spread of HIV-1 and evasion of neutralizing antibodies. *Vaccine*, *31*(49), 5789-5797.
- Schmidt, O., & Teis, D. (2012). The ESCRT machinery. *Current Biology*, *22*(4), R116-120. doi: 10.1016/j.cub.2012.01.028
- Schmidt, S., Fritz, J. V., Bitzegeio, J., Fackler, O. T., & Keppler, O. T. (2011). HIV-1 Vpu blocks recycling and biosynthetic transport of the intrinsic immunity factor CD317/tetherin to overcome the virion release restriction. *Mbio*, *2*(3), e00036-00011. doi: 10.1128/mBio.00036-11
- Schmitt, K., Guo, K., Algaier, M., Ruiz, A., Cheng, F., Qiu, J., . . . Stephens, E. B. (2011). Differential virus restriction patterns of rhesus macaque and human APOBEC3A: implications for lentivirus evolution. *Virology*, *419*(1), 24-42. doi: 10.1016/j.virol.2011.07.017
- Schmitt, K., Guo, K., Katuwal, M., Wilson, D., Prochnow, C., Bransteitter, R., . . . Stephens, E. B. (2013). Lentivirus restriction by diverse primate APOBEC3A proteins. *Virology*, *442*(1), 82-96. doi: 10.1016/j.virol.2013.04.002
- Schmitt, T. J., Clark, J. E., & Knotts, T. A. (2009). Thermal and mechanical multistate folding of ribonuclease H. *Journal of Chemical Physics*, *131*(23).
- Schubert, U., FerrerMontiel, A. V., OblattMontal, M., Henklein, P., Strebel, K., & Montal, M. (1996). Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells. *Febs Letters*, *398*(1), 12-18. doi: Doi 10.1016/S0014-5793(96)01146-5
- Schwantes, A., Truyen, U., Weikel, J., Weiss, C., & Lochelt, M. (2003). Application of chimeric feline foamy virus-based retroviral vectors for the induction of antiviral immunity in cats. *Journal of Virology*, *77*(14), 7830-7842.
- Schwefel, D., Groom, H. C., Boucherit, V. C., Christodoulou, E., Walker, P. A., Stoye, J. P., . . . Taylor, I. A. (2014). Structural basis of lentiviral subversion of a cellular protein degradation pathway. *Nature*, *505*(7482), 234-238. doi: 10.1038/nature12815
- Sedore, S. C., Byers, S. A., Biglione, S., Price, J. P., Maury, W. J., & Price, D. H. (2007). Manipulation of P-TEFb control machinery by HIV: recruitment of P-TEFb from the large form by Tat and binding of HEXIM1 to TAR. *Nucleic Acids Research*, *35*(13), 4347-4358. doi: 10.1093/nar/gkm443
- Serra-Moreno, R., Jia, B., Breed, M., Alvarez, X., & Evans, D. T. (2011). Compensatory changes in the cytoplasmic tail of gp41 confer resistance to tetherin/BST-2 in a pathogenic nef-deleted SIV. *Cell Host & Microbe*, *9*(1), 46-57. doi: 10.1016/j.chom.2010.12.005

- Serra-Moreno, R., Zimmermann, K., Stern, L. J., & Evans, D. T. (2013). Tetherin/BST-2 antagonism by Nef depends on a direct physical interaction between Nef and tetherin, and on clathrin-mediated endocytosis. *Plos Pathogens*, *9*(7), e1003487. doi: 10.1371/journal.ppat.1003487
- Shah, A. H., Sowrirajan, B., Davis, Z. B., Ward, J. P., Campbell, E. M., Planelles, V., & Barker, E. (2010). Degranulation of Natural Killer Cells Following Interaction with HIV-1-Infected Cells Is Hindered by Downmodulation of NTB-A by Vpu. *Cell Host & Microbe*, *8*(5), 397-409. doi: 10.1016/j.chom.2010.10.008
- Sharifi, H. J., Furuya, A. M., & de Noronha, C. M. (2012). The role of HIV-1 Vpr in promoting the infection of nondividing cells and in cell cycle arrest. *Current Opinion in Hiv and Aids*, *7*(2), 187-194. doi: 10.1016/j.coviro.2013.08.004  
10.1097/COH.0b013e32835049e0
- Sheehy, A. M., Gaddis, N. C., Choi, J. D., & Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*, *418*(6898), 646-650. doi: 10.1038/nature00939
- Sheehy, A. M., Gaddis, N. C., & Malim, M. H. (2003). The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med*, *9*(11), 1404-1407. doi: 10.1038/nm945
- Shen, Q. T., Schuh, A. L., Zheng, Y. Q., Quinney, K., Wang, L., Hanna, M., . . . Audhya, A. (2014). Structural analysis and modeling reveals new mechanisms governing ESCRT-III spiral filament assembly. *Journal of Cell Biology*, *206*(6), 763-777. doi: 10.1083/jcb.201403108
- Shibata, R., Sakai, H., Kawamura, M., Tokunaga, K., & Adachi, A. (1995). Early Replication Block of Human-Immunodeficiency-Virus Type-1 in Monkey Cells. *Journal of General Virology*, *76*, 2723-2730.
- Shinohara, M., Io, K., Shindo, K., Matsui, M., Sakamoto, T., Tada, K., . . . Takaori-Kondo, A. (2012). APOBEC3B can impair genomic stability by inducing base substitutions in genomic DNA in human cells. *Sci Rep*, *2*, 806. doi: 10.1038/srep00806
- Simon, J. H., Gaddis, N. C., Fouchier, R. A., & Malim, M. H. (1998). Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nat Med*, *4*(12), 1397-1400. doi: 10.1038/3987
- Simon, V., Zennou, V., Murray, D., Huang, Y., Ho, D. D., & Bieniasz, P. D. (2005). Natural variation in Vif: differential impact on APOBEC3G/3F and a potential role in HIV-1 diversification. *Plos Pathogens*, *1*(1), e6. doi: 10.1371/journal.ppat.0010006
- Singh, P. K., Plumb, M. R., Ferris, A. L., Iben, J. R., Wu, X. L., Fadel, H. J., . . . Levin, H. L. (2015). LEDGF/p75 interacts with mRNA splicing factors and targets HIV-1 integration to highly spliced genes. *Genes & Development*, *29*(21), 2287-2297.
- Smith, H. C., Bennett, R. P., Kizilyer, A., McDougall, W. M., & Prohaska, K. M. (2012). Functions and regulation of the APOBEC family of proteins. *Semin Cell Dev Biol*, *23*(3), 258-268. doi: 10.1016/j.semcdb.2011.10.004
- Smith, J. L., & Pathak, V. K. (2010). Identification of specific determinants of human APOBEC3F, APOBEC3C, and APOBEC3DE and African green monkey APOBEC3F that interact with HIV-1 Vif. *Journal of Virology*, *84*(24), 12599-12608. doi: 10.1128/JVI.01437-10
- Smith, R. A., Loeb, L. A., & Preston, B. D. (2005). Lethal mutagenesis of HIV. *Virus Research*, *107*(2), 215-228. doi: 10.1016/j.virusres.2004.11.011
- Song, B. (2009). TRIM5alpha. *Curr Top Microbiol Immunol*, *339*, 47-66. doi: 10.1007/978-3-

642-02175-6\_3

- Soros, V. B., Yonemoto, W., & Greene, W. C. (2007). Newly synthesized APOBEC3G is incorporated into HIV virions, inhibited by HIV RNA, and subsequently activated by RNase H. *Plos Pathogens*, 3(2), e15. doi: 10.1371/journal.ppat.0030015
- Sova, P., & Volsky, D. J. (1993). Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with vif-negative human immunodeficiency virus type 1. *Journal of Virology*, 67(10), 6322-6326.
- Sowd, G. A., Serrao, E., Wang, H., Wang, W., Fadel, H. J., Poeschla, E. M., & Engelman, A. N. (2016a). A critical role for alternative polyadenylation factor CPSF6 in targeting HIV-1 integration to transcriptionally active chromatin. *Proc Natl Acad Sci U S A*, 113(8), E1054-1063. doi: 10.1073/pnas.1524213113
- Sowd, G. A., Serrao, E., Wang, H., Wang, W. F., Fadel, H. J., Poeschla, E. M., & Engelman, A. N. (2016b). A critical role for alternative polyadenylation factor CPSF6 in targeting HIV-1 integration to transcriptionally active chromatin. *Proceedings of the National Academy of Sciences of the United States of America*, 113(8), E1054-E1063.
- Stanley, B. J., Ehrlich, E. S., Short, L., Yu, Y. K., Xiao, Z. X., Yu, X. F., & Xiong, Y. (2008). Structural insight into the human immunodeficiency virus Vif SOCS box and its role in human E3 ubiquitin ligase assembly. *Journal of Virology*, 82(17), 8656-8663. doi: 10.1128/JVI.00767-08
- Starrett, G. J., Luengas, E. M., McCann, J. L., Ebrahimi, D., Temiz, N. A., Love, R. P., . . . Harris, R. S. (2016). The DNA cytosine deaminase APOBEC3H haplotype I likely contributes to breast and lung cancer mutagenesis. *Nature Communications*, 7, 12918. doi: 10.1038/ncomms12918
- Stauch, B., Hofmann, H., Perkovic, M., Weisel, M., Kopietz, F., Cichutek, K., . . . Schneider, G. (2009). Model structure of APOBEC3C reveals a binding pocket modulating ribonucleic acid interaction required for encapsidation. *Proc Natl Acad Sci U S A*, 106(29), 12079-12084. doi: 10.1073/pnas.0900979106
- Stavrou, S., Crawford, D., Blouch, K., Browne, E. P., Kohli, R. M., & Ross, S. R. (2014). Different modes of retrovirus restriction by human APOBEC3A and APOBEC3G in vivo. *Plos Pathogens*, 10(5), e1004145. doi: 10.1371/journal.ppat.1004145
- Stenglein, M. D., Burns, M. B., Li, M., Lengyel, J., & Harris, R. S. (2010). APOBEC3 proteins mediate the clearance of foreign DNA from human cells. *Nature Structural & Molecular Biology*, 17(2), 222-229. doi: 10.1038/nsmb.1744
- Stevenson, M. (2003). HIV-1 pathogenesis. *Nat Med*, 9(7), 853-860. doi: 10.1038/nm0703-853
- Stopak, K., de Noronha, C., Yonemoto, W., & Greene, W. C. (2003). HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Molecular Cell*, 12(3), 591-601.
- Strebel, K. (2003). Virus-host interactions: role of HIV proteins Vif, Tat, and Rev. *Aids*, 17 Suppl 4, S25-34.
- Strebel, K. (2007). HIV accessory genes Vif and Vpu. *Adv Pharmacol*, 55, 199-232. doi: 10.1016/s1054-3589(07)55006-4
- Strebel, K. (2013). HIV accessory proteins versus host restriction factors. *Curr Opin Virol*, 3(6), 692-699. doi: 10.3389/fmicb.2014.00127  
10.1016/j.coviro.2013.08.004
- Strebel, K. (2014). HIV-1 Vpu - an ion channel in search of a job. *Biochim Biophys Acta*, 1838(4), 1074-1081. doi: 10.1016/j.bbamem.2013.06.029

- Stremlau, M. (2007). GE Prize-winning essay. Why Old World monkeys are resistant to HIV-1. *Science*, 318(5856), 1565-1566. doi: 10.1126/science.1152905
- Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., . . . Sodroski, J. (2006). Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5 alpha restriction factor. *Proceedings of the National Academy of Sciences of the United States of America*, 103(14), 5514-5519.
- Stremlau, M., Perron, M., Welikala, S., & Sodroski, J. (2005). Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *Journal of Virology*, 79(5), 3139-3145. doi: 10.1128/JVI.79.5.3139-3145.2005
- Sundquist, W. I., & Krausslich, H. G. (2012). HIV-1 Assembly, Budding, and Maturation. *Cold Spring Harb Perspect Med*, 2(7). doi: Artn A006924  
10.1101/Cshperspect.A006924
- Suzuki, Y., Misawa, N., Sato, C., Ebina, H., Masuda, T., Yamamoto, N., & Koyanagi, Y. (2003). Quantitative analysis of human immunodeficiency virus type 1 DNA dynamics by real-time PCR: integration efficiency in stimulated and unstimulated peripheral blood mononuclear cells. *Virus Genes*, 27(2), 177-188.
- Swiecki, M., Omattage, N. S., & Brett, T. J. (2013). BST-2/tetherin: structural biology, viral antagonism, and immunobiology of a potent host antiviral factor. *Molecular Immunology*, 54(2), 132-139. doi: 10.1016/j.molimm.2012.11.008
- Swiecki, M., Scheaffer, S. M., Allaire, M., Fremont, D. H., Colonna, M., & Brett, T. J. (2011). Structural and biophysical analysis of BST-2/tetherin ectodomains reveals an evolutionary conserved design to inhibit virus release. *Journal of Biological Chemistry*, 286(4), 2987-2997. doi: 10.1074/jbc.M110.190538
- Switzer, W. M., Salemi, M., Shanmugam, V., Gao, F., Cong, M. E., Kuiken, C., . . . Heneine, W. (2005). Ancient co-speciation of simian foamy viruses and primates. *Nature*, 434(7031), 376-380. doi: 10.1038/nature03341
- Taggart, R. T., Kannisto, E. D., Enriquez, S. M., Gollnick, P., Baysal, B. E., Land, A. M., . . . Harris, R. S. (2013). Endogenous APOBEC3A DNA cytosine deaminase is cytoplasmic and nongenotoxic. *Nature Communications*, 288(24), 17253-17260. doi: 10.1038/ncomms7881  
10.1074/jbc.M113.458661
- Takaori-Kondo, A. (2006). APOBEC family proteins: novel antiviral innate immunity. *Int J Hematol*, 83(3), 213-216. doi: 10.1532/ijh97.05187
- Tang, C., Ndassa, Y., & Summers, M. F. (2002). Structure of the N-terminal 283-residue fragment of the immature HIV-1 Gag polyprotein. *Nature Structural Biology*, 9(7), 537-543. doi: 10.1038/nsb806
- Tang, J., Lin, Y., Co, E., Hartsuck, J. A., & Lin, X. (1992). Understanding Hiv Protease - Can It Be Translated into Effective Therapy against Aids. *Scandinavian Journal of Clinical & Laboratory Investigation*, 52, 127-135.
- Tedbury, P. R., & Freed, E. O. (2014). The role of matrix in HIV-1 envelope glycoprotein incorporation. *Trends in Microbiology*, 22(7), 372-378. doi: 10.1016/j.tim.2014.04.012
- Thielen, B. K., McNevin, J. P., McElrath, M. J., Hunt, B. V., Klein, K. C., & Lingappa, J. R. (2010). Innate immune signaling induces high levels of TC-specific deaminase activity in primary monocyte-derived cells through expression of APOBEC3A isoforms. *Journal of Biological Chemistry*, 285(36), 27753-27766. doi: 10.1074/jbc.M110.102822

- Thieu, K. P., Morrow, M. P., Shedlock, D. J., Schoenly, K. A., Mallilankaraman, K., Choo, A. Y., . . . Muthumani, K. (2009). HIV-1 Vpr: regulator of viral survival. *Current Hiv Research*, 7(2), 153-162.
- Tian, C., Yu, X., Zhang, W., Wang, T., Xu, R., & Yu, X. F. (2006). Differential requirement for conserved tryptophans in human immunodeficiency virus type 1 Vif for the selective suppression of APOBEC3G and APOBEC3F. *Journal of Virology*, 80(6), 3112-3115. doi: 10.1128/JVI.80.6.3112-3115.2006
- Toohey, M. G., & Jones, K. A. (1989). In vitro formation of short RNA polymerase II transcripts that terminate within the HIV-1 and HIV-2 promoter-proximal downstream regions. *Genes Dev*, 3(3), 265-282.
- Trobridge, G. D. (2009). Foamy virus vectors for gene transfer. *Expert Opin Biol Ther*, 9(11), 1427-1436. doi: 10.1517/14712590903246388
- Tsuboi, M., Yamane, A., Horiguchi, J., Yokobori, T., Kawabata-Iwakawa, R., Yoshiyama, S., . . . Nishiyama, M. (2016). APOBEC3B high expression status is associated with aggressive phenotype in Japanese breast cancers. *Breast Cancer*, 23(5), 780-788. doi: 10.1007/s12282-015-0641-8
- Tungaturthi, P. K., Sawaya, B. E., Ayyavoo, V., Murali, R., & Srinivasan, A. (2004). HIV-1 Vpr: genetic diversity and functional features from the perspective of structure. *DNA and Cell Biology*, 23(4), 207-222. doi: 10.1089/104454904773819798
- Tungaturthi, P. K., Sawaya, B. E., Singh, S. P., Tomkowicz, B., Ayyavoo, V., Khalili, K., . . . Srinivasan, A. (2003). Role of HIV-1 Vpr in AIDS pathogenesis: relevance and implications of intravirion, intracellular and free Vpr. *Biomed Pharmacother*, 57(1), 20-24.
- UNAIDS. (2015a). AIDS by the Numbers.
- UNAIDS. (2015b). How AIDS Changed Everything.
- UNAIDS. (2016). Global AIDS update. Geneva, Switzerland
- Usami, Y., Popov, S., Popova, E., Inoue, M., Weissenhorn, W., & H, G. G. (2009). The ESCRT pathway and HIV-1 budding. *Biochem Soc Trans*, 37(Pt 1), 181-184. doi: 10.1042/BST0370181
- Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., . . . Guatelli, J. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host & Microbe*, 3(4), 245-252. doi: 10.1016/j.chom.2008.03.001
- Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F., & Debyser, Z. (2006). Cellular co-factors of HIV-1 integration. *Trends in Biochemical Sciences*, 31(2), 98-105. doi: 10.1016/j.tibs.2005.12.002
- Vartanian, J. P., Guetard, D., Henry, M., & Wain-Hobson, S. (2008). Evidence for editing of human papillomavirus DNA by APOBEC3 in benign and precancerous lesions. *Science*, 320(5873), 230-233. doi: 10.1126/science.1153201
- Vercruysse, T., & Daelemans, D. (2013). HIV-1 Rev Multimerization: Mechanism and Insights. *Current Hiv Research*, 11(8), 623-634.
- VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J., & Carter, C. A. (2001a). Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc Natl Acad Sci U S A*, 98(14), 7724-7729. doi: 10.1073/pnas.131059198
- VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J., & Carter, C. A.



- (2001b). Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proceedings of the National Academy of Sciences of the United States of America*, 98(14), 7724-7729. doi: Doi 10.1073/Pnas.131059198
- Viswanathan, K., Smith, M. S., Malouli, D., Mansouri, M., Nelson, J. A., & Fruh, K. (2011). BST2/Tetherin enhances entry of human cytomegalovirus. *Plos Pathogens*, 7(11), e1002332. doi: 10.1371/journal.ppat.1002332
- Vodicka, M. A., Koepp, D. M., Silver, P. A., & Emerman, M. (1998). HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. *Genes Dev*, 12(2), 175-185.
- von Kleist, L., Stahlschmidt, W., Bulut, H., Gromova, K., Puchkov, D., Robertson, M. J., . . . Haucke, V. (2011). Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell*, 146(3), 471-484. doi: 10.1016/j.cell.2011.06.025
- von Schwedler, U. K., Stray, K. M., Garrus, J. E., & Sundquist, W. I. (2003). Functional surfaces of the human immunodeficiency virus type 1 capsid protein. *Journal of Virology*, 77(9), 5439-5450.
- vonderHelm, K. (1996). Retroviral proteases: Structure, function and inhibition from a non-anticipated viral enzyme to the target of a most promising HIV therapy. *Biological Chemistry*, 377(12), 765-774.
- Votteler, J., & Sundquist, W. I. (2013). Virus Budding and the ESCRT Pathway. *Cell Host & Microbe*, 14(3), 232-241. doi: 10.1016/j.chom.2013.08.012
- Wang, Y., Schmitt, K., Guo, K., Santiago, M. L., & Stephens, E. B. (2016). Role of the single deaminase domain APOBEC3A in virus restriction, retrotransposition, DNA damage and cancer. *Journal of General Virology*, 97(1), 1-17. doi: 10.1099/jgv.0.000320
- Wang, Y., Wang, Z., Pramanik, A., Santiago, M. L., Qiu, J., & Stephens, E. B. (2016). A chimeric human APOBEC3A protein with a three amino acid insertion confers differential HIV-1 and adeno-associated virus restriction. *Virology*, 498, 149-163. doi: 10.1016/j.virol.2016.08.001
- Ward, A. B., & Wilson, I. A. (2015). Insights into the trimeric HIV-1 envelope glycoprotein structure. *Trends in Biochemical Sciences*, 40(2), 101-107.
- Warren, C. J., Xu, T., Guo, K., Griffin, L. M., Westrich, J. A., Lee, D., . . . Pyeon, D. (2015). APOBEC3A functions as a restriction factor of human papillomavirus. *Journal of Virology*, 89(1), 688-702. doi: 10.1128/jvi.02383-14
- Wei, B. L., Arora, V. K., Foster, J. L., Sodora, D. L., & Garcia, J. V. (2003). In vivo analysis of Nef function. *Current Hiv Research*, 1(1), 41-50.
- Weiss, R. A. (1993). How does HIV cause AIDS? *Science*, 260(5112), 1273-1279.
- Wichroski, M. J., Ichiyama, K., & Rana, T. M. (2005). Analysis of HIV-1 viral infectivity factor-mediated proteasome-dependent depletion of APOBEC3G: correlating function and subcellular localization. *Journal of Biological Chemistry*, 280(9), 8387-8396. doi: 10.1074/jbc.M408048200
- Wiegand, H. L., & Cullen, B. R. (2007). Inhibition of alpharetrovirus replication by a range of human APOBEC3 proteins. *Journal of Virology*, 81(24), 13694-13699. doi: 10.1128/JVI.01646-07
- Wijesinghe, P., & Bhagwat, A. S. (2012). Efficient deamination of 5-methylcytosines in DNA by human APOBEC3A, but not by AID or APOBEC3G. *Nucleic Acids Research*, 40(18), 9206-9217. doi: 10.1093/nar/gks685

- Wildurn, S., Schindler, M., Munch, J., & Kirchhoff, F. (2006). Contribution of Vpu, Env, and Nef to CD4 down-modulation and resistance of human immunodeficiency virus type 1-infected T cells to superinfection. *Journal of Virology*, *80*(16), 8047-8059. doi: 10.1128/JVI.00252-06
- Wilén, C. B., Tilton, J. C., & Doms, R. W. (2012a). HIV: cell binding and entry. *Cold Spring Harb Perspect Med*, *2*(8). doi: 10.1101/cshperspect.a006866
- Wilén, C. B., Tilton, J. C., & Doms, R. W. (2012b). Molecular Mechanisms of HIV Entry. *Viral Molecular Machines*, *726*, 223-242.
- Willey, R. L., Rutledge, R. A., Dias, S., Folks, T., Theodore, T., Buckler, C. E., & Martin, M. A. (1986). Identification of Conserved and Divergent Domains within the Envelope Gene of the Acquired-Immunodeficiency-Syndrome Retrovirus. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(14), 5038-5042.
- Wissing, S., Montano, M., Garcia-Perez, J. L., Moran, J. V., & Greene, W. C. (2011). Endogenous APOBEC3B restricts LINE-1 retrotransposition in transformed cells and human embryonic stem cells. *Journal of Biological Chemistry*, *286*(42), 36427-36437. doi: 10.1074/jbc.M111.251058
- Witkowski, W., & Verhasselt, B. (2013). Contributions of HIV-1 Nef to immune dysregulation in HIV-infected patients: a therapeutic target? *Expert Opin Ther Targets*, *17*(11), 1345-1356. doi: 10.1517/14728222.2013.830712
- Wlodawer, A., & Erickson, J. W. (1993). Structure-Based Inhibitors of Hiv-1 Protease. *Annual Review of Biochemistry*, *62*, 543-585.
- Worthylake, D. K., Wang, H., Yoo, S. H., Sundquist, W. I., & Hill, C. P. (1999). Structures of the HIV-1 capsid protein dimerization domain at 2.6 angstrom resolution. *Acta Crystallographica Section D-Biological Crystallography*, *55*, 85-92. doi: 10.1107/S09074444998007689
- Xiao, X., Li, S. X., Yang, H., & Chen, X. S. (2016). Crystal structures of APOBEC3G N-domain alone and its complex with DNA. *Nature Communications*, *7*, 12193. doi: 10.1038/ncomms12193
- Xue, B., Mizianty, M. J., Kurgan, L., & Uversky, V. N. (2012). Protein intrinsic disorder as a flexible armor and a weapon of HIV-1. *Cellular and Molecular Life Sciences*, *69*(8), 1211-1259. doi: 10.1007/s00018-011-0859-3
- Yamashita, M., & Emerman, M. (2004). Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. *Journal of Virology*, *78*(11), 5670-5678. doi: 10.1128/JVI.78.11.5670-5678.2004
- Yamashita, M., & Emerman, M. (2006). Retroviral infection of non-dividing cells: Old and new perspectives. *Virology*, *344*(1), 88-93. doi: 10.1016/j.virol.2005.09.012
- Yamashita, M., Perez, O., Hope, T. J., & Emerman, M. (2007). Evidence for direct involvement of the capsid protein in HIV infection of nondividing cells. *Plos Pathogens*, *3*(10), 1502-1510. doi: ARTN e15610.1371/journal.ppat.0030156
- Yamauchi, K., Wada, K., Tanji, K., Tanaka, M., & Kamitani, T. (2008). Ubiquitination of E3 ubiquitin ligase TRIM5 alpha and its potential role. *Febs Journal*, *275*(7), 1540-1555.
- Yan, J., Hao, C., DeLucia, M., Swanson, S., Florens, L., Washburn, M. P., . . . Skowronski, J. (2015). CyclinA2-Cyclin-dependent Kinase Regulates SAMHD1 Protein Phosphohydrolase Domain. *Journal of Biological Chemistry*, *290*(21), 13279-13292. doi: 10.1074/jbc.M115.646588

- Yan, J., Kaur, S., DeLucia, M., Hao, C., Mehrens, J., Wang, C., . . . Skowronski, J. (2013). Tetramerization of SAMHD1 is required for biological activity and inhibition of HIV infection. *Journal of Biological Chemistry*, 288(15), 10406-10417. doi: 10.1074/jbc.M112.443796
- Yang, B., Chen, K., Zhang, C., Huang, S., & Zhang, H. (2007). Virion-associated uracil DNA glycosylase-2 and apurinic/apurimidinic endonuclease are involved in the degradation of APOBEC3G-edited nascent HIV-1 DNA. *Journal of Biological Chemistry*, 282(16), 11667-11675. doi: 10.1074/jbc.M606864200
- Yi, E. H., Yoo, H., Noh, K. H., Han, S., Lee, H., Lee, J. K., . . . Ye, S. K. (2013). BST-2 is a potential activator of invasion and migration in tamoxifen-resistant breast cancer cells. *Biochem Biophys Res Commun*, 435(4), 685-690. doi: 10.1016/j.bbrc.2013.05.043
- Yoo, H., Park, S. H., Ye, S. K., & Kim, M. (2011). IFN-gamma-induced BST2 mediates monocyte adhesion to human endothelial cells. *Cellular Immunology*, 267(1), 23-29. doi: 10.1016/j.cellimm.2010.10.011
- Yu, F. H., Chou, T. A., Liao, W. H., Huang, K. J., & Wang, C. T. (2015). Gag-Pol Transframe Domain p6\* Is Essential for HIV-1 Protease-Mediated Virus Maturation. *Plos One*, 10(6), e0127974. doi: 10.1371/journal.pone.0127974
- Yu, Q., Chen, D., Konig, R., Mariani, R., Unutmaz, D., & Landau, N. R. (2004). APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. *Journal of Biological Chemistry*, 279(51), 53379-53386. doi: 10.1074/jbc.M408802200
- Zapp, M. L., Hope, T. J., Parslow, T. G., & Green, M. R. (1991). Oligomerization and Rna-Binding Domains of the Type-1 Human-Immunodeficiency-Virus Rev Protein - a Dual Function for an Arginine-Rich Binding Motif. *Proceedings of the National Academy of Sciences of the United States of America*, 88(17), 7734-7738.
- Zeinalipour-Loizidou, E., Nicolaou, C., Nicolaidis, A., & Kostrikis, L. G. (2007). HIV-1 integrase: From biology to chemotherapeutics. *Current Hiv Research*, 5(4), 365-388.
- Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., & Gao, L. (2003). The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature*, 424(6944), 94-98. doi: 10.1038/nature01707
- Zhang, T., Cai, J., Chang, J., Yu, D., Wu, C., Yan, T., . . . Lin, D. (2013). Evidence of associations of APOBEC3B gene deletion with susceptibility to persistent HBV infection and hepatocellular carcinoma. *Hum Mol Genet*, 22(6), 1262-1269. doi: 10.1093/hmg/dd513
- Zhang, W., Du, J., Evans, S. L., Yu, Y., & Yu, X. F. (2011). T-cell differentiation factor CBF-beta regulates HIV-1 Vif-mediated evasion of host restriction. *Nature*, 481(7381), 376-379. doi: 10.1038/nature10718
- Zhang, W., Zhang, X., Tian, C., Wang, T., Sarkis, P. T., Fang, Y., . . . Xu, R. (2008). Cytidine deaminase APOBEC3B interacts with heterogeneous nuclear ribonucleoprotein K and suppresses hepatitis B virus expression. *Cellular Microbiology*, 10(1), 112-121. doi: 10.1111/j.1462-5822.2007.01020.x
- Zhang, Z., Gu, Q., Jaguva Vasudevan, A. A., Jeyaraj, M., Schmidt, S., Zielonka, J., . . . Munk, C. (2016). Vif Proteins from Diverse Human Immunodeficiency Virus/Simian Immunodeficiency Virus Lineages Have Distinct Binding Sites in A3C. *Journal of Virology*, 90(22), 10193-10208. doi: 10.1128/JVI.01497-16
- Zhao, R. Y., Li, G., & Bukrinsky, M. I. (2011). Vpr-host interactions during HIV-1 viral life cycle. *J Neuroimmune Pharmacol*, 6(2), 216-229. doi: 10.1186/1742-4690-8-25  
10.1007/s11481-011-9261-z

- Zhen, A., Du, J., Zhou, X., Xiong, Y., & Yu, X. F. (2012). Reduced APOBEC3H variant anti-viral activities are associated with altered RNA binding activities. *Plos One*, 7(7), e38771. doi: 10.1371/journal.pone.0038771
- Zhen, A., Wang, T., Zhao, K., Xiong, Y., & Yu, X. F. (2010). A single amino acid difference in human APOBEC3H variants determines HIV-1 Vif sensitivity. *Journal of Virology*, 84(4), 1902-1911. doi: 10.1128/jvi.01509-09
- Zheng, L., Yang, Y. D., Lu, G. C., & Salvato, M. S. (2005). Extracellular HIV Tat and Tat cysteine rich peptide increase CCR5 expression in monocytes. *J Zhejiang Univ Sci B*, 6(7), 668-672. doi: 10.1631/jzus.2005.B0668
- Zielonka, J., Bravo, I. G., Marino, D., Conrad, E., Perkovic, M., Battenberg, M., . . . Munk, C. (2009). Restriction of equine infectious anemia virus by equine APOBEC3 cytidine deaminases. *Journal of Virology*, 83(15), 7547-7559. doi: 10.1128/JVI.00015-09