HIV-1 Evasion of Human TRIM5α via Cyclophilin A

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HIV-1 EVASION OF HUMAN TRIM5α VIA CYCLOPHILIN A

A Dissertation Presented

By

Kyusik Kim

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JULY 17TH, 2020

INTERDISCIPLINARY GRADUATE PROGRAM
HIV-1 EVASION OF HUMAN TRIM5α VIA CYCLOPHILIN A

A Dissertation Presented
By
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF COPYRIGHTED MATERIALS PRODUCED BY THE AUTHOR</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF THIRD PARTY COPYRIGHTED MATERIALS</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER I. GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. HIV-1 STRUCTURE AND GENOME</td>
<td>4</td>
</tr>
<tr>
<td>1.1.1. HIV-1 Virion Structure</td>
<td>4</td>
</tr>
<tr>
<td>1.1.2. HIV-1 Genome Structure</td>
<td>5</td>
</tr>
<tr>
<td>1.1.2.1. HIV-1 Structural Proteins</td>
<td>6</td>
</tr>
<tr>
<td>1.1.2.2. HIV-1 Regulatory Proteins</td>
<td>10</td>
</tr>
<tr>
<td>1.1.2.3. HIV-1 Accessory Proteins</td>
<td>12</td>
</tr>
<tr>
<td>1.2. HIV-1 LIFE CYCLE</td>
<td>16</td>
</tr>
<tr>
<td>1.2.1. HIV-1 Entry</td>
<td>17</td>
</tr>
<tr>
<td>1.2.2. HIV-1 in the Cytoplasm: Reverse Transcription and Uncoating</td>
<td>18</td>
</tr>
<tr>
<td>1.2.3. Nuclear Import of HIV-1</td>
<td>20</td>
</tr>
<tr>
<td>1.2.4. HIV-1 Integration</td>
<td>23</td>
</tr>
<tr>
<td>1.2.5. Expression of HIV-1 Provirus</td>
<td>25</td>
</tr>
<tr>
<td>1.2.6. Assembly and Maturation of Progeny HIV-1 Particles</td>
<td>28</td>
</tr>
</tbody>
</table>
1.3. HOST INNATE AND INTRINSIC DEFENSE AGAINST HIV-1 ...............31
1.3.1. Innate Immune Sensing of HIV-1 .........................................................34
1.3.2. Intrinsic Restriction Factors against HIV-1 ........................................40
  1.3.2.1. APOBEC3G ..................................................................................40
  1.3.2.2. SAMHD1 ......................................................................................45
  1.3.2.3. BST-2/Tetherin ..............................................................................51
  1.3.2.4. Myxovirus Resistance Protein 2 (Mx2/MxB) ...................................56
  1.3.2.5. SERINC and T-cell Immunoglobulin and Mucin Domain (TIM) .....59
  1.3.2.6. Other Restriction Factors ................................................................62

CHAPTER II. CYCLOPHILIN A PROTECTS HIV-1 FROM HUMAN TRIM5α ....67
2.1. SUMMARY .................................................................................................68
2.2. INTRODUCTION ..........................................................................................69
  2.2.1. The Roles of Cyclophilin A in HIV-1 Infection ......................................69
    2.2.1.1. Cyclophilins ..................................................................................69
    2.2.1.2. Cyclophilin A (CypA) ....................................................................71
    2.2.1.3. Interaction of CypA with HIV-1 CA ..............................................72
    2.2.1.4. CypA Promotes HIV-1 Infection .....................................................77
  2.2.2. Capsid-binding Restriction Factor TRIM5α and HIV-1 .....................78
    2.2.2.1. Tripartite Motif-containing (TRIM) Family of Proteins .................80
    2.2.2.2. TRIM5: Isoforms, Structure and Binding to HIV-1 CA .................86
    2.2.2.3. Antiviral Activities of TRIM5α: Premature Disassembly of Retroviral
              CA .........................................................................................................96
2.2.2.4. Antiviral Activities of TRIM5α: Innate Immune Response to Retroviral CA .................................................................100

2.2.3. The Relationship between TRIM5α and Cyclophilin A in HIV-1 Infection ..................................................................................105

2.3. RESULTS ........................................................................................................107

2.4. DISCUSSION ..................................................................................................131

2.5. MATERIALS AND METHODS .......................................................................133

CHAPTER III. DISCUSSION ..................................................................................150

APPENDIX I. REGULATION OF HIV-1 INFECTIVITY BY CYCLOPHILIN A AND HUMAN TRIM5α IN CANCER CELL LINES ......................................................170

APPENDIX II. HIV-1 CAPSID MUTATION CONFERS RESISTANCE TO RESTRICTION BY HUMAN TRIM5α .................................................................192

BIBLIOGRAPHY ....................................................................................................200
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor, Dr. Jeremy Luban, for his guidance, support, and advice throughout my graduate career. I have learned ways of thinking and attitudes about science from his critical questions and comments during discussions. His enthusiasm has also encouraged me not to be depressed or frustrated, in an optimistic way, whenever I had a difficult time during projects. My graduate study with him was invaluable time in my scientific career and in my life.

I appreciate my committee members, Drs. Erik Sontheimer, Kate Fitzgerald, Oliver Rando, Egil Lien, and Melissa Moore. They have provided me with their insightful advice and comments over 4 years since our first TRAC meeting in 2017. I also appreciate Dr. Sara Sawyer for serving as an external committee member of my dissertation defense.

I would like to thank the past and present members of the Luban lab, in particular to Dr. William (Ted) Diehl, Dr. Sean McCauley, Dr. Leonid Yurkovetskiy, Ann Dauphin, Claudia Carbone, Mehmet (Hakan) Guney, Noah Silverstein, and Dr. Yetao Wang. I have learned a number of experimental techniques from them. They have also helped me to develop scientific communication skills, as well as provided me with advice for my future career.

I thank Korean community at UMass Medical School and in Boston for their support throughout my life in the US.

Lastly, I would like to express my deepest gratitude to my parents, family, and friends in Korea for all of their support.
The abundant cellular protein Cyclophilin A (CypA) was found to bind to HIV-1 capsid (CA) in 1993. Since that time, several complementary methods, including disruption of the binding interface by cyclosporine A, CA mutants, and CypA mutants, have been used to demonstrate that CypA acts within human target cells to promote HIV-1 infection. In contrast, in cells from non-human primates, CypA in target cells decreases HIV-1 infectivity, and it does so by promoting TRIM5α-mediated restriction. Using human cancer cell lines and the genetic methods available at the time, attempts to obtain evidence that CypA inhibits HIV-1 restriction by the human TRIM5α ortholog, let alone that human TRIM5α restricts HIV-1, were unsuccessful.

Here we revisit the question of the mechanism by which CypA increases HIV-1 infectivity by exploiting lentiviral vectors optimized for primary human blood cells that serve as HIV-1 targets. Disruption of CA–CypA interaction is demonstrated to render HIV-1 vulnerable to endogenous human TRIM5α-mediated recognition and restriction, which occur prior to completion of reverse transcription. Identical findings were acquired with single-cycle vectors or with replication-competent viruses. Consistently, a previously identified, cyclosporine-resistant CA mutation A92E is also shown to confer resistance against restriction by human TRIM5α. Therefore, the results presented in this thesis reveal that HIV-1 exploits a host protein CypA bound to its CA to evade potent restriction by human TRIM5α. This finding not only answers a long-standing question
regarding the role of CypA in HIV-1 infection, but also may reinvigorate the development of CypA inhibitors for treatment of HIV-1.
LIST OF FIGURES

Figure 1.1. Genome organization and virion structure of HIV-1 .......................15
Figure 1.2. Overview of HIV-1 life cycle .........................................................16
Figure 1.3. Host intrinsic restriction factors against HIV-1 and viral antagonisms .................................................................66
Figure 2.1. CA−CypA interaction ..................................................................74
Figure 2.2. CsA−CypA complex blocks T cell signaling ...............................76
Figure 2.3. Domain structure of TRIM proteins .............................................81
Figure 2.4. Regulation of activities of TRIM proteins by homo- and hetero- multimerization .................................................................85
Figure 2.5. TRIM5 isoforms expressed in human cells ...............................87
Figure 2.6. Structure and higher-order assembly of TRIM5α over CA ........90
Figure 2.7. Ubiquitin-mediated restriction activity of TRIM5α .........................103
Figure 2.8. Assessment of shRNA−mediated knockdown in primary human blood cells ........................................................................108
Figure 2.9. Disruption of the CA−CypA interaction in primary human blood cells renders HIV-1 susceptible to restriction by TRIM5 ..................................................111
Figure 2.10. The interaction between HIV-1 CA and CPSF6 is independent of restriction activity of human TRIM5α .................................................................117
Figure 2.11. Human TRIM5α is sufficient to explain the inhibition of reverse transcription that results from disruption of CA−CypA interaction ....................121
Figure 2.12. Endogenous TRIM5α in primary human macrophages associates
with HIV-1 CA after acute challenge but only when the CA–CypA interaction is disrupted ........................................................................................................................................................................... 125

Figure 2.13. Endogenous TRIM5α suppresses the spread of HIV-1 infection in primary human macrophages and CD4+ T cells when the CA–CypA interaction is disrupted ........................................................................................................................................................................................................ 129

Figure A.I.1. CypA and TRIM5α independently regulate HIV-1 infectivity in the TE671, RD, and CEM-SS cell lines .................................................................................................................................................................................................. 172

Figure A.I.2. CypA protects HIV-1 from endogenous TRIM5α in the Jurkat cell line as observed in primary blood cells ........................................................................................................................................................................ 176

Figure A.I.3. Rescued expression of human TRIM5α in SupT1 cell line is capable of restricting HIV-1 with disruption of CA–CypA interaction .............................................................................................................................................................. 180

Figure A.I.4. CA–CypA interaction shields HIV-1 replication from human TRIM5α in the Jurkat and SupT1 cell lines .................................................................................................................................................................................................. 183

Figure A.I.5. Human TRIM5α is able to associate with HIV-1 CA when CA–CypA interaction is disrupted in the U2OS cell line .................................................................................................................................................................................................. 187

Figure A.II.1. CA-A92E mutation renders HIV-1 resistant to restriction by human TRIM5α ........................................................................................................................................................................................................... 195

Figure A.II.2. Human TRIM5α is not able to inhibit the replication of HIV-1 bearing the CA-A92E mutation ........................................................................................................................................................................................................... 198
LIST OF TABLES

Table 2.1. Plasmids used in this study .........................................................145
Table 2.2. Drugs and reagents ....................................................................148
Table 2.3. qPCR primers and probes for Late RT quantification ..............149
LIST OF COPYRIGHTED MATERIALS PRODUCED BY THE AUTHOR

LIST OF THIRD PARTY COPYRIGHTED MATERIALS

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CHAPTER I.

GENERAL INTRODUCTION
Human immunodeficiency virus type 1 (HIV-1) is a causative agent of acquired immunodeficiency syndrome (AIDS) (Weiss 1993). The virus targets immune cells, mainly CD4+ T cells, which damages the human immune system to allow opportunistic infection by other pathogens and results in increased mortality. As a blood-borne pathogen, HIV-1 can be transmitted through sexual contact, blood transfusion, needle-sharing, and maternal-infant exposure (Shaw and Hunter 2012).

Historically, AIDS was first clinically reported in 1981 (Gottlieb et al. 1981). Two years later, two independent groups led by Luc Montagnier and Robert C. Gallo isolated the responsible virus from an AIDS patient (Barre-Sinoussi et al. 1983; Gallo et al. 1984). The virus was originally named lymphadenopathy-associated virus or human T-cell leukemia-lymphoma virus-3 (HTLV-III), but was later renamed HIV. In 1986, as the second virus causing AIDS was discovered, the first virus was named HIV-1 and the second as HIV-2 to distinguish them (Clavel et al. 1986; Case 1986). Around this period, a T-cell tropic HTLV-III-like retrovirus was isolated from rhesus macaque, a non-human primate, having simian AIDS, which was the first finding of simian immunodeficiency virus (SIV) (Gravell et al. 1984; Daniel et al. 1984, 1985). Genomic sequence analysis of this virus showed similarity between HIV and SIV, suggesting an evolutionary relationship (Chakrabarti et al. 1987). Phylogenetic studies based on further isolated HIVs and SIVs showed that the HIVs originated from zoonotic transmission of SIV from non-human primates. HIV-1 emerged from SIVcpz,
which infects chimpanzees (Gao et al. 1999; Keele et al. 2006), whereas HIV-2 evolved from SIVsm, which was found in sooty mangabeys (Hirsch et al. 1989; Gao et al. 1994; Santiago et al. 2005).

Both HIV-1 and HIV-2 cause AIDS, but they show many dissimilarities to each other, as they originate from viruses infecting different species as described above. Although their genomes have a similar genetic structure, they have low overall nucleotide sequence identity, at about 55%, as well as low amino acid sequence identity of gag, env, and pol (Motomura, Chen, and Hu 2008). In addition, HIV-1 is highly transmittable and occurs prevalently worldwide as a pandemic, whereas HIV-2 is less transmittable and is geographically confined to Western Africa (Reeves and Doms 2002; Campbell-Yesufu and Gandhi 2011). This thesis focuses mainly on HIV-1 virology and host–HIV-1 interaction.

According to a recent report from the World Health Organization (WHO) (https://www.who.int/), 37.9 million people were living with HIV-1 infection at the end of 2018. Also, in that year, 1.7 million people were newly infected and 770,000 people died from HIV-related causes. Additionally, the Centers for Disease Control and Prevention (CDC) reported that, in the US, 1.14 million individuals had HIV in 2016 (https://www.cdc.gov). Highly active antiretroviral therapy (HAART), a combination therapy that uses a drug cocktail of multiple compounds targeting the early stage of HIV-1 infection, is currently used to treat HIV-1. Continuous treatment with HAART is effective at decreasing the viral load of patients, and thus, HIV-1–infected individuals are now expected to live much
like those without HIV. However, currently there is still no cure or vaccine for HIV-1. Also, a recent report from the WHO showed that only approximately 62% of HIV-1 patients were receiving this treatment globally in 2018 (https://www.who.int/). Thus, comprehensive development of effective and cost-efficient therapeutics, such as drugs and vaccines, and of strategies to provide patients with medication is needed to address the HIV-1 pandemic.

1.1. HIV-1 STRUCTURE AND GENOME

HIV-1 belongs to the genus Lentivirus in the Retroviridae family (King et al. 2011). Retroviruses have RNA genomes and an enzyme called reverse transcriptase to generate the viral DNA genome from the template RNA genome during viral infection. This process is called reverse transcription and is required for the subsequent integration of the viral DNA genome into the host cell genome. This integrated viral genome, called the provirus, is expressed for the generation of progeny virion particles by the gene expression machinery of the host cells. As a member of the Retroviridae family, HIV-1 also shares these characteristics with other retroviruses.

1.1.1. HIV-1 Virion Structure

As depicted in Figure 1.1, the HIV-1 virion forms a spherical particle with a diameter of approximately 100–120 nm (Gelderblom, Ozel, and Pauli 1989; Briggs et al. 2003; German Advisory Committee Blood (Arbeitskreis Blut),
Subgroup “Assessment of Pathogens Transmissible by Blood” 2016). The virion has two copies of a single-stranded RNA genome, which interact with nucleocapsid. This nucleocapsid–RNA complex is enclosed by a cone-shaped capsid core structure. In this capsid core, there are also viral accessory proteins like Vpr and enzymatic proteins such as reverse transcriptase, integrase, and protease, which are required for virus particle maturation and HIV-1 infection (Gelderblom, Ozel, and Pauli 1989; Pornillos, Ganser-Pornillos, and Yeager 2011). These proteins will be described more detailed later in this section. As an enveloped virus, the capsid core of HIV-1 is surrounded by a lipid bilayer acquired from the infected host cell membrane. This envelope membrane interacts with viral matrix protein at the inner side and contains viral glycoproteins comprising gp41 transmembrane proteins and gp120 surface glycoproteins that project out of the lipid membrane, like spikes, and are required for binding to the receptors of host target cells for infection (Gelderblom, Ozel, and Pauli 1989; Checkley, Luttge, and Freed 2011).

1.1.2. HIV-1 Genome Structure

As described above, HIV-1 harbors two identical copies of a single-stranded, positive-sense RNA molecule as the viral genome. These RNA genomes have a size of approximately 10 kilo base-pair and contain 5'-cap with 7-methylguanosine and 3’-polyadenylated tails, like cellular messenger RNAs (mRNAs) (Wain-Hobson et al. 1985; Ratner et al. 1985; Castelli and Levy 2002).
As shown in Figure 1.1, they have approximately 600-nucleotide long-terminal repeats (LTRs) at both the 5’ and 3’ ends, which are critical for reverse transcription and integration. The 5’-LTR primarily functions as a promoter for transcription of the provirus, whereas the 3’-LTR is responsible for polyadenylation of the viral transcripts (Brown, Tiley, and Cullen 1991; Klaver and Berkhout 1994). Both LTRs consist of three compartments, U3, R, and U5. The U3 region contains the promoter, enhancer, and regulatory sequences, including transcription factor-binding sites. The R region in the 5’-LTR has the transcription start site and trans-activation response (TAR) element for trans-activation of the LTR promoter upon interaction with a viral protein Tat, while the R region in the 3’-LTR has the polyadenylation site. The R sequence also serves as a self-priming site during reverse transcription.

In addition to the flanking LTRs, the HIV-1 genome contains cis-acting elements such as the primer-binding site (PBS), the packaging signal (ψ), the Rev responsive element (RRE), and the central polypurine tract (cPPT), which are necessary for HIV-1 infection cycles. Furthermore, the genome encodes nine viral proteins, including three structural proteins (gag, pol, and env), two regulatory proteins (tat, and rev), and four accessory proteins (vif, vpr, vpu, and nef) (Frankel and Young 1998).

1.1.2.1. HIV-1 Structural Proteins

The structural proteins are responsible for building virus particles and play
critical roles in each step of the HIV-1 infection cycle from virus−target recognition, reverse transcription, and integration to the production of progeny virus particles. The gag gene produces the 55-kDa precursor group-specific antigen (Gag) polyprotein (Pr55), which contains six domains, matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), p6, and two spacer peptides, one located between CA and NC and another between NC and p6. This precursor protein is cleaved by viral protease into three proteins, MA, CA, and NC, during maturation of the progeny virus particle after its release from infected host cells. MA (p17) binds to the membrane via an N-terminally attached myristoyl group (Göttlinger, Sodroski, and Haseltine 1989; Bryant and Ratner 1990; Spearman et al. 1994; Ono, Orenstein, and Freed 2000; Hermida-Matsumoto and Resh 2000; Saad et al. 2006) and aids the incorporation of glycoprotein spikes into the HIV-1 envelope (Dorfman et al. 1994; Cosson 1996; Murakami and Freed 2000). CA (p24) forms multiple hexamer and pentamer lattices to build a conical core structure enclosing viral RNA genomes and proteins (Casey et al. 1985; Campbell and Hope 2015b). This CA core plays an essential role in early steps of HIV-1 infection through interactions with host cellular proteins such as cyclophilin A (Yamashita and Engelman 2017). This also protects the HIV-1 genome from detection by innate immune sensors like cyclic GMP-AMP synthase (cGAS) (Rasaiyaah et al. 2013; Lahaye et al. 2013; Gao et al. 2013). NC (p7) is a basic protein that interacts with viral RNA genomes via its two zinc-finger motifs (Skripkin et al. 1994; Dannull et al. 1994; De Guzman et al. 1998).
This protein functions as a nucleic acid chaperone, remodeling nucleic acid structure into the most thermodynamically stable structure, which regulates reverse transcription upon HIV-1 infection and viral genome packaging into HIV-1 particles (De Rocquigny et al. 1992; Allain et al. 1994; You and McHenry 1994; Rodríguez-Rodríguez et al. 1995; Guo et al. 1997; Guo et al. 2000; Levin et al. 2005). P6 protein recruits the endosomal complex required for transport (ESCRT) machinery through its interaction with tumor susceptibility gene 101 (Tsg101) and apoptosis-linked gene 2-interacting protein X (ALIX) for budding of HIV-1 particles from the host cell membrane (Garrus et al. 2001; Martin-Serrano, Zang, and Bieniasz 2001; Strack et al. 2003). Tsg101 and ALIX proteins are subunits of the ESCRT complex. This protein also mediates the interaction between Gag polyprotein and a viral protein Vpr for the incorporation of Vpr into the virus particles (Bachand et al. 1999).

The pol gene encodes three enzymatic proteins, protease (PR), reverse transcriptase (RT), and integrase (IN). Because the pol gene is located downstream of the gag gene from the 5' LTR promoter, the HIV-1 genome produces a 160-kDa Gag-Pol polyprotein to express the pol gene via a programmed ribosomal translation strategy (Jacks et al. 1988; Wills, Gesteland, and Atkins 1994; Dulude, Baril, and Brakier-Gingras 2002). The p6 sequence at the 3' end of the gag gene contains a slippery site consisting of repetitive U nucleotides immediately upstream of a stem-loop structure. The stem-loop induces ribosomal stalling on the slippery sequence, and the ribosome then
moves backward by one nucleotide, which triggers ribosomal frameshifting to continue translation of the following pol region. This happens in 5% of translation events. Interestingly, this ratio of Gag-Pol to Gag at approximately 1:20 is important for virus assembly, based on findings that disruption of this ratio by increasing the expression of Gag-Pol or PR causes inhibition of the assembly due to immature proteolytic processing of Gag (Kräusslich 1991; Park and Morrow 1991; Rosé, Babé, and Craik 1995; Shehu-Xhilaga, Crowe, and Mak 2001). PR is responsible for proteolytic maturation of virus particles by cleaving the viral Gag and Gag-Pol polyproteins into the functional parts (Kohl et al. 1988; Peng et al. 1989; Göttlinger, Sodroski, and Haseltine 1989; Wiegers et al. 1998). As described previously, RT generates the viral complementary DNA (cDNA) genome using the RNA genome as the template (Temin and Mizutani 1970; Baltimore 1970; Roberts, Bebenek, and Kunkel 1988; Telesnitsky and Goff 2011; Hu and Hughes 2012). This protein possesses three enzymatic functions. It acts as a RNA-dependent DNA polymerase to synthesize a DNA strand based on a viral RNA strand, as a RNase H to remove a RNA strand from RNA:DNA hybrid intermediates, and as a DNA-dependent DNA polymerase to generate viral double-stranded DNA genome. IN integrates the viral cDNA genome into the host cell genome, after the import of the viral genome−IN complex (Gallay et al. 1997; Bouyac-Bertoia et al. 2001), called the pre-integration complex, into the nucleus, resulting in the establishment of provirus (Fujiwara and Mizuuchi 1988; Bushman, Fujiwara, and Craigie 1990).
The *env* gene expresses 160-kDa polyprotein (gp160), which is cotranslationally glycosylated in the rough endoplasmic reticulum. This glycosylated protein is cleaved by the host cell protease furin into the transmembrane protein (TM, gp41) and the surface protein (SU, gp120) (McCune et al. 1988; Willey et al. 1988; Hallenberger et al. 1992). The SU protein protrudes from the viral envelope membrane and is responsible for the interaction with the host cell receptor cluster of differentiation 4 (CD4), and thus, it determines the specificity and cell tropism of the virus (Dalgleish et al. 1984; Klatzmann et al. 1984; Kwong et al. 1998). This protein has been investigated as a vaccine target. However, attempts for neutralization of this protein by antibodies have failed due to the glycosylated modification of the protein residues and variable regions covering the conserved regions that the antibodies target (Labrijn et al. 2003). The TM protein is buried in the envelope membrane and mediates the membrane fusion between HIV-1 and target cells upon the SU−CD4 interaction to infect cells (Weissenhorn et al. 1997; Chan et al. 1997; Blumenthal, Durell, and Viard 2012).

1.1.2.2. HIV-1 Regulatory Proteins

The regulatory proteins promote the expression of viral RNAs and proteins essential for HIV-1 infection. The *tat* gene produces a trans-activator of transcription (Tat) protein (Sodroski et al. 1985a; Arya et al. 1985). This factor interacts with the stem-loop structured trans-activation response (TAR) element located in the 5’-LTR promoter region (Sodroski et al. 1985b; Feng and Holland
1988; Berkhout, Silverman, and Jeang 1989; Dingwall et al. 1989, 1990), which recruits the transcription elongation complex to largely increase HIV-1 transcription (Kao et al. 1987; Herrmann and Rice 1995; Zhu et al. 1997; Mancebo et al. 1997; Wei et al. 1998; Bieniasz et al. 1999).

Regulator of virion gene expression (Rev) encoded by the gene \textit{rev} (Sodroski et al. 1986a; Feinberg et al. 1986) binds to the cis-acting element Rev response element (RRE) located within the \textit{env} gene (Rosen et al. 1988; Hadzopoulou-Cladaras et al. 1989; Malim et al. 1989; Cochrane, Chen, and Rosen 1990; Holland et al. 1990; Kjems et al. 1991) to facilitate nuclear export of unspliced or partially spliced HIV-1 RNAs (Malim et al. 1989; Felber et al. 1989; Emerman, Vazeux, and Peden 1989; Brighty and Rosenberg 1994; Fischer et al. 1995; Churchill et al. 1996). These RNAs are translated into the structural proteins to build newly generated virus particles. Particularly, the unspliced HIV-1 RNAs form the HIV-1 RNA genome of the new viruses. Thus, this Rev-dependent export is important for the generation of progeny HIV-1 virus particles. This also prevents unspliced HIV-1 RNAs from being detected by innate immune sensing (McCabe et al. 2018; Akiyama et al. 2018). In addition, Rev binding to the RRE in the nucleus inhibits splicing of the viral RNAs to generate partially spliced RNAs (Michael et al. 1991; Stutz and Rosbash 1994; Kalland et al. 1994; Powell et al. 1997), which increases structural and accessory protein expression but decreases the expression of regulatory proteins such as Tat and Rev from fully spliced viral RNAs. Thus, Rev plays a role to shift the expression pattern of
HIV-1 proteins to prepare for the production of progeny virus particles in the late phase of infection.

1.1.2.3. HIV-1 Accessory Proteins

The viral accessory proteins are not required for HIV-1 infection in some cell types but provide a more permissive environment for viral infection by regulating the expression levels of cellular proteins including antiviral factors. The 23-kDa viral infectivity factor (Vif) protein encoded by the \textit{vif} gene promotes HIV-1 infection (Sodroski et al. 1986\textsuperscript{b}; Strebel et al. 1987; Fisher et al. 1987) by antagonizing an antiviral factor apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) (Sheehy et al. 2002), which functions as a cytidine deaminase to introduce a G-to-A mutation into the HIV-1 RNA genome (Lecossier et al. 2003; Harris et al. 2003; Mangeat et al. 2003; Zhang et al. 2003; Suspène et al. 2004; Bishop et al. 2004).

The viral protein R (Vpr) produced by the \textit{vpr} gene is a 14-kDa protein incorporated into HIV-1 virion particles (Wong-Staal, Chanda, and Ghrayeb 1987; Ogawa et al. 1989; Cohen et al. 1990). Upon HIV-1 infection, Vpr enhances import of the pre-integration complex (PIC) containing the viral cDNA genome into the nucleus through interaction with Importin \(\alpha\) (Kamata et al. 2005; Nitahara-Kasahara et al. 2007). Vpr also increases the cellular Ca\(_{2+}\) level to trigger nuclear factor of activated T cells (NFAT)-mediated signaling to activate the infected T cells, which upregulates HIV-1 transcription (Lahti, Manninen, and
Saksela 2003; Höhne et al. 2016). This protein also induces proteasome-mediated degradation of host proteins involved in DNA repair pathways, including uracil-DNA glycosylase (UNG2) (Bouhamdan et al. 1996; Hansen et al. 2016; Hrecka et al. 2016), helicase-like transcription factor (HLTF) DNA helicase (Hrecka et al. 2016; Lahouassa et al. 2016; Yan et al. 2019), tet methylcytosine dioxygenase 2 (TET2) (Lv et al. 2018) and exonuclease 1 (Exo1) (Yan et al. 2018), resulting in G2 cell cycle arrest linked to an increase in LTR-driven transcription and immune evasion of HIV-1. Recent studies have shown that these four proteins indeed function as antiviral factors against HIV-1, demonstrating that Vpr also antagonizes them like other accessory proteins.

The vpu gene encodes the 9-kDa viral protein U (Vpu), which facilitates the release of HIV-1 virion particles from infected cells (Cohen et al. 1988; Strebel, Klimkait, and Martin 1988). This type I integral membrane protein decreases the surface level of CD4 (Wille et al. 1992; Margottin et al. 1998; Levesque, Zhao, and Cohen 2003) and bone marrow stromal antigen 2 (BST-2)/Tetherin (Neil, Zang, and Bieniasz 2008; Van Damme et al. 2008) that block the release of virus particles from the host cellular membrane via direct interaction. To do so, Vpu induces proteasomal degradation of newly generated CD4 in the golgi apparatus (Wille et al. 1992; Margottin et al. 1998; Levesque, Zhao, and Cohen 2003) as well as removes BST-2/Tetherin through the lysosomal degradation pathway (Iwabu et al. 2009; Douglas et al. 2009; Bastien Mangeat et al. 2009; Mitchell et al. 2009; Dubé et al. 2010).
The 27-kDa negative factor (Nef) protein generated from the nef gene is a protein associated with the membrane via its myristoylated residues (Terwilliger et al. 1986; Luciw, Cheng-Mayer, and Levy 1987; Guy et al. 1987). Nef counteracts the antiviral activity of serine incorporator (SERINC) 3 and SERINC5 that incorporate into the progeny HIV-1 particles and then block virus entry into the next target cells (Usami, Wu, and Göttlinger 2015; Rosa et al. 2015). Studies showed that the interaction of Nef with SERINC proteins on the plasma membrane induces endocytosis of the proteins followed by lysosome-mediated degradation (Rosa et al. 2015; Shi et al. 2018). Nef also downregulates surface levels of CD4 and major histocompatibility class (MHC)-I to evade the antiviral activities of cytotoxic T cells (Garcia and Miller 1991; Craig, Pandori, and Guatelli 1998; Blagoveshchenskaya et al. 2002; Landi et al. 2011). Upon the infection of macrophages, Nef stimulates the cells to produce chemokines to recruit lymphocytes to the infection site, which expedites the spread of the virus (Swingler et al. 1999). Lastly, primary HIV-1 isolates containing defective nef were found in patients showing long-term non-progress of HIV-1 without antiretroviral therapy treatment (Deacon et al. 1995; Dyer et al. 1997), suggesting the importance of Nef in HIV-1 progression and pathogenicity.
Figure 1.1. Genomic organization and virion structure of HIV-1

Reproduced with permission from (Kirchhoff 2013).

(A) Genomic organization of HIV-1.

(B) Mature virion structure of HIV-1
1.2. HIV-1 LIFE CYCLE

Figure 1.2. Overview of HIV-1 life cycle

Reproduced with permission from (Rambaut et al. 2004).
1.2.1. HIV-1 Entry

HIV-1 infection begins with the recognition of host target cells by viruses. This occurs when the viral SU glycoprotein gp120 spike on the HIV-1 envelope binds the host receptor CD4 present at the surface of target cells (Dalgleish et al. 1984; Klatzmann et al. 1984; Kwong et al. 1998). CD4 is expressed mainly on CD4+ T cells that serve as the main target of HIV-1 and at low levels on macrophages and dendritic cells. Therefore, HIV-1 can also infect the innate immune myeloid cells but less efficiently than CD4+ T cells (Chauveau et al. 2017). In addition to the receptor CD4, HIV-1 gp120 also interacts with co-receptor C-C chemokine receptor type 5 (CCR5) (Alkhatib et al. 1996; Deng et al. 1996; Doranz et al. 1996; Dragic et al. 1996) or C-X-C chemokine receptor type 4 (CXCR4) (Feng et al. 1996). This interaction of gp120 with these receptors determines virus specificity and tropism. The tropism of HIV-1 using CCR5 for virus entry is called R5-tropic, and that utilizing CXCR4 is called X4-tropic. R5-tropic viruses are found in most primary HIV-1 isolates from patients and also called macrophage-tropic viruses because they can infect macrophages. X4-tropic viruses are mainly found in laboratory-adapted strains. HIV-1 having both forms of tropism, R5X4-tropic virus, is also common in viruses isolated from patients, like R5-viruses (Berger et al. 1998; Keele and Derdeyn 2009).

The binding of gp120 to CD4 triggers a conformational change of gp120 to additionally interact with the co-receptor (Kwong et al. 1998). This gp120−CD4−CCR5 (or CXCR4) interaction sequentially causes the TM
glycoprotein gp41 to insert its fusion peptide into the host target cell membrane, which results in fusion between the HIV-1 envelope and the host cell membrane by forming the fusion pore (Weissenhorn et al. 1997; Chan et al. 1997; Blumenthal, Durell, and Viard 2012). The viral CA core encasing the RNA genome and proteins then enters into the host cell cytoplasm through the pore.

1.2.2. HIV-1 in the Cytoplasm: Reverse Transcription and Uncoating

In the cytoplasm, the HIV-1 RNA genome in the CA core undergoes reverse transcription by the viral RT (reverse transcriptase) protein to generate the cDNA genome (Temin and Mizutani 1970; Baltimore 1970; Roberts, Bebenek, and Kunkel 1988; Telesnitsky and Goff 2011; Hu and Hughes 2012). Briefly, the RNA-dependent DNA polymerase function of RT first synthesizes a minus-sense, single-stranded DNA with the positive sense RNA genome as the template. The RNase H part of RT then removes the original RNA genomic strand from the RNA:DNA hybrid intermediate. Finally, the DNA-dependent DNA polymerase function of RT synthesizes positive sense DNA strands to generate double-stranded viral DNA genomes. The CA core enclosing the genome plays important roles in HIV-1 infection during this reverse transcription in the cytoplasm (Campbell and Hope 2015b). First, the CA core is critically involved in the reverse transcription based on reports showing that disruption of the CA core stability blocks the reverse transcription (Forshey et al. 2002). Also, cytoplasmic deoxynucleotides (dNTPs) as building blocks of the reverse transcription are
imported into the CA core through core pores (Jacques et al. 2016). Secondly, the CA core protects the viral genome from the innate immune sensing of the host cells, such as cGAS, which detects exogenous RNA:DNA hybrids or double-stranded DNAs (Rasaiyaah et al. 2013; Lahaye et al. 2013; Gao et al. 2013). Third, the CA core interacts with host proteins essential for HIV-1 infection (Yamashita and Engelman 2017). For example, interaction with microtubule proteins guides the CA core to the nucleus (McDonald et al. 2002; Arhel et al. 2006; Sabo et al. 2013). In addition, the CA–cyclophilin A (CypA) interaction safeguards the CA core from inhibition by an antiviral factor tripartite motif-containing protein 5α (TRIM5α) (Kim et al. 2019; Selyutina et al. 2020). This finding will be discussed with the thesis research presented in Chapter II.

During or after reverse transcription, the CA core becomes disassembled, so that the viral DNA genome is delivered to the nucleus and further to the host genomic DNAs. This disassembly step is called uncoating. The precise timing of uncoating remains to be determined. Early studies implied that the CA core is disassembled early after virus entry (Miller, Farnet, and Bushman 1997; Fassati and Goff 2001). Since then, two uncoating models have been proposed: 1) the CA core is loosen and disassembled during cytoplasmic transport (Hulme, Perez, and Hope 2011; Xu et al. 2013; Yang, Fricke, and Diaz-Griffero 2013; Lukic et al. 2014; Cosnefroy, Murray, and Bishop 2016; Francis et al. 2016; Mamede et al. 2017), and 2) disassembly of the CA core occurs at the nuclear pore complex after CA binding to the complex (Arhel et al. 2007; Rasaiyaah et al.
2013; Lahaye et al. 2013; Francis et al. 2016; Burdick et al. 2017; Francis and Melikyan 2018; Fernandez et al. 2019). Furthermore, a recent study has suggested that the intact CA core is imported into the nucleus and undergoes uncoating near the integration site on the host genome (Burdick et al. 2020). In addition to uncoating, the completion timing of the reverse transcription is not well understood. Many researchers believe the reverse transcription is completed in the cytoplasm, before nuclear import of the viral genome. However, some studies have shown that the reverse transcription is not completed in the cytoplasm and is still ongoing in the nucleus (Bukrinsky et al. 1993a; Barbosa et al. 1994; Galvis et al. 2014). Recently, a research group has also demonstrated that the completion occurs in the intact CA core in the nucleus (Burdick et al. 2020). Thus, further studies are needed to clarify how the reverse transcription and uncoating processes are organized and arranged for better understanding of the early phase of HIV-1 infection. For the following description of the HIV-1 life cycle, I consider a model in which the reverse transcription is completed in the cytoplasm.

### 1.2.3. Nuclear Import of HIV-1

After viral double-stranded cDNA genomes are synthesized, viral proteins IN (integrase) (Gallay et al. 1997; Bouyac-Bertoia et al. 2001), MA (matrix) (Bukrinsky et al. 1993b), and Vpr (Heinzinger et al. 1994; Vodicka et al. 1998; Fouchier et al. 1998) interact with the viral DNA genome to form the PIC for
integration. Interaction of the CA core with microtubule-associated proteins escorts the PIC to the nucleus (McDonald et al. 2002; Arhel et al. 2006; Sabo et al. 2013), particularly to the nuclear pore complexes (NPCs). Then, the PIC, covered by the partially disassembled CA core or the intact CA core, is translocated into the nucleus through the NPCs. Two components of NPCs, nucleoporin 358 (Nup358) and nucleoporin 153 (Nup153), are known to be important for this nuclear import. These Nups were identified as factors that facilitate HIV-1 infection, by three independent large-scale, genome-wide small interfering RNA (siRNA) screening experiments (Brass et al. 2008; König et al. 2008; Zhou et al. 2008). Nup358 is localized to the cytoplasmic side, whereas Nup153 has the nucleoplasmic position in the NPC structure. Nup358 has a C-terminal CypA-homology domain that binds to the CA core and isomerizes peptide bonds of the CA (Schaller et al. 2011; Bichel et al. 2013; Lin et al. 2013). Therefore, Nup358 is thought to function as a docking site of the CA core with the PIC, as well as to participate in the uncoating of the CA core through its isomerization function. However, this role of Nup358 needs to be clarified given that data have shown that the contribution of Nup358 to HIV-1 infection is independent on the CypA-homology domain (Meehan et al. 2014). Nup153 is suggested to be responsible for nuclear import of the PIC based on reports showing that Nup153 binds to HIV-1 CA (Matreyek et al. 2013), as well as others showing that the depletion of Nup153 decreases the nuclear entry of HIV-1 (Matreyek and Engelman 2011; Di Nunzio et al. 2012, 2013). Additionally,
researchers have suggested that the CA–CypA interaction influences the nuclear import mediated by the two Nups and the subsequent integration site selection of HIV-1, based on experiments employing a mutant CA or a chemical compound that disrupts the CA interaction with CypA (Schaller et al. 2011).

In addition to the Nups, the host proteins binding to HIV-1 CA also contribute to the nuclear import. Transportin proteins involved in the translocation of nuclear proteins from the cytoplasm to the nucleus are thought to be exploited by HIV-1 for the nuclear import. Transportin-1 (TRN-1 or TNPO1) is a karyopherin that transports nuclear proteins into the nucleus via interaction with nuclear localization signal (NLS) sequences (Twyffels, Gueydan, and Kruys 2014). A recent study reported that TRN-1 binds to the newly identified NLS of HIV-1 CA, which induces the CA uncoating and promotes the nuclear entry of the virus (Fernandez et al. 2019). Like TRN-1, transportin-3 (TNPO3) is a nuclear import receptor that mediates translocation of serine/arginine-rich mRNA splicing factor proteins into the nucleus (Kataoka, Bachorik, and Dreyfuss 1999). This protein was shown to directly interact with HIV-1 IN (Christ et al. 2008; Larue et al. 2012; Tsirkone et al. 2017) and HIV-1 CA (Zhou et al. 2011; Valle-Casuso et al. 2012). Also, depletion of TNPO3 was found to inhibit the nuclear import of HIV-1 (Christ et al. 2008; Valle-Casuso et al. 2012), suggesting that TNPO3 transports the interacting HIV-1 into the nucleus. Around the same time, cleavage and polyadenylation specific factor 6 (CPSF6), one of the proteins transported by TNPO3, was found to bind to HIV-1 CA (Lee et al. 2010; Price et
The following studies reported that HIV-1 inhibition by TNPO3 depletion is mainly due to the interaction with CPSF6 proteins trapped and accumulated in the cytoplasm (De Iaco et al. 2013; Fricke et al. 2013). Moreover, CA–CPSF6 interaction was recently reported to mediate the translocation of HIV-1 at the NPC based on the observed accumulation of HIV-1 molecules at the nuclear envelope when CPSF6 binding to CA was disrupted (Bejarano et al. 2019). Therefore, these studies imply that CPSF6 regulated by TNPO3 is important in the nuclear import of HIV-1, or that the CA–CPSF6 interaction enables HIV-1 to take advantage of TNPO3 to enter into the nucleus. Given that TNPO3 is capable of binding to HIV-1 IN and CA, the protein may also have its own role in the nuclear entry of HIV-1, independent of CPSF6.

1.2.4. HIV-1 Integration

Upon transportation of the PIC into the nucleus, IN of the PIC cleaves both ends of the viral DNA genome to expose 3′-hydroxyl groups (Fujiwara and Mizuuchi 1988; Bushman, Fujiwara, and Craigie 1990). This processing triggers the strand transfer reaction in which the reactive 3′-hydroxyl groups attack phosphodiester bonds of the host genomic DNA. Then, IN ligates the viral and host genomic DNAs, generating single-stranded regions flanking the viral genomes. The host DNA repair system is then recruited to complete the HIV-1 integration by filling these overhang regions, which results in the provirus establishment (Fujiwara and Mizuuchi 1988; Bushman, Fujiwara, and Craigie 1990).
In some cases, HIV-1 genomic DNAs fails to be integrated into the host genome. The linear double-stranded viral DNAs can either form 1-LTR circles by homologous recombination of LTRs at both ends or undergo non-homologous end joining to generate 2-LTR circles (Li et al. 2001). Thus, researchers measure the amount of 2-LTR circles to assess nuclear import of HIV-1 in experimental conditions.

Genomic analyses for the integration sites have shown that HIV-1 is preferentially integrated in actively transcribed regions (Schröder et al. 2002; Mitchell et al. 2004; Han et al. 2004). This is important for the expression of HIV-1 provirus, since proviruses located in heterochromatin regions are epigenetically repressed by host silencing machinery.

HIV-1 exploits host proteins to guide the PIC to the genomic regions. CPSF6, one of the CA-binding proteins, is a subunit of the CPSF complex that plays a role in the cleavage of the 3’-end of newly synthesized RNA transcripts and the subsequent formation of 3’-polyadenylated tails, which indicates that CPSF6 is recruited to the transcribed regions for RNA processing. Studies have shown that CA−CPSF6 interaction directs the CA-interacting PIC to the actively expressed genomic regions (Chin et al. 2015; Sowd et al. 2016; Achuthan et al. 2018). Moreover, lens epithelium-derived growth factor (LEDGF/p75) is a transcription factor that induces gene expression via binding to the promoter regions and is also one of the PIC components (Cherepanov et al. 2003; Emiliani et al. 2005). This transcription factor was thought to bring the interacting PIC to
the transcribed area of the host genome (Maertens et al. 2003; Ciuffi et al. 2005; Llano et al. 2006; Marshall et al. 2007; Shun et al. 2007). However, recent studies have reported that this protein indeed does not largely influence the localization of HIV-1 integration into the actively transcribed regions but rather the integration sites within the gene bodies in the regions, following the activity of CPSF6 (Sowd et al. 2016; Achuthan et al. 2018). These studies have suggested two distinct phases of HIV-1 integration: in the first stage, CPSF6 leads HIV-1 to the actively transcribed regions, and the second stage, LEDGF/p75 dictates the integration sites within those regions.

Furthermore, HIV-1 also utilizes NPCs for biased integration, in addition to a simple tunnel for nuclear import. The translocated promoter region (TPR) is a component of NPCs localized to the inner nuclear basket structure. This protein regulates chromatin organization by recruiting actively transcribed genes toward NPCs while excluding heterochromatin regions from NPCs. Therefore, this factor is suggested to promote viral integration by providing a more HIV-1-favorable chromatin condition, although this factor is not required for nuclear import of HIV-1 (Lelek et al. 2015; Wong, Mamede, and Hope 2015). The fact that the TPR stabilizes LEDGF/p75 at the nuclear periphery, and vice versa (Lelek et al. 2015), supports the role of the TPR in HIV-1 infection.

1.2.5. Expression of HIV-1 Provirus

The integrated HIV-1 provirus is expressed by the host transcription and
translation machinery. As described in section 1.1.2. HIV-1 Genome Structure, the 5’-LTR is a promoter for HIV-1 transcription. The 5’-LTR contains the TATA box (Garcia et al. 1989) sequence and binding sites for cellular transcription factors such as specificity protein 1 (Sp1) (Jones et al. 1986), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Nabel and Baltimore 1987), NFAT (Kinoshita et al. 1997; Cron et al. 2000), activator protein 1 (AP-1) (Van Lint, Burny, and Verdin 1991; Kharroubi and Verdin 1994), and TATA-binding protein (TBP) (Zenzie-Gregory et al. 1993). The RNA polymerase II-driven transcription by the binding of these cellular proteins on the 5’-LTR can be initiated but is inefficient. This produces a low level of RNA transcripts, most of which are short and incomplete and thus degraded quickly (Kao et al. 1987). However, a viral protein Tat can be expressed during this transcription stage (Kao et al. 1987). Tat interacts with a stem-loop structure of the regulatory element called TAR within the 5’-LTR region (Sodroski et al. 1985b; Feng and Holland 1988; Berkhout, Silverman, and Jeang 1989; Dingwall et al. 1989, 1990), which recruits positive transcriptional elongation factor b (P-TEFb) to the transcription complex (Zhu et al. 1997; Wei et al. 1998; Bieniasz et al. 1999). P-TEFb then phosphorylates the C-terminal domain of RNA polymerase II, which largely improves the transcription efficiency (O’Brien et al. 1994; Herrmann and Rice 1995; Chun and Jeang 1996; Okamoto et al. 1996; Yang, Herrmann, and Rice 1996; Mancebo et al. 1997).

The transcription of HIV-1 provirus generates three classes of transcripts:
i) multiply (fully) spliced transcripts encoding Tat, Rev, or Nef; ii) partially spliced transcripts encoding Vif, Vpr, or Vpu; and iii) unspliced transcripts encoding Gag, or Gag-Pol polyproteins (Kim et al. 1989; Purcell and Martin 1993; Karn and Stoltzfus 2012). Multiply spliced transcripts can be exported to the cytoplasm by cellular proteins like other cellular mRNAs, while unspliced or partially spliced transcripts cannot and are further spliced or degraded in the nucleus. Thus, in the beginning stage of provirus expression, only Tat, Rev, and Nef proteins are translated. After the Rev protein is produced, Rev enters the nucleus and binds to the regulatory element RRE of unspliced and partially spliced HIV-1 transcripts (Rosen et al. 1988; Hadzopoulou-Cladaras et al. 1989; Malim et al. 1989; Cochrane, Chen, and Rosen 1990; Holland et al. 1990; Kjems et al. 1991). This binding not only prevents the activity of cellular splicing factors on the RNA transcripts (Michael et al. 1991; Stutz and Rosbash 1994; Kalland et al. 1994; Powell et al. 1997) but also allows the RNAs to be exported from the nucleus via the chromosomal maintenance 1 (CRM1)-dependent pathway mediated by the interaction between Rev binding to the RNAs and CRM1 (Malim et al. 1989; Felber et al. 1989; Emerman, Vazeux, and Peden 1989; Brighty and Rosenberg 1994; Fischer et al. 1995; Churchill et al. 1996; Cullen 2003). Thus, upon Rev expression, the viral protein expression is shifted from regulatory proteins to structural proteins and accessory proteins. Therefore, Rev is a key protein in the progression of HIV-1 infection from early to late stage.
1.2.6. Assembly and Maturation of Progeny HIV-1 Particles

The exported HIV-1 RNA transcripts are translated to produce viral proteins as described in section 1.1.2. HIV-1 Genome Structure. Briefly, the Gag and Gag-Pol polyproteins are generated from one unspliced transcript by the programmed ribosomal translation frameshift over the slippery sequence as described (Jacks et al. 1988; Wills, Gesteland, and Atkins 1994; Dulude, Baril, and Brakier-Gingras 2002). Gp160 Env is produced in the endoplasmic reticulum and cleaved into gp120 SU and gp41 TM by cellular protease furin in the golgi apparatus (McCune et al. 1988; Willey et al. 1988; Hallenberger et al. 1992). The newly produced viral RNAs and proteins from HIV-1 provirus are assembled to generate the progeny virion particles. Among the products, Gag proteins play a central role in the virus assembly process. Gag and Gag-Pol are associated with lipid raft regions of the host cell plasma membrane through their MA parts (Bryant and Ratner 1990; Spearman et al. 1994; Ono, Orenstein, and Freed 2000; Hermida-Matsumoto and Resh 2000; Ono and Freed 2001; Saad et al. 2006). The MA also recruits Env proteins to the membrane regions via the MA–gp41 interaction when the Env proteins are bound to the plasma membrane through the host cell secretory pathway from the golgi apparatus (Dorfman et al. 1994; Cosson 1996; Murakami and Freed 2000). Moreover, the NC part of Gag and Gag-Pol proteins interacts with unspliced HIV-1 RNA genome by binding to the packaging signal (ψ) of the viral RNAs upon dimerization of the two viral RNA genome transcripts (Skripkin et al. 1994; Dannull et al. 1994; De Guzman et al.
This RNA–Gag interaction is also thought to facilitate the positioning of Gag at the host cell membrane (Kutluay and Bieniasz 2010). The p6 part of Gag assists the incorporation of Vpr into the virus particles (Bachand et al. 1999), which is necessary for the nuclear import of the PIC and viral evasion of host antiviral factors in the infection of the next target cells. In addition to the recruitment and packaging of other viral factors for virus assembly, Gag proteins also function in the budding and release of virus particles from the host cell membrane. They recruit the host ESCRT machinery via the interaction of their p6 parts with Tsg101 and ALIX, subunits of the cellular ESCRT complex (Garrus et al. 2001; Martin-Serrano, Zang, and Bieniasz 2001; Strack et al. 2003). This complex stabilizes HIV-1 bud formation induced by membrane-associated Gag proteins and excises the viral and cellular membranes to liberate the virus particles from the host producer cells (Bieniasz 2009).

During virus assembly and budding, viral accessory proteins are also recruited to the assembly sites to promote particle formation and block the incorporation of antiviral factors into the particles. Vif prevents the incorporation of APOBEC3G that largely mutates viral RNA genomes to inhibit HIV-1 infection (Sheehy et al. 2002). Vpu, a type I integral membrane protein, inhibits the activity of BST-2/Tetherin that binds to the viral membrane to block the virus release (Neil, Zang, and Bieniasz 2008; Van Damme et al. 2008). Vpu also degrades CD4 proteins that interfere with proper formation of the HIV-1 Env complex in the golgi apparatus (Willey et al. 1992; Margottin et al. 1998; Levesque, Zhao, and
Cohen 2003). Nef, a membrane-associated protein, thwarts the association of virus particles with SERINC3 and 5 proteins that hinder the entry of HIV-1 virions into the next target cells (Usami, Wu, and Göttlinger 2015; Rosa et al. 2015). Like Vpu, Nef also down-regulates the surface level of CD4 on the host cell membrane to increase the efficiency of virus budding and release (Garcia and Miller 1991; Craig, Pandori, and Guatelli 1998).

Soon after release from the host plasma membrane, the virus particles are immature and non-infectious (Wright et al. 2007; Briggs et al. 2009). They must undergo a maturation process to become mature, infectious virion particles. This process is primarily governed by the activity of viral PR, which is cleaved by autoproteolysis of the Gag-Pol proteins. Then, Gag and Gag-Pol are cleaved into functional forms by the PR-catalyzed proteolytic process (Kohl et al. 1988; Peng et al. 1989; Göttlinger, Sodroski, and Haseltine 1989; Wiegers et al. 1998). MA, CA, NC, and p6 are generated from Gag proteins, and RT and IN are produced from Pol proteins. This PR-mediated cleavage process triggers major rearrangement of the proteins inside the virus particle. MA continues to be associated with the envelope membrane in the interaction with gp41 TM proteins. CA forms pentamer and hexamer lattices to make a cone-shaped core structure encasing the viral RNA genome and proteins (Gross et al. 2000; Ganser et al. 1999). NC remains bound to the viral RNA genome inside the CA core to maintain the conformation for efficient infection and subsequent reverse transcription (Sundquist and Krausslich 2012; Freed 2015). This mature virion
particle infects new host target cells and undergoes the HIV-1 life cycle described above.

1.3. HOST INNATE AND INTRINSIC DEFENSE AGAINST HIV-1

During the replication cycle of HIV-1, there are a number of interactions between HIV-1 and host factors, some of which promote HIV-1 infection. For example, the HIV-1 CA core interacts with CypA in order to evade an endogenous antiviral factor, TRIM5α, which will be discussed in Chapter II (Kim et al. 2019). Interactions between CPSF6 and HIV-1 CA as well as between LEDGF/p75 and the viral PIC contribute to HIV-1 integration into actively transcribed regions of the host genome (Chin et al. 2015; Sowd et al. 2016; Achuthan et al. 2018). The ESCRT machinery is also used by HIV-1 to facilitate virus budding out of the host cells through the interaction between HIV-1 Gag p6 and subunits of the ESCRT (Garrus et al. 2001; Martin-Serrano, Zang, and Bieniasz 2001; Strack et al. 2003). HIV-1 exploits these host cellular proteins for its successful replication. On the other hand, some other interactions inhibit HIV-1 infection. These antiviral interactions mainly consist of two classes of interactions with pattern recognition receptors (PRRs) and intrinsic restriction factors.

PRRs are a class of proteins that trigger innate immune signaling upon their detection of invading pathogen-derived factors, such as viral nucleic acids and bacterial cell wall components, as a danger signal. The pathogen factors
recognized by PRRs are called pathogen-associated molecular patterns (PAMPs). PRRs are designed to efficiently detect common types of PAMPs shared by pathogens. For example, Toll-like receptor 4 (TLR4) detects lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria (Chow et al. 1999; O’Neill and Bowie 2007). Also, intracellular double-stranded RNAs found in infection by a variety of RNA viruses are recognized by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Loo et al. 2008; Loo and Gale Jr. 2011). PAMP recognition by PRRs stimulates the innate immune response. The LPS–TLR4 interaction activates the myeloid differentiation primary response 88 (MyD88)-mediated NF-κB and Toll/interleukin receptor (TIR)-domain-containing adaptor inducing interferon-β (TRIF)-mediated interferon regulatory factor 3 (IRF3) signaling pathways (Chow et al. 1999; O’Neill and Bowie 2007). RNA detection by either RIG-I or MDA5 also triggers the mitochondrial antiviral-signaling protein (MAVS)-mediated NF-κB and IRF3 signaling cascades (Loo et al. 2008; Loo and Gale Jr. 2011). These immune signaling events result in the production of cytokines including interferon (IFN) and interleukin 6 (IL-6). Therefore, PRRs do not directly block the infections but serve to establish cellular and environmental conditions resistant to the invading pathogens through the activation of immune responses upon PAMP detection.

The intrinsic restriction factors, on the other hand, mostly interact with viral factors, which directly inhibit specific steps of infection cycle. There are common
features shared among these factors (Malim and Bieniasz 2012; Duggal and Emerman 2012; Blanco-Melo, Venkatesh, and Bieniasz 2012; Harris, Hultquist, and Evans 2012). First, they possess autonomous and dominant activities that block virus infection. Thus, expression of the factors in cells largely inhibits viral infection. Secondly, they are constitutively expressed and/or their expression is further induced by activation of innate immune signaling. Third, they use their own unique mechanisms to inhibit specific stages of viral replication. Fourth, they are often antagonized by viral proteins. For example, as described in 1.1.2.3. **HIV-1 Accessory Proteins**, HIV-1 has strategies to evade restriction factors using viral accessory proteins that are not required for viral infection but counteract the host antiviral factors. These viral proteins directly interact with the host restriction factors, which induces degradation of the factors. Fifth, restriction factors display features of positive selection as a result of coevolution with viruses. The host experiences continued pressure from invading pathogens for a long time. Accordingly, host restriction factors attempt to avoid interactions with viral proteins in order to evade the antagonism by the viral factors. This process spontaneously selects variants of the host factor proteins, which results in a high ratio of non-synonymous mutations to synonymous mutations in regions within the interaction interface for viral proteins.

In this section, the innate and intrinsic defense systems of the host against HIV-1 infection will be discussed, with a specific focus on the restriction factors.
1.3.1. Innate Immune Sensing of HIV-1

As introduced above, the interactions of PRRs with HIV-1 PAMPs stimulate the innate immune response to produce an antiviral environment. The HIV-1 PAMPs detected by the PRRs cover a range of viral factors including glycoproteins, CA, and viral nucleic acids. Dependent on a PRR and the detected PAMP of HIV-1, the interactions occur at the cell surface or in the endosome, cytoplasm, or nucleus.

The TLRs are one type of PRRs recognizing HIV-1. TLR2 and TLR4 expressed on the cell surface detect HIV-1 glycoprotein gp120 as a PAMP to trigger NF-κB−mediated proinflammatory cytokine signaling (Nazli et al. 2013). Heparan sulfate, a noncanonical attachment receptor for HIV-1, is also required for this TLR−gp120 interaction. This was observed particularly in primary genital epithelial cells, and thus, researchers suggested that the detecting cells warn nearby cells of the HIV-1 invasion, even though these cells are not the main target of HIV-1 (Nazli et al. 2013). Additionally, TLR7 and TLR8 function to detect HIV-1 infection through the interaction with viral single-stranded RNAs in the endosome (Beignon et al. 2005; Meier et al. 2007; Gringhuis et al. 2010; O’Brien et al. 2011; Guo et al. 2014; Khatamzas et al. 2017; Meås et al. 2020). TLR7 is expressed in plasmacytoid dendritic cells, whereas TLR8 is present in myeloid dendritic cells, macrophages, and CD4+ T cells. These cells, with the exception of CD4+ T cells, express a limited level of surface CD4, and therefore, HIV-1 entry through CD4−gp120 interaction is less efficient. Instead, gp120 interacts
with a surface molecule, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), inducing internalization of the HIV-1 particle (Kwon et al. 2002). During this endocytosis, TLR7 or TLR8 located on the endosomal membrane binds to single-stranded RNA molecules of HIV-1. This detection activates the MyD88-mediated cytokine and IRF7-mediated IFN signaling pathways. In addition to these well-known TLRs, TLR10 was also recently reported to be among the TLRs that act against HIV-1 (Henrick et al. 2019). This TLR was shown to be expressed at higher levels in the breast milk cells of HIV-1–infected women compared with the cells of uninfected women. In subsequent experiments using human cell lines, TLR10 was found to activate IL-8 and NF-κB signaling upon its detection of HIV-1 gp41.

Viral nucleic acids can also be detected by PRRs in the cell cytoplasm. The HIV-1 RNA genome harbors many secondary structures (Watts et al. 2009), which can be detected by intracellular RNA sensors. Several reports have shown that RIG-I, but not MDA5, described above recognizes HIV-1 genomic RNAs to trigger MAVS-dependent innate immune signaling in monocytes and macrophage cell lines (Solis et al. 2011; Berg et al. 2012). HIV-1 PR was also shown to decrease the level of cytoplasmic RIG-I via lysosome-mediated degradation for viral evasion (Solis et al. 2011). However, because these studies were mainly performed by transfection of HIV-1 RNAs, examination with virion particles is required to confirm the role of RIG-I in HIV-1 infection. In addition to RIG-I, DEAD box-containing RNA helicase DDX3 also recognizes HIV-1 RNAs in
primary dendritic cells (Gringhuis et al. 2016). This PRR detects ‘abortive’ RNAs transcribed from HIV-1 provirus, rather than the invading HIV-1 genomic RNAs. Specifically, upon HIV-1 integration into the host genome, the initial provirus transcription produces short, abortive transcripts because of the very low efficiency (Kao et al. 1987; Gringhuis et al. 2010). These transcripts can be detected by DDX3, which activates the MAVS-dependent signaling pathway. However, HIV-1 has already established its evasion strategy when the virus begins to infect dendritic cells. During virus entry, HIV-1 gp120 interacts with the surface DC-SIGN of dendritic cells for viral endocytosis, due to low surface expression of CD4. This gp120–DC-SIGN interaction induces Raf1-dependent activation of the mitotic kinase polo-like kinase 1 (PLK1). The activated PLK1 binds to MAVS, preventing the recruitment of downstream signaling factors, such as tumor necrosis factor receptor-associated factor 3 (TRAF3), and thus, blocking subsequent immune signaling via MAVS.

In addition to viral RNAs, HIV-1 DNA generated by reverse transcription is also a viral PAMP. cGAS is known to play a main role in detecting this PAMP. cGAS is a cytosolic nucleic acid sensor that recognizes exogenous double-stranded DNAs and RNA:DNA hybrids and then synthesizes cyclic GMP-AMPs (cGAMPs), as the secondary messenger, in order to activate the stimulator of interferon genes (STING)-mediated pathway to produce type I IFNs (Sun et al. 2013; Wu et al. 2013; Mankan et al. 2014). cGAS sensing of HIV-1 DNA was first described in a study showing that cGAS mediates IFN-β induction by HIV-1
infection, which can be blocked by the inhibition of reverse transcription (Gao et al. 2013). This study also examined macrophages and dendritic cells expressing a restriction factor called sterile alpha motif (SAM) domain and histidine aspartate (HD) domain-containing protein 1 (SAMHD1) that possesses enzymatic activity to decrease cytoplasmic dNTP levels. This function largely inhibits HIV-1 reverse transcription in the cells (Lagouette et al. 2011; Hrecka et al. 2011). The study also found that SAMHD1 inhibition in macrophages and dendritic cells enables cGAS to detect HIV-1 DNA. Moreover, three prime repair exonuclease 1 (TREX1) was also identified to contribute to HIV-1 evasion of cGAS by digesting accumulated HIV-1 DNAs that are sensed by the PRR (Yan et al. 2010; Gao et al. 2013; Kumar et al. 2018). All of these findings support that cGAS detects HIV-1 DNA as the product of reverse transcription. Furthermore, polyglutamine binding protein 1 (PQBP1) was revealed to be a cofactor for the recognition of HIV-1 DNA by cGAS (Yoh et al. 2015).

Viral CA has been suggested to be involved in cGAS sensing of HIV-1 DNA. Researchers have observed that HIV-2 CA is more sensitive to recognition by cGAS than HIV-1 CA in dendritic cells (Lahaye et al. 2013). This was supported by a recent study showing that non-POU domain-containing octamer-binding protein (NONO) is an essential cofactor for cGAS sensing of HIV in the nucleus, and that NONO binds to HIV-2 CA with higher affinity than HIV-1 CA in macrophages and dendritic cells (Lahaye et al. 2018). Recent studies have shown that the HIV-1 CA destabilization by a chemical compound causes cGAS
to detect HIV-1 DNA, suggesting that HIV-1 CA protects the viral DNA genome from cGAS (Siddiqui et al. 2019; Sumner et al. 2019). This may imply that NONO binding to HIV CA negatively affects the stability of CA to result in detection by cGAS. In addition, a report suggested that HIV-1 was detected by cGAS when the CA–CypA interaction was disrupted. Considering that CA–CypA interaction stabilizes HIV-1 CA, loss of this interaction would cause CA destabilization, which could enable cGAS to sense HIV-1 DNA, in accordance with the studies described above. Furthermore, the first study described in this paragraph also showed that substitutions of several residues in HIV-1 CA with ones of HIV-2 CA renders the HIV-1 CA vulnerable to cGAS sensing (Lahaye et al. 2013). Intriguingly, this substitution increases CypA binding to the mutant HIV-1 CA. This seems counterintuitive, because CypA is known to stabilize HIV-1 CA and disruption of CypA binding to the CA was found to render HIV-1 detectable by cGAS. However, given that the ratio of CypA to HIV-1 CA can change the in vitro CA morphology (Liu et al. 2016), disruption of the optimal ratio due to the increased CypA interaction caused by the mutation may affect CA stability. Hence, all of these observations suggest that CA plays an important role in shielding HIV-1 from detection by cGAS.

Interferon gamma-inducible protein 16 (IFI16), a cellular DNA sensor, is additionally suggested to detect HIV-1 DNA, resulting in the induction of a STING-dependent immune response like cGAS (Jakobsen et al. 2013). Particularly, this protein was also observed to increase cGAMP production upon
exogenous DNA stimulation, which indicates that IFI16 and cGAS may function together to detect HIV-1 DNA (Jønsson et al. 2017). Further investigations are needed to examine the collaborative relationship between IFI16 and cGAS in HIV-1 infection and the mechanism by which IFI16 sensing regulates cGAMP production by cGAS.

In addition to the research focused on known PRRs described above, reports have demonstrated innate immune responses against HIV-1 by unidentified PRRs. First, binding of CypA to newly generated viral CA proteins expressed from provirus induces immune signaling during HIV-1 infection of dendritic cells and T cells (Manel et al. 2010). Moreover, the loss of HIV-1 CA interaction with CPSF6 was shown to activate NF-κB- and IRF3-mediated signaling pathways independently of cGAS (Rasaiyaah et al. 2013). However, the PRRs determining the immune response in these two studies have yet to be identified.

The antiviral immune response induced upon detection of HIV-1 by PRRs is important to defend against the infection. This is the reason why HIV-1 becomes a poor IFN inducer in antigen-presenting cells like dendritic cells (Cingöz and Goff 2019). As described above, HIV-1 utilizes its CA and host proteins, including SAMHD1, TREX1 and DC-SIGN-mediated signaling proteins, to evade the PRRs. These strategies inhibit viral infection of dendritic cells but enable HIV-1 to be hidden from the host immune system, in contrast to HIV-2, which can infect and activate these cells. This may explain why HIV-1, but not
HIV-2, has caused a pandemic. However, at the same time, HIV-1 indeed exploits this immune activation to enhance its proviral transcription in CD4+ T cells (Nabel and Baltimore 1987; Brooks et al. 2003; Kim et al. 2006). Thus, HIV-1 ingeniously takes advantage of the host immune system, in a cell type-specific manner, for its successful infection and progress.

1.3.2. Intrinsic Restriction Factors against HIV-1

In this section, each of the known intrinsic restriction factors will be described and discussed. A restriction factor TRIM5α will be discussed in 2.2.2.

Capsid-binding Restriction Factor TRIM5α and HIV-1.

1.3.2.1. APOBEC3G

A HIV-1 accessory protein Vif is required for viral replication in CD4+ T cells, monocyte-derived macrophages, and a limited number of cell lines that are non-permissive. HIV-1 particles devoid of Vif are non-infectious when they are produced by these non-permissive cells, whereas the HIV-1 particles produced by the permissive cell lines are infectious (Gabuzda et al. 1992; Schwedler et al. 1993; Madani and Kabat 1998; Simon et al. 1998). Particularly, studies showed that heterokaryon cells made by fusion of non-permissive cells with permissive cells turn out to be non-permissive to Vif-deficient HIV-1, suggesting that non-permissive cells express an antiviral factor that possesses a dominant activity and is counteracted by Vif (Madani and Kabat 1998; Simon et al. 1998). A
subsequent study identified APOBEC3G (also known as CEM15) as the factor and also found that Vif prevents incorporation of this protein into newly produced HIV-1 particles (Sheehy et al. 2002).

Since then, researchers have reported that Vif specifically interacts with APOBEC3G prior to incorporation into virus particles and subsequently recruits an E3 ubiquitin ligase complex comprised of Cullin-5, elongin B, elongin C, and RING-box protein 1, which promotes ubiquitination of the target APOBEC3G and subsequent proteasomal degradation (Yu et al. 2003; Mariani et al. 2003; Sheehy, Gaddis, and Malim 2003; Mehle et al. 2004, 2006). The interaction of HIV-1 Vif with a host protein core binding factor β (CBF-β) was also shown to be essential for Vif-mediated degradation of APOBEC3G by enhancing the stability of Vif and the activity of Vif-associated E3 ubiquitin ligase complex (Jäger et al. 2011; Zhang et al. 2011; Hultquist et al. 2012; Kim et al. 2013).

APOBEC3G belongs to the APOBEC family of cytidine deaminase proteins (Harris, Petersen-Mahrt, and Neuberger 2002; Jarmuz et al. 2002). Among the seven known APOBEC3 proteins, APOBEC3B, APOBEC3D, APOBEC3F, APOBEC3H, and APOBEC3G have an antiviral activity against HIV-1 infection (Desimmie et al. 2014). HIV-1 Vif is capable of counteracting all of these antiviral APOBECs, particularly via its different motifs specific to each APOBEC, which implies that HIV-1 evolves to efficiently evade the host APOBEC-mediated restriction (Simon et al. 2005; Russell and Pathak 2007; Ooms et al. 2013; Refsland 2013; Desimmie et al. 2014). APOBEC3G is the
most well understood among the anti-HIV-1 APOBECs, because it has been the focus of most related studies since its discovery (Sheehy et al. 2002). The APOBEC proteins are expressed at a high level in CD4+ T cells, macrophages, mature dendritic cells, and other cells that are non-permissive. The most abundant APOBEC protein is APOBEC3G. Their expression is also induced by IFN stimulation (Koning et al. 2009; Refsland et al. 2010).

As described above, APOBEC3G in non-permissive cells is incorporated into HIV-1 particles through its interaction with viral nucleocapsid and nucleic acids when it is not antagonized by Vif (Zennou et al. 2004; Apolonia et al. 2015). Upon infection by the HIV-1 particles, APOBEC3G, along with viral proteins and the RNA genome, is translocated from virions into the cytoplasm of target cells. This factor then initiates its restriction activity during viral reverse transcription (Mariani et al. 2003; Lecossier et al. 2003; Harris et al. 2003; Mangeat et al. 2003; Zhang et al. 2003; Suspène et al. 2004; Bishop et al. 2004). APOBEC3G introduces C-to-U mutations into the newly synthesized minus-strand HIV-1 DNA through its cytidine deaminase function. These U nucleotides are matched to A nucleotides in the complementary plus strand of the viral DNA generated by DNA-dependent DNA polymerase activity of HIV-1 RT. Thus, APOBEC3G-mediated deamination results in a high frequency of G-to-A hypermutations in the HIV-1 DNA genome at completion of viral reverse transcription. The aberrant sequences resulting from the mutations in HIV-1 DNAs can be detected and degraded by cellular factors. Furthermore, these mutations are highly likely to
cause premature stop codons as well as missense mutations in the HIV-1 genome. Thus, even though HIV-1 DNAs affected by APOBEC3G can establish the proviruses in the host genome, they fail to produce infectious virus particles due to those mutations (Lecossier et al. 2003; Harris et al. 2003; Mangeat et al. 2003; Zhang et al. 2003; Suspène et al. 2004; Bishop et al. 2004). An investigation also showed that this activity of APOBEC3G can also introduce G-to-A mutations into the region of an RNA regulatory element TAR, resulting in inhibition of transcription of the HIV-1 provirus (Nowarski et al. 2014). In addition, studies suggested that the host uracil base excision repair (UBER) proteins are involved in the degradation of HIV-1 DNAs that contain APOBEC3G-derived hypermutations (Yang et al. 2007; Pollpeter et al. 2018). However, this is in conflict with other studies (Kaiser and Emerman 2006; Langlois and Neuberger 2008), and thus, this role of UBER enzymes remains to be determined.

APOBEC3G is also suggested to possess antiviral activities independent of its deaminase function (Newman et al. 2005; Luo et al. 2007; Guo et al. 2007a; Iwatani et al. 2007; Bishop et al. 2008; Gillick et al. 2013; Chaurasiya et al. 2014). These activities inhibit: i) elongation of viral reverse transcription (Iwatani et al. 2007; Bishop et al. 2008; Gillick et al. 2013; Chaurasiya et al. 2014); ii) annealing of transfer RNA_{Lys} (tRNA_{Lys}) to the primer binding site in HIV-1 genomic RNA (Guo et al. 2007a); and iii) integration of HIV-1 DNAs into the host genome (Luo et al. 2007). In contrast, other studies demonstrated that the catalytic deamination activity of APOBEC3G is required for its HIV-1 restriction
based on experiments with mutant APOBEC3Gs lacking this enzymatic function (Miyagi et al. 2007; Schumacher et al. 2008; Browne, Allers, and Landau 2009). Thus, further investigations are needed to clarify the deaminase-independent activity of APOBEC3G.

Previous studies reported that hypermutated HIV-1 provirus genomes are found in acutely and chronically HIV-1 infected patients and vertically infected infants. The results from those studies imply that a small amount of APOBEC3G could be incorporated into HIV-1 particles even in the presence of HIV-1 Vif during HIV-1 infection (Desimmie et al. 2014; Kim et al. 2014). This might be due to the presence of Vif variants that differ in their ability to counteract APOBEC3G or the IFN-induced high expression of APOBEC3G that would be sufficient to overcome antagonism by Vif. Indeed, certain levels of mutagenesis on the HIV-1 genome by the small amount of APOBEC3G, which are not lethal to the virus, seem to be beneficial to the virus, because these mutations could provide HIV-1 with genetic diversity and thus enable HIV-1 to acquire resistance to anti-HIV-1 drugs. This hypothesis is supported by several observations that antiretroviral drug-resistant HIV-1 emerged due to the APOBEC3G-mediated mutagenesis in experiments with cultured cells (Mulder, Harari, and Simon 2008; Kim et al. 2010), and that failure of antiretroviral treatment in some HIV-1-infected patients was associated with the appearance of drug-resistant viruses bearing partially defective Vif variants (Fourati et al. 2010). Therefore, HIV-1 may exploit a host restriction factor APOBEC3G to obtain genetic variability to facilitate its evolution.
However, this is possible only if the deaminase function of APOBEC3G introduces a sublethal level of mutations into the HIV-1 genome. In addition, the fact that all primate lentiviruses encode Vif may imply that APOBEC3G-mediated restriction is fatal to replication of the lentiviruses including HIV-1 (Strebel et al. 1987). Thus, therapeutic approaches that use a potent activity of APOBEC3G via disruption of the Vif-mediated counteraction may have a high potential to effectively suppress HIV-1 infection, as well as to prevent the APOBEC3G-derived evolution of HIV-1. Considering that some reported drug compounds can disrupt the interaction between Vif and E3 ubiquitin ligase complex (Nathans et al. 2008; Huang et al. 2013a; Huang et al. 2013b; Zhang et al. 2015; Miyakawa et al. 2015), the use of such compounds could contribute to the development of APOBEC3G-based therapeutics for treatment of HIV-1.

1.3.2.2. SAMHD1

HIV-1 targets cells that express surface CD4 molecules. These cells include CD4+ T cells and myeloid cells such as dendritic cells and macrophages. Although activated CD4+ T cells are susceptible to HIV-1 infection, resting CD4+ T cells and the myeloid cells are indeed resistant to HIV-1 infection (Stevenson et al. 1990; Zack et al. 1990; Nègre et al. 2000; Pierson et al. 2002; Ganesh et al. 2003; Plesa et al. 2007; Kaushik et al. 2009; Dai et al. 2009). In contrast, other lentiviruses, especially SIVmac and HIV-2 are able to efficiently infect the cells that are refractory to HIV-1. Intriguingly, infection of those cells by SIVmac and
HIV-2 was shown to be blocked when a viral accessory protein viral protein X (Vpx) was deleted in these two viruses (Guyader et al. 1989; Yu et al. 1991). In line with this observation, transfer of a Vpx protein was observed to render these cells susceptible to HIV-1 infection (Goujon et al. 2006). Thus, these results indicate that Vpx enables the viruses to overcome an inhibitory activity present in resting CD4+ T cells and myeloid cells. The Vpx proteins expressed in cells comprise a E3 ubiquitin ligase complex with cellular DNA damage-binding protein 1 (DDB1), Cullin4 (CUL4), and DDB1 and CUL4-associated factor 1 (DCAF1) that promotes ubiquitination of target proteins to induce proteasome-mediated degradation of the targets (Le Rouzic et al. 2007; Srivastava et al. 2008). This suggests that Vpx may antagonize an activity of a host protein by specifically binding to the protein and facilitating its degradation. Two independent groups using tandem affinity chromatography or immunoprecipitation followed by mass spectrometry-based analysis identified SAMHD1 as the inhibitory factor targeted by the viral Vpx (Laguette et al. 2011; Hrecka et al. 2011). Vpx was further shown to interact with the C-terminal region of SAMHD1 and then to recruit the E3 ligation complex in order to remove the interacting SAMHD1 (Ahn et al. 2012).

SAMHD1 is expressed at a high level in resting CD4+ T cells and myeloid cells (Laguette et al. 2011; Descours et al. 2012; Baldauf et al. 2012). SAMHD1 consists of an N-terminal sterile alpha motif (SAM) domain that functions for protein–protein interaction and protein–nucleic acid interaction and a C-terminal histidine aspartate (HD) domain responsible for the following catalytic activities:
deoxyguanosine triphosphate (dGTP)-activated deoxynucleoside triphosphate phosphohydrolase (dNTPase), exonuclease, and phosphodiesterase (Aviv et al. 2003; White et al. 2013a). The dNTPase activity of SAMHD1 is thought to be essential for its restriction activity against lentiviral infection in resting CD4+ T cells and myeloid cells. The pool of intracellular dNTPs is required for reverse transcription of lentiviruses including HIV-1, HIV-2, and SIVmac. The catalytic activity of SAMHD1 hydrolyzes all of four cellular deoxynucleoside triphosphate (dNTPs) to deoxynucleosides and triphosphate, which results in a large decrease in the dNTP pool in the cells (Goldstone et al. 2011; Powell et al. 2011; Lahouassa et al. 2012). Thereby, the dNTPase activity of SAMHD1 is able to inhibit viral infection (Baldauf et al. 2012; Lahouassa et al. 2012). The presence of Vpx could thus rescue the inhibited infection by promoting degradation of SAMHD1, as previously observed (Lagouette et al. 2011; Hrecka et al. 2011; Lahouassa et al. 2012). The importance of the dNTPase function in SAMHD1-mediated restriction is also supported by a study showing that an increase in the intracellular dNTP concentration by addition of exogenous deoxynucleosides rescues HIV-1 infectivity in dendritic cells (Baldauf et al. 2012; Lahouassa et al. 2012). An investigation also consistently reported that this activity of SAMHD1 is capable of restricting a variety of retroviruses except a prototype foamy virus that can complete its reverse transcription predominantly in the virus particle prior to virus entry into target cells (Gramberg et al. 2013).

The dNTPase function controlling the amount of intracellular dNTPs plays
an important role in the cell cycle, because cells need the dNTPs as building blocks of their genome during proliferation. Thus, the expression level of SAMHD1 protein is regulated according to the status of cells (Dragin et al. 2015; Ruffin et al. 2015). In addition to the monomer or dimer forms, SAMHD1 exists as a tetramer for its full dNTPase activity in the presence of dGTP, and this tetramerization turns SAMHD1 into a stable and long-lived protein (Amie, Bambara, and Kim 2013; Brandariz-Nuñez et al. 2013; White et al. 2013b; Hansen et al. 2014; Ji et al. 2014; Koharudin et al. 2014; Li et al. 2015; Cardamone et al. 2017). Thus, the activity of SAMHD1 present in cells is also dependent on the cell cycle stage. Accordingly, SAMHD1 proteins were observed to be phosphorylated by cyclin-dependent kinases (CDKs) in dividing cells, such as activated CD4+ T cells, that do not have the restriction activity of SAMHD1. The phosphorylation was found on the threonine residue at position 592 within its catalytic HD domain (Cribier et al. 2013; White et al. 2013b; Pauls et al. 2014; Yan et al. 2015; Ruiz et al. 2015) and could be reversed by a CDK inhibitor called p21Cip1/Waf1 (Valle-Casuso et al. 2017). This post-translational modification by phosphorylation is suggested to hamper the stability of SAMHD1 by disrupting the tetramer forms, causing the loss of its dNTPase function linked to its restriction activity (Cribier et al. 2013; Pauls et al. 2014; St. Gelais et al. 2014; Arnold et al. 2015; Tang et al. 2015; Patra, Bhattacharya, and Bhattacharya 2017). However, this is controversial, because some studies reported that the phosphorylation of SAMHD1 does not affect the dNTPase activity (White et al.
2013b; Welbourn et al. 2013). The results from the latter studies proposed that another enzymatic function of SAMHD1 is also involved in its antiviral activity, in addition to the dNTPase function.

SAMHD1 was observed to possess an exonuclease function against single-stranded RNAs and single-stranded DNAs, which was suggested to participate in its antiviral activity (Goncalves et al. 2012; Tüngler et al. 2013; Beloglazova et al. 2013; Welbourn and Strebel 2016). In a study, researchers introduced either of two point mutations into SAMHD1 to separate its dNTPase and RNase functions, in order to examine which function contributes to the restriction activity. Interestingly, they observed that a mutant SAMHD1 deficient for dNTPase function still inhibited HIV-1, whereas the other mutant without RNase function lost its antiviral activity against the virus, suggesting that the RNase function, not the dNTPase function, of SAMHD1 is important for the restriction activity (Ryoo et al. 2014). This could imply that SAMHD1 targets the genomic RNAs or reverse transcription intermediates of incoming HIV-1 for degradation. However, this hypothesis is in conflict with observations that addition of Vpx or exogenous deoxynucleosides at 6 to 24 hours after infection rescues HIV-1 infection previously inhibited by SAMHD1 (Hofmann et al. 2013; Mlcochova et al. 2014), considering the timing of reverse transcription, which initiates within minutes after virus entry (Perez-Caballero et al. 2005a).

Furthermore, other researchers could not observe the importance of the RNase function in the restriction activity of SAMHD1 (Seamon et al. 2015; Antonucci et
al. 2016). Thus, the involvement of the exonuclease function in SAMHD1-mediated restriction remains unclear.

Recently, host proteins that contribute to virus evasion of SAMHD1-mediated restriction were found. CD81 plays an important role in T-cell activation (Rocha-Perugini et al. 2013), and its expression is increased in activated CD4+ T cells that are permissive to HIV-1 infection (Witherden, Boismenu, and Havran 2000). This molecule was recently identified to promote proteasomal degradation of SAMHD1 via direct interaction, which enhances intracellular dNTP levels and thus, reverse transcription of HIV-1 (Rocha-Perugini et al. 2017). Additionally, the proviral integration sites for the moloney murine leukemia virus (PIM) family of proto-oncogene serine/threonine-protein kinases, including PIM1 and PIM3, were discovered to phosphorylate viral Vpx, which increases the antagonistic activity against SAMHD1 (Miyakawa et al. 2019).

Even though SAMHD1 is a potent restriction factor expressed in the target cells, HIV-1 does not evolve to have a Vpx that degrades the factor, differently from HIV-2. Considering the difference in the prevalence of these two viruses (Reeves and Doms 2002; Campbell-Yesufu and Gandhi 2011), HIV-1 may have an advantage over HIV-2 based on its inhibition by SAMHD1. In accordance with this hypothesis, previous studies demonstrated that the restriction activity of SAMHD1 that blocks viral reverse transcription enables HIV-1 to evade sensing by cGAS, antigen presentation by infected cells, and the activity of cytotoxic T cells in vivo (Gao et al. 2013; Ayinde et al. 2015; Maelfait et al. 2016). In addition,
recent studies also demonstrated that SAMHD1 itself is able to downregulate the innate immune signaling pathways induced by invading viruses including HIV-1 (Chen et al. 2018; Qin et al. 2020). Considering these results, unsuccessful HIV-1 infection in myeloid cells may be a strategy of the virus to circumvent the host immune system. As a part of this strategy, HIV-1 may evolve without Vpx so that the virus exploits the high level of SAMHD1 expression in myeloid cells.

1.3.2.3. BST-2/Tetherin

Previous studies showed that HIV-1 particles devoid of Vpu have a smaller number of surface glycoproteins on their envelope and accumulate at the surface of infected cells, rather than being released from the cells (Strebel et al. 1989; Terwilliger et al. 1989; Klimkait et al. 1990). Subsequent investigations found that CD4 molecules interact with viral glycoproteins in the golgi apparatus, which interrupts proper formation of the surface glycoproteins. Vpu was shown to rescue these glycoproteins by inducing proteasomal degradation of the CD4s to prevent their interactions with the viral proteins (Willey et al. 1992; Margottin et al. 1998; Levesque, Zhao, and Cohen 2003; Bour and Strebel 2003). This function of Vpu was shown to be separate from the second observation of HIV-1 particles entangled on the cell surface based on mutational analysis of Vpu. The inhibitory activity against release of HIV-1 devoid of Vpu was more potent upon IFN stimulation (Neil et al. 2007) and found to vary among different cell lines (Neil et al. 2006; Hussain et al. 2008). Particularly, the heterokaryon cells resulting
from fusion of cells that are permissive and ones that are non-permissive to HIV-1 without Vpu became non-permissive, which indicates that this antiviral activity is dominant (Varthakavi et al. 2003). In addition, HIV-1 particles dangling on the cell membrane were released by a protease treatment (Neil et al. 2007). Thus, these results suggested that Vpu counteracts the antiviral activity of an unknown factor that blocks release of progeny HIV-1 virus particles from infected cells.

Two independent studies identified BST-2/Tetherin (also known as CD317) as the Vpu-sensitive antiviral factor, based on a comparison of gene expression profiles between the permissive and non-permissive cells (Neil, Zang, and Bieniasz 2008; Van Damme et al. 2008). These studies confirmed the IFN-inducible expression of the factor and its dominant restriction activity by observing that transfection of permissive cells with BST-2/Tetherin-encoding DNA converted the cells to be non-permissive to Vpu-deficient HIV-1.

BST-2/Tetherin is a type II transmembrane protein that contains a short N-terminal cytoplasmic domain, a transmembrane domain, a coiled-coil alpha helical extracellular domain that undergoes homodimerization, and a C-terminal glycosylphosphatidylinositol (GPI) membrane anchor (Hinz et al. 2010; Venkatesh and Bieniasz 2013). When virus budding occurs, this protein is incorporated into virus particles. One end of the protein remains on the viral envelope membrane, and the other end simultaneously attaches to the infected cell membrane, which prevents release of HIV-1 particles from cells. This process was demonstrated to be dependent on the topology, rather than on the
primary sequence of BST-2/Tetherin, based on mutational analyses and domain replacement experiments (Perez-Caballero et al. 2009). Additionally, BST-2/Tetherin was shown to preferentially bind to the viral membrane via its C-terminal GPI anchor domain, rather than with a random orientation of binding to the two membranes (Venkatesh and Bieniasz 2013).

The HIV-1 particles entrapped by BST-2/Tetherin undergo endocytosis, followed by lysosomal degradation (Neil et al. 2006; Miyakawa et al. 2009). To counteract this antiviral activity, HIV-1 Vpu binds to BST-2/Tetherin through the transmembrane domain-to-transmembrane domain interaction. This promotes ubiquitination of the transmembrane domain of BST-2/Tetherin but also induces internalization of the protein from the cell membrane to the trans-Golgi network and subsequent sequestration in a perinuclear endosomal compartment for proteasomal and/or lysosomal degradation (Iwabu et al. 2009; Douglas et al. 2009; Mangeat et al. 2009; Mitchell et al. 2009; Dubé et al. 2010; Tervo et al. 2011). A non-canonical autophagy pathway was also reported to be involved in the activity of Vpu against BST-2/Tetherin linked to the lysosomal degradation (Madjo et al. 2016). This process driven by Vpu in turn downregulates the surface level of BST-2/Tetherin to enable HIV-1 particles to evade the restriction activity. Recent studies identified additional factors involved in the activities of BST-2/Tetherin and HIV-1 Vpu: the lymphocyte-specific protein 1 that supports BST-2/Tetherin by promoting endocytosis of the trapped HIV-1 (Kulkarni et al. 2020); and the V0 subunit C of vacuolar ATPase that assists HIV-1 Vpu by
facilitating sequestration of BST-2/Tetherin (Waheed et al. 2020). Indeed, BST-2/Tetherin has two isoforms. The short isoform lacks the first 12 residues found in the long isoform. Both isoforms form homo- and heterodimers and are able to entangle HIV-1 particles. Compared with the long isoform, the short isoform was shown to be less sensitive to Vpu antagonism due to its deficiency of N-terminal residues, and thus, to more effectively prevent HIV-1 release (Cocka and Bates 2012).

BST-2/Tetherin also functions to activate innate immune signaling during its entrapment of virus particles, in addition to the blockage of virus release. When the protein interacts with the virus particles for the restriction, the dual tyrosine motif within its cytoplasmic domain is phosphorylated by the tyrosine kinase Syk (Galão et al. 2012; Cocka and Bates 2012; Tokarev et al. 2013; Galão et al. 2014). This phosphorylated BST-2/Tetherin recruits a signaling complex consisting of TRAF2, TRAF6, and Ubc13 to activate the transforming growth factor-β-activated kinase 1 (TAK1), which triggers NF-κB–dependent signaling to produce proinflammatory cytokines and type I IFN (Galão et al. 2012; Tokarev et al. 2013; Galão et al. 2014). However, this activity of BST-2/Tetherin as an innate immune sensor can also be counteracted by HIV-1 Vpu, which reduces the protein on the cell surface (Galão et al. 2012; Tokarev et al. 2013; Galão et al. 2014). Given that the short isoform of BST-2/Tetherin is less susceptible to the activity of Vpu, this isoform may be able to activate the immune signaling by avoiding the Vpu, differently from the long isoform. This
short BST-2/Tetherin, however, is not able to induce the innate immune response, because the dual tyrosine motif required for this activity is missing in this isoform (Cocka and Bates 2012).

The HIV-1 evasion of BST-2/Tetherin–mediated restriction is suggested to be critical for HIV-1 spread in vivo and the transmission that contributes to the pandemic. Genomic analyses of vpu gene repertoires from HIV-1 patients showed that Vpu maintained the functions of downregulating CD4 and counteracting BST-2/Tetherin, despite a large increase in variations in the amino acid sequences of Vpu over the course of infection (Pickering et al. 2014). In addition, an epidemiological study suggested that the anti-BST-2/Tetherin activity of Vpu is important for human-to-human transmission based on findings that all the Vpus of pandemic, transmitted/founder HIV-1 in this study possessed the counteracting activity against BST-2/Tetherin (Iwami et al. 2015). The importance of this Vpu activity was also experimentally demonstrated with a humanized mouse model in which the anti-BST-2/Tetherin function is required for efficient spread during the early phase of HIV-1 infection in vivo (Yamada et al. 2018). Furthermore, the antiviral activity of BST-2/Tetherin was observed to render HIV-1–infected cells susceptible to antibody-dependent cellular toxicity, which can be counteracted by HIV-1 Vpu. This finding may also support the crucial role of this restriction factor in the spread of HIV-1, with consideration of adaptive immunity in vivo (Arias et al. 2014; Richard et al. 2017). Therefore, BST-2/Tetherin and HIV-1 Vpu represent therapeutic targets with high potential for the efficient
1.3.2.4. Myxovirus Resistance Protein 2 (Mx2/MxB)

Mx2 was identified as a restriction factor against HIV-1 by three independent studies, based on results from gene expression profiling of cell lines that show differential levels of HIV-1 inhibition by IFN stimulation (Goujon et al. 2013; Kane et al. 2013), as well as from an examination of the restriction activity of Mx proteins against HIV-1 infection (Liu et al. 2013). The studies confirmed that Mx2 possesses dominant antiviral activity via experiments with cells that overexpress Mx2, and that endogenous Mx2 expression is not detectable but induced by IFN stimulation. They also showed that Mx2-mediated restriction does not affect reverse transcription but reduces the amount of nuclear HIV-1 DNA, indicating that Mx2 inhibits nuclear import of HIV-1 (Goujon et al. 2013; Kane et al. 2013). In addition, the restriction activity of Mx2 was observed to be dependent on HIV-1 CA based on differential levels of restriction against CA containing mutations, including one that disrupts CA–CypA interaction (Goujon et al. 2013; Kane et al. 2013). Accordingly, a study also suggested that Mx2 acts in a CypA-dependent mechanism by demonstrating that HIV-1 restriction by Mx2 is inhibited when CA–CypA interaction is disrupted by a CypA inhibitor compound or CypA knockdown, in addition to the CA mutation (Liu et al. 2013).

Subsequent investigations of the in vitro assembled CA structure found that Mx2 is capable of directly binding to HIV-1 CA and its oligomerization is
required for this binding (Fricke et al. 2014; Fribourgh et al. 2014; Buffone et al. 2015; Dicks et al. 2016). The results from experiments using the CA-binding assays additionally suggested that Mx2 inhibits uncoating of HIV-1 CA (Fricke et al. 2014).

Mx2 belongs to the dynamin and guanosine triphosphatase (GTPase) family of proteins. Like dynamins, Mx proteins contain a GTPase domain (G), middle domain (MD), and a GTPase effector domain (GED) from N- to C-terminus (Haller et al. 2010). Mx2 has two isoforms generated via an alternative start codon in its coding sequence. A long isoform of Mx2 additionally contains a small N-terminal domain with a length of 25 amino acids, upstream of the G domain, that is not found in the short isoform (Melén et al. 1996). This N-terminal domain, particularly the triple-arginine motif at positions 11–13 within the domain, was demonstrated to be essential for binding of Mx2 with CA and the subsequent restriction activity (Kane et al. 2013; Busnadiego et al. 2014; Fricke et al. 2014; Matreyek et al. 2014; Goujon et al. 2014, 2015; Schulte et al. 2015; Betancor et al. 2019). This domain is also known to bear a nuclear localization signal, with the key residues being lysine at position 20 and tyrosine at position 21, but this function is not believed to be important for restriction (Schulte et al. 2015). Accordingly, the short isoform of Mx2 that does not contain this domain did not show an antiviral activity against HIV-1 (Matreyek et al. 2014; Goujon et al. 2014). Recently, in addition to this small N-terminal domain, the downstream G domain was also shown to interact with HIV-1 CA (Betancor et al. 2019). That
research also showed that the short Mx2 is thus able to bind to CA via the G domain competitively with the long Mx2, which negatively regulates Mx2-mediated restriction of HIV-1. Given that the restriction was identified to be independent of the GTPase activity of Mx2 (Kane et al. 2013; Goujon et al. 2013, 2014), the G domain seems to primarily act to bind to HIV-1 CA in HIV-1 restriction.

Recent studies reported relationships of Mx2 with other host proteins. First, NPC and nuclear import machinery were demonstrated to be required for the full antiviral activity of Mx2. Nucleoporins (Nups) were observed to be involved in Mx2-mediated restriction of HIV-1 at differential levels, dependent on cell type, cell cycle, and CA (Kane et al. 2018). Particularly, Nup214 and TNPO1 were shown to directly interact with Mx2, which determines the position of Mx2 at the nuclear envelope and participates in the restriction activity (Dicks et al. 2018).

Secondly, a report showed that SAMHD1-mediated restriction activity is dependent on the presence of Mx2. Although Mx2 did not directly interact with SAMHD1, nor affect the cellular localization, phosphorylation, and dNTPase activity of SAMHD1, the depletion of Mx2 abolished the restriction capacity of the protein (Buffone et al. 2019). Further investigations are needed to better understand how Nups and nuclear import machinery regulate Mx2-mediated restriction, as well as the relationship between SAMHD1 and Mx2. In addition to these questions, the mechanism by which Mx2 restricts HIV-1 CA remains unknown. Given that the binding of Mx2 to HIV-1 CA inhibits the uncoating
(Fricke et al. 2014), Mx2 may seize the CA core enclosing the viral PIC at the nuclear envelope, which prevents programmed uncoating of CA and further blocks nuclear import of virus. Further studies are required to identify the mechanism of restriction by Mx2.

1.3.2.5. SERINC and T-cell Immunoglobulin and Mucin Domain (TIM)

A HIV-1 accessory protein Nef is known to enhance the infectivity of virus particles. HIV-1 virus particles produced in the absence of Nef were observed to have decreased infectivity, depending on the producer and target cell type (Chowers et al. 1994; Miller et al. 1994). Consistently, the enhancement of HIV-1 infectivity by Nef was also shown to require the presence of Nef in the producer cells (Münch et al. 2007). These observations indicated that Nef may prevent some inhibitory factor from incorporation into newly produced HIV-1 particles in producer cells. Two subsequent studies independently identified SERINC5 and SERINC3 (further studies have focused on SERINC5, which possesses stronger activity), membrane-associated proteins containing 11 transmembrane domains, as the factors counteracted by Nef. For these identifications, one of the studies used a proteomics comparison between wild-type, Nef- HIV-1, and Nef- HIV-1 produced by T lymphoid cells (Usami, Wu, and Göttlinger 2015) and the other study performed a comparison of transcription profiles between high and low Nef-responsive cells (Rosa et al. 2015). Thus, these investigations demonstrated that HIV-1 Nef inhibits incorporation of the SERINC5s into newly generated HIV-1
particles. They also confirmed the dominant restriction activity of the SERINC5s against HIV-1 in experiments using cells that overexpress the proteins. Moreover, Nef is suggested to decrease the surface level of the SERINC5s through endolysosomal degradation (Rosa et al. 2015; Shi et al. 2018), as this viral protein downregulates CD4 and MHC molecules on the surface of host cells (Garcia and Miller 1991; Craig, Pandori, and Guatelli 1998; Blagoveshchenskaya et al. 2002; Landi et al. 2011). Recently, studies showed that the intracellular loop 4 region of SERINC5 determines its vulnerability to Nef-mediated antagonism (Dai et al. 2018) and reported that natural Nef polymorphisms display different levels of SERINC5 downregulation (Jin et al. 2019). The results from these studies support a more complete understanding of the mechanism by which HIV-1 Nef specifically antagonizes SERINC5.

SERINC5 is constitutively expressed and not further induced by IFN stimulation (Usami, Wu, and Göttlinger 2015; Rosa et al. 2015; Beitari et al. 2017). SERINC proteins function to synthesize sphingolipid and phosphatidylserine (PS) in the cellular plasma membrane (Inuzuka, Hayakawa, and Ingi 2005). This feature raised a hypothesis that incorporation of SERINC5 into HIV-1 particles alters the lipid composition of the viral envelope to decrease infectivity. This hypothesis, however, was disproved (Trautz et al. 2017). Instead, the incorporated SERINC5 was observed to inhibit formation of the fusion pore when virus particles attach to the target cells, as well as to render the HIV-1 sensitive to broadly neutralizing antibodies targeting the conserved gp41
domains of the viral Env protein (Sood et al. 2017). In addition, SERINC5-mediated restriction depends on the Env proteins of HIV-1 particles. Studies reported that the restriction capacity differs among Env proteins of HIV-1 strains (Usami, Wu, and Göttlinger 2015; Rosa et al. 2015; Beitari et al. 2017) and further demonstrated that the variable regions 1 and 2 (Usami, Wu, and Göttlinger 2015), or variable region 3 (Beitari et al. 2017), of gp120 are determinants of the restriction. Replacement of the Env regions was shown to change the sensitivity of HIV-1 to SERINC5. These results suggest that Env also possesses antagonistic activity against SERINC5 (Beitari et al. 2017). Further investigations are needed to understand how SERINC5-mediated restriction is determined by different sequences in the variable regions of Env and to determine the definite mechanism by which SERINC5 inhibits fusion between the HIV-1 envelope and target cell membrane.

In addition to SERINC5, the T-cell immunoglobulin and mucin domain (TIM)-1 protein has been identified recently as a membrane-associated restriction factor prevented by HIV-1 Nef (Li et al. 2019). The TIM-family proteins are also transmembrane proteins like the SERINC proteins and are known to play roles in the cellular immune response (Kuchroo et al. 2003; Freeman et al. 2010). They are also constitutively expressed and not IFN-inducible proteins (Li et al. 2014a). TIM-1, TIM-3, and TIM-4 were previously shown to inhibit HIV-1 release via their incorporation into HIV-1 particles and simultaneously binding to the PS on the host cell membrane via their PS-binding sites (Li et al. 2014a). A
recent study demonstrated that HIV-1 Nef blocks the TIM-1–mediated restriction of HIV-1 by downregulating the surface level of the protein via induction of TIM-1 endocytosis, as well as that SERINC3 or SERINC5 enhances the restriction capacity of TIM-1 by stabilizing the protein (Li et al. 2019). Additionally, researchers reported that another TIM-family protein, TIM-3, is counteracted by HIV-1 Vpu, which induces intracellular sequestration of TIM-3 to reduce its surface level (Prévost et al. 2020).

1.3.2.6. Other Restriction Factors

There are more restriction factors against HIV-1 infection, in addition to the factors discussed above. This section provides brief introductions of several of those restriction factors.

The interferon-induced transmembrane proteins (IFITMs) are IFN-inducible membrane-associated proteins that inhibit a broad range of enveloped viruses. Among the members of the IFITM family, IFITM1, IFITM2, and IFITM3 are known to restrict HIV-1 infection (Lu et al. 2011). IFITM1 is located on the cell plasma membrane, whereas IFITM2 and IFITM3 are located on membranes of late endosomes and lysosomes (Feeley et al. 2011; Jia et al. 2014; Weston et al. 2016). The IFITMs present on the host membrane do not prevent viral attachment or endocytosis of virus particles, but block fusion of the viral envelope and cell membrane through regulation of membrane cholesterol to decrease the membrane fluidity (Li et al. 2013; Amini-Bavil-Olyaee et al. 2013; Desai et al.
In addition, the IFITMs are incorporated into the newly produced virus particles, which inhibits fusion of the virus particles with target cells. Thus, this incorporation of IFITMs decreases the infectivity of the virus particles (Tartour et al. 2014; Compton et al. 2014).

The zinc-finger antiviral protein (ZAP) inhibits the expression of viral products by promoting degradation of viral mRNAs through the following strategies. First, ZAP recruits the RNA processing exosome to target mRNAs upon its direct interaction with the viral mRNAs via its CCCH zinc-finger motif (Guo et al. 2004, 2007b). Second, ZAP cooperates with mRNA degradation machinery to specifically remove multiply spliced HIV-1 RNA transcripts (Zhu et al. 2011). To do so, the antiviral factor recruits cellular poly(A)-specific ribonuclease (PARN) to trim the 3’ poly(A) tail of target mRNAs, and the RNA exosome to subsequently degrade the mRNAs from the 3’ end. Additionally, ZAP also recruits decapping enzymes to remove the 5’-cap of target mRNAs, followed by degradation from the 5’ end by an exoribonuclease. Third, ZAP is able to suppress translation of viral mRNAs by blocking interactions between translation initiation factors and target mRNAs (Zhu et al. 2012). A recent study reported that ZAP is capable of detecting non-self RNAs like viral RNAs, based on the host CG suppression (Takata et al. 2017). That study demonstrated that viral transcripts containing CG-enriched sequences, which are rarely present in the vertebrate host due to CG suppression, are inhibited specifically by ZAP.

The human silencing hub (HUSH) complex was recently identified as a
restriction factor against HIV-1 infection that acts by repressing transcription of the provirus through its silencing machinery with SET domain bifurcated histone lysine methyltransferase 1 (SETDB1). This complex is counteracted by viral accessory proteins Vpx and Vpr from primate lentiviruses (Chougui et al. 2018; Yurkovetskiy et al. 2018). A recent study also demonstrated that the nuclear protein 220 (NP220) recognizes unintegrated retroviral DNAs in the nucleus of infected cells, which recruits the HUSH complex. Then, this complex subsequently recruits SETDB1 to silence expression of the viral DNAs (Zhu et al. 2018).

The membrane-associated RING-CH2 (MARCH2) and MARCH8 both have E3 ubiquitin ligase activity against viral infection. MARCH8 prevents HIV-1 Env proteins from incorporating into newly produced virus particles by downregulating the surface protein level through intracellular sequestration of the protein (Tada et al. 2015). This activity does not inhibit the production of virus particles but decreases the infectivity of the particles. The antagonism by HIV-1 accessory proteins was not observed. MARCH2 is upregulated upon HIV-1 infection and promotes ubiquitination of viral Env to induce the selective lysosomal degradation (Zhang, Lu, and Liu 2018).

Schlafen11 (SLFN11) is one of the IFN-stimulated genes (ISGs) and inhibits the translation of HIV-1 proteins expressed from the viral provirus (Li et al. 2012). Due to differences in codon usage of encoded proteins between the host and virus, HIV-1 requires a transition of the transfer RNA (tRNA) pool for
efficient production of its proteins in the host cells (Weringh et al. 2011). SLFN11 prevents the change in the tRNA pool through its binding to tRNAs. Thus, this factor was suggested to specifically inhibit HIV-1 protein synthesis in infected cells. Recent studies have suggested that SLFN11 is indeed an antiviral factor against a broad range of viruses, not only HIV-1, by demonstrating that the activity affects translation of non-optimized codon-containing RNA transcripts from non-viral and viral sources (Stabell et al. 2016; Lin et al. 2016; Valdez et al. 2019).
Figure 1.3. Host intrinsic restriction factors against HIV-1 and viral antagonisms.
Adapted from (Seissler, Marquet, and Paillart 2017). Overview of host restriction factors (red boxes) and viral accessory proteins (blue boxes) that counteract these factors.
CHAPTER II.

CYCLOPHILIN A PROTECTS HIV-1 FROM HUMAN TRIM5α

Author Contributions
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This chapter is published in Nature Microbiology.

2.1. SUMMARY

The HIV-1 capsid (CA) protein lattice encases viral genomic RNA and regulates steps essential to target-cell invasion (Yamashita and Engelman 2017). Cyclophilin A (CypA) has interacted with the CA of lentiviruses related to HIV-1 for millions of years (Luban et al. 1993; Katzourakis et al. 2007; Gilbert et al. 2009; Goldstone et al. 2010; Malfavon-Borja et al. 2013; Mu et al. 2014). Disruption of the CA–CypA interaction decreases HIV-1 infectivity in human cells (Franke, Yuan, and Luban 1994; Thali et al. 1994; Braaten, Franke, and Luban 1996a; Braaten and Luban 2001; Sokolskaja, Sayah, and Luban 2004) but stimulates infectivity in non-human primate cells (Towers et al. 2003; Sayah et al. 2004a; Berthoux et al. 2005a). Genetic and biochemical data suggest that CypA protects HIV-1 from a CA-specific restriction factor in human cells (Sayah and Luban 2004b; Sebastian and Luban 2005; Sebastian, Sokolskaja, and Luban 2006; Stremlau et al. 2006; Luban 2007). Discovery of the CA-specific restriction factor tripartite-containing motif 5α (TRIM5α) (Stremlau et al. 2004) and multiple, independently derived, TRIM5–CypA fusion genes (Sayah et al. 2004; Brennan, Kozyrev, and Hu 2008; Newman et al. 2008; Virgen et al. 2008; Wilson et al. 2008; Malfavon-Borja et al. 2013; Mu et al. 2014; Boso et al. 2019) pointed to human TRIM5α being the CypA-sensitive restriction factor. However, HIV-1 restriction by human TRIM5α in tumor cell lines is minimal (Stremlau et al. 2004) and inhibition of such activity by CypA has not been detected (Sokolskaja, Berthoux, and Luban 2006). Here, by exploiting reverse genetic tools optimized
for primary human blood cells, we demonstrate that disruption of the CA–CypA interaction renders HIV-1 susceptible to potent restriction by human TRIM5α, with the block occurring before reverse transcription. Endogenous TRIM5α associated with virion cores as they entered the cytoplasm, but only when the CA–CypA interaction was disrupted. These experiments resolve the long-standing mystery of the role of CypA in HIV-1 replication by demonstrating that this ubiquitous cellular protein shields HIV-1 from previously inapparent restriction by human TRIM5α.

2.2. INTRODUCTION

2.2.1. The Roles of Cyclophilin A in HIV-1 Infection

Upon HIV-1 entry, the viral CA core enclosing the viral RNA genome enters into the host target cell cytoplasm. In the early stage of HIV-1 infection, from virus entry to nuclear import, HIV-1 interacts with many host factors through its CA for successful infection (Yamashita and Engelman 2017). One of the host proteins is cyclophilin A, a member of the cyclophilin family.

2.2.1.1. Cyclophilins

Cyclophilins are a protein group that belongs to a large protein family of immunophilins. Immunophilins mostly possess an enzymatic activity of peptidyl-prolyl isomerase (PPIase) that catalyzes the change in conformation between cis and trans forms of peptide bonds adjacent to proline residues. Historically, a
protein harboring the PPIase activity was first identified from a porcine kidney in 1984 (Fischer, Bang, and Mech 1984). In the same year, another group independently isolated a protein as a binding partner of an immunosuppressive drug compound cyclosporine A (CsA) from bovine thymocytes (Handschumacher et al. 1984). This protein was termed cyclophilin (Cyp) based on its characteristics of high affinity with CsA. Five years later, the two proteins, PPIase and Cyp, were discovered to be the same protein (Takahashi, Hayano, and Suzuki 1989; Fischer et al. 1989). Other protein subfamilies of the large immunophilin family include FK506-binding proteins (FKBPs) and parvulins. These protein subfamilies also possess PPIase activity, as described above, and directly interact with chemical compounds; FKBPs interact with FK506 (Siekierka et al. 1989; Harding et al. 1989) and rapamycin (Brown et al. 1994; Sabatini et al. 1994), whereas parvulins bind to juglone (Rahfeld et al. 1994a, 1994b; Hennig et al. 1998). However, they are structurally distinct from cyclophilins.

Cyclophilins are found in a broad range of species, including bacteria, plants, insects, and mammals (Galat 1999). Humans have 17 cyclophilins including cyclophilin A (Wang and Heitman 2005; Davis et al. 2010). They share a common structural domain called cyclophilin-like domain (CLD) that is responsible for the PPIase activity. Cyclophilins can be classified into two groups: i) single-domain cyclophilins, including cyclophilin A, which consist of a single CLD; and ii) multi-domain cyclophilins, which contain additional, functional domains such as an RNA recognition motif (RRM) domain, WD40 domain, and
tetratricopeptide repeat (TPR) domain, which determine their subcellular localization and specific functions (Wang and Heitman 2005; Davis et al. 2010). Thus, cyclophilins are located in subcellular compartments including the cytoplasm, mitochondria (Rassow et al. 1995; Woodfield, Price, and Halestrap 1997), endoplasmic reticulum (Price et al. 1991; Hasel et al. 1991; Friedman, Trahey, and Weissman 1993), and nucleus (Mi et al. 1996; Nestel et al. 1996; Bourquin et al. 1997; Horowitz, Kobayashi, and Krainer 1997). They are also involved in diverse biological contexts, including protein folding (Baker, Colley, and Zuker 1994; Rassow et al. 1995; Ferreira et al. 1996), cellular signaling (Brazin et al. 2002; Colgan et al. 2004), mRNA splicing and processing (Bourquin et al. 1997; Horowitz et al. 2002; Xu et al. 2006), apoptosis (Lin and Lechleiter 2002), and host–virus interaction (Luban et al. 1993; Braaten, Franke, and Luban 1996; Watashi et al. 2005; Hanoulle et al. 2009). Cyclophilin A, one of the cyclophilins, and its role in HIV-1 infection will be described in detail in the following sections.

2.2.1.2. Cyclophilin A (CypA)

The ubiquitous, abundant cytoplasmic protein is a peptidyl prolyl isomerase (PPIase) that facilitates interconversion of cis to trans conformation of proline residues of target proteins (Fischer et al. 1989; Takahashi, Hayano, and Suzuki 1989; Bosco et al. 2002; Colgan et al. 2004; Davis et al. 2010). CypA binds to proline-containing peptides through its hydrophobic pocket as the
catalytic site for its enzymatic activity, which plays roles in a variety of cellular functions, including protein folding, subcellular localization, and cell signaling (Wang and Heitman 2005; Nigro, Pompilio, and Capogrossi 2013). For example, in yeast, CypA regulates meiosis and the nuclear export of zinc finger protein 1 (Zpr1) (Ansari, Greco, and Luban 2002). In human CD4+ T cells, CypA modulates T-cell signaling by tuning kinase activity of IL-2-inducible T-cell kinase (Itk) (Colgan et al. 2004). CypA is also involved in many human diseases including infectious diseases caused by viral infection (Zhou et al. 2012; Nigro, Pompilio, and Capogrossi 2013). Researchers have reported important roles of CypA in the formation of reactive oxygen species (Jin et al. 2000; Satoh et al. 2009; Satoh, Nigro, and Berk 2010), in pro-inflammatory pathways and inflammation (Kim et al. 2005; Ramachandran et al. 2012; Bell et al. 2012), as well as in diabetes (Ramachandran et al. 2012), rheumatoid arthritis (Billich et al. 1997; Kim et al. 2005; Yang et al. 2008; Wang et al. 2010), and neurodegenerative diseases such as Alzheimer's disease (Bell et al. 2012; Kanyenda et al. 2011) and amyotrophic lateral sclerosis (Lee et al. 1999; Basso et al. 2009; Nardo et al. 2011; Tanaka et al. 2011). In addition, CypA has been reported to regulate infectivity of hepatitis B or C viruses (Chatterji et al. 2009; Tian et al. 2010), vesicular stomatitis virus (Bose et al. 2003), influenza A virus (Shaw et al. 2008; Liu et al. 2009), coronaviruses (Luo et al. 2004), and so on.

2.2.1.3. Interaction of CypA with HIV-1 CA
In 1993, CypA was first identified as a host protein that directly binds to HIV-1 CA by yeast two-hybrid screening (Luban et al. 1993). The interaction between CA and target cell cytoplasmic CypA was shown to promote HIV-1 replication in human cells. HIV-1 CA monomer uses its proline-rich motif, between helices 4 and 5, to directly interact with the hydrophobic pocket of CypA (Gamble et al. 1996; Gitti et al. 1996; Vajdos et al. 1997) (Figure 2.1). This motif forms an unstructured loop that protrudes from the CA body, known as the CypA-binding loop. Within this loop motif, the glycine at position 89 (Gly89) and the proline at position 90 (Pro90) are the primary determinants for CA–CypA interaction (Franke, Yuan, and Luban 1994; Gamble et al. 1996; Gitti et al. 1996; Braaten, Franke, and Luban 1996a; Vajdos et al. 1997). The peptide bond between Gly89 and Pro90 is the enzymatic target of CypA, based on the observation that the Pro90 residue mostly retains in trans conformation in the presence of CypA (Bosco et al. 2002). Furthermore, these two residues are highly conserved in HIV-1 sequences, which indicates the importance of CA–CypA interaction based on positive selection during evolution (Luban et al. 1993; Katzourakis et al. 2007; Gilbert et al. 2009; Goldstone et al. 2010; Malfavon-Borja et al. 2013; Mu et al. 2014). The interaction between HIV-1 CA and CypA is blocked by mutations of either of the two residues (Franke, Yuan, and Luban 1994; Braaten, Franke, and Luban 1996a; Braaten et al. 1996b; Braaten and Luban 2001) as well as by chemical inhibitor compounds targeting CypA, such as cyclosporine A (CsA) (Handschumacher et al. 1984; Thali et al.
Figure 2.1. CA–CypA interaction
Adapted with permission from (Mascarenhas and Musier-Forsyth 2009). The CypA binding loop (red) of the N-terminal domain of CA (CA-NTD) (gray) interacts with the hydrophobic pocket of CypA (cyan). The Pro90 residue (blue) of CA and the Arg55 (black) of CypA form a hydrogen bond (Howard et al. 2003).
1994; Franke and Luban 1996). CsA binds to the hydrophobic pocket with high affinity and thereby efficiently disrupts CA–CypA interaction. Intriguingly, CsA has an immunosuppressive function as a side effect in addition to CypA inhibition due to the structure of the CsA–CypA complex. This complex can interact with calcineurin, a calmodulin and Ca\textsubscript{2+}-dependent phosphatase, to inhibit dephosphorylation and translocation of NFAT. As a result, the CsA–CypA complex blocks the NFAT signaling pathway, and thus, inhibits activation of T cells treated with CsA (Handschumacher et al. 1984; Liu et al. 1991; Flanagan et al. 1991; O’Keefe et al. 1992; Clipstone and Crabtree 1992) (Figure 2.2).

In addition to the Gly89 and Pro90 residues of HIV-1 CA, a recent structural study using cryo-electron microscopy identified a non-canonical, secondary interaction site between CA and CypA (Liu et al. 2016). Through this site, CypA bridges adjacent CA monomers, which contributes to further CA stabilization. However, the following investigation reported that mutations of the predicted key residues of CypA within this secondary binding site did not have a significant effect on HIV-1 infectivity nor the binding affinity between CypA and HIV-1 CA (Peng et al. 2019). Thus, further studies are needed to resolve this inconsistent observation.
Figure 2.2. CsA–CypA complex blocks T cell signaling
Reproduced from (Flores et al. 2019). Upon activation of T cell signaling, calcineurin dephosphorylates a transcription factor NFAT to induce the NFAT translocation into the nucleus, resulting in production of IL-2 that is essential for T cell proliferation. CsA specifically forms a complex with CypA in the cytoplasm. The CsA–CypA complex inhibits the activity of calcineurin via a direct interaction, which blocks T cell activation.
2.2.1.4. CypA Promotes HIV-1 Infection

As CypA of target cells binds to the incoming HIV-1 CA in the cytoplasm, CypA proteins of producer cells are also incorporated into newly generated HIV-1 virus particles through CA–CypA interaction at a CypA/CA ratio of approximately 1:10 (Franke, Yuan, and Luban 1994; Thali et al. 1994). However, the role of these incorporated CypAs is still unknown. Previous experiments showed that CypA depletion in producer cells did not affect HIV-1 infection of target cells, and HIV-1 infectivity was decreased only when CypA was depleted in the target cells (Sokolskaja, Sayah, and Luban 2004). Given that the conformation and stability of the CA core are altered with changes in the ratio of CypA to CA in vitro (Liu et al. 2016), the incorporated CypA may function to maintain the optimal shape of the CA core in mature virion particles. However, this hypothesis is in conflict with the fact that mature, conical CA core was observed in HIV-1 virus particles containing the CA-P90A mutation (Li, Kar, and Sodroski 2009). Further investigations are needed to identify the roles of CypA from producer cells in HIV-1 infection.

Target cell CypA enhances HIV-1 infectivity (Franke, Yuan, and Luban 1994; Thali et al. 1994; Franke and Luban 1996; Braaten, Franke, and Luban 1996a; Braaten et al. 1996b; Braaten and Luban 2001; Sokolskaja, Sayah, and Luban 2004; Hatzioannou et al. 2005; De Iaco and Luban 2014), especially during the early stage of the infection, by elevating the viral reverse transcription level (Braaten, Franke, and Luban 1996a; De Iaco and Luban 2014), which
indicates that CypA in these cells acts prior to the completion of the reverse transcription. In particular, disruption of CA–CypA interaction reduces HIV-1 infectivity (Franke, Yuan, and Luban 1994; Thali et al. 1994; Franke and Luban 1996; Braaten, Franke, and Luban 1996a; Braaten et al. 1996b; Braaten and Luban 2001; Sokolskaja, Sayah, and Luban 2004; Hatziioannou et al. 2005; De Iaco and Luban 2014), which suggests that CypA protects HIV-1 CA from the unknown anti-HIV-1 restriction factor. However, the unidentified restriction factor and the underlying mechanisms by which CypA increases viral reverse transcription and infectivity in human cells remain unclear.

Moreover, the role of CA–CypA interaction in HIV-1 infection indeed varies among species. The disruption of CypA binding to HIV-1 CA inhibits viral infection in human cells, as described above, but increases viral infection in cells from non-human primates (Towers et al. 2003; Berthoux et al. 2004; Sayah et al. 2004a; Hatziioannou et al. 2004a; Berthoux et al. 2005a). Thus, despite much progress achieved over two decades of research since the discovery of the CA–CypA interaction in 1993 (Luban et al. 1993), many questions regarding the role of CypA in HIV-1 infection remain to be resolved.

2.2.2. Capsid-binding Restriction Factor TRIM5α and HIV-1

Cells from non-human primate species are non-permissive to HIV-1 infection (Münk et al. 2002; Cowan et al. 2002; Besnier, Takeuchi, and Towers 2002). This dominant and saturable restriction against HIV-1 occurs after virus
entry but before reverse transcription. (Münk et al. 2002; Cowan et al. 2002; Besnier, Takeuchi, and Towers 2002; Hatzioannou et al. 2003). Researchers have attempted to discover the determinant of this HIV-1 resistance in the monkey genes. In 2004, two groups independently identified capsid-specific restriction factors, TRIM5α and TRIMCyp, via screening of cDNA libraries from Old World rhesus macaque and New World owl monkey, respectively, in human cells (Stremlau et al. 2004; Sayah et al. 2004a). In the first study, TRIM5α was observed to inhibit HIV-1 infection prior to viral reverse transcription. Its SPRY domain at the C-terminus was also demonstrated to be specific to HIV-1 CA for the restriction (Stremlau et al. 2004). TRIMCyp, discovered in the second study, is a TRIM5-CypA fusion protein, containing a CypA sequence at the C-terminus, instead of the TRIM5α PRYSPRY domain. This chimeric gene is generated by the LINE1-mediated retrotransposition of CypA cDNA into the TRIM5 locus in the South American owl monkey (Sayah et al. 2004a). Subsequent studies also found these independently generated fusion genes in Asian macaques (Brennan, Kozyrev, and Hu 2008; Newman et al. 2008; Virgen et al. 2008; Wilson et al. 2008). The C-terminal CypA domain is responsible for recognition of HIV-1 CA, as CypA directly interacts with HIV-1 CA (Luban et al. 1993; Franke, Yuan, and Luban 1994). Studies in both species thus indicated that TRIM5, a member of the TRIM family of proteins, is the determinant for the species-specific restriction activity in non-human primate cells.
2.2.2.1. Tripartite Motif-containing (TRIM) Family of Proteins

TRIM proteins are a large family of proteins that includes approximately 100 members (Reymond et al. 2001; Han, Lou, and Sawyer 2011) involved in a wide range of cellular functions including apoptosis (Wang et al. 1998; Chae et al. 2003), autophagy (Pineda et al. 2015; Missiroli et al. 2016; Wang et al. 2018), transcription (Friedman et al. 1996; Le Douarin et al. 1996; Shimono et al. 2000), differentiation (Harada et al. 1999; Spencer et al. 2000; Beer et al. 2002), and innate immune response (Uchil et al. 2013; Versteeg et al. 2013). They are characterized by a common domain architecture, consisting of a really interesting new gene (RING) domain, one or two B-box domains, and a coiled-coil domain from the N- to C-terminus. This TRIM-specific architecture is also called the RBCC domain (Reddy, Etkin, and Freemont 1992; Reymond et al. 2001) (Figure 2.3).

The N-terminal RING domain possesses the activity of an E3 ubiquitin ligase that adds ubiquitin molecules to substrate proteins from recruited E2 ubiquitin-conjugating enzymes. With this RING domain, TRIM proteins generate two major forms of ubiquitination. The first is Lys48-linked ubiquitination, which mostly results in recruitment of proteasome for degradation of the ubiquitinated target proteins (Xu et al. 2009). The other one is Lys63-linked ubiquitination, which is associated with cellular signaling (Pertel et al. 2011; Fletcher et al. 2018), modulation of protein activity (Sorrentino et al. 2008; Lamb et al. 2009; Li et al. 2011a), and subcellular localization of protein (Huang et al. 2003; Lauwers,
### Figure 2.3. Domain structure of TRIM proteins

Reproduced with permission from (Gent, Sparrer, and Gack 2018). TRIM proteins consist of a RING domain, a B-Box 1 and/or B-Box 2 domain, a coiled-coil domain (CCD), and C-terminal domain: SPRY-associated domain (PRY), SPIa and the ryanodine receptor domain (SPRY), C-terminal subgroup one signature domain (Cos), fibronectin type 3 domain (FN3), plant homeodomain (PHD), bromodomain (BROMO), acid-rich region (AR), filamin domain, NHL repeats (NHL), transmembrane domain (TM), ADP-ribosylation factor domain (ARF), and meprin and tumor necrosis factor receptor–associated factor homology domain (MATH). Numbers on the right indicate individual TRIM proteins.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Numbers</th>
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<tbody>
<tr>
<td>RING</td>
<td>5, 6, 7, 10, 11, 15, 17, 21, 26, 27, 35, 38, 39, 41, 50, 58, 60, 62, 68, 72, 75</td>
</tr>
<tr>
<td>SPRY</td>
<td>4, 22, 34, 43, 47, 64, 65</td>
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<tr>
<td>SPRY</td>
<td>48, 49, 51, 53, 77</td>
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<tr>
<td>Cos-FN3-SPRY</td>
<td>1, 9, 36, 67</td>
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<tr>
<td>Cos-FN3-SPRY</td>
<td>31, 40, 73, 74</td>
</tr>
<tr>
<td>Cos-FN3-SPRY</td>
<td>8, 19, 56</td>
</tr>
<tr>
<td>PHD-BROMO</td>
<td>24, 28, 33</td>
</tr>
<tr>
<td>Cos-AR</td>
<td>54, 55, 63</td>
</tr>
<tr>
<td>Filamin-NHL</td>
<td>2, 3</td>
</tr>
<tr>
<td>TM</td>
<td>13, 59</td>
</tr>
<tr>
<td>PRY-SPRY</td>
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<tr>
<td>PRY-SPRY</td>
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<td>Pyrin</td>
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<tr>
<td>FN3-SPRY</td>
<td>76</td>
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</table>
Jacob, and André 2009). In addition to ubiquitination, the RING domain also acts to modify target proteins by catalyzing the conjugation of ubiquitin-like (UBL) proteins such as small ubiquitin modifier (SUMO) (Chu and Yang 2011) and ISG15 (Zou and Zhang 2006; Zou, Wang, and Zhang 2007). Moreover, the RING domain contains conserved cysteine and histidine residues constituting zinc-binding motifs that coordinate two zinc ions, which facilitates protein–protein interaction, especially between the RING domain and E2 ubiquitin-conjugating enzymes (Barlow et al. 1994; Borden et al. 1995; Lorick et al. 1999; Yin et al. 2009; Metzger et al. 2014).

TRIM proteins contain one or two B-box domains immediately downstream of the N-terminal RING domain. There are type 1 and type 2 B-box domains (B-box 1 and B-box 2). Nearly all TRIM proteins harbor a B-box 2 domain, and some have an additional B-box 1 domain, always in a sequence of type 1 to type 2 from N-terminus, which suggests a conserved evolutionary function based on the B-box structure (Reymond et al. 2001). Similar to the RING domain, they also contain zinc-binding motifs consisting of cysteine and histidine residues that coordinate two zinc atoms, which potentially promotes protein–protein interaction (Massiah et al. 2006, 2007). Although intact B-box domains are required for the function of TRIM proteins, the functional role in many TRIM proteins is still unclear. However, in the case of TRIM5α, which will be discussed in this thesis, the B-box 2 domain is required for the higher-order self-assembly (Li and Sodroski 2008; Ganser-Pornillos et al. 2011; Goldstone et
al. 2014). Given that the B-box2 domain contains zinc-binding motifs (Massiah et al. 2006, 2007), the motifs may be important in this higher-order association of TRIM5α by facilitating interactions between TRIM5α proteins.

The coiled-coil domain functions to allow self-association of TRIM proteins (Reymond et al. 2001; Koliopoulos et al. 2016). The self-interaction is based on antiparallel dimerization, which places a RING domain and B-box domains at each end of the dimer, according to studies with several TRIM proteins (Sanchez et al. 2014; Goldstone et al. 2014; Li et al. 2014b; Weinert et al. 2015; Dawidziak et al. 2017). In TRIM5α, this position of the two domains is suggested to contribute to further higher-order association (Keown et al. 2016; Li et al. 2016; Wagner et al. 2016). Thus, the coiled-coil domain that mediates dimerization is critical for further assembly of TRIM proteins. In particular, given that sequences of the coiled-coil domains are highly conserved across TRIM proteins, dimerization in an antiparallel fashion may be a common feature of the TRIM family proteins (Li et al. 2014b). In addition to the RBCC domain described above, TRIM proteins have a C-terminal domain responsible for specific functions (Vitale, Moss, and Vaughan 1996; Vichi et al. 2005), interaction with target proteins or nucleic acids (Stremlau et al. 2004; Short and Cox 2006; Keeble et al. 2008; Choudhury et al. 2017), and subcellular localization (Reymond et al. 2001; Short and Cox 2006). This domain varies among members of the TRIM family proteins. The most prevalent C-terminal domain is the PRYSPRY domain (also known as B30.2 domain) (Sardiello et al. 2008).
TRIM proteins can be classified on the basis of their domain configurations and C-terminal domains (Short and Cox 2006; Ozato et al. 2008) (Figure 2.3).

TRIM proteins undergo higher-order homomultimerization, which promotes the activity of E3 ubiquitin ligase (Yudina et al. 2015; Fletcher et al. 2018). Additionally, they are also able to form heteromultimers with other TRIM proteins, especially with closely related TRIM proteins, notwithstanding the considerably lower efficiency compared to the homologous multimerization. In the case of TRIM5α, heterologous multimerization with TRIM4, TRIM6, and TRIM34 was observed (Reymond et al. 2001; Zhang et al. 2006; Li et al. 2011b). A recent study also demonstrated a functional activity of the heteromultimeric TRIM34−TRIM5α that restricts CPSF6-deficient HIV-1 CA and SIV CA in human cells (Ohainle et al. 2020). Thus, the heterologous association between different TRIM proteins can be required for their functions. On the other hand, this association can also negatively regulate the activity of TRIM proteins, as observed in the example of TRIM proteins forming heteromultimers with other TRIM proteins that do not contain the RING domain (Urano et al. 2009).

Furthermore, considering splicing variants of TRIMs, the association of TRIM proteins with their isoforms missing the C-terminal domains is also likely to inhibit their functional activity. This was demonstrated by a study with TRIM5 isoforms (Battivelli et al. 2011), which will be discussed in the next section. Thus, the activities of TRIM proteins are sophisticatedly regulated by the homo- and heteromultimerization with isoforms and other TRIM proteins (Figure 2.4).
Figure 2.4. Regulation of activities of TRIM proteins by homo- and hetero-multimerization

Adapted with permission from (Napolitano and Meroni 2012). Multimers are represented as dimers, for simplicity. E2 indicates E2 ubiquitin-conjugating enzymes that interact with RING domains of TRIM proteins.

(A) Homo-multimerization of TRIM proteins.
(B) Hetero-multimerization of TRIM proteins.
(C) Hetero-multimerization of a TRIM protein and a RING-less TRIM protein.
(D) Hetero-multimerization of TRIM isoforms.
2.2.2.2. TRIM5: Isoforms, Structure and Binding to HIV-1 CA

TRIM5 is a member of the TRIM family of proteins and shares a common structure with other family members, including a RING domain, B-box 2 domain, and coiled-coil domain. TRIM5 is expressed as multiple isoforms generated by alternative splicing: TRIM5α, TRIM5γ, TRIM5δ, TRIM5ε, TRIM5ι, TRIM5κ, TRIM5h, and TRIM5e (Reymond et al. 2001; Battivelli et al. 2011; Gent, Sparrer, and Gack 2018) (Figure 2.5). TRIM5α is the most abundant TRIM5 isoform, representing approximately 45–55% of total TRIM5 mRNAs in cells, including CD4+ T cells and macrophages (Battivelli et al. 2011; Rahmberg et al. 2017), with the longest length and containing a C-terminal domain not found in other isoforms. The domain is called the PRYSPRY (B30.2) domain and was shown to recognize the TRIM5α-sensitive retroviral CA core (Stremlau et al. 2004). Other isoforms are not able to inhibit the retroviral infection due to lack of the C-terminal PRYSPRY domain. However, they share the other domains with TRIM5α, including the N-terminal RING domain, B-box 2 domain, and coiled-coil domain (Reymond et al. 2001; Battivelli et al. 2011; Gent, Sparrer, and Gack 2018), which contributes to the formation of heterodimers with the α isoform (Berthoux et al. 2005b; Perez-Caballero et al. 2005b; Maegawa et al. 2008) (Figure 2.5). This results in dominant-negative activity of the TRIM5 isoforms against TRIM5α (Stremlau et al. 2004; Maegawa et al. 2008; Battivelli et al. 2011), suggesting that the presence of these isoforms modulates the restriction activity of TRIM5α. Given that the relative proportion of TRIM5 isoforms was reported to vary slightly
Figure 2.5. TRIM5 isoforms expressed in human cells
Adapted from (Battivelli et al. 2011). In splicing profiles, gray boxes indicate coding regions, whereas empty boxes indicate noncoding regions. Numbers above the profiles indicate exon numbers. The splicing profile of TRIM5α is presented with exons encoding the domains, including RING, linker 1 (L1), B-box 2 (B-box), coiled-coil (CC), linker 2 (L2), and PRYSPRY (SPRY), at the top of the figure. The given codes between the list of TRIM5 isoforms and the corresponding splicing profiles are representative nucleotide accession numbers.
among cell types (Battivelli et al. 2011; Rahmberg et al. 2017), the antiviral activity of TRIM5α may also differ among cell types due to the differential expression patterns of the dominant-negative TRIM5 isoforms. The expression levels of the TRIM5 isoforms may also be regulated to modulate the activity of TRIM5α in cells upon specific stimuli, although a previous study reported that several forms of stimulation including IFN treatment and viral infection did not alter the relative abundance of TRIM5α in CD4+ T cells (Rahmberg et al. 2017).

The N-terminal RING domain possesses E3 ubiquitin ligase activity to attach ubiquitin to the substrate from recruited E2 ubiquitin-conjugated enzyme proteins (Diaz-Griffero et al. 2006a; Langelier et al. 2008; Kar et al. 2008; Pertel et al. 2011). This activity induces the proteasome-mediated degradation pathway to remove the target proteins or generates intermediate ubiquitinated materials to trigger signal transduction. The ubiquitination activity of TRIM5α is connected to the mechanism by which TRIM5α restricts retroviruses, which will be discussed in detail in the next sections: 2.2.2.3. Antiviral Activities of TRIM5α: Premature Disassembly of Retroviral CA and 2.2.2.4. Antiviral Activities of TRIM5α: Innate Immune Response to Retroviral CA.

The B-box 2 and coiled coil domains are responsible for the oligomerization and further formation of higher-order assemblies of TRIM5α (Li and Sodroski 2008; Ganser-Pornillos et al. 2011; Sastri et al. 2014; Sanchez et al. 2014; Goldstone et al. 2014; Li et al. 2016; Wagner et al. 2016; Keown et al. 2016; Wagner et al. 2018; Skorupka et al. 2019). First, TRIM5α dimerizes
through the coiled-coil interactions between two antiparallel TRIM5α proteins. In this TRIM5α dimer, a RING domain and a B-box 2 domain are positioned at both ends and two PRYSPRY domains are located in the middle of the coiled-coil domains. These PRYSPRY domains face against the coiled-coil domains. The linker 2 region that connects the coiled-coil domain and C-terminal PRYSPRY domain is suggested to mediate the disposition of the PRYSPRY domain (Sanchez et al. 2014; Goldstone et al. 2014; Roganowicz et al. 2017). The B-box 2 domain at each end functions to form a trimer with the TRIM5 dimer by interacting with the other B-box 2 domains of two nearby TRIM5α dimers, which contributes to the formation of higher-order structures such as hexagonal lattices (Figure 2.6). The importance of these two constructing domains are supported by the observation that they are indispensable for TRIM5α-mediated restriction against retroviruses (Perez-Caballero et al. 2005b; Javanbakht et al. 2006; Perron et al. 2007; Diaz-Griffero et al. 2009). In vitro studies using purified TRIM5α proteins have shown that TRIM5α proteins are spontaneously assembled (Ganser-Pornillos et al. 2011; Li et al. 2016; Wagner et al. 2016). This feature is believed to cause the formation of cytoplasmic bodies when TRIM5α is overexpressed in the cells (Reymond et al. 2001; Ganser-Pornillos et al. 2011; Fletcher et al. 2018), even though the clustered bodies are not required for the restriction activity (Song et al. 2005a; Perez-Caballero et al. 2005a, 2005b). Indeed, TRIM5α proteins do not bind to CA monomers but efficiently interact with CA assemblies in vitro (Sebastian and Luban 2005; Diaz-Griffero et al. 2006b;
Kim et al., Figure 2.6
Figure 2.6. Structure and higher-order assembly of TRIM5α over CA
Adapted with permission from (Ganser-Pornillos and Pornillos 2019).

(A) Domain structure of TRIM5α. TRIM5α consists of RING, B-box 2, coiled-coil (Coil), and C-terminal PRYSPRY (SPRY) domains. There are two flexible linker regions between RING and B-box 2 domains (linker 1) and between Coil and SPRY domains (linker 2).

(B) Structure of TRIM5α dimer that interacts with CA. TRIM5α forms an antiparallel dimer via its coiled-coil domain (Goldstone et al. 2014) (Protein Data Bank (PDB) code 4TN3). The RING domains are placed at both ends, and face against CA. C-terminal SPRY domains are positioned in the middle of coiled-coil domain, and directly interact with CA, mainly via their V1 loops (Yang et al. 2012; Biris et al. 2012). CTD, carboxy-terminal domain; NTD, amino-terminal domain.

(C) Trimer of TRIM5α dimers. The B-box 2 domains mediate the formation of trimers of TRIM5α dimers with the neighboring dimers (Wagner et al. 2016) (PDB code 5IEA). The continuous trimer formation contributes to the generation of higher-order hexagonal lattice of TRIM5α over CA.

(D) Model of a CA core coated by hexagonal lattice of TRIM5α. The black circles indicate a TRIM5α dimer (top) and a trimer of TRIM5α dimers (middle) for (B) and (C), respectively. This structural figure is generated based on crystal structures of the HIV-1 CA hexamer (Pornillos et al. 2009) (PDB code 3H47) and pentamer (Pornillos, Ganser-Pornillos, and Yeager 2011) (PDB code 3P05) and rhesus monkey TRIM5α coiled-coil dimer (Goldstone et al. 2014), B-box 2 trimer (Wagner et al. 2016) and SPRY domain (Biris et al. 2012) (PDB codes 3H47, 3P05, 4TN3, 5EIA and 2LM3).
Stremlau et al. 2006; Langelier et al. 2008; Black and Aiken 2010), which results in the formation of hexagonal lattices, as described above, over the CA structures as the templates (Figure 2.6). Researchers have also reported that the presence of CA assemblies facilitates the self-association of TRIM5α (Ganser-Pornillos et al. 2011). This multimerization by TRIM5α is thought to be critical for TRIM5α recognition of the retroviral CA core. The binding affinity of a PRYSPRY domain for the assembled CAs is very weak (Yang et al. 2012; Biris et al. 2012, 2013), but TRIM5α is able to amplify the binding strength through its multimerization over the assembled CA structure.

Structural studies have provided insight into how TRIM5α forms the hexagonal lattice over the CA core. A recent study using molecular simulation with structural analyses proposed a sequence of steps for the higher-order TRIM5α assembly (Yu et al. 2020): i) free TRIM5α dimers rapidly interact with or dissociate from CA; ii) TRIM5α dimers bound to the CA diffuse on the CA surface; iii) these dimers interact with other TRIM5α dimers bound to the CA through their B-box 2 domains, resulting in the formation of trimers of the TRIM5α dimers; and iv) the trimer formation spreads over the CA surface from the previously formed trimers of dimers. In this predicted course, the second step involving the motion of dimers bound to CA is consistent with a previous structural study showing small movement of the coiled-coil domain regions of the trimers (Wagner et al. 2016). Moreover, the simulation based on structural analysis also showed that non-hexagonal defects, such as pentameric and
heptameric defects, could be present in the TRIM5α lattice over CA (Yu et al. 2020), as previous studies observed size differences of individual rings in the lattice (Li et al. 2016; Wagner et al. 2016). The flexibility of the interaction between TRIM5α and CA, based on the motion of TRIM5α dimers bound to CA and non-hexagonal defect structures in the lattice, is suggested to optimize the ability of hexagonal net formed by TRIM5α to fully cover the retroviral CA, considering the curvatures of the CA surface as well as the irregularity of the CA shape, despite the typical cone shape, in HIV-1 virions (Welker et al. 2000; Briggs et al. 2003, 2006; Mattei et al. 2016).

Although retroviral CA recognition by TRIM5α via its higher-order lattice is relatively well understood, the interaction interface between TRIM5α and CA at a molecular level has yet to be characterized. TRIM5α has been suggested to interact with multiple regions of CA. Determinants of CA sensitivity to TRIM5α-mediated restriction were mapped to various regions of the CA (Owens et al. 2004; Hatziioannou et al. 2004a; Ylinen et al. 2005; Song et al. 2007; Mortuza et al. 2008; Kono et al. 2010; Kuroishi et al. 2010; Ohkura et al. 2011; McCarthy et al. 2013; Ohkura and Stoye 2013). Moreover, structural studies also suggested that the four loop regions (v1, v2, v3, and v4) of the C-terminal PRYSPRY domain of TRIM5α are involved in the interaction with regions of one CA hexamer as well as two CA hexamers at once (Yang et al. 2012; Biris et al. 2012, 2013; Morger et al. 2018). In particular, an investigation using cryo-electron tomography recently demonstrated that TRIM5α contacts CA in four different
ways while forming a hexagonal lattice over CA (Skorupka et al. 2019). In detail, PRYSPRY domains of the TRIM5α dimers in the lattice structure are placed between two CA hexamers, on a lopsided region of a CA hexamer, or at the center of a CA hexamer. Thus, all of these findings indicate that TRIM5α interacts with CA at multiple regions. Given that the binding affinity of TRIM5α with CA is weak (Yang et al. 2012; Biris et al. 2012, 2013), this binding mode of TRIM5α at various sites on CA may provide the flexibility of interaction needed for formation of the optimal structures of a higher-order lattice over retroviral CAs that bear variable curvatures. In addition, this may also be a strategy by which TRIM5α counteracts attempts by the retrovirus to evolve to evade TRIM5α through mutation of its CA. CA recognition by weak interactions at multiple regions may enable TRIM5α to retain its capability to detect CA containing a mutation that disrupts the interaction at one or two regions. In contrast to this binding mode, however, TRIM5α is also suggested to primarily interact with the CypA-binding region of CA (Owens et al. 2004; Hatzioannou et al. 2004a; Ylinen et al. 2005; Kono et al. 2010; Quinn et al. 2018; Yu et al. 2020). In addition, several studies have suggested that among the four loop regions of the PRYSPRY domain, the v1 region mainly determines its interaction with CA (Sawyer et al. 2005; Song et al. 2005b; Yap, Nisole, and Stoye 2005; Li et al. 2006; Yang et al. 2012; Biris et al. 2012, 2013; Morger et al. 2018). Therefore, further research is required to gain a deeper understanding of how TRIM5α binds to CA at the molecular level.
As a species-specific restriction factor, TRIM5α is believed to be a shield against cross-species transmission of retroviruses. TRIM5α tends to inhibit retroviruses from different species, as opposed to those from the same species (Hatziioannou et al. 2004b, 2006). For instance, TRIM5α from rhesus macaque strongly restricts HIV-1 (Stremlau et al. 2004), but the human TRIM5α ortholog does not show efficient restriction of HIV-1 (Hatziioannou et al. 2003, 2004b). The human ortholog instead restricts N-murine leukemia virus (MLV) and equine infectious anemia virus (Hatziioannou et al. 2003, 2004b). In addition, evolutionary analyses have shown that TRIM5α has rapidly evolved, especially at the sequences of variable regions within the PRYSPRY domain that recognizes the viral CA cores (Sawyer et al. 2005; Song et al. 2005b). A 13-residue patch, including a key residue 332, in the variable regions was found to be critical for CA recognition by TRIM5α. When the 13-residue patch sequences in human TRIM5α were replaced with the sequences of rhesus TRIM5α, the engineered human ortholog gained the restriction capacity against HIV-1 of the wild-type rhesus TRIM5α (Sawyer et al. 2005; Stremlau et al. 2005). Furthermore, the human ortholog with the substitution of one amino acid, residue 332, also exhibited restriction activity against HIV-1 (Yap, Nisole, and Stoye 2005; Li et al. 2006). This indicates that the patch within the PRYSPRY domain has been under positive selection for the CA specificity of TRIM5α.
2.2.2.3. Antiviral Activities of TRIM5α: Premature Disassembly of Retroviral CA

There are two known antiviral activities of TRIM5α: i) premature CA disassembly; and ii) triggering of the innate immune signaling against HIV-1. The accurate mechanism of TRIM5α-mediated premature CA disassembly still remains unclear, despite a long history of related research. In the first study reporting TRIM5α as a restriction factor, TRIM5α expression was shown to decrease HIV-1 cDNA levels and subsequent infectivity, demonstrating that TRIM5α acts against HIV-1, prior to HIV-1 reverse transcription and early after virus entry (Stremlau et al. 2004). Since then, experiments using an in vitro ‘fate-of-capsid’ assay showed that TRIM5α significantly reduces the level of particulate CA but increases the level of soluble CA compared to the control condition, which indicates that the CA core is disassembled by TRIM5α-mediated recognition (Stremlau et al. 2006; Diaz-Griffero et al. 2007). These observations suggest that TRIM5α destabilizes the incoming CA cores to disrupt the optimally programmed HIV-1 infection stages, considering that the timing of HIV-1 uncoating is critical for reverse transcription and the subsequent nuclear import of the PIC.

The mechanism by which TRIM5α binding causes the premature disassembly of the viral CA core is still unknown. The RING domain of TRIM5α has been suggested to participate in this CA dismantlement through its ubiquitinating enzymatic activity. This has been supported by studies using
proteasome inhibitors or RING domain mutant TRIM5α. The presence of a proteasome inhibitor, MG132, was observed to restore reverse transcription of HIV-1 that had been decreased by TRIM5α (Anderson et al. 2006; Wu et al. 2006). Also, experiments using the ‘fate-of-capsid’ assay showed that the decrease in particulate CA by TRIM5α was not observed with the proteasome inhibitor or RING domain mutation (Diaz-Griffero et al. 2007; Roa et al. 2012; Kutluay, Perez-Caballero, and Bieniasz 2013). Moreover, the CA−TRIM5α interaction was stably detected in cells treated with a proteasome inhibitor, but not in non-treated cells (Campbell et al. 2008). A recent study additionally demonstrated that an immunoproteasome is involved in restriction against HIV-1 by TRIM5α in IFN-stimulated cells and also showed that the RING domain is necessary for this activity (Jimenez-Guardeño et al. 2019). In line with this idea, researchers proposed that the CA core would be ruptured by proteasome-mediated degradation following the ubiquitination. However, they were unable to detect ubiquitinated CA proteins (Stremlau et al. 2006; Kutluay, Perez-Caballero, and Bieniasz 2013).

TRIM5α has a short life-time due to its auto-ubiquitination by the RING domain (Diaz-Griffero et al. 2006a). TRIM5α in the presence of proteasome inhibitors or RING domain mutation was observed to have slower turnover compared to the wild-type protein (Diaz-Griffero et al. 2006a). In the trimer formation of TRIM5α dimers, the RING domains are gathered alongside the next positioned B box 2 domains, which activates the RING domains to potentially
augment the auto-ubiquitination (Yudina et al. 2015). In accordance with the report that the formation of high-order TRIM5α structures is promoted in the presence of CA assemblies (Ganser-Pornillos et al. 2011), researchers have observed that the TRIM5α-sensitive retroviral CA core induces the destabilization of cellular TRIM5α proteins (Rold and Aiken 2008). In addition, a recent report showed that TRIM5α assembly on retroviral CA induces the formation of a N-terminally anchored K63-linked polyubiquitin chain to recruit proteasomes (Fletcher et al. 2015, 2018). The ubiquitination activity of TRIM5α will be described in more detail in the next section 2.2.2.4. Antiviral Activities of TRIM5α: Innate Immune Response to Retroviral CA. These features have raised a model in which the premature CA disassembly is driven by the proteasome-mediated degradation of auto-ubiquitinated TRIM5α bound to the HIV-1 CA core. Although the binding strength of TRIM5α with CA is weak (Yang et al. 2012; Biris et al. 2012, 2013), the simultaneous degradation of TRIM5α proteins at multiple sites over the CA core may have enough force to dismantle the CA structure. The degradation of TRIM5α by proteasome may also continue to the interacting CA protein without direct ubiquitination.

Experiments with proteasome inhibitors indeed suggested that an additional layer of TRIM5α-mediated restriction occurs after the viral reverse transcription (Wu et al. 2006; Anderson et al. 2006). In the experiments, proteasome inhibition rescued HIV-1 reverse transcription but failed to recover the infectivity or the decreased level of 2-LTR circles that indicates nuclear import
of viral genome. This could be due to the stabilization of CAs by TRIM5α coating in the absence of proteasome activity (Quinn et al. 2018), which presumably prevents the CA uncoating required for nuclear import. However, this hypothesis was disproven by several reports showing that HIV-1 is able to generate a functional PIC even with proteasome inhibition (Anderson et al. 2006) and that a comparable level of HIV-1 2-LTR circles is detected in cells expressing RING domain mutant TRIM5α (Roa et al. 2012), compared to the control condition. These findings suggest that TRIM5α indeed possesses proteasome-independent activity after reverse transcription prior to viral integration, in addition to proteasome-dependent activity prior to reverse transcription. Further research is required to reveal this additional restriction capacity of TRIM5α.

Furthermore, TRIM5α binding to the viral CA core is also thought to be involved in the autophagy-mediated inhibition of HIV-1 infection (O’Connor et al. 2010; Mandell et al. 2014; Keown et al. 2018; Saha, Chisholm, and Mandell 2020). This is in accordance with the identification of interaction between TRIM5α and proteins related to the autophagy pathway (O’Connor et al. 2010; Mandell et al. 2014; Keown et al. 2018). These observations seem to conflict with the model of proteasome-mediated inhibition described above. However, this may imply that TRIM5α indeed utilizes both mechanisms to restrict HIV-1 upon its recognition, or that either of the two mechanisms of TRIM5α-mediated inhibition is dependent on cell type or cellular and environmental conditions, considering the interplay between the ubiquitin–proteasome system and autophagy (Ji and
Researchers have reported that TRIM5 is involved in innate immune signaling. Overexpression of TRIM5 orthologs from human, non-human primate, and murine species was shown to activate TAK1 to trigger NF-κB− and AP-1−mediated signaling pathways in human cells (Pertel et al. 2011; Tareen and Emerman 2011; Chang, Yoshimi, and Ozato 2015; Campbell et al. 2015a; Lascano et al. 2016), as well as to potentiate the innate immune response to immunogenic stimulations such as LPS (Pertel et al. 2011; Versteeg et al. 2013). The activation of immune signaling pathways by TRIM5 is dependent on higher-order assembly of TRIM5 and E3-ubiquitin ligase activity of the N-terminal RING domain (Pertel et al. 2011; Tareen and Emerman 2011; Uchil et al. 2013; Yudina et al. 2015; Fletcher et al. 2015, 2018; Na et al. 2018).

At a low endogenous expression level, cellular TRIM5 dimers have a limitation to form higher-order structure. The TRIM5 proteins recruit an E2 ubiquitin-conjugating enzyme Ube2W (also known as UBC16), which mono-ubiquitinates the TRIM5 molecules at their N-termini. This self-ubiquitination of TRIM5 results in its rapid turnover via proteasome-mediated degradation (Fletcher et al. 2015, 2018) (Figure 2.7). This prevents undesirable activation of innate immune signaling in the absence of retrovirus infection. On the other hand, upon
retrovirus infection, endogenous TRIM5α proteins are assembled to form a hexagonal lattice over the invading retroviral CA core (Goldstone et al. 2014; Li et al. 2016; Wagner et al. 2016, 2018; Skorupka et al. 2019). Additionally, when TRIM5 is overexpressed in cells, the proteins undergo higher-order self-assembly that can be observed as cytoplasmic bodies (Reymond et al. 2001; Ganser-Pornillos et al. 2011; Fletcher et al. 2018). The formation of a higher-order TRIM5 assembly in either condition contributes to arrangement of trivalent RING domains, as described in section 2.2.2.2. TRIM5: Isoforms, Structure and Binding to HIV-1 CA. In the conditions, TRIM5 first recruits the E2-ubiquitin enzyme Ube2W for its self-monoubiquitination as described above. The trimer of the RING domains in the higher-order TRIM5 assembly then acts to extend Lys63-linked ubiquitin chains from the mono-ubiquitin at the N-termini of TRIM5. The heterodimers of E2 ubiquitin-conjugating enzymes Ube2N (also known as UBC13)–Ube2V1 (also known as UEV1A) or Ube2N–Ube2V2 (also known as UEV2) are recruited to the TRIM5 proteins for this ubiquitin chain elongation. A structural study recently found that a tri-ionic anchor motif of the RING domain that consists of the Glu11, Glu12, and Glu20 residues plays a critical role in this ubiquitination through interaction with the Arg6 and Arg14 residues of Ube2N (Kiss et al. 2019). The resulting polyubiquitination of TRIM5 not only promotes activation of innate immune signaling, but also recruits proteasome for turnover of TRIM5 as well as premature disassembly of retroviral CA, which inhibits viral reverse transcription (Fletcher et al. 2015, 2018) (Figure 2.7). Furthermore, the
higher-order TRIM5 assembly also synthesizes unanchored Lys63-linked polyubiquitin chains with the recruited heterodimer of E2 enzymes Ube2N−Ube2V1 or Ube2N−Ube2V2 (Pertel et al. 2011; Yudina et al. 2015; Fletcher et al. 2018). The TAK1 kinase complex is activated by these free polyubiquitin chains through its ubiquitin binding components TAK1-binding protein 2 (TAB2) and TAB3, which promotes auto-phosphorylation of TAK1 (Xia et al. 2009). This leads to the induction of the downstream NF-κB− and AP-1−mediated signaling cascades (Figure 2.7). Moreover, TRIM5 also catalyzes polyubiquitination of TAK1 (Na et al. 2018). The polyubiquitinated TAK1 is suggested to play a role in activation of innate immune signaling (Sorrentino et al. 2008; Lamb et al. 2009; Li et al. 2011a), although a recent study showed that this activity of TRIM5 is not sufficient for AP-1−mediated signaling induced by TRIM5 (Na et al. 2018). Thus, TAK1 ubiquitination may be responsible specifically for NF-κB−dependent signaling through ubiquitination-induced conformational change of TAK1 that increases interaction with, and subsequent phosphorylation of, the IκB kinase (IKK) complex. The IKK activated by this phosphorylation then induces phosphorylation and degradation of the NF-κB inhibitor IκB, which leads to the activation of NF-κB (Israël 2010; Hinz and Scheidereit 2014).

In addition to TRIM5α, exogenously expressed TRIM5γ, TRIM5δ, and a recombinant TRIM5, all of which do not contain a C-terminal PRYSPRY domain, were also shown to induce NF-κB−mediated signaling (Tareen and Emerman...
Figure 2.7. Ubiquitin-mediated restriction activity of TRIM5α
Adapted with permission from (Hage and Rajsbaum 2019). In the absence of retroviral CA, TRIM5α dimer is N-terminally mono-ubiquitinated with a recruited E2 ubiquitin-conjugating enzyme Ube2W, and undergoes its rapid turnover via proteasome-mediated degradation (Fletcher et al. 2015, 2018). Upon retroviral infection, TRIM5α dimers form higher-order hexagonal lattice over the viral CA. The trivalent RING domains in this structure generate the N-terminal Lys63-linked polyubiquitination of TRIM5α from the attached mono-ubiquitin molecule, as well as produce unanchored Lys63-linked polyubiquitin chain, with heterodimers of E2 ubiquitin-conjugating enzymes Ube2N–Ube2V1 or Ube2N–Ube2V2 (Pertel et al. 2011; Fletcher et al. 2015, 2018). The potential mechanisms of TRIM5α-mediated restriction include: (A) proteasomal degradation; (B) premature disassembly of retroviral CA that inhibits viral reverse transcription; and (C) TAK1-mediated innate immune signaling activated by the unanchored polyubiquitin chain.
2011; Versteeg et al. 2013; Na et al. 2018). This indicates that the N-terminal RING domain, B-box 2 domain, and coiled-coil domain responsible for TRIM5 assembly, but not the C-terminal PRYSPRY domain, are essential for TRIM5α-mediated NF-κB activation. However, a recent study showed that the PRYSPRY domain is necessary for activation of AP-1–dependent, but not NF-κB–dependent, signaling (Na et al. 2018). That study additionally demonstrated that TRIM5α with mutations in its PRYSPRY domain, including a substitution of a key residue serine at position 453 with a proline, loses its capabilities of auto-ubiquitination and restriction against HIV-1, but retains the activity of TAK1 polyubiquitination. These results suggest that TRIM5α polyubiquitination is important for AP-1 activation, but not for NF-κB activation. They also imply that the C-terminal PRYSPRY domain potentially contributes to the formation of a higher-order assembly harboring trivalent RING arrangement when TRIM5α is overexpressed, in addition to when TRIM5α recognizes the retroviral CA core. Further studies are needed to identify the mechanisms by which the NF-κB– and AP-1–mediated signaling pathways are differentially activated by TRIM5α and its domains. Intriguingly, the result showing that TRIM5α polyubiquitination is not essential for NF-κB–mediated signaling (Na et al. 2018) is in conflict with other studies reporting the opposite result (Campbell et al. 2015a; Fletcher et al. 2018). Thus, further examinations of the relationship between the ubiquitination of TRIM5α and NF-κB activation are also necessary.

The activation of AP-1– and NF-κB–driven signaling pathways by TRIM5α
leads to the production of cytokines such as IFNs followed by the expression of IFN-stimulated genes, including restriction factors, TRIM5α itself (Sakuma, Mael, and Ikeda 2007; Carthagena et al. 2008), and Mx2 (Goujon et al. 2013; Kane et al. 2013). Thus, TRIM5α plays a role in making the cellular environment non-permissive to retroviral infection through the activation of innate immune signaling. However, NF-κB activation by TRIM5α was also shown to increase the expression of integrated HIV-1 provirus, given that the transcription factor NF-κB binds to the promoter region within the HIV-1 LTR to enhance transcription of the provirus (Uchil et al. 2013). This implies that infection by TRIM5α-sensitive retroviruses could reactivate latent HIV-1 reservoirs. Therefore, given that human TRIM5α does not efficiently restrict HIV-1, HIV-1 may exploit the activity of human TRIM5α that activates innate immune signaling for reactivation of its latent reservoirs in opportunism with other retroviral infection.

2.2.3. The Relationship between TRIM5α and Cyclophilin A in HIV-1 Infection

Disruption of CA–CypA interaction largely inhibits HIV-1 infection in human cells (Franke, Yuan, and Luban 1994; Thali et al. 1994; Franke and Luban 1996; Braaten, Franke, and Luban 1996a; Braaten et al. 1996b; Braaten and Luban 2001; Sokolskaja, Sayah, and Luban 2004; Hatzioannou et al. 2005; De Iaco and Luban 2014). The discovery of TRIM5α and TRIMCyp (Stremlau et al. 2004; Sayah et al. 2004a), CA-specific restriction factors, raised the
hypothesis that CA–CypA interaction inhibits TRIM5α-mediated restriction against HIV-1 in human cells. Several studies, prior to the identification of
TRIM5α, supported this hypothesis by showing that the loss of CA–CypA
interaction renders HIV-1 sensitive to Ref-1 activity (Towers et al. 2003), and that
HIV-1 infection is independent of CA–CypA interaction in cells without Ref-1
activity (Sayah and Luban 2004b). The factor responsible for this Ref-1 activity
was identified later as TRIM5α (Stremlau et al. 2004). In addition, the relationship
between TRIM5α and CypA in HIV-1 infection was found in non-human primate
cells, even though CypA enhances the anti-HIV-1 restriction activity of the
TRIM5α orthologs in these cells (Towers et al. 2003; Berthoux et al. 2005a).
Furthermore, studies showed that determinants of HIV-1 CA sensitivity to
restriction by TRIM5α are located within the CypA-binding region on the CA,
suggesting that TRIM5α interacts with the CA at one or more sites proximal to
the CypA-binding region (Ikeda et al. 2004; Hatzioannou et al. 2004a; Owens et

However, in 2006, two independent groups demonstrated that CypA and
human TRIM5α act independently to regulate HIV-1 infectivity through a series of
experiments using human cancer cell lines, such as TE671 and CEM-SS, and a
feline CRFK cell line, which many researchers have used to study HIV-1
(Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006).
Considering that these cells are not physiologically relevant to HIV-1 infection
and that the effect of CA–CypA interaction on HIV-1 infectivity is dependent on
cell type (Sokolskaja, Sayah, and Luban 2004; Hatzioannou et al. 2005; Li, Kar, and Sodroski 2009; De Iaco and Luban 2014), further examination of these two factors in primary human blood cells (specifically CD4+ T cells), which serve as HIV-1 targets, was required. With the development of genetic tools to deliver genes of interest to primary human cells (Pertel et al. 2011; Fellmann et al. 2013; McCauley et al. 2018; Yurkovetskiy et al. 2018), we were able to carry out this further investigation in primary human cells.

2.3. RESULTS

To assess the role of TRIM5α and CypA in the primary human blood cell types that serve as targets for HIV-1 infection in vivo, lentiviral vectors were optimized for titer and knockdown efficiency in these cells (Sokolskaja, Berthoux, and Luban 2006; Pertel et al. 2011; Fellmann et al. 2013; McCauley et al. 2018; Yurkovetskiy et al. 2018). Primary human macrophages, dendritic cells (DCs) and CD4+ T cells were transduced with lentivectors bearing a puromycin resistance cassette and short hairpin RNAs (shRNAs) targeting either TRIM5 or luciferase (Luc) as a control. After three days of selection in puromycin, knockdown was confirmed by quantitative PCR with reverse transcription (RT−qPCR) for TRIM5 mRNA and by rescue of N-tropic MLV restriction (Figure 2.8A−C), as done previously (Sokolskaja, Berthoux, and Luban 2006; Pertel et al. 2011). TRIM5 and Luc control knockdown cells were then challenged with single-cycle, vesicular stomatitis virus glycoprotein (VSV G)-pseudotyped, HIV-1−green
A. Macrophages

B. Dendritic cells

C. CD4+ T cells + MLV

D. shRNA Target

E. shRNA Target
Figure 2.8. Assessment of shRNA-mediated knockdown in primary human blood cells.

(A–C) Lentiviral vectors containing puromycin N-acetyltransferase (PuroR) and shRNA targeting TRIM5 or Luc were used to transduce macrophages (A), dendritic cells (B), or CD4+ T cells (C). At 3 days post-transduction, cells were selected with puromycin for 3 days. Total RNA was isolated from the macrophages and dendritic cells, followed by cDNA synthesis, and qPCR with TaqMan detection of TRIM5 and the housekeeping gene OAZ1, for normalization (mean ± s.e.m., n = 3 independent samples). Significance was determined by two-tailed, unpaired t-test (A and B). The selected CD4+ T cells were challenged with N- or B-MLV vector harboring GFP reporter for 3 days. Flow cytometry was used to assess the percentage of GFP+ cells. The infectivity of each vector in TRIM5 knockdown cells was normalized to the Luc control condition. Shown is mean ± s.d. (n = 3 donors for each). Significance was determined by two-tailed, paired t-test (C).

(D and E) Macrophages were simultaneously transduced with two lentiviral vectors, the first expressing shRNA targeting TRIM5 or Luc with PuroR, and the second expressing blasticidin S-deaminase and shRNA targeting CypA or Luc (D), or CypA, CPSF6 or Luc (E). After selection with both antibiotics, CypA, CPSF6, and β-actin proteins were detected by western blot. Data shown is representative of three independent experiments using cells from three blood donors.
fluorescent protein (GFP) reporter vectors. Three days later, the percentage of GFP+ cells was assessed by flow cytometry as a measure of infectivity.

Compared to Luc control knockdown, TRIM5 knockdown had minimal effect on wild-type (WT) HIV-1 transduction efficiency in macrophages, dendritic cells or CD4+ T cells (Figure 2.9A−E). The infectivity of HIV-1 CA-P90A, a mutant that disrupts CypA binding (Braaten, Franke, and Luban 1996a; Franke, Yuan, and Luban 1994), was attenuated compared to the WT in control knockdown cells generated with all three cell types (Figure 2.9A−E). The effect was evident in cells from all blood donors tested (at least three blood donors per condition) and over a 100-fold range in challenge vector titer (Figure 2.9E). TRIM5 knockdown in macrophages, dendritic cells or CD4+ T cells increased CA-P90A infectivity (Figure 2.9A−E). Results were the same whether the challenge was with a three-plasmid vector system based on the clade B HIV-1 NL4-3 lab strain (Adachi et al. 1986; McCauley et al. 2018) (Figure 2.9A−C) or a two-plasmid vector system based on the clade C HIV-1 ZM249M transmission-founder strain from Zambia (Salazar-Gonzalez et al. 2009; McCauley et al. 2018) (Figure 2.9D).

Given previous reports that endogenous human TRIM5α in immortalized cell lines has a modest effect on HIV-1 infectivity, and that CypA and TRIM5α act independently to regulate HIV-1 transduction (Sokolskaja, Berthoux, and Luban 2006), the relatively large magnitude rescue of CA-P90A infectivity by TRIM5 knockdown in primary human blood cells was surprising. Complementary pharmacologic and reverse genetic approaches were therefore used to disrupt
Figure 2.9. Disruption of the CA−CypA interaction in primary human blood cells renders HIV-1 susceptible to restriction by TRIM5.

(A–D) Macrophages (A), dendritic cells (B) or CD4+ T cells (C and D) were selected after transduction with a lentivirus expressing shRNA targeting TRIM5 or Luc (control) and challenged with single-cycle, VSV G-pseudotyped, HIV-1_{NL4-3−GFP} (A–C) or HIV-1_{ZM249M−GFP} (D), bearing WT CA or CA-P90A (mean ± s.e.m., n = 3 donors for each).

(E) Raw infectivity data for single cycle viruses, before normalization of infectivity to control condition. Shown are representative of three independent experiments using cells from three blood donors for each condition.

(F) TRIM5 knockdown or Luc knockdown macrophages were challenged with HIV-1_{NL4-3−GFP} in the presence of 8 μM CsA or DMSO solvent (mean ± s.e.m., n = 4 donors).

(G) Macrophages expressing shRNA targeting TRIM5 or Luc were challenged with single-cycle, VSV G-pseudotyped, HIV-1_{NL4-3−GFP} in the presence of 8 μM CsA, 8 μM CsH, or DMSO solvent (mean ± s.e.m., n = 2 donors).

(H–J) TRIM5 knockdown or Luc knockdown CD4+ T cells were challenged with single-cycle HIV-1_{NL4-3−GFP} (H), HIV-1_{ZM249M−GFP} (I), or HIV-1_{Z331M-TF−GFP} (J) in the presence of 2.5 μM GS-CypAi3 or DMSO solvent (mean ± s.e.m., n = 3 donors).

(K) HIV-1_{NL4-3−GFP} was used to challenge TRIM5 or Luc knockdown CD4+ T cells with 2.5 μM GS-CypAi48 or DMSO solvent alone (mean ± s.e.m., n = 2 donors).

(L) Macrophages were transduced simultaneously with two vectors expressing shRNAs, as indicated, and selected with puromycin and blasticidin. Cells were then challenged with HIV-1_{NL4-3−GFP} (mean ± s.e.m., n = 3 donors). The percentage of GFP+ cells was assessed by flow cytometry and normalized to the WT in Luc control knockdown cells in all cases. Significance was determined by
two-tailed, paired Student’s $t$-test for data generated with at least three donors ($n = 3$)

(M) Luc or TRIM5 knockdown macrophages were simultaneously challenged with a constant amount of single-cycle, VSV G-pseudotyped HIV-1$^{\text{NL4-3}}$–GFP containing CA-P90A and the indicated quantities of HIV-1$^{\text{NL4-3}}$ VLPs harboring either WT CA or CA-P90A. Flow cytometry was used to measure the percentage of GFP$^+$ cells at day 3 post-challenge. Data shown here are representative of four independent experiments performed on cells from four blood donors.
the CA–CypA interaction. For pharmacologic disruption, cells were incubated in media containing small molecules that compete with CA for binding to CypA (Luban et al. 1993; Thali et al. 1994; Franke, Yuan, and Luban 1994; Braaten, Franke, and Luban 1996a; Mackman et al. 2018). Compared to dimethylsulfoxide (DMSO) solvent alone, cyclosporine A (CsA) reduced HIV-1 transduction efficiency in Luc control knockdown macrophages (Figure 2.9F). In contrast, cyclosporine H (CsH), an analogue with 1,000-fold lower affinity for CypA (Husi and Zurini 1994), caused only a slight increase in HIV-1 infection (Figure 2.9G). Since CsA blocks T cell proliferation, two non-immunosuppressive CypA inhibitors derived from sanglifehrin A, GS-CypAi3 and GS-CypAi48 (Mackman et al. 2018), were used instead on this cell type; these drugs decreased HIV-1 transduction efficiency in primary CD4+ T cells (Figure 2.9H–K). TRIM5 knockdown reversed the HIV-1 inhibition in macrophages caused by CsA (Figure 2.2F), or in CD4+ T cells caused by the sanglifehrin A-derivatives (Figure 2.9H–K).

To disrupt the CA–CypA interaction using a genetic approach, macrophages were transduced with two vectors for the knockdown of either TRIM5 or CypA, or both. The first vector conferred puromycin resistance and expressed shRNAs targeting either TRIM5 or Luc. The second vector conferred blasticidin resistance and expressed shRNAs targeting either CypA or Luc. After simultaneous transduction with pairs of these vectors, macrophages were selected for three days in both puromycin and blasticidin and then challenged
with single-cycle, VSV G-pseudotyped, HIV-1–GFP reporter vector bearing WT CA. Compared to the Luc control knockdown, CypA knockdown reduced CypA protein levels ~70% (Figure 2.8D). As with CA-P90A and the small molecule inhibitors, CypA knockdown decreased transduction efficiency (Figure 2.9L). This effect was rescued by simultaneous knockdown of TRIM5 (Figure 2.9L) without restoring CypA protein levels (Figure 2.8D). Taken together, these data indicate that endogenous human TRIM5 is required for HIV-1 restriction in the absence of the CA–CypA interaction. Consistent with the previously reported CA-specific saturation of TRIM5 restriction activity (Towers et al. 2003; Sokolskaja, Berthoux, and Luban 2006), the effective titer of HIV-1–GFP reporter vector bearing CA-P90A was increased in a dose-dependent manner by the addition of virus-like particles (VLPs) bearing CA-P90A, but not by VLPs bearing WT CA (Figure 2.9M).

Given that TRIM5α forms a lattice over the HIV-1 CA core complex for its antiviral activity, binding of host factors, like CypA, to CA may prevent the antiviral activity of TRIM5α. Mx2 and cleavage and polyadenylation specificity factor subunit 6 (CPSF6) have been identified as CA-binding factors, which restrict viral infection (Goujon et al. 2013; Kane et al. 2013) and aid integration of the viral genome into actively transcribed regions in the host genome (Chin et al. 2015; Sowd et al. 2016; Burdick et al. 2020), respectively. Considering that Mx2 is an interferon (IFN)-inducible gene (Goujon et al. 2013; Kane et al. 2013) and that human TRIM5α-mediated restriction against HIV-1 was observed in cells without IFN
stimulation, HIV-1 infectivity with disruption of CA−CPSF6 interaction was examined. First, the CA-N74D mutation (Lee et al. 2010) was used to disrupt this interaction in DCs or macrophages expressing a shRNA targeting luciferase control or TRIM5 (Figure 2.10A, B). Surprisingly, like the CA-P90A mutant virus used as a control in this experiment, the CA-N74D mutation decreased HIV-1 infectivity in Luc control knockdown cells but TRIM5 knockdown restored this infectivity in both cell types. These results suggest that CA−CPSF6 interaction may protect HIV-1 CA from restriction by human TRIM5α or that CA-N74D mutation may negatively affect CA−CypA interaction. Thus, two further experiments using virus bearing the CA-A77V mutation, an additional mutation found to disrupt CA−CPSF6 interaction (Saito et al. 2016), or CPSF6 knockdown were performed to ascertain whether CPSF6 binding to HIV-1 CA inhibits the antiviral activity of human TRIM5α in macrophages (Figure 2.10C, D). After macrophages were transduced with either shRNA targeting TRIM5 or CypA, or both as described above, a single-cycle, VSV G-pseudotyped, HIV-1−GFP reporter vector harboring WT CA, CA-N74D, or CA-A77V to disrupt CA−CPSF6 interaction were used to challenge the knockdown cells (Figure 2.10C). In Luc control knockdown cells, both the CA-N74D and CA-P90A mutations decreased the infectivity of HIV-1, whereas HIV-1 transduction with the CA-A77V mutation was comparable to that with WT CA. TRIM5 knockdown rescued the infectivity reduced by CA-N74D or CA-P90A toward the level of WT virus but showed minor effects on both WT and CA-A77V viruses. The different effects between CA-
Figure 2.10. The interaction between HIV-1 CA and CPSF6 is independent of restriction activity of human TRIM5α.

(A and B) Dendritic cells (A) or macrophages (B) expressing shRNA targeting TRIM5 or Luc control were challenged with single-cycle, VSV G-pseudotyped HIV-1NL4-3−GFP containing WT CA, CA-N74D, or CA-P90A (mean ± s.e.m., n = 2 donors).

(C) Macrophages were transduced with two vectors expressing shRNAs targeting TRIM5 or Luc, and CypA or Luc, respectively, followed by selection with puromycin and blasticidin. Cells were then challenged with HIV-1NL4-3−GFP bearing WT CA, CA-N74D, CA-A77V, or CA-P90A (mean ± s.e.m., n = 2 donors).

(D and E) Simultaneous transduction of macrophages was performed to generate TRIM5/CPSF6 (D) or TRIM5/TNPO3 (E) double knockdown conditions including respective single knockdown and Luc control knockdown conditions, as indicated. HIV-1NL4-3−GFP was used to challenge these cells (mean ± s.e.m., n = 2 donors). The percentage of GFP-expressing cells was assessed by flow cytometry and normalized to the WT in Luc control knockdown cells in all cases.
N74D and CA-A77V mutations on HIV-1 transduction imply that human TRIM5α-mediated restriction against HIV-1 CA occurs independently of CA–CPSF6 interaction, as well as that the N74D mutation in HIV-1 CA abates CA–CypA interaction. The latter point is consistent with the observation that the infectivity of CA-N74D virus was less sensitive to CypA knockdown, compared to WT or CA-A77V viruses (Figure 2.10C). Lastly, macrophages were transduced with vectors for knockdown of TRIM5, CPSF6, or both (Figure 2.10D). The CPSF6 protein level was largely decreased in CPSF6 knockdown cells compared to the Luc control knockdown cells (Figure 2.8E). Macrophages expressing shRNA targeting transportin 3 (TNPO3), instead of CPSF6, along with TRIM5 shRNA, were also generated (Figure 2.10E). TNPO3 imports CPSF6 into the nucleus, and thus, CPSF6 resides only in the cytoplasm when TNPO3 is knocked down. HIV-1 infection is inhibited by the interaction between CA and cytoplasmic CPSF6 in TNPO3 knockdown cells (De Iaco et al. 2013; Fricke et al. 2013). These knockdown cells were challenged with WT HIV-1-GFP vectors. CPSF6 knockdown showed a minimal effect on HIV-1 infectivity (Figure 2.10D), similar to the observation for the CA-A77V mutation (Figure 2.10C). This is different from CypA knockdown, which consistently reduced HIV-1 infectivity (Figure 2.9L). The inhibition of HIV-1 transduction due to CA–CPSF6 trapped in the cytoplasm by TNPO3 knockdown, the effects of which were not affected by TRIM5 knockdown, was also observed (Figure 2.10E). Therefore, these data demonstrate that CA–CPSF6 interaction is independent of the restriction activity of human TRIM5α
against HIV-1 CA.

Although the shRNA that targets TRIM5 is distinguished from the next most similar sequence in the human genome (GRCh38) by multiple mismatches, off-target effects are theoretically possible. To test whether TRIM5α is sufficient to explain the HIV-1 restriction activity associated with CA–CypA disruption, a vector was designed based on the ubiquitin fusion technique (Varshavsky 2005) that expresses a tripartite fusion of puromycin N-acetyl transferase (PuroR), the K48R mutant of ubiquitin (UbK48R) and the coding sequence for a protein of interest, in addition to an shRNA (Figure 2.11A). Four variants of the plasmid were engineered in which the shRNA targeted either TRIM5 or Luc, with or without a TRIM5α coding sequence bearing mismatches in the shRNA target sequence (Figure 2.11A).

Macrophages (Figure 2.11B, C) and CD4+ T cells (Figure 2.11D) were transduced with each of the four variants of the shRNA tripartite fusion vector and selected for three days with puromycin. TRIM5 expression level was largely increased in cells transduced with vectors containing TRIM5α coding sequence (Figure 2.11B). The expression level of endogenous TRIM5 was decreased by shRNA targeting TRIM5, regardless of the expression of exogenous TRIM5α in cells (Figure 2.11B). Cells were then challenged with the three-plasmid HIV-1–GFP reporter vector and assessed by flow cytometry for percentage of GFP+ cells three days later. As in Figure 2.9, the infectivity of the vector bearing WT CA was minimally affected by TRIM5 knockdown or by TRIM5α overexpression.
Figure 2.11. Human TRIM5α is sufficient to explain the inhibition of reverse transcription that results from disruption of CA–CypA interaction.

(A) A schematic representation of an all-in-one shRNA-rescue lentivector, in which the spleen focus-forming virus (SFFV) promoter expresses a tripartite fusion of PurOR, UbK48R and an open reading frame (ORF) for a gene of interest, as well as a microRNA30-based shRNA (miR30).

(B–D) All-in-one lentivectors encoding empty control (no ORF) or non-targetable, shRNA-resistant TRIM5α coding sequence (ntTRIM5α), along with shRNA targeting Luc or TRIM5, as indicated in (A), were used to transduce macrophages (B and C) or CD4+ T cells (D). Total RNA was isolated from the macrophages, followed by cDNA synthesis, and TaqMan-based qPCR to detect both endogenous and exogenous TRIM5, or only endogenous TRIM5, with the housekeeping gene OAZ1, for normalization (mean ± s.e.m., n = 3 technical replicate). The data shown are representative of two independent experiments using cells from two blood donors for each condition (B). The transduced cells were challenged with HIV-1NL4-3−GFP bearing WT CA or CA-P90A for 3 days, as indicated. The percentage of GFP-expressing cells was measured by flow cytometry and normalized to the values for no ORF/Luc knockdown cells challenged with WT CA; mean ± s.e.m., n = 3 donors for each. Significance was determined by two-tailed, paired Student’s t-test (C and D).

(E–H) TRIM5 knockdown or Luc knockdown macrophages (E) or CD4+ T cells (F), macrophages (G) or CD4+ T cells (H) transduced with the all-in-one shRNA-rescue lentivectors described in (A) were challenged with HIV-1NL4-3−GFP containing WT CA or CA-P90A, as indicated. DNA was extracted 20 h post challenge and late products of reverse transcription were assessed by qPCR (mean ± s.e.m., n = 3 biologically independent samples). RT-D185K/D186L mutant virus was used as a control for background signal. Significance was determined by two-tailed, unpaired Student’s t-test.
(Figure 2.11C, D). Compared to the WT, the infectivity of vector bearing CA-P90A was decreased (Figure 2.11C, D) and the infectivity of this mutant was rescued by TRIM5 shRNA (Figure 2.11C, D). In the presence of shRNA targeting TRIM5, delivery of TRIM5α coding sequence bearing shRNA target-site mismatches restored restriction activity to the control level (Figure 2.11C, D). These results demonstrate that, in primary human blood cells, human TRIM5α is sufficient to restrict HIV-1 transduction, but only when the CA–CypA interaction is disrupted.

To determine at which step in the virus life cycle human TRIM5α inhibits HIV-1 when the CA–CypA interaction is disrupted, HIV-1 complementary DNA (cDNA) resulting from reverse transcription was assessed by qPCR. Macrophages and CD4+ T cells were stably transduced and selected with vector expressing shRNAs targeting TRIM5 or Luc (control) (Figure 2.11E, F), or with each of the four variants of the shRNA tripartite fusion vector (Figure 2.11G, H). Cells were then challenged with a HIV-1 reporter vector that had the 34 base pair loxP sequence in the U3 region of the 3' long terminal repeat to distinguish reporter vector transcripts from those of the shRNA lentivector (De Iaco and Luban 2011). DNA was collected 20 h post challenge and qPCR was performed using primers specific for full-length linear HIV-1 cDNA (late RT). In all experiments, reporter vector bearing the RT D185K/D186L loss-of-function mutation (De Iaco and Luban 2011) was included as a control for background signal not due to nascent reverse transcription (Figure 2.11E–H).
In Luc control knockdown macrophages and CD4+ T cells, viral cDNA was reduced by CA-P90A and this reduction was reversed by TRIM5 knockdown (Figure 2.11E, F). Viral cDNA was also reduced by CA-P90A in either cell type transduced with the control shRNA tripartite fusion vector (Figure 2.11G, H). TRIM5 shRNA rescued the cDNA (Figure 2.11E–H) and rescue of TRIM5α with the non-targetable coding sequence again decreased the CA-P90A cDNA (Figure 2.11G, H). These results demonstrate that, when the CA–CypA interaction is disrupted, human TRIM5α blocks HIV-1 at an early step of viral infection, before the completion of reverse transcription.

To determine whether TRIM5α associates with HIV-1 CA in cells when the CA–CypA interaction is disrupted, primary human macrophages were stably transduced with TRIM5 shRNA or Luc shRNA and then challenged for 2 h with WT HIV-1 reporter vector in the presence or absence of CsA, or with HIV-1 vectors bearing WT CA or CA-P90A. Cells were fixed and subjected to the proximity ligation assay (PLA) with antibodies specific for HIV-1 CA and endogenous human TRIM5α. When cells were challenged with WT HIV-1 in the absence of CsA, very few punctae were detected (Figure 2.12A–D). Similarly, few puncta were detected when cells were treated with CsA in the absence of HIV-1 challenge (Figure 2.12A, B). By contrast, when cells were challenged with WT HIV-1 in the presence of CsA or with HIV-1 CA-P90A, multiple puncta were detected (Figure 2.12A–D), an increase of at least 10- and 20-fold in the average number of puncta per cell over the background, respectively (Figure 2.12B, D).
**A**

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Figure 2.12. Endogenous TRIM5α in primary human macrophages associates with HIV-1 CA after acute challenge but only when the CA–CypA interaction is disrupted.

(A–D) Macrophages were transduced and selected with vector bearing shRNA targeting either TRIM5 or Luc. Cells were then challenged for 2 h with VSV G-pseudotyped HIV-1_{NL4-3−GFP} in the presence of 5 μM CsA or DMSO solvent (A and B) or challenged with HIV-1_{NL4-3−GFP} harbouring WT CA or CA-P90A (C and D), as indicated. Cells were fixed and PLA was performed with antibodies against HIV-1 CA and TRIM5α. The representative images in (A) and (C) show PLA punctae (red), nuclei stained with Hoechst (blue) and actin filaments stained with phalloidin (green). The graphs in (B) and (D) show the number of PLA punctae per cell with mean ± s.e.m. For (B): Luc knockdown + CsA, no virus, n = 45 cells analysed; Luc knockdown + DMSO + HIV-1, n = 45; Luc knockdown + CsA + HIV-1, n = 69 (donor 1) or 80 (donor 2); TRIM5 knockdown + CsA + HIV-1, n = 45. For (D): Luc knockdown + WT HIV-1, n = 20; Luc knockdown + CA-P90A HIV-1, n = 25 (donor 1) or 30 (donor 2); TRIM5 knockdown + CA-P90A HIV-1, n = 20; TRIM5 knockdown + WT HIV-1, n = 20. Significance was determined by two-tailed, unpaired Student’s t-test. The data shown are representative of four independent experiments using cells from four blood donors for each condition. Scale bars in (A) and (C), 5 μm.

(E) Luc control knockdown macrophages treated with 5 μM CsA were challenged with VSV G-pseudotyped HIV-1_{NL4-3−GFP} in the presence of 2 μM MG132 or DMSO solvent. Cells were fixed and proximity ligation assay (PLA) was performed with anti-CA (p24) and anti-TRIM5α antibodies. Representative images show PLA puncta (red), nuclei stained with Hoechst (blue), and actin filaments stained with phalloidin (green). Scale bars are 5 μm. The graph on the right shows the number of puncta per cell in the PLA, after analysis of 30 cells per condition (mean ± s.e.m.). Significance was determined by two-tailed, unpaired Student’s t-test.
TRIM5 knockdown eliminated the punctae (Figure 2.12A–D); this indicated that
the PLA signal was dependent on TRIM5 expression. The PLA signal was also
dependent on the proteasome inhibitor MG132 (Figure 2.12E), a result consistent
with the reported involvement of the proteasome in the inhibition of reverse
transcription by TRIM5α (Wu et al. 2006). These results indicate that, in the
infection of primary human cells, endogenous TRIM5α associates with HIV-1 CA
when the CA–CypA interaction is disrupted.

The above experiments used single-cycle HIV-1 vectors. The effect of
CypA on HIV-1 restriction by human TRIM5α was therefore evaluated next using
replication-competent HIV-1 in a context where the virus spreads from cell to cell.
Primary human macrophages were challenged with clade B HIV-1 bearing a
macrophage-tropic env (HIV-1MAC) and replication was monitored for 14 days by
measuring the accumulation of reverse transcriptase activity in the supernatant.
As in the single-cycle experiments (Figure 2.9), TRIM5 knockdown itself had little
effect on WT HIV-1 replication (Figure 2.13A, B). Disruption of the CA–CypA
interaction with CsA (Figure 2.13A) or with shRNA targeting CypA (Figure 2.13B)
effectively suppressed viral spread in the culture and, in both cases, replication
kinetics were completely restored to the control level by shRNA targeting TRIM5
(Figure 2.13A, B). Primary CD4+ T cells were then challenged with a clade C
transmission-founder virus (HIV-1ZM249M). As observed in macrophages, TRIM5
knockdown alone had minimal effect on WT HIV-1 replication (Figure 2.13C, D).
No viral replication was detectable when the CA–CypA interaction was disrupted.
Kim et al., Figure 2.6
Figure 2.13. Endogenous TRIM5α suppresses the spread of HIV-1 infection in primary human macrophages and CD4+ T cells when the CA–CypA interaction is disrupted.

(A and B) The spread of HIV-1MAC infection in TRIM5 or Luc knockdown macrophages with 5 μM CsA (A) or with vectors bearing shRNAs targeting CypA or Luc (B), as indicated.

(C and D) The spread of HIV-1ZM249M infection in CD4+ T cells expressing shRNA targeting TRIM5 or Luc with 2.5 μM GS-CypAi3 (C) or when challenged with virus bearing CA-P90A (D), as indicated.

(E) The spread of HIV-1MAC infection in macrophages transduced with the all-in-one shRNA-rescue lentivectors described in Figure 3.4, as indicated. HIV-1 replication was monitored by measuring reverse transcriptase activity (RTU/μl) in the culture supernatant over time. The data shown are representative of two independent experiments using cells from two blood donors for each condition.
by the small molecule GS-CypAi3 (Figure 2.13C) or by the presence of CA-P90A in HIV-1 (Figure 2.13D); in both cases shRNA targeting TRIM5 rescued replication kinetics to the level of the controls (Figure 2.13C, D). Furthermore, the shRNA tripartite fusion vectors were exploited to rule out off-target effects of the shRNA and to demonstrate that TRIM5α is sufficient to restrict HIV-1 replication under conditions in which the CA–CypA interaction is interrupted (Figure 2.13E).

2.4. DISCUSSION

The abundant cellular protein CypA interacts with the incoming HIV-1 CA core upon viral infection. This interaction promotes HIV-1 infection by enhancing viral reverse transcription levels in human cells, but the underlying mechanism remained unclear. The experiments presented here demonstrate that, in primary human blood cells, HIV-1 exploits CypA to evade CA recognition/restriction by endogenous human TRIM5α. This answers the long-standing question of how CypA promotes HIV-1 infection and clearly establishes that, in the absence of CypA, human TRIM5α potently restricts HIV-1. The regulation of HIV-1 infectivity by CypA and human TRIM5α is also demonstrated to be independent of the interaction of CA with another CA-binding factor CPSF6. Conservation of the lentiviral CA–CypA interaction across millions of years of evolution (Luban et al. 1993; Katzourakis et al. 2007; Gilbert et al. 2009; Goldstone et al. 2010; Malfavon-Borja et al. 2013; Mu et al. 2014) is probably a result of selective
pressure applied by TRIM5 orthologs encoded by host species that are otherwise permissive for lentiviral replication.

Given that CypA and TRIM5α both bind to HIV-1 CA, the simplest model for how CypA prevents TRIM5α-mediated recognition is that CypA sterically blocks TRIM5α from binding to CA. Studies have suggested that TRIM5α interacts with the CypA-binding loop of HIV-1 CA (Owens et al. 2004; Hatzioannou et al. 2004a; Ylinen et al. 2005; Kono et al. 2010; Quinn et al. 2018; Yu et al. 2020). In addition, an evolutionary analysis of the binding site of lentiviral CA for CypA and TRIM5α also showed that the two factors have bound to the proximal sites on CA for over 10 million years (McCarthy et al. 2015). These results imply that CA–CypA interaction is highly likely to interfere with TRIM5α binding to CA. The discovery of the secondary binding site of CypA for HIV-1 CA (Liu et al. 2016) would also support this model, because this additional interaction is likely to increase the chance for sterical hindrance of the formation of the multimeric TRIM5α lattice over the CA core. Alternatively, because CypA possesses peptidyl-prolyl isomerase activity (Colgan et al. 2004) and HIV-1 CA-P90 is a validated substrate (Bosco et al. 2002), CypA might shift the CA conformation and thereby protect HIV-1 CA from recognition by TRIM5α.

In contrast to the results here, previous reports showed that non-human primate TRIM5α orthologs are capable of restricting HIV-1 when expressed in human cells (Stremlau et al. 2004) and that CypA promotes such restriction in non-human primate cells (Towers et al. 2003; Sayah et al. 2004a; Berthoux et al. 2005).
These observations likely reflect a different mode of CA recognition by TRIM5α orthologs from these species.

Moreover, the current study in primary human cells gave opposite results compared with previous studies in human cancer cell lines, which found that CypA and human TRIM5α independently regulate HIV-1 infectivity (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). This difference may be due to differences in metabolic status between the cell types or the differential expression of the unknown cofactor involved in human TRIM5α-mediated restriction. The recent discovery of the role of immunoproteasome in the restriction activity of human TRIM5α in IFN-stimulated cells implies the presence of the cofactor (Jimenez-Guardeño et al. 2019). Further investigations are required to identify what causes this difference in the CypA–TRIM5α relationship in HIV-1 infectivity between primary human cells and cell lines.

Finally, the results here indicate that, by rendering HIV-1 susceptible to the potent antiviral activity of TRIM5α, non-immunosuppressive CypA inhibitors have the potential to make an important contribution to anti-HIV-1 drug cocktails.

2.5. MATERIALS AND METHODS

Plasmids. All the plasmids used here are described in Table 3.1 and are available, along with full sequences, at https://www.addgene.org/Jeremy_Luban/.

Human blood. Leukopaks were obtained from anonymous, healthy blood donors
(New York Biologics). These experiments were reviewed by the University of Massachusetts Medical School Institutional Review Board and declared to be non-human subjects research, according to National Institutes of Health (NIH) guidelines (http://grants.nih.gov/grants/policy/hs/faqs_aps_definitions.htm).

**Cell culture.** All cells were cultured in humidified, 5% CO$_2$ incubators at 37 °C. HEK293 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 20 mM GlutaMAX-I, 1× MEM non-essential amino acids and 25 mM HEPES, pH 7.2 (DMEM–FBS complete). Peripheral blood mononuclear cells (PBMCs) were isolated from leukopaks by gradient centrifugation on Lymphoprep (Axis-Shield Poc AS, catalogue no. AXS-1114546). To generate dendritic cells or macrophages, CD14$^+$ mononuclear cells were enriched by positive selection using anti-CD14 antibody microbeads (Miltenyi, catalogue no. 130-050-201). Enriched CD14$^+$ cells were plated in RPMI-1640, supplemented with 5% heat-inactivated human AB$^+$ serum (Omega Scientific), 1 mM sodium pyruvate, 20 mM GlutaMAX-I, 1× MEM non-essential amino acids and 25 mM HEPES pH 7.2 (RPMI–HS complete), at a density of $10^6$ cells/ml for macrophages or $2 \times 10^6$ cells/ml for dendritic cells. To differentiate CD14$^+$ cells into macrophages, 1:100 human granulocyte–macrophage colony stimulating factor (hGM-CSF)-conditioned media was added. To differentiate CD14$^+$ cells into dendritic cells, 1:100 cytokine-conditioned media containing hGM-CSF and human interleukin-4


(hIL-4) was added. hGM-CSF and hIL-4 were produced from HEK293 cells transduced with pAIP-hGMCSFco (Addgene no. 74168) or pAIP-hIL4-co (Addgene no. 74169), as previously described (Pertel et al., 2004; McCauley et al., 2018). CD4+ T cells were isolated from CD14-depleted PBMCs using anti-CD4 antibody microbeads (Miltenyi, catalogue no. 130-045-101); enrichment was typically >90%, as assessed by measuring the percentage of CD3+/CD4+ cells via flow cytometry with fluorescein isothiocyanate (FITC)-anti-CD3 (Biolegend, catalogue no. 317306) and allophycocyanin (APC)-anti-CD4 (Biolegend, catalogue no. 317416). The cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 20 mM GlutaMAX-I, 1× MEM non-essential amino acids and 25 mM HEPES pH 7.2 (RPMI–FBS complete) with 50 U/ml hIL-2 (NIH AIDS Reagent Program, catalogue no. 136).

**Virus production.** 24 h before transfection, 6 × 10⁵ HEK293 cells were plated per well in six-well plates. All transfections used 2.49 μg plasmid DNA with 6.25 μl TransIT LT1 transfection reagent (Mirus) in 250 μl Opti-MEM (Gibco). 2.49 μg of replication-competent HIV-1 provirus DNA was transfected. For two-part, single-cycle vector, or for HIV-1 VLPs lacking a packageable genome, 2.18 μg of env-defective HIV-1 provirus or p8.9 NΔSB gag-pol plasmid was cotransfected with 0.31 μg pMD2.G VSV G plasmid, respectively. Three-part, single-cycle vectors were produced by cotransfecting 1.25 μg minimal lentivector genome plasmid (pALPS-GFP, pWPTS-GFP, pLXIN-GFP, pAPM-D4-miR30, pABM-D4-
miR30 or pPU-ORF-miR30), 0.93 μg gag-pol plasmid (psPAX2, p8.9 NΔSB, pCIG3-N or pCIG3-B (Bock et al. 2000)) and 0.31 μg pMD2.G VSV G plasmid. Vpx-containing simian immunodeficiency virus (SIV)−VLPs were produced by the transfection of 2.18 μg pSIV3+ and 0.31 μg pMD2.G plasmid. 16 h post transfection, the culture media was changed to the media specific for the cells to be transduced. Viral supernatant was harvested at 72 hour post-transfection, passed through a 0.45 μm filter and stored at −80 °C.

**Exogenous reverse transcriptase assay.** A total of 5 μl transfection supernatant was mixed with 5 μl 0.25% Triton X-100, 50 mM KCl, 100 mM Tris−HCl pH 7.4 and 0.4 U/μl RiboLock RNase inhibitor and then diluted 1:100 in 5 mM (NH₄)₂SO₄, 20 mM KCl and 20 mM Tris−HCl pH 8.3. 10 μl of this was then added to a single-step, RT−PCR assay with 35 nM bacteriophage MS2 RNA (Integrated DNA Technologies) as a template, 500 nM of each primer (5′-TCCTGCTCAACTTCTGTGCAG-3′ and 5′-CACAGGTCAAACCTCTAGGAATG-3′) and 0.1 μl hot-start Taq DNA polymerase (Promega) in 20 mM Tris−HCl pH 8.3, 5 mM (NH₄)₂SO₄, 20 mM KCl, 5 mM MgCl₂, 0.1 mg/ml BSA, 1/20,000 SYBR Green I (Invitrogen) and 200 μM of deoxynucleotides (dNTPs) in a total reaction volume of 20 μl. The RT−PCR reaction was carried out in a Bio-Rad CFX96 real-time PCR detection system with the following parameters: 42 °C for 20 min, 95 °C for 2 min and 40 cycles (95 °C for 5 sec, 60 °C for 5 sec, 72 °C for 15 sec and acquisition at 80 °C for 5
Transduction with lentiviral knockdown vectors. For dendritic cells, $2 \times 10^6$ CD14$^+$ monocytes/ml were transduced with a 1:4 volume of SIV$^-$VLPs and a 1:4 volume of knockdown lentivector. For macrophages, $10^6$ CD14$^+$ monocytes/ml were transduced with a 1:8 volume of SIV$^-$VLPs and a 1:8 volume of knockdown lentivector. The Vpx-containing SIV$^-$VLPs were added to these cultures to overcome a SAMHD1 block to lentiviral transduction (Laguet et al. 2011; Hrecka et al. 2011). Transduced cells were selected with 3 μg/ml puromycin (InvivoGen, catalogue no. ant-pr-1), 10 μg/ml blasticidin (InvivoGen, catalogue no. ant-bl-1) or both, for 3 days, starting 3 day post-transduction.

Following isolation with magnetic beads, human CD4$^+$ T cells were cultured at 2 to $3 \times 10^6$ cells/ml in RPMI–FBS complete, supplemented with 50 U/ml hIL-2 and stimulated with 5 μg/ml PHA-P (Sigma-Aldrich, catalogue no. L-1668). Alternatively, CD4$^+$ T cells at $10^6$ cells/ml were stimulated with 25 μl/ml ImmunoCult Human CD3/CD28 T Cell Activator (STEMCELL Technologies, catalogue no. 10991). At day 3 post stimulation, T cells were replated at 2 to $3 \times 10^6$ cells/ml in RPMI–FBS complete, with 50 U/ml hIL-2. Cells were transduced with $10^8$ reverse transcription units (RTUs) of viral vector per $10^6$ cells for 3 days, followed by selection with 2 μg/ml puromycin. After selection for 3 days, cells were restimulated with PHA-P or ImmunoCult Human CD3/CD28 T Cell Activator for 3 days. The stimulated cells were then replated at 2 to $3 \times 10^6$ cells/ml (PHA)
or at $10^6$ cells/ml (CD3/CD28) in RPMI–FBS complete with 50 U/ml hIL-2 and challenged with lentiviral vectors for the assessment of single-cycle infectivity or with replication competent HIV-1 for spreading infection. Fresh media containing hIL-2 was replenished every 2–3 days.

**Infectivity assay using single-cycle viruses.** For human dendritic cells, $2.5 \times 10^5$ cells were seeded per well, in a 48-well plate, on the day of virus challenge. Media containing VSV G-pseudotyped lentiviral vector expressing GFP (HIV-1–GFP) was added to challenge cells in a total volume of 250 μl. For human macrophages, $2.5 \times 10^5$ cells were seeded per well in a 24-well plate and challenged with HIV-1–GFP in a total volume of 500 μl. The cells were also simultaneously challenged with HIV-1 VLPs, as indicated. A 1:50 volume of SIV–VLPs was also added to the medium during the virus challenge of dendritic cells or macrophages. To challenge human CD4+ cells activated with PHA, $5 \times 10^5$ cells were plated per well in a 96-well plate 3 days after the second PHA stimulation. For CD4+ cells stimulated with CD3/CD28 activator, $2 \times 10^5$ cells were plated in each well of a 96-well plate, 3 days after secondary stimulation. Cells were then challenged with GFP reporter viruses in a total volume of 200 μl. For all three cell types, four dilutions of viral stocks, from $10^5$ to $10^8$ RTU/ml, were used to challenge cells. Where indicated, cells were pretreated with 8 μM CsA, 8 μM CsH or 2.5 μM of non-immunosuppressive CypA inhibitors from Gilead (GS-CypAi3 or GS-CypAi48) (Mackman et al. 2018), for 1 hour before virus challenge.
In experiments using CsH treatment, the media was replaced after 16 hours of treatment to avoid CsH toxicity (Petrillo et al. 2018).

At 48 hours post challenge with two-part HIV-1 vectors, or at 72 hours post challenge with three-part lentiviral vectors, cells were harvested for flow cytometric analysis by pipetting (CD4+ T cells) or scraping (dendritic cells and macrophages). Cells were pelleted at 500g for 5 min and fixed in a 1:4 dilution of BD Cytofix Fixation Buffer with phosphate-buffered saline (PBS) without Ca2+ and Mg2+, supplemented with 2% FBS and 0.1% NaN3.

**Flow cytometry.** Data were collected on an Accuri C6 (BD Biosciences) and plotted with FlowJo software v.10. Infectivity at each dilution, in each condition (CA mutant, CypA inhibitor or CypA knockdown) was compared to the infectivity of WT CA in the control condition. Dilutions yielding infectivity greater than 30% GFP+ cells were excluded from the analysis on the assumption that these were out of the linear range, according to the Poisson distribution.

**Statistical analysis.** Experimental n values and information regarding the statistical tests can be found in the figure legends. The data for infectivity assays using single-cycle viruses including at least three independent donors were statistically analysed using two-tailed paired t-tests compared to the control condition or the indicated condition for each donor. The qPCR data for experiments measuring viral cDNA levels with three biologically independent
samples for each condition were analysed using two-tailed, unpaired t-tests for the comparison of two conditions as indicated in Figure 3.4. The data from PLA quantification were assessed for statistical significance using two-tailed unpaired t-tests to compare two conditions as indicated in Figure 3.5. All statistical analyses were performed using PRISM 8.2 (GraphPad Software).

**qPCR for viral late reverse transcriptase product.** Total DNA was extracted from cells using DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer’s instructions. Late reverse transcription products were detected with the TaqMan system using the primers pWPTS J1B fwd and pWPTS J2 rev with the late reverse transcription probe (LRT-P) (Reinhard et al. 2014). Mitochondrial DNA was used for normalization with the following primer/probe set: MH533, MH534 and Mito probe (Butler, Hansen, and Bushman 2001). The primer and probe sequences are specified in Table 3.3. The qPCR was performed in 20 μl reaction mix containing 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 900 nM each primer, 250 nM TaqMan probe and 30 to 50 ng template DNA. After an initial incubation at 50 °C for 2 min and a second incubation at 95 °C for 10 min, 45 cycles of amplification were carried out at 95 °C for 15 sec followed by 1 min and 30 sec at 60 °C. Real-time PCR reactions were run on a CFX96 thermal cycler (Bio-Rad).

**RT-qPCR.** Total RNA was isolated in TRIzol reagent followed by RNA
purification with RNeasy Plus Mini kit (Qiagen). First-strand cDNA was generated using SuperScript VILO Master Mix (Thermo Fisher) with random hexamers, in accordance with the manufacturer’s instructions. Duplex qPCR was performed in 20 μl reaction mix containing 1× TaqMan Gene Expression Master Mix, 1× TaqMan Gene Expression Assay targeting the exonic region of TRIM5 mRNA to detect both exogenous and endogenous TRIM5 (FAM dye-labelled, TaqMan probe ID no. Hs01552559_m1) or the 5’ untranslated region (UTR) of TRIM5 mRNA to detect only endogenous TRIM5 (FAM dye-labelled, TaqMan probe ID no. Hs00263630_m1), 1× TaqMan Gene Expression Assay targeting a housekeeping gene OAZ1 (VIC dye-labelled, primer-limited, TaqMan probe ID no. Hs00427923_m1). Amplification was on a Bio-Rad CFX96 real-time PCR detection system, using 50 °C for 2 min, 95 °C for 10 min, then 45 cycles of 95 °C for 15 sec and 60 °C for 60 sec.

Western blot. Cells were lysed in Hypotonic Lysis Buffer: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% NP-40, 0.1% Triton X-100 and cOmplete mini protease inhibitor (Sigma-Aldrich) for 20 min on ice. The lysates were mixed 1:1 with 2× Laemmli buffer containing 1:20-diluted 2-mercaptoethanol, boiled for 10 min and centrifuged at 16,000g for 5 min at 4 °C. Samples were run on 4–20% SDS–PAGE and transferred to nitrocellulose membranes. Membrane blocking and antibody binding were in TBS Odyssey Blocking Buffer (Li-Cor). Primary antibodies used were rabbit anti-CypA (1:10,000 dilution; Enzo Life
Sciences, catalogue no. BML-SA296), rabbit anti-CPSF6 (1:2,000 dilution; Novus Biologicals, catalogue no. NB100-61596), and mouse anti-β-actin (1:1,000 dilution; Abcam, catalogue no. ab3280). Goat anti-mouse-680 (Li-Cor, catalogue no. 925-68070) and goat anti-rabbit-800 (Li-Cor, catalogue no. 925-32211) as secondary antibodies were used at 1:10,000 dilutions. Blots were scanned on the Li-Cor Odyssey CLx.

**PLA.** 2.5 × 10⁵ macrophages were plated on 12 mm coverslips (Warner Instrument, catalogue no. CS-12R15) in 24-well plates. Cells were spinoculated at 1,200g using 6 × 10⁸ RT unit/ml of three-part lentiviral vector (pALPS-GFP, p8.9 NΔSB and pMD2.G, generated as above) at 13 °C for 2 hours. The media was replaced with RPMI–HS complete containing 2 μM MG132 and either DMSO or 5 μM CsA; cells were incubated at 37 °C for 2 hours. Coverslips were fixed with 3.7% formaldehyde (Thermo Fisher) in 0.1 M PIPES, pH 6.8, for 5 min at room temperature and then incubated at room temperature for 1 hour in PBS containing 0.1% saponin, 10% donkey serum, 0.01% sodium azide, mouse anti-TRIM5α antibody (NIH AIDS Reagent Program, catalogue no. 12271) at a 1:750 dilution and rabbit anti-HIV-1 CA (p24) antibody (Abcam, catalogue no. ab32352) at a 1:400 dilution.

The samples were processed further using a Duolink In Situ Red kit (Sigma-Aldrich), following the instructions of the manufacturer. Next, samples were incubated with 10 μM phalloidin (fluorescein isothiocyanate; Enzo Life
Science) and 1 mg/ml Hoechst 33342 (Invitrogen), in PBS containing 10% donkey serum and 0.01% sodium azide for 30 min at room temperature.

Coverslips were mounted on slides and stored at −20 °C. Interaction was detected as fluorescent spots (λ\text{excitation/emission} at 598/634 nm). λ\text{excitation/emission} at 475/523 nm and λ\text{excitation/emission} at 390/435 nm were used to detect phalloidin and Hoechst, respectively. z-stack images were collected with a DeltaVision wide-field fluorescent microscope (Applied Precision, GE) and deconvolved with SoftWoRx deconvolution software v.7.0.0 (Applied Precision, GE). All images were acquired under identical acquisition conditions and analysed by Imaris 8.3.1 (Bitplane). Three-dimensional representations were constructed by using the Easy 3D function (Imaris 8.3.1).

**Challenge with replication-competent HIV-1.** 5 × 10⁵ macrophages per well in 12-well plates were challenged with 10⁸ RT units of HIV-1 for 2 hours, in the presence of CsA or DMSO solvent, as indicated. Macrophage experiments used NL4-3\text{MAC}; pNL4-3, in which env was replaced from the end of the signal peptide to the env stop codon, with macrophage-tropic env from GenBank (no. U63632.1). 3 days after secondary stimulation with CD3/CD28, 10⁶ CD4⁺ T cells per well in 48-well plates were challenged with 2 × 10⁷ RT units of HIV-1 for 2 hours, in the presence of GS-CypAi3 or DMSO solvent, as indicated. CD4⁺ T cell experiments used HIV-1\text{ZM249M}, a clade C transmission-founder strain. After HIV-1 challenge, cells were washed with fresh media and resuspended in 1 ml of
RPMI–HS complete for macrophages or RPMI–FBS complete containing 50 U/ml hIL-2 for CD4+ T cells. Where indicated, culture media also contained CsA, GS-CypAi3 or DMSO solvent. Every 2–3 days, culture supernatant was harvested to measure RT activity.
Table 2.1. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Purpose</th>
<th>Notes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAIP- hGMCSF-co</td>
<td>Stable 293 cell line expressing human cytokine GM-CSF</td>
<td>SFFV promoter expresses codon-optimized human GM-CSF and puromycin N-acetyltransferase (Pertel et al. 2011; McCauley et al. 2018)</td>
<td>Addgene #74168</td>
</tr>
<tr>
<td>pAIP-hIL4-co</td>
<td>Stable 293 cell line expressing human cytokine IL4</td>
<td>SFFV promoter expresses codon-optimized human IL4 and puromycin N-acetyltransferase (Pertel et al. 2011; McCauley et al. 2018)</td>
<td>Addgene #74169</td>
</tr>
<tr>
<td>pSIV-∆psi/∆env/∆Vif/∆Vpr</td>
<td>SIVMAC251 gag-pol/vpx</td>
<td>Production of SIV-VLPs containing Vpx protein</td>
<td>Pending</td>
</tr>
<tr>
<td>pMD2.G</td>
<td>VSV Glycoprotein</td>
<td>Pseudotype HIV-1 vectors with VSV Glycoprotein</td>
<td>Addgene #12259</td>
</tr>
<tr>
<td>psPAX2</td>
<td>HIV-1 gag-pol</td>
<td>Encodes gag structural proteins and pol enzymes to generate virion particles to generate 3-part lentiviral vector</td>
<td>Addgene #12260</td>
</tr>
<tr>
<td>p8.9 NΔSB</td>
<td>HIV-1 gag-pol</td>
<td>Minimal HIV-1 packaging plasmid for gag and pol expression (Berthoux et al. 2004)</td>
<td>Pending</td>
</tr>
<tr>
<td>p8.9 NΔSB N74D</td>
<td>HIV-1 gag-pol</td>
<td>p8.9 NΔSB bearing N74D mutant capsid sequence (De Iaco and Luban 2011)</td>
<td>Pending</td>
</tr>
<tr>
<td>p8.9 NΔSB A77V</td>
<td>HIV-1 gag-pol</td>
<td>p8.9 NΔSB bearing A77V mutant capsid sequence</td>
<td>Pending</td>
</tr>
<tr>
<td>p8.9 NΔSB P90A</td>
<td>HIV-1 gag-pol</td>
<td>p8.9 NΔSB bearing P90A mutant capsid sequence (De Iaco and Luban 2011)</td>
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<tr>
<td>p8.9 NΔSB D185K/D186L</td>
<td>HIV-1 gag-pol</td>
<td>p8.9 NΔSB bearing RT-D185K/D186L mutant reverse transcriptase sequence</td>
<td>Pending</td>
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<tr>
<td>pALPS GFP</td>
<td>GFP reporter of lentiviral vector</td>
<td>SFFV promoter expresses GFP (McCauley et al. 2018)</td>
<td>Addgene #101323</td>
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<tr>
<td>pWPTS GFP</td>
<td>Lentivector for quantification of HIV-1 cDNA</td>
<td>Contains a unique loxP sequence within a region of the 3' LTR U3. EF1α promoter expresses GFP (De Iaco and Luban 2011)</td>
<td>Addgene #12255</td>
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<tr>
<td>pAPM-D4-miR30-L1221</td>
<td>Lentivector for luciferase (control) knockdown</td>
<td>SFFV promoter expresses puromycin N-acetyltransferase and miR30-based shRNA targeting luciferase. Target sequence: 5'-TACAAAAGCTCTCAGACAAG-3'; negative control for knockdown</td>
<td>Addgene #115846</td>
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<tr>
<td>pAPM-D4-miR30-TRIM5</td>
<td>Lentivector for TRIM5 knockdown</td>
<td>SFFV promoter expresses puromycin N-acetyltransferase and miR30-based shRNA targeting TRIM5. Target sequence: 5'-TGCCAAGCATGCTCACTGCAA-3'</td>
<td>Pending</td>
</tr>
<tr>
<td>---------------------</td>
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<td>-------------------------------------------------------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>pABM-D4-miR30-L1221</td>
<td>Lentivector for luciferase (control) knockdown</td>
<td>SFFV promoter expresses blasticidin S-deaminase and miR30-based shRNA targeting luciferase. Target sequence: 5'-TACAAACGCTCTCATCGACAAG-3'; negative control for knockdown</td>
<td>Pending</td>
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<tr>
<td>pABM-D4-miR30-CypA</td>
<td>Lentivector for CypA knockdown</td>
<td>SFFV promoter expresses blasticidin S-deaminase and miR30-based shRNA targeting cyclophilin A. Target sequence: 5'-CTGGATTGCAGAGTTAAGTTTA-3'</td>
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<tr>
<td>pABM-D4-miR30-CPSF6</td>
<td>Lentivector for CPSF6 knockdown</td>
<td>SFFV promoter expresses blasticidin S-deaminase and miR30-based shRNA targeting CPSF6. Target sequence: 5'-TGGAAAGAGAATTGCATTATAT-3'</td>
<td>Pending</td>
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<tr>
<td>pABM-D4-miR30-TNPO3</td>
<td>Lentivector for TNPO3 knockdown</td>
<td>SFFV promoter expresses blasticidin S-deaminase and miR30-based shRNA targeting TNPO3. Target sequence: 5'-TGGACAGTAACTTCATGGCTAA-3'</td>
<td>Pending</td>
</tr>
<tr>
<td>pPU-Empty-miR30-L1221</td>
<td>All-in-one knockdown-rescue lentivector for luciferase (control) knockdown</td>
<td>SFFV promoter expresses puromycin N-acetyltransferase and miR30-based shRNA targeting luciferase. Target sequence: 5'-TACAAACGCTCTCATCGACAAG-3'; negative control in knockdown-rescue experiment</td>
<td>Pending</td>
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<tr>
<td>pPU-Empty-miR30-TRIM5</td>
<td>All-in-one knockdown-rescue lentivector for TRIM5 knockdown</td>
<td>SFFV promoter expresses puromycin N-acetyltransferase and miR30-based shRNA targeting TRIM5. Target sequence: 5'-TGCCAAGCATGCTCACTGCAA-3'</td>
<td>Pending</td>
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<tr>
<td>pPU-huTRIM5α-miR30-L1221</td>
<td>All-in-one knockdown-rescue lentivector for huTRIM5α expression and luciferase (control) knockdown</td>
<td>SFFV promoter expresses puromycin N-acetyltransferase, exogenous huTRIM5α protein and miR30-based shRNA targeting luciferase. Target sequence: 5'-TACAAACGCTCTCATCGACAAG-3'</td>
<td>Pending</td>
</tr>
<tr>
<td>pPU- huTRIM5α- miR30- TRIM5</td>
<td>All-in-one knockdown-rescue lentivector for huTRIM5α expression and TRIM5 knockdown</td>
<td>SFFV promoter expresses puromycin N-acetyltransferase, exogenous huTRIM5α protein and miR30-based shRNA targeting TRIM5. Target sequence: 5’-TGCCAAGCATGCCTCACTGCAA-3’</td>
<td>Pending</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>---------</td>
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<tr>
<td>pNL4-3/Mac-tropic Env</td>
<td>Full-length infectious HIV-1&lt;sub&gt;MAC&lt;/sub&gt;</td>
<td>Encodes HIV-1&lt;sub&gt;NL4-3&lt;/sub&gt; proviral sequences containing macrophage-tropic JR-FL env</td>
<td>Neagu et al., 2009</td>
</tr>
<tr>
<td>pLXIN GFP</td>
<td>GFP reporter of MLV-based vector</td>
<td>MLV-based transfer vector expressing GFP and neomycin resistance protein</td>
<td>Pending</td>
</tr>
<tr>
<td>pCIG3 N</td>
<td>N-tropic MLV gag-pol</td>
<td>MLV packaging plasmid for N-tropic MLV gag-pol expression (Bock et al. 2000)</td>
<td>Pending</td>
</tr>
<tr>
<td>pCIG3 B</td>
<td>B-tropic MLV gag-pol</td>
<td>MLV packaging plasmid for B-tropic MLV gag-pol expression (Bock et al. 2000)</td>
<td>Pending</td>
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<tr>
<td>pUC57mini ZM249M</td>
<td>HIV-1 clade C molecular clone</td>
<td>Molecular clone of transmitted/founder virus HIV-1&lt;sub&gt;ZM249M&lt;/sub&gt; (Salazar-Gonzalez et al. 2009)</td>
<td>NIH AIDS Reagent #12416</td>
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<tr>
<td>pUC57mini ZM249M P90A</td>
<td>HIV-1 clade C molecular clone</td>
<td>Molecular clone of transmitted/founder virus HIV-1&lt;sub&gt;ZM249M&lt;/sub&gt; bearing P90A capsid mutation</td>
<td>NIH AIDS Reagent #12416</td>
</tr>
<tr>
<td>pUC57mini ZM249M Δenv eGFP</td>
<td>HIV-1 clade C molecular clone</td>
<td>Molecular clone of transmitted/founder virus HIV-1&lt;sub&gt;ZM249M&lt;/sub&gt; with deletion of 79 nucleotides following the env signal peptide and GFP in place of nef (Salazar-Gonzalez et al. 2009; McCauley et al. 2018)</td>
<td>Addgene #101321</td>
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<tr>
<td>pUC57mini ZM249M P90A Δenv eGFP</td>
<td>HIV-1 clade C molecular clone</td>
<td>Molecular clone of transmitted/founder virus HIV-1&lt;sub&gt;ZM249M&lt;/sub&gt; containing P90A capsid mutation, with deletion of 79 nucleotides following the env signal peptide and GFP in place of nef</td>
<td>Pending</td>
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<tr>
<td>pUC57mini Z331M-TF Δenv eGFP</td>
<td>HIV-1 clade C molecular clone</td>
<td>Molecular clone of transmitted/founder virus HIV-1&lt;sub&gt;Z331M-TF&lt;/sub&gt; with deletion of 79 nucleotides following the env signal peptide and GFP in place of nef (Deymier et al. 2015; McCauley et al. 2018)</td>
<td>Addgene #101320</td>
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### Table 2.2. Drugs and reagents.

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<th>Reagents</th>
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<tr>
<td>DMEM, high glucose</td>
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<tr>
<td>Fetal Bovine Serum</td>
<td>GE Healthcare Life Sciences</td>
<td>SH30087.03</td>
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<tr>
<td>RPMI-1640</td>
<td>ThermoFisher</td>
<td>11875119</td>
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<tr>
<td>Human AB+ Serum</td>
<td>Omega Scientific</td>
<td>HS-20</td>
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<tr>
<td>HEPES</td>
<td>Corning</td>
<td>25-060-Cl</td>
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<tr>
<td>MEM Non-Essential Amino Acids</td>
<td>Corning</td>
<td>25-025-Cl</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>Corning</td>
<td>25-000-Cl</td>
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<td>GlutaMAX™-I</td>
<td>ThermoFisher</td>
<td>35050-061</td>
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<tr>
<td>Opti-MEM</td>
<td>ThermoFisher</td>
<td>31985-062</td>
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<tr>
<td>TransIT-LT1</td>
<td>Mirus Bio</td>
<td>MIR2306</td>
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<tr>
<td>BD Cytofix Fixation Buffer</td>
<td>BD Bioscience</td>
<td>554655</td>
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<tr>
<td>cOmplete mini protease inhibitor</td>
<td>Roche</td>
<td>11836170001</td>
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<tr>
<td>2-mercaptoethanol</td>
<td>Invitrogen</td>
<td>21985-023</td>
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<tr>
<td>Laemmli Buffer</td>
<td>BioRad</td>
<td>161-0737</td>
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<tr>
<td>4-20% gradient SDS-PAGE gels, 12 well</td>
<td>BioRad</td>
<td>456-1095</td>
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<td>0.45 mM nitrocellulose membrane</td>
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<td>162-0112</td>
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<tr>
<td>TBS Odyssey Blocking Buffer</td>
<td>Li-Cor</td>
<td>927-50000</td>
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<td>Pierce™ 16% Formaldehyde (w/v), Methanol-free</td>
<td>ThermoFisher</td>
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<td>PIPES</td>
<td>Sigma-Aldrich</td>
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<td>Phalloidin (FITC)</td>
<td>Enzo Life Science</td>
<td>ALX-350-268-MC01</td>
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<td>Hoeschst 33342</td>
<td>Invitrogen</td>
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<td>Cyclosporine A</td>
<td>Sigma-Aldrich</td>
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<td>GS-CypAi48</td>
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<td>MG132</td>
<td>Sigma-Aldrich</td>
<td>M7449</td>
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### Table 2.3. qPCR primers and probes for Late RT quantification

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<thead>
<tr>
<th>Primer or Probe name</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWPT J1B Fwd</td>
<td>5'-GCATACATTATACGAAGTTATGCTGC-3'</td>
<td>Primer to detect late RT product</td>
</tr>
<tr>
<td>pWPT J2 Rev</td>
<td>5'-GCCGTGCGCGCTTCAGCAAGC-3'</td>
<td>Primer to detect late RT product</td>
</tr>
<tr>
<td>LRT-P (Probe)</td>
<td>5'-(FAM)-CAGTGGCGCCCAGACAGGGA-(TAMRA)-3'</td>
<td>Probe to detect late RT product</td>
</tr>
<tr>
<td>MH533</td>
<td>5'-ACCCACTCCCTCTTAGCCAATATT-3'</td>
<td>Primer to detect mitochondrial DNA</td>
</tr>
<tr>
<td>MH534</td>
<td>5'-GTAGGGCTAGGCCACCG-3'</td>
<td>Primer to detect mitochondrial DNA</td>
</tr>
<tr>
<td>Mito-P (Probe)</td>
<td>5'-(FAM) CTAGTCTTTGCGCTGCAAGCA (TAMRA)-3'</td>
<td>Probe to detect mitochondrial DNA</td>
</tr>
</tbody>
</table>
CHAPTER III.

DISCUSSION
Upon entry of HIV-1 into the cell through fusion of the HIV-1 envelope with host cell plasma membrane, the CA core enclosing the viral RNA genome and proteins required for the infection is translocated into the cytoplasm. This CA core plays essential roles in the early stages of the HIV-1 replication cycle (Yamashita and Engelman 2017). As it traverses the cytoplasm, HIV-1 CA interacts with host proteins to advance the infection steps. CA–CypA interaction not only enhances viral reverse transcription levels but also stabilizes the CA. Cytoskeleton-associated proteins bound to the CA guide HIV-1 to host NPCs. Interactions of the CA with CPSF6, TNPOs, and Nups facilitate nuclear import of HIV-1. In particular, the CA itself and its interactions with these factors safeguard HIV-1 from detection by host PRRs such as cGAS. In the nucleus, CPSF6 bound to the CA directs the viral PIC to actively transcribed regions of the host genome for establishment of the provirus.

Among the CA-binding host factors, a ubiquitous, abundant protein CypA was first identified to bind to HIV-1 CA in the early 1990s (Luban et al. 1993). Subsequent investigations suggested that CypA shields HIV-1 from host antiviral protein(s), because loss of the CA–CypA interaction was observed to largely decrease HIV-1 infectivity in human cells (Braaten, Franke, and Luban 1996a; Franke, Yuan, and Luban 1994; Thali et al. 1994; Braaten and Luban 2001; Sokolskaja, Sayah, and Luban 2004). Ten years later, in the mid-2000s, a CA-targeting restriction factor TRIM5α was discovered (Stremlau et al. 2004). Researchers proposed that the unknown antiviral protein prevented by CypA
would be TRIM5α in human cells, based on the following reasons: i) TRIM5α-like antiviral activity called Ref-1 was shown to be dependent on the CA−CypA interaction in human cells (Towers et al. 2003; Sayah and Luban 2004b); ii) a relationship between the restriction activity of TRIM5α and CypA was observed in cells from non-human primate species (Berthoux et al. 2005a); iii) an evolutionary linkage between CypA and TRIM5α was suggested by the discovery of TRIMCyp in New World monkey species (Sayah et al. 2004a); iv) human TRIM5α showed only minimal restriction activity against HIV-1, in contrast to non-human primate TRIM5α orthologs (Hatzioannou et al. 2003, 2004a, 2004b; Stremlau et al. 2004); and v) both CypA and TRIM5α function through the interaction with HIV-1 CA at a similar timing prior to reverse transcription (Braaten, Franke, and Luban 1996a; Stremlau et al. 2004; Anderson et al. 2006; Wu et al. 2006; De Iaco and Luban 2014). However, in 2006, two years after the identification of TRIM5α, the hypothesis that proposed the connection between CypA and TRIM5α in regulation of HIV-1 infectivity was disproven by two independent studies based on a series of experiments using human cancer-derived cell lines (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). Intriguingly, 13 years later, this previously refuted hypothesis has been reversely proven to be correct by the thesis research presented in Chapter II (Kim et al. 2019).

Lentiviral vectors have been engineered to acquire greater efficiency of transduction in primary human cells (Pertel et al. 2011; McCauley et al. 2018; Yurkovetskiy et al. 2018). These developed vectors were used in this thesis
research of HIV-1 infection in primary human blood cells that serve as actual targets of HIV-1. In this research, virologic, genetic, and pharmacological approaches were utilized to disrupt the activities of CypA and TRIM5α by introducing mutations into HIV-1 CA, using gene knockdown by lentivirally transferred shRNAs and adding chemical compounds targeting CypA, respectively, to investigate the roles of the two factors. We found that CypA shields HIV-1 CA from recognition and restriction by human TRIM5α during the early step of HIV-1 infection prior to completion of reverse transcription. Therefore, the main role of CypA in HIV-1 infection has been finally uncovered 26 years after the identification of CypA as the first CA-binding host protein (Luban et al. 1993). In addition, human TRIM5α has been demonstrated to possess potent restriction capacity against HIV-1 for the first time since its discovery in 2004 (Stremlau et al. 2004). This finding is also consistent with previously observed activities of CypA in the enhancement of reverse transcription (Braaten, Franke, and Luban 1996a; De Iaco and Luban 2014), as the current research also shows that CypA prevents the restriction activity of TRIM5α that induces premature CA disassembly prior to completion of reverse transcription. Our finding is also supported by a recent study that used an in vitro CA-binding assay and showed that binding of human TRIM5α to assembled CA tubes is dependent on the presence of CA–CypA interaction (Selyutina et al. 2020).

Our finding raises further questions that need to be resolved. First, our finding from primary human blood cells including CD4+ T cells and macrophages
is a contrast to the observation in previous studies showing HIV-1 regulation by CypA independent of human TRIM5α in human cancer cell lines such as TE671 and CEM-SS (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). The subsequent investigation using cancer cell lines described in Appendix I demonstrates that the CypA–TRIM5α relationship in HIV-1 infection varies among cell lines, as different relationships were observed among cell lines. In this study, CypA was observed to protect HIV-1 CA from TRIM5α in Jurkat and U2OS cell lines, whereas CypA and TRIM5α were shown to be less dependent on each other in TE671 and CEM-SS cells, which is consistent with previous studies (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). In particular, endogenous TRIM5α of primary blood cells and Jurkat cells showed a potent restriction capacity against HIV-1 CA without the CA–CypA interaction, whereas this capacity of TRIM5α was minimal in the TE671 and CEM-SS cell lines. This may suggest the presence of an unknown cofactor that is involved in the restriction activity of human TRIM5α and differentially expressed among the cell lines. The factor may facilitate association of TRIM5α with HIV-1 CA or premature disassembly of the CA by TRIM5α.

Considering that the TRIM5α-mediated restriction activity is proposed to connect with the proteasome- (Anderson et al. 2006; Wu et al. 2006; Diaz-Griffero et al. 2007; Roa et al. 2012; Kutluay, Perez-Caballero, and Bieniasz 2013; Fletcher et al. 2018; Jimenez-Guardeño et al. 2019) or autophagy-mediated degradation pathway (O’Connor et al. 2010; Mandell et al. 2014; Keown et al. 2018; Saha,
Chisholm, and Mandell 2020), the factor may be involved in either of the pathways. In particular, a recent discovery of immunoproteasome as a novel factor that participates in the antiviral activity of TRIM5α in an IFN-stimulated condition supports the possible presence of the cofactor (Jimenez-Guárdeño et al.2019). Further investigations using gene knockdown- or knockout-based screening in comparisons of the cells will identify the cofactor(s), which will contribute to a better understanding of TRIM5α-mediated restriction against HIV-1 CA.

In addition to the different levels of TRIM5α-mediated restriction, HIV-1 infection of CEM-SS and TE671 cells with disruption of CA–CypA interaction was observed to remain inhibited even when TRIM5α was depleted (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). This implies that there may be an additional, unidentified restriction factor dependent on CA–CypA interaction in the two cell lines, but not in primary blood cells and the other cell lines. Thus, further research including the screening experiment proposed above will contribute to the identification of this factor. Additionally, the spontaneously occurring CA-A92E mutation in HIV-1 that is resistant to cyclosporine treatment has recently been reported to render HIV-1 CA insensitive to human TRIM5α (Selyutina et al. 2020). Thus, the use of HIV-1 bearing the CA-A92E mutation will also be useful for investigating whether the unidentified restriction activity is independent of the endogenous TRIM5α.

Second, our finding poses a question regarding the mechanism by which
CA–CypA interaction prevents association of human TRIM5α with HIV-1 CA. The simplest model is that CA–CypA interaction sterically hinders binding of human TRIM5α to HIV-1 CA. TRIM5α has been suggested to interact with the CypA-binding loop of HIV-1 CA (Owens et al. 2004; Hatzioannou et al. 2004a; Ylinen et al. 2005; Kono et al. 2010; Quinn et al. 2018; Yu et al. 2020). Consistently, two independent studies, including the research presented in Appendix II, have found that CA mutation of the alanine residue at position 92 within the CypA-binding loop renders HIV-1 CA invulnerable to TRIM5α, implying that TRIM5α interacts with this loop region (Selyutina et al. 2020). In addition to these studies, evolutionary analysis also showed that TRIM5α interacts with lentiviral CA at a site proximal to the CypA-binding region over 10 million years (McCarthy et al. 2015). This study also described that some mutations that sensitize SIVmac to a subset of TRIM5α are located in regions responsible for interaction with host factors such as CypA and CPSF6, which implies that the loss of CA–factor interaction by the mutations renders the lentiviruses susceptible to TRIM5α-mediated restriction. Thus, these studies support the model in which CypA bound to HIV-1 CA induces steric hindrance of CA–TRIM5α association based on competitive interaction of the two host factors with the CypA-binding loop. Indeed, the sites on HIV-1 CA that interact with the PRYSPRY domain of TRIM5α have not been clearly determined. Instead, studies have suggested multiple regions on CA as determinants of CA sensitivity to TRIM5α-mediated restriction (Owens et al. 2004; Hatzioannou et al. 2004a; Ylinen et al. 2005;
Song et al. 2007; Mortuza et al. 2008; Kono et al. 2010; Kuroishi et al. 2010; Ohkura et al. 2011; McCarthy et al. 2013; Ohkura and Stoye 2013). Additionally, TRIM5α is also suggested to bind to multiple, adjacent CA hexamers on CA (Yang et al. 2012; Biris et al. 2013; Kovalskyy and Ivanov 2014; Morger et al. 2018). Consistently, a recent structural study employing cryo-electron tomography showed that TRIM5α forms four different modes of interaction with CA when multimeric TRIM5α proteins cover HIV-1 CA (Skorupka et al. 2019). This implies that TRIM5α uses different interaction sites on CA, in addition to the CypA-binding loop, dependent on the mode. However, given that the binding strength between individual TRIM5α and HIV-1 CA is weak, inhibition of some CA–TRIM5α interactions by CypA may present a sufficient obstacle to the formation of a high-order TRIM5α assembly on CA. Particularly, considering the multiple CA-binding modes of TRIM5α, the discovery of a secondary CypA-binding site on HIV-1 CA may support this ‘steric hindrance’ model, because this additional binding of CypA could increase the likelihood that the interaction between TRIM5α and CA is blocked by CypA (Liu et al. 2016).

The alternative model is that CA–CypA interaction modifies the binding regions on CA to prevent binding of TRIM5α to CA. CypA possesses a peptidyl-prolyl isomerase activity (Colgan et al. 2004). CypA targets proline-containing motifs and induces interconversion from the cis- to trans-formation of the peptide bonds. In particular, the peptide bond between Gly89 and Pro90 of HIV-1 CA was identified as a target of the enzymatic activity of CypA (Bosco et al. 2002).
Thus, the conformational change of CA induced by CypA may thwart CA–TRIM5α interaction. In addition to this catalytic activity of CypA, CA–CypA interaction has been reported to alter the dynamics of the interacting region (Lu et al. 2015). A study using magic-angle spinning NMR showed that the CypA-binding loop of HIV-1 CA has a highly dynamic structure, but its motion becomes significantly attenuated when CypA binds to the loop, independent of the isomerase activity of CypA. Intriguingly, in CA containing the CA-A92E mutation, the loop displays increased rigidity regardless of interaction with CypA. Given that human TRIM5α does not bind to the CA-A92E–bearing HIV-1 CA (Selyutina et al. 2020) and that TRIM5α is suggested to interact with the CypA-binding loop (Owens et al. 2004; Hatzioannou et al. 2004a; Ylinen et al. 2005; Kono et al. 2010; Quinn et al. 2018; Yu et al. 2020), the mobility of the loop structure may be the determinant of CA–TRIM5α association. In other words, the CypA-binding loop with dynamic motion may be required for the interaction between CA and human TRIM5α. The interaction is inhibited when the motion of the loop is reduced by CA–CypA interaction or by introduction of the CA-A92E mutation. Thus, HIV-1 may exploit this fine-tuning activity of CypA to evade restriction by endogenous human TRIM5α.

Overall, the CypA-binding loop of HIV-1 CA is thought to be important for the association of human TRIM5α with CA. Further investigations of the interaction between human TRIM5α and the assembled CA tube or CA core from a structural viewpoint will prove the models proposed here. These studies will
provide deeper mechanistic insight into HIV-1 evasion of TRIM5α using a host protein CypA.

Third, in contrast to human TRIM5α, non-human primate TRIM5α orthologs from Old World monkeys are capable of restricting HIV-1 CA in the presence of CypA (Stremlau et al. 2004; Yap, Nisole, and Stoye 2005; Li et al. 2006; Richardson et al. 2008). Furthermore, CA–CypA interaction is known to inhibit HIV-1 infection by enhancing the restriction activity of the TRIM5α (Towers et al. 2003; Berthoux et al. 2004, 2005a). Considering that CypA binding to CA prevents association of human TRIM5α with CA, these results imply that human TRIM5α binds to HIV-1 CA in a different manner than the monkey TRIM5α orthologs. As described above, TRIM5α is suggested to interact with the CypA-binding loop of HIV-1 CA (Owens et al. 2004; Hatzioannou et al. 2004a; Ylinen et al. 2005; Kono et al. 2010; Quinn et al. 2018; Yu et al. 2020). However, the dependency of human and monkey TRIM5α orthologs on the CypA-binding region in the CA–TRIM5α association seems to differ. First, despite a small conflict with a previous report (Owens et al. 2004; Hatzioannou et al. 2004a), a monkey TRIM5α ortholog was observed to bind to HIV-1 CA at a similar level in the presence or absence of CA–CypA interaction (Burse, Shi, and Aiken 2017). In that study, CA–CypA interaction was shown to rather enhance the second stage of TRIM5α-mediated restriction after reverse transcription to block nuclear entry of HIV-1 (Burse, Shi, and Aiken 2017). In contrast, human TRIM5α is unable to interact with HIV-1 CA when CA–CypA interaction is present (Kim et al.
Secondly, recent studies demonstrated that the CA-A92E mutation in HIV-1 prevents association of human TRIM5α with CA, as well as the subsequent restriction by human TRIM5α (Selyutina et al. 2020). On the other hand, previous studies showed that CA-A92E-bearing HIV-1 was inhibited in the same way as WT HIV-1 in either cells from the non-human primate species (Hatziioannou et al. 2004a; Kuroishi et al. 2010) or human cells expressing TRIM5α from rhesus macaque (Li et al. 2006). Moreover, in a NMR-based study, human TRIM5α was shown to interact with the CypA-binding loop when it had a high mobility (Quinn et al. 2018). Thus, decreased motion of the loop by CA–CypA interaction or CA-A92E mutation is suggested to inhibit the interaction between CA and human TRIM5α. In contrast, the studies described above imply that the monkey TRIM5α orthologs are able to bind to the CypA-binding region, regardless of the dynamics. Therefore, these observations suggest that the CypA-binding loop is critical for the CA recognition by human TRIM5α, but not by monkey TRIM5α orthologs, although all these TRIM5α orthologs are suggested to interact with the loop in the CA–TRIM5α association. Given that TRIM5α is thought to interact with multiple CA regions including the CypA-binding loop (Owens et al. 2004; Hatziioannou et al. 2004a; Ylinen et al. 2005; Song et al. 2007; Mortuza et al. 2008; Kono et al. 2010; Kuroishi et al. 2010; Ohkura et al. 2011; McCarthy et al. 2013; Ohkura and Stoye 2013), non-human primate TRIM5α orthologs may utilize another CA region, such as the loop 3 region, as a key interacting interface, distant from the CypA-binding loop (Hatziioannou et al. 2019; Selyutina et al. 2020).
2004a; Kuroishi et al. 2010). This difference in the binding modes between human and monkey TRIM5α orthologs would determine the sensitivity of TRIM5α activity to the CA–CypA interaction.

Most of the amino acid sequence differences between these TRIM5α orthologs are found in the C-terminal PRYSPRY domain that determines the CA specificity (Song et al. 2005b; Sawyer et al. 2005). In particular, the replacement of a 13-residue patch sequence in human TRIM5α with the sequence of non-human primate TRIM5α orthologs, or even a substitution of a key residue arginine at position 332 with a proline of the monkey orthologs, enables the engineered human TRIM5α to restrict HIV-1 CA in the presence of CA–CypA interaction as the monkey orthologs do (Stremlau et al. 2004; Yap, Nisole, and Stoye 2005; Li et al. 2006; Richardson et al. 2008). Furthermore, consistent with the observation for non-human primate TRIM5α orthologs, the engineered human TRIM5α containing the primate sequence was also capable of inhibiting HIV-1 bearing the CA-A92E mutation (Li et al. 2006). Therefore, these results not only indicate that the 13-residue patch including the key residue at position 332 within the PRYSPRY domain is important in the determination of the binding modes of TRIM5α, but also suggest that the patch has been under positive selection for CA specificity of TRIM5α.

Further investigations of the differences in the CA-binding modes of TRIM5α between human and non-human primate species, along with an engineered human TRIM5α bearing the 13-residue patch, are expected to define
the major and minor binding sites of TRIM5α on HIV-1 CA, as well as to elucidate how the residues within the PRYSPRY domain determine the interaction between TRIM5α and CA. In addition to an understanding of how TRIM5α recognizes HIV-1 CA, this research will also contribute to further gene therapy studies in which endogenous TRIM5α is modified to treat HIV-1, as researchers have attempted (Neagu et al. 2009; Walker et al. 2012; Dufour et al. 2018).

Fourth, TRIM5α is known to function as a PRR to trigger TAK1-dependent innate immune signaling upon its recognition of retroviral CA, such as N-tropic MLV CA (Pertel et al. 2011; Fletcher et al. 2015, 2018). Considering our finding that human TRIM5α is capable of recognizing and restricting HIV-1 CA when CA–CypA interaction is disrupted, TRIM5α is highly likely to act as a PRR against HIV-1 CA. Indeed, a recent preliminary report has shown human TRIM5α-mediated restriction and innate immune signaling against CA-P90A–mutant HIV-1 (Saha, Chisholm, and Mandell 2020). In that study, challenge of a macrophage cell line with the CA-P90A–mutant virus induced IFN and nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing protein 1 (NLRP1) expression, and pre-challenge with this mutant HIV-1 rendered the cells non-permissive to subsequent challenge with WT HIV-1 due to the cellular environment primed by the immune response. In addition, a previous study also reported that NF-κB- and IRF3-mediated immune signaling is triggered by HIV-1 infection in disruption of the CA–CypA interaction (Rasaiyaah et al. 2013). The findings of that study suggested that cGAS plays a
role in the immune response by showing that inhibition of reverse transcription
prevents this immune signaling, as well as that the level of cellular cGAMP
increases upon infection of CA-P90A-mutant HIV-1. Given that cGAS was
reported to detect HIV-1 DNA upon destabilization of HIV-1 CA (Siddiqui et al.
2019; Sumner et al. 2019), all the studies described here suggest a model in
which cGAS senses HIV-1 DNA exposed from the disassembled CA by the
restriction activity of human TRIM5α when CA–CypA interaction is disturbed.
Considering that both TRIM5α-mediated restriction and HIV-1 reverse
transcription occur within minutes after virus entry (Perez-Caballero et al. 2005a;
Campbell et al. 2008), cGAS may sense shortly generated RNA:DNA hybrid
intermediates or HIV-1 DNAs as products of the early reverse transcription. In
this model, human TRIM5α could also trigger TAK1-mediated signaling, in
addition to CA disassembly, upon its recognition of CA. Thus, the induced
immune response against HIV-1 without CA–CypA interaction may be a result of
cooperation of human TRIM5α- and cGAS-mediated activation of immune
signaling pathways. Further studies to examine the synthesis of unanchored
Lys63-linked polyubiquitin chain and ubiquitination of TRIM5α are needed to
illuminate innate immune sensing of HIV-1 CA by human TRIM5α in disruption of
CA–CypA interaction, in addition to the previously reported HIV-1 detection by
cGAS. Moreover, in addition to these two PRRs, it is also possible that an
intracellular sensor RIG-I recognizes remaining HIV-1 genomic RNAs exposed
from CA as it is rapidly dismantled by TRIM5α, which induces MAVS-dependent
innate immune signaling (Solis et al. 2011; Berg et al. 2012). Further investigations of these immune sensors in HIV-1 infection when CA−CypA interaction is disrupted will elucidate the collaborative innate immune sensing of HIV-1 by multiple PRRs upon virus entry.

Researchers have recently proposed the involvement of autophagy machinery in innate immune activation by TRIM5α. The ubiquitination activity of TRIM5α required for activation of immune signaling was shown to depend on its association with an autophagic adaptor protein p62 and autophagosome membranes (Imam et al. 2019). Additionally, in response to retroviral infection and stress, both TRIM5α and TAK1 kinase complex were shown to be capable of switching the function of p62, by promoting the formation of cytoplasmic p62 bodies, to a signaling platform (Kehl et al. 2019) from an autophagy receptor that recognizes protein targets including TAK1 and facilitates their degradation by autophagy (Pankiv et al. 2007; Clausen et al. 2010; Komatsu et al. 2010). The formation of p62 bodies induced by the two factors thus prevents p62-driven TAK1 degradation by autophagy. Importantly, these p62 bodies contribute to the assembly of activated TAK1 kinase complex to promote the downstream signaling (Kehl et al. 2019). These studies suggest that the autophagy-independent function of the autophagy factor p62 has an important role in innate immune activation by TRIM5α−TAK1. However, the result showing that the association of TRIM5α with autophagosome membrane is required for its ubiquitination activity still implies the role of autophagy machinery in innate
immune signaling by TRIM5α. In addition, another study demonstrated that autophagy factors, including Beclin-1 and autophagy related 7 (ATG7), mediate the interaction between human TRIM5α and TAK1 and the subsequent TAK1 activation that is essential for the downstream immune signaling cascade (Saha, Chisholm, and Mandell 2020). Interestingly, previous investigations found that TAB2 and TAB3 interact with Beclin-1 in resting conditions, which prevents TAK1 activation and autophagy. In response to stress conditions inducing autophagy, Beclin-1 is dissociated from the complex with TAB2 and TAB3 and then stimulates autophagy, whereas the disengaged TAB proteins bind to TAK1 to form the TAK1 kinase complex that activates cellular signaling (Criollo et al. 2011; Takaesu, Kobayashi, and Yoshimura 2012; Niso-Santano et al. 2012).

These studies suggest that invasion of a TRIM5α-sensitive retroviral CA, including the HIV-1 CA when CA–CypA interaction is disrupted, triggers the dissociation of the Beclin-1–TAB complex, which activates autophagy machinery and TAK1 kinase complex. TRIM5α may facilitate this dissociation by ubiquitylating Beclin-1 upon its recognition of retroviral CA, which is consistent with accumulating evidence showing that cellular Beclin-1 is regulated by ubiquitination (Boutouja et al. 2017). Subsequently, the activated autophagy machinery with the dissociated Beclin-1 would mediate the interaction of TRIM5α with TAK1. In parallel, the TAB proteins would activate TAK1 by binding to free polyubiquitin chains synthesized by TRIM5α upon the formation of TAK1 kinase complex. Further studies are required to obtain a deeper understanding of the
coordinated functions of TRIM5α and the autophagy machinery in innate immune sensing of retroviral CA.

Lastly, this thesis research demonstrates that the use of chemical compounds to disrupt CA–CypA interaction renders HIV-1 susceptible to endogenous human TRIM5α. This discovery will encourage researchers to develop CypA inhibitor compounds to treat HIV-1. The current HAART used to treat HIV-1 patients contains multiple drug compounds that directly target viral factors. Considering the observed effect of CypA inhibitor compounds on HIV-1 infection and replication in primary human blood cells, addition of CypA inhibitors into an anti-HIV-1 drug cocktail will produce more effective HAART by taking advantage of activity of the host endogenous antiviral factor.

Indeed, in addition to the HIV-1 CA–CypA interaction, interaction between CypA and nonstructural protein 5A (NS5A) of HCV has been shown to be critical in hepatitis C virus (HCV) replication (Hanoulle et al. 2009; Chatterji et al. 2009; Kaul et al. 2009; Coelmont et al. 2010; Fernandes, Ansari, and Striker 2010; Watashi and Shimotohno 2007). In accordance with these reports, CypA inhibitor compounds have been developed for HCV treatment. Even though cyclosporine A (CsA) is a CypA inhibitor approved by the Food and Drug Administration (FDA), this compound is used mainly as an immunosuppressant medication to treat autoimmune diseases or to prevent organ transplant rejection, based on its side effect described in 2.2. INTRODUCTION. Therefore, non-immunosuppressive CypA inhibitors have been developed and produced, and
are currently in clinical trials. These compounds include alisporivir (DEB-025), CRV431 (same as CPI-431-32), NIM811, SCY-635, NV556 (same as NVP-018), and others (Baugh, Garcia-Rivera, and Gallay 2013; Gallay et al. 2015). The compounds GS-CypAi3 and GS-CypAi48, which were used in this thesis research, are also included among the developed non-immunosuppressive CypA inhibitors (Mackman et al. 2018; Kim et al. 2019). Given that these compounds have shown an inhibitory effect against HIV-1 infection in addition to HCV infection, they have the potential to make an important contribution to improved anti-HIV-1 drug cocktails.

Compared with the drug compounds currently used in the anti-HIV-1 drug cocktail, CypA inhibitor compounds are a different type of drug, because they target a host protein, rather than HIV-1 factors such as viral protease and reverse transcriptase. Drugs targeting the host would have advantages over those targeting viral factors. First, the genetic barriers to viral resistance to drug treatment are higher for the host-targeting drugs. RNA viruses such as HIV-1 and HCV can evade the antiviral drugs that target them via a high mutation rate during their replication. This is less likely to be a concern for compounds targeting host proteins. Secondly, host-targeting compounds function regardless of the serotype or genotype of the viruses, because they target host proteins. Third, these compounds can target multiple viruses that exploit the host protein targeted by the drugs. For instance, because CypA has been shown to regulate the infectivity of many viruses, including HIV-1, HCV, and coronaviruses (Luo et
al. 2004), the developed CypA inhibitors can be used in infectious diseases caused by these viruses. Accordingly, these drug compounds can potentially be used to treat patients with co-infection of two or more viruses that are regulated by CypA. Therefore, once non-immunosuppressive CypA inhibitor compounds are approved by the FDA, they will be valuable in the development of therapeutics for infectious diseases including the HIV-1 pandemic.

Conclusively, this thesis research has found that HIV-1 exploits CA–CypA interaction to escape from restriction by endogenous human TRIM5α. This discovery has not only solved the long-standing enigma of the role of CypA, but also revealed the previously hidden, potent restriction capacity of TRIM5α against HIV-1 CA. This discovered HIV-1 evasion of a restriction factor TRIM5α via an interaction with a host protein CypA represents an unusual strategy, given that HIV-1 mostly utilizes viral accessory proteins such as Vif and Vpu to avoid intrinsic antiviral factors. The findings of the current research open opportunities to potentially identify unrevealed cofactor(s) involved in TRIM5α-mediated restriction and additional undisclosed restriction factors, considering the differences observed between primary human blood cells and several cancer-derived cell lines such as TE671 and CEM-SS cells. Moreover, according to the proposed difference in binding modes between human and non-human primate TRIM5α orthologs, these results suggest that the interaction of human TRIM5α with HIV-1 CA in the absence of CypA represents an important focus of future research. Such studies will advance our understanding of how the PRYSPRY
domain of TRIM5α interacts with CA, which will contribute to the development of gene therapy strategies based on the restriction activity of TRIM5α. Moreover, further investigations of the innate immune detection of HIV-1 triggered by human TRIM5α will broaden our knowledge of how host PRRs are cooperatively organized to efficiently sense the invading retrovirus. Finally, with respect to the CypA inhibitor compounds currently in clinical trials, the results of this research suggest that these host-targeting drugs have great potential to improve the efficacy of HIV-1 therapies, which until now have employed virus-targeting compounds only.
APPENDIX I.

REGULATION OF HIV-1 INFECTIVITY BY CYCLOPHILIN A AND HUMAN TRIM5α IN CANCER CELL LINES

Contributors: Kyusik Kim, Ann Dauphin, Sevnur Komrulu, Edward Campbell, and Jeremy Luban

The thesis research presented in Chapter II demonstrates that HIV-1 exploits CA–CypA interaction to evade endogenous TRIM5α–mediated restriction activity in primary human blood cells as HIV-1 targets (Kim et al. 2019). The roles of CypA and TRIM5α in HIV-1 infection are consistent in all cell types, including innate immune myeloid cells such as dendritic cells and macrophages and CD4+ T cells, a type of lymphocytes. In contrast, researchers previously reported that CypA and TRIM5α independently regulate HIV-1 infectivity in cancer cell lines such as TE671 and CEM-SS (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). Considering the sources of the cell lines, it is interesting that the CEM-SS cell line derived from T-cell leukemia showed a different CypA–TRIM5α relationship from primary CD4+ T cells, as did the TE671 cell line, which originated from a muscle tumor, rhabdomyosarcoma (Stratton et al. 1989). Given that there were minor, but perhaps important, experimental differences in the transduction of the cells and
shRNA-mediated knockdown between this thesis research and the previous studies, the roles of CypA and TRIM5α in HIV-1 infection of the cancer cell lines were re-examined.

As in the experiments with primary human blood cells described in Chapter II, TE671 and CEM-SS cells were transduced with lentiviral vectors containing shRNA targeting Luc control or TRIM5. In addition to these two cell lines, the RD cell line, previously identified to be identical to the TE671 cell line (Stratton et al. 1989), was also investigated to confirm the results obtained in TE671 cells. After 3 days of selection with puromycin, TRIM5 knockdown was confirmed by the loss of N-tropic MLV restriction (Figure A.I.1A). The Luc control or TRIM5 knockdown cells were then challenged with VSV glycoprotein-pseudotyped, single-cycle, HIV-1-derived lentiviral vectors encoding a GFP reporter (HIV-1–GFP), followed by assessment of HIV-1 infectivity by flow cytometry at day 3 post-challenge.

First, TE671 and RD cells expressing the shRNA targeting Luc control or TRIM5 were challenged with HIV-1–GFP in the presence of DMSO solvent or CsA (Figure A.I.1B). Disruption of CA–CypA interaction by CsA inhibited HIV-1 infectivity in both cell lines. TRIM5 knockdown did not show rescue of this infectivity to the control wild-type (WT) level with DMSO solvent (Figure A.I.1B). In addition to CsA treatment, the Luc control or TRIM5 knockdown cells were secondly transduced with lentiviral vectors expressing shRNA specific to Luc control or CypA. These cells were then selected with blasticidin for 3 days and
Kim et al., Figure A.I.1

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Figure A.I.1. CypA and TRIM5α independently regulate HIV-1 infectivity in the TE671, RD, and CEM-SS cell lines.

(A) TE671, RD, and CEM-SS cells were transduced with lentiviral vectors expressing shRNA targeting Luc control or TRIM5. After antibiotic selection, the knockdown cells were challenged with VSV G-pseudotyped, single-cycle MLV-GFP vectors containing N- or B-tropic MLV CA (For CEM-SS, mean ± s.e.m., n = 2 technical replicates).

(B) TE671 and RD cells expressing Luc control or TRIM5 shRNA were challenged with single-cycle, VSV G-pseudotyped HIV-1\textsubscript{NL4-3}−GFP in the presence of 2.5 µM CsA or DMSO solvent (mean ± s.e.m., n = 2 technical replicates).

(C) TE671 and RD cells were sequentially transduced and selected with two lentiviral vectors for the knockdown conditions, as indicated. The cells were then challenged with HIV-1\textsubscript{NL4-3}−GFP (mean ± s.e.m., n = 2 technical replicates).

(D) CEM-SS cells transduced with shRNA-expressing lentiviral vectors for Luc control or TRIM5 knockdown, followed by challenge with HIV-1\textsubscript{NL4-3}−GFP bearing WT CA or CA-P90A (mean ± s.e.m., n = 2 technical replicates).

(E) Sequential transductions of CEM-SS cells with two lentiviral vectors were performed to generate the indicated knockdown conditions. The cells were then challenged with HIV-1\textsubscript{NL4-3}−GFP (mean ± s.e.m., n = 2 technical replicates).

The percentage of GFP-positive cells was measured by flow cytometry at day 3 post-challenge and normalized to B-tropic MLV (A) or to WT (B-E) in Luc control knockdown cells in the absence of CsA.
challenged with HIV-1-GFP. CypA knockdown genetically disrupting CA–CypA interaction was observed to decrease HIV-1 infectivity compared to Luc control knockdown (Figure A.I.1C). Consistently, the addition of TRIM5 knockdown to CypA knockdown slightly rescued or did not increase the infectivity (Figure A.I.1C).

In addition to the TE671 and RD cell lines, CEM-SS cells expressing Luc control or TRIM5 shRNA were challenged with HIV-1–GFP bearing WT CA or CA-P90A (Figure A.I.1D). The knockdown cells were also used for the second transduction with lentiviral vectors for Luc control or CypA knockdown, as described above. These double knockdown cells were challenged with HIV-1-GFP containing WT CA (Figure A.I.1E). HIV-1 infectivity was decreased when CA–CypA interaction was disrupted by either CA-P90A or CypA knockdown in CEM-SS cells (Figure A.I.1D, E). TRIM5 knockdown minimally increased this reduced infectivity (Figure A.I.1D, E). Thus, all of these data indicate that the relationship between CypA and TRIM5α in TE671, RD, and CEM-SS cell lines is different from that in primary blood cells (Figure 3.2). The results here are consistent with the previous studies described above, implying that regulation of HIV-1 infection by CypA and TRIM5α differs between cancer cell lines and primary blood cells.

To explore this finding, the CypA–TRIM5α relationship in HIV-1 infection was investigated in two additional cell lines, Jurkat and SupT1, derived from T-cell leukemia and T-cell lymphoma, respectively. In the Jurkat cell line, TRIM5α-
mediated restriction activity against N-tropic MLV and decreased HIV-1 infectivity
with CA-P90A mutation were observed (Figure A.I.2A). To examine the
relationship between CypA and TRIM5α in HIV-1 infection, Luc control or TRIM5
knockout Jurkat cells were generated by CRISPR/Cas9-based technology. Jurkat
cells were transduced with lentiviral vectors encoding a Cas9 protein and a
guideRNA (gRNA) cassette targeting Luc control or TRIM5. Two target sites
were used for each gene knockout. The transduced cells were selected with
puromycin for 2 weeks. TRIM5 knockout generated by either of the two different
gRNAs was confirmed by challenge of the cells with N-tropic MLV, as performed
for TRIM5 knockdown (Figure A.I.2B). Then, HIV-1−GFP harboring WT CA or
CA-P90A was used to challenge these knockout cells. As observed in Figure
A.I.2A, disruption of CA–CypA interaction reduced HIV-1 infectivity in both of the
Luc control knockout cells (Figure A.I.2C). This infectivity was recovered toward
the level of WT HIV-1 in Luc control knockout cells in both TRIM5 knockout cells
(Figure A.I.2C). Secondly, the knockout Jurkat cells were challenged with HIV-1-
GFP having WT CA in the presence or absence of CsA. HIV-1 infectivity was
coherently decreased by the pharmacologic disturbance of CA–CypA interaction
but rescued to WT virus level in control knockout cells treated with DMSO solvent
by TRIM5 knockout (Figure A.I.2D). Furthermore, to genetically interrupt
CA–CypA interaction, Luc control or TRIM5 knockout Jurkat cells were secondly
transduced with lentiviral vectors containing a Cas9 gene and a gRNA cassette
specific to Luc control or CypA. Two target sites for each gene were used for
Figure A.I.2. CypA protects HIV-1 from endogenous TRIM5α in the Jurkat cell line as observed in primary blood cells.

(A) Jurkat cells were challenged with MLV-GFP harboring N- or B-tropic MLV CA (left), or with HIV-1\textsubscript{NL4-3}−GFP containing WT CA or CA-P90A (right; mean ± s.e.m., $n = 6$ biologically independent experiments). The graph with MLV-GFP shown here is a representative of the results from two independent experiments (left).

(B) Jurkat cells were transduced and selected with lentiviral vectors expressing a Cas9 and a guideRNA cassette targeting Luc control or TRIM5, followed by challenge with MLV-GFP containing N- or B-tropic MLV CA. The graph shown here is a representative of two independent experiments.

(C and D) Jurkat cells expressing a Cas9 and a guideRNA specific to Luc control or TRIM5 were challenged with HIV-1\textsubscript{NL4-3}−GFP bearing WT CA or CA-P90A (C) (mean ± s.e.m., $n = 2$ biologically independent experiments), or WT HIV-1\textsubscript{NL4-3}−GFP in the presence of 5 µM CsA or DMSO solvent (D) (mean ± s.e.m., $n = 2$ technical replicates).

(E) Jurkat cells were sequentially transduced with two lentiviral vectors to achieve the indicated knockout conditions. The cells were then challenged with WT HIV-1\textsubscript{NL4-3}−GFP (mean ± s.e.m., $n = 2$ biologically independent experiments).

(F and G) Lentiviral vectors containing shRNA specific to Luc control or TRIM5 were used to transduce Jurkat cells. The knockdown cells were challenged with HIV-1\textsubscript{NL4-3}−GFP bearing WT CA or CA-P90A (F), or WT HIV-1\textsubscript{NL4-3}−GFP in the presence of 5 µM CsA or DMSO solvent (G) (mean ± s.e.m., $n = 2$ biologically independent experiments for each).

The percentage of GFP-expressing cells was assessed by flow cytometry 3 days after the virus challenge, and normalized to the WT in Luc control knockout or knockdown cells without CsA treatment (A and C-G).
knockout. After 2 weeks of the selection with blasticidin, cells were challenged with WT HIV-1−GFP. HIV-1 infectivity was decreased in CypA knockout cells compared to the control knockout cells but was restored to the control level when TRIM5 knockout was added with CypA knockout (Figure A.I.2E). Therefore, a similar CypA−TRIM5α relationship in HIV-1 infection was observed in the Jurkat cell line and primary blood cells.

Because all of these data from Jurkat cells were based on CRISPR/Cas9-mediated gene knockout, to confirm the acquired results, Luc control or TRIM5 knockdown Jurkat cells were generated by transduction with the shRNA-containing lentiviral vectors used in previous experiments. These knockdown cells were challenged with HIV-1−GFP bearing WT CA or CA-P90A (Figure A.I.2F), or with WT HIV-1−GFP in the presence of DMSO solvent or CsA (Figure A.I.2G). TRIM5 knockdown was observed to rescue the HIV-1 infectivity inhibited by CA-P90A or CsA (Figure A.I.2F, G), consistently with the knockout experiments (Figure A.I.2C–E). All the data here demonstrate that CA−CypA interaction safeguards HIV-1 from restriction by endogenous TRIM5α in Jurkat cells, similarly with the observation in primary blood cells, but differently from that in the previous three cell lines used in Figure A.I.1. Thus, this suggests that the relationship between CypA and TRIM5α in the regulation of HIV-1 also varies among cell lines.

SupT1 cells were also used to investigate the relationship between the two host proteins in HIV-1 infection. Intriguingly, SupT1 cells displayed the loss of
TRIM5α-mediated restriction activity based on the observed high infectivity of N-tropic MLV and of CA-P90A HIV-1 (Figure A.I.3A). Similar infectivity between WT and CA-P90A–containing HIV-1 in the SupT1 cell line was indeed observed in previous investigations (Vozzolo et al. 2010; Meehan et al. 2014). In accordance with this observation, RNA-Seq–based gene expression profiling data provided by two studies (Barretina et al. 2012; Rausell et al. 2016) have shown that SupT1 has a more than 3-fold lower level of TRIM5 expression compared to Jurkat cells, which have potent TRIM5α activity (Figure A.I.3B). This was also confirmed by quantitative reverse transcription PCR-based measurement of the TRIM5 mRNA levels in both the Jurkat and SupT1 cell lines (Figure A.I.3B). These data indicate that SupT1 cells have a marginal restriction activity of TRIM5α due to the low expression level of this protein. To determine whether the exogenous expression of TRIM5α restricts HIV-1 CA in the disruption of CA–CypA interaction in SupT1 cells, these cells were transduced with lentiviral vectors encoding human TRIM5α or control vectors lacking cDNA. The transduced cells were selected with puromycin for 3 days. The exogenous expression of human TRIM5α was confirmed by the observation of the N-tropic MLV inhibition (Figure A.I.3C). The cells were then challenged with HIV-1–GFP having WT CA or CA-P90A. The infectivity was similar between WT CA and CA-P90A viruses in SupT1 cells without exogenous TRIM5α expression (Figure A.I.3D), consistent with the observation in Figure A.I.3A. WT HIV-1 was minimally affected, whereas infection by HIV-1 with CA-P90A was inhibited by the expression of human TRIM5α.
Figure A.I.3. Rescued expression of human TRIM5α in SupT1 cell line is capable of restricting HIV-1 with disruption of CA–CypA interaction.

(A) SupT1 cells were challenged with MLV-GFP containing N- or B-tropic MLV CA (left), or with HIV-1 NL4-3−GFP bearing WT CA or CA-P90A (right; mean ± s.e.m., n = 6 biologically independent experiments). The graph with MLV-GFP presented here is a representative of two independent experiments (left).

(B) RNA expression level of TRIM5 in Jurkat and SupT1 cell lines, according to two published studies (left and middle), were visualized with units of transcripts per kilobase million (TPM) and reads per kilobase million (RPKM), respectively. Right: Total RNA was isolated from Jurkat and SupT1 cells and the TRIM5 mRNA level was assessed by RT–qPCR with the TaqMan probe system. The TRIM5 expression level was normalized to the level of OAZ1, a housekeeping gene.

(C and D) SupT1 cells were transduced with lentiviral vectors encoding human TRIM5α or control vectors lacking cDNA, followed by the antibiotic selection. The cells were then challenged with MLV-GFP harboring N- or B-tropic MLV CA (C), or HIV-1 NL4-3−GFP containing WT CA or CA-P90A (D) (mean ± s.e.m., n = 3 biologically independent experiments).
These observations demonstrate that human TRIM5α is capable of restricting HIV-1 when CA–CypA interaction is disrupted, but this ability depends on the cell line.

The experiments described above used single-cycle HIV-1–GFP vectors to assess HIV-1 infectivity. To define whether CypA protects HIV-1 replication from restriction by human TRIM5α in Jurkat cells, as observed in primary blood cells, Luc control or TRIM5 knockout Jurkat cells were infected with replication-competent HIV-1. The media supernatants from the infected cell culture were harvested over time to monitor HIV-1 replication by quantifying the viruses in the media based on the viral reverse transcriptase activity. In the experiment using HIV-1 containing WT CA or CA-P90A, CA-P90A–containing HIV-1 was unable to replicate in control knockout cells (Figure A.I.4A). This HIV-1 was shown to replicate in TRIM5 knockout cells, although the replication was slower than that of WT HIV-1 (Figure A.I.4A). The second experiment with or without CsA treatment displayed that the presence of CsA decreased the replication level of HIV-1, but TRIM5 knockout restored the replication toward that observed in the absence of CsA (Figure A.I.4B). Additionally, when CypA knockout cells were infected with WT HIV-1, the virus was observed to replicate slowly compared to the replication rate in Luc control or TRIM5 knockout cells (Figure A.I.4C). However, the addition of TRIM5 knockout with CypA knockout rescued the replication level toward the level seen in the control knockout cells (Figure A.I.4C).
A. Jurkat + HIV-1\textsubscript{NL4-3}

B. Jurkat + HIV-1\textsubscript{NL4-3}

C. Jurkat + HIV-1\textsubscript{NL4-3}

D. ALH-CCR5 Jurkat

E. Jurkat

F. Jurkat + HIV-1\textsubscript{ZM249M}

G. SupT1 + HIV-1\textsubscript{NL4-3}
Figure A.I.4. CA–CypA interaction shields HIV-1 replication from human TRIM5α in the Jurkat and SupT1 cell lines.

(A-C) The spread of HIV-1_{NL4-3} infection in Luc control or TRIM5 knockout Jurkat cells with virus containing CA-P90A (A), with 5 µM CsA (B), or after transduction with vectors bearing Cas9 and guideRNA targeting Luc control or CypA (C). The data shown here are representative of four (A) or two (B and C) independent experiments.

(D and E) A lentiviral vector encoding CCR5 was used to transduce Luc control or TRIM5 knockout Jurkat cells, followed by flow cytometry-based assessment of surface CCR5 levels (D) or by challenge with single-cycle HIV-1_{NL4-3−GFP} harboring WT CA or CA-P90A (E) (mean ± s.e.m., n = 2 biologically independent experiments).

(F) The spread of HIV-1_{ZM249M} (left) or HIV-1_{Z331M-TF} (right) infection in CCR5-expressing Luc control or TRIM5 knockout Jurkat cells in the presence of 5 µM CsA. The data are representative of two independent experiments.

(G) Replication of HIV-1_{NL4-3} bearing WT CA or CA-P90A in SupT1 cells transduced with lentiviral vectors expressing human TRIM5α or control vectors lacking cDNA.

HIV-1 replication in (A-C), (F), and (G) was monitored over time based on the reverse transcriptase activity of viruses (RTU/µl) in the culture media supernatant.
The HIV-1 used in the experiments above was a clade B HIV-1\textsubscript{NL4-3} lab strain. Hence, the replications of primary HIV-1 isolates from patients in the knockout Jurkat cells were also monitored to examine whether CypA also protects these different HIV-1 strains from TRIM5α activity. Because these HIV-1 viruses are R5-tropic, unlike X4-tropic HIV-1\textsubscript{NL4-3}, the knockout Jurkat cells were transduced with lentiviral vectors encoding CCR5, prior to the viral infection. After 3 days of selection with hygromycin, CCR5 expression on the cell surface was confirmed by flow cytometry (Figure A.I.4D). The recovery of CA-P90A-bearing HIV-1 infectivity by TRIM5 knockout was also confirmed in these cells to confirm that this additional CCR5 expression did not have an effect on HIV-1 regulation by CypA and TRIM5α (Figure A.I.4E). Then, the two clade C HIV-1\textsubscript{ZM249M} or HIV-1\textsubscript{Z331M-TF} transmission/founder strains from Zambia (Salazar-Gonzalez et al. 2009; Deymier et al. 2015; McCauley et al. 2018) were used to infect these CCR5-expressing Luc control or TRIM5 knockout cells in the presence of CsA or DMSO solvent. The replication levels of the two HIV-1s were both decreased with the addition of CsA to disturb CA–CypA interaction, although the two viruses showed different levels of sensitivity to CsA (Figure A.I.4F). These hindered replication levels were restored toward the level in cells without CsA by TRIM5 knockout (Figure A.I.4F).

In addition to Jurkat cells, SupT1 cells expressing human TRIM5α were infected with HIV-1\textsubscript{NL4-3} harboring WT CA or CA-P90A. Consistent with the experiments using single-cycle, HIV-1-GFP in Figure A.I.3D, CA-
P90A–containing HIV-1 was able to replicate at a comparable level to WT HIV-1 but showed a decreased replication level with expression of human TRIM5α (Figure A.I.4G). Thus, all the data from these replication experiments indicate that CypA shields HIV-1 from restriction by human TRIM5α during viral replication in Jurkat and SupT1 cell lines, as observed in primary blood cells in Chapter II.

The restriction activity of TRIM5α requires its association with HIV-1 CA. To test whether CypA thwarts TRIM5α interaction with HIV-1 CA, a proximity ligation assay (PLA) with antibodies against HIV-1 CA or TRIM5α was performed. U2OS cells transduced with lentiviral vectors expressing shRNAs specific for Luc control or TRIM5 were used for this microscopy-based assay, after confirmation that the decreased infectivities of N-tropic MLV and CA-P90A–harboring HIV-1 were both recovered in the TRIM5 knockdown cells (Figure A.I.5A, B). To evaluate the CA–TRIM5α association with disruption of CA–CypA interaction, Luc control or TRIM5 knockdown cells were challenged with HIV-1-GFP having WT CA, with or without CsA treatment, for 2 hours. All these virus challenges were performed in the presence of MG132, a proteasome inhibitor, which is known to facilitate the detection of CA–TRIM5α complexes (Wu et al. 2006; Campbell et al. 2008). The cells were then fixed and used for the PLA. Interaction of CA with TRIM5α was detected almost only when CA–CypA was disrupted in Luc control knockdown cells (Figure A.I.5C, D). The CA–TRIM5α complex was rarely detected in the absence of CsA or in TRIM5 knockdown
Figure A.I.5. Human TRIM5α is able to associate with HIV-1 CA when CA–CypA interaction is disrupted in the U2OS cell line.

(A and B) U2OS cells transduced with lentiviral vectors harboring shRNA specific to Luc control or TRIM5 were challenged with MLV-GFP containing N- or B-tropic MLV CA (A), or HIV-1_{NL4-3}−GFP having WT CA or CA-P90A (B) (mean ± s.e.m., $n = 2$ technical replicates).

(C and D) Luc control or TRIM5 knockdown U2OS cells were challenged with HIV-1_{NL4-3}−GFP containing WT CA for 2 hours in the presence of 5 µM CsA or DMSO solvent. The cells were then fixed, and a PLA using antibodies against HIV-1 CA and TRIM5α was performed. The representative images in (C) display PLA puncta (red) and nuclei stained with Hoechst (blue). The graph in (D) shows the number of PLA puncta per cell in each condition (mean ± s.e.m.; Luc knockdown + CsA, no virus, $n = 35$ cells analyzed; Luc knockdown + DMSO + HIV-1, $n = 35$; Luc knockdown + CsA + HIV-1, $n = 53$; TRIM5 knockdown + CsA + HIV-1, $n = 37$). The data here are representative of four independent experiments.
cells (Figure A.I.5C, D). These data demonstrate that CA–CypA interaction blocks the association of endogenous TRIM5α with the incoming HIV-1 CA in the U2OS cell line, consistent with the observation in primary macrophages described in Chapter II.

In this section, the relationship between CypA and TRIM5α in HIV-1 infection in six cancer cell lines were investigated. In the TE671, RD, and CEM-SS cell lines, endogenous TRIM5α did not show efficient restriction against HIV-1 in the disruption of CA–CypA interaction, suggesting that the regulation of HIV-1 infectivity by CypA is independent of TRIM5α activity, consistent with the conclusions of previous studies (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006). On the other hand, the CypA–TRIM5α relationship in the Jurkat and U2OS cell lines were observed to be equivalent to that in primary blood cells, showing that CypA protects HIV-1 from recognition/restriction by human TRIM5α, in accordance with recent studies (Kim et al. 2019; Selyutina et al. 2020). Moreover, SupT1 cells were shown to be permissive to HIV-1 even when CA–CypA interaction was disrupted, due to the low gene expression of TRIM5. Exogenous expression of human TRIM5α in SupT1 cells also demonstrated that human TRIM5α is able to restrict HIV-1 when CA–CypA interaction is disrupted. Interestingly, the relationship between CypA and TRIM5α in HIV-1 infection was found to vary among the CEM-SS, Jurkat, and SupT1 cell lines, although these lines are all derived from T cells. Thus, the data presented here indicate that the CypA–TRIM5α relationship in HIV-1
infection varies depending on cell line, rather than on cell type.

The difference in HIV-1 regulation by CypA and TRIM5α among cell lines may be due to the different restriction capacities of TRIM5α against HIV-1 CA. Minimal rescue of HIV-1 infectivity with TRIM5 knockdown in disruption of CA–CypA interaction was observed in the TE671, RD, and CEM-SS cells, in contrast to the observations in Jurkat, U2OS, and primary blood cells. Given that endogenous TRIM5α of all these cells showed potent restriction activity against N-tropic MLV, TRIM5α protein itself in the TE671 and CEM-SS cell lines is less likely to contain amino acid differences causing the weak restriction capacity. Thus, this may imply the presence of an unknown cofactor involved in the restriction activity of human TRIM5α against HIV-1 CA. This factor that would be differentially expressed in the cell lines could either promote the association of TRIM5α with HIV-1 CA or enhance the restriction activity upon the CA–TRIM5α interaction. Further experiments examining the CA–TRIM5α association when CypA is blocked from binding to CA in the TE671 and CEM-SS cell lines will show whether the factor acts on the interaction of TRIM5α with CA or on the restriction activity of TRIM5α. Given that TRIM5α is suggested to restrict HIV-1 CA via a proteasome- (Anderson et al. 2006; Wu et al. 2006; Diaz-Griffero et al. 2007; Roa et al. 2012; Kutluay, Perez-Caballero, and Bieniasz 2013; Fletcher et al. 2018; Jimenez-Guardeño et al. 2019) or autophagy-mediated degradation pathway (O’Connor et al. 2010; Mandell et al. 2014; Keown et al. 2018; Saha, Chisholm, and Mandell 2020), the factor could be involved in these pathways.
The different levels of TRIM5α-mediated HIV-1 restriction can explain how, without the CA–CypA interaction, HIV-1 infectivity was not rescued by TRIM5 knockdown in some cell lines. However, the HIV-1 infectivity still remained decreased by disruption of CA–CypA interaction in the cell lines with TRIM5α knockdown. This could suggest that CA–CypA interaction shields HIV-1 CA from the unidentified restriction factor in addition to TRIM5α. Therefore, considering the differences in the CypA–TRIM5α relationship among the cell lines, gene knockdown/knockout screening experiments using the cell lines with HIV-1 in conditions disturbing CA–CypA interaction, along with a comparison of gene expression profiles among cell lines, will be useful to identify these unknown factors. Comparisons of CEM-SS and TE671 cells to the Jurkat and SupT1 cells expressing human TRIM5α would contribute to the discovery of the cofactor of TRIM5α-mediated restriction of HIV-1, as well as to the identification of the additional restriction factor.
APPENDIX II.

HIV-1 CAPSID MUTATION CONFRMS RESISTANCE TO RESTRICTION BY HUMAN TRIM5α

Contributors: Kyusik Kim, Ann Dauphin, and Jeremy Luban

In Appendix I, the relationship between CypA and TRIM5α in the regulation of HIV-1 infectivity was examined in several human cancer cell lines. Consistent with previously published studies (Sokolskaja, Berthoux, and Luban 2006; Keckesova, Ylinen, and Towers 2006), CypA and TRIM5α were observed to independently regulate HIV-1 infectivity in the TE671, RD, and CEM-SS cell lines. However, the ability of CypA to protect HIV-1 from TRIM5α was observed in other cell lines including Jurkat and U2OS, similarly to the observation in primary human blood cells described in Chapter II, which suggests that host–virus interactions can have different outcomes in different cell lines as well as cell types.

In the experiments described above, the CA-P90A mutation was used to disrupt CA–CypA interaction. In the primary human blood cells used in Chapter II and Jurkat cells used in Appendix I, this mutation was found to decrease HIV-1 infectivity, which could be rescued toward the WT virus level upon TRIM5 knockdown or knockout. Interestingly, researchers previously reported that the
CA-A92E mutation spontaneously occurs in HIV-1-infected cells in culture in the presence of cyclosporines, which are CypA inhibitor compounds (Aberham, Weber, and Phares 1996; Braaten et al. 1996b). Subsequent studies showed that CA-A92E mutant HIV-1 was less vulnerable to the disruption of CA–CypA interaction by CsA or the loss of CypA expression (Braaten and Luban 2001; Sokolskaja, Sayah, and Luban 2004; Hatziioannou et al. 2005; De Iaco and Luban 2014). Thus, our finding, with consideration of these studies, raises the hypothesis that the CA-A92E mutation renders HIV-1 resistant to restriction by human TRIM5α.

To assess HIV-1 infectivity, VSVg-pseudotyped, single-cycle, HIV-1–derived lentiviral vectors containing a GFP reporter (HIV-1–GFP) were used to challenge cells, followed by flow cytometry-based measurement of the percentage of GFP-positive cells at day 3 post-challenge. Jurkat cells were first utilized to examine the hypothesis (Figure A.II.1A, B). To generate cells with knockout of TRIM5, CypA, or both, Jurkat cells were transduced with lentiviral vectors containing a puromycin-resistance marker, Cas9, and guideRNA (gRNA) targeting luciferase control (Luc) or TRIM5, followed by selection under puromycin from day 3 post-transduction. Two weeks later, some of these knockout cells were challenged with HIV-1–GFP bearing wild-type CA (WT CA), CA-A92E, CA-P90A, or CA-P90A/A92E (Figure A.II.1A). The rest of the knockout cells were secondly transduced with lentiviral vectors expressing a blasticidin-resistance marker, Cas9, gRNA specific to Luc or CypA. The cells were then
selected under both puromycin and blasticidin at day 3 post-transduction with CypA vectors. TRIM5 knockout and CypA knockout were confirmed by assessing MLV infectivity and the protein level of CypA, respectively, using the same methods described in Chapter II. These knockout cells were then challenged with HIV-1−GFP containing WT CA or CA-A92E mutation (Figure A.II.1B).

Comparable infectivity was observed between the WT CA and CA-A92E viruses in all the knockout cells except CypA knockout cells, indicating that the A92E mutation in HIV-1 CA does not have an apparent effect on HIV-1 infectivity (Figure A.II.1A, B). The infectivity was decreased by the CA-P90A mutation or CypA knockout, but this reduction was restored toward the control level by TRIM5 knockout or, intriguingly, addition of the CA-A92E mutation (Figure A.II.1A, B), which suggests that the CA-A92E mutation confers resistance against TRIM5α to HIV-1 CA.

In Appendix I, the SupT1 cell line showed a TRIM5α-null phenotype in HIV-1 infection, but its CypA was shown to protect HIV-1 CA from exogenously expressed TRIM5α. As used in those experiments, SupT1 cells were transduced with lentiviral vectors to express human TRIM5α or with control vectors lacking cDNA, followed by antibiotic selection. As in Figure A.II.1A, the SupT1 cells were challenged with HIV-1−GFP harboring WT CA, CA-P90A, CA-A92E, or CA-P90A/A92E (Figure A.II.1C). Viruses containing WT CA or CA-A92E mutation showed comparable infectivity in both conditions. CA-P90A mutant virus was observed to be susceptible to the exogenous expression of human TRIM5α.
Figure A.II.1

A

Jurkat + HIV-1NL4-3

Relative infectivity (%)

WT A92E P90A P90A A92E

B

Jurkat + HIV-1NL4-3

Relative infectivity (%)

WT A92E

No cDNA huTRIM5α

SupT1 + HIV-1NL4-3

Relative infectivity (%)

WT A92E P90A P90A A92E

D

Dendritic cells + HIV-1NL4-3

Relative infectivity (%)

WT A92E P90A P90A A92E

Luc KD TRIM5 KD

Kim et al., Figure A.II.1
Figure A.II.1. CA-A92E mutation renders HIV-1 resistant to restriction by human TRIM5α.

(A) Jurkat cells were transduced and selected with lentiviral vectors for Luc control or TRIM5 knockout, followed by challenge with single-cycle, VSV G-pseudotyped HIV-1<sub>NL4-3</sub>−GFP containing WT CA, CA-P90A, CA-A92E, or CA-P90A/A92E (mean ± s.e.m., n = 2 biologically independent experiments).

(B) Jurkat cells were sequentially transduced with two vectors to achieve the indicated knockout conditions. The knockout cells were then challenged with HIV-1<sub>NL4-3</sub>−GFP containing WT CA, or CA-A92E (mean ± s.e.m., n = 2 biologically independent experiments).

(C) SupT1 cells were transduced with lentiviral vectors expressing human TRIM5α or control vectors lacking cDNA, followed by antibiotic selection. HIV-1<sub>NL4-3</sub>−GFP containing WT CA, CA-P90A, CA-A92E, or CA-P90A/A92E was used to challenge these cells (mean ± s.e.m., n = 2 technical replicates).

(D) Dendritic cells were selected after transduction with a lentiviral vector expressing shRNA specific to TRIM5 or Luc, and challenged with HIV-1<sub>NL4-3</sub>−GFP containing WT CA, CA-P90A, CA-A92E, or CA-P90A/A92E (mean ± s.e.m., n = 2 technical replicates).

The percentage of GFP-expressing cells was assessed by flow cytometry at day 3 post-challenge and normalized to the WT in Luc control knockout/knockdown or No cDNA-control cells.
However, this sensitivity could be overcome by the addition of the CA-A92E mutation (Figure A.II.1C), consistently with the previous experiments using Jurkat cells (Figure A.II.1A, B).

Primary human dendritic cells were used to confirm that the CA-A92E mutation conferred resistance to human TRIM5α, in addition to the cancer cell lines described above. Transduction of lentiviral vectors, followed by antibiotic selection, was performed to generate the dendritic cells expressing shRNAs targeting Luc control or TRIM5. These cells were challenged with the four viruses having WT CA or mutant CA, used in the previous experiments (Figure A.II.1A, C). Consistently, adding the CA-A92E mutation recovered the infectivity of CA-P90A mutant viruses reduced by TRIM5α-mediated restriction in Luc knockdown cells (Figure A.II.1D). All of these data demonstrate that HIV-1 bearing the CA-A92E mutation is invulnerable to human TRIM5α.

Lastly, the resistance conferred by the CA-A92E mutation was also transferred to HIV-1 during viral replication (Figure A.II.2). Luc or TRIM5 knockout Jurkat cells were infected by replication-competent HIV-1 NL4-3 harboring WT CA, CA-P90A, CA-A92E, or CA-P90A/A92E. Viral replication was monitored over time by quantifying the virus levels in the culture media supernatant. Replication of CA-P90A mutant virus was largely inhibited, compared to that of WT HIV-1. HIV-1 with the CA-A92E mutation was able to replicate at a comparable level to the WT virus, but at a higher level, up to 1,400-fold greater, than CA-P90A-containing virus. Importantly, the removal of TRIM5 or the
Figure A.II.2. Human TRIM5α is not able to inhibit the replication of HIV-1 bearing the CA-A92E mutation.

Replication of HIV-1\textsubscript{NL4-3} bearing WT CA, CA-P90A, CA-A92E, or CA-P90A/A92E in Jurkat cells transduced with lentiviral vectors for Luc control or TRIM5 knockout. HIV-1 replication was monitored over time by assessing reverse transcriptase activity in the culture supernatant. The graph presented here is a representative of two independent experiments.
addition of the CA-A92E mutation was observed to restore the replication of CA-P90A–bearing HIV-1 toward the replication level of WT HIV-1, consistently with the previous data and studies. This indicates that the CA-A92E mutation blocks restriction of HIV-1 by TRIM5α during the replication.

All the data here demonstrate that HIV-1 can spontaneously acquire the CA-A92E mutation in order to safeguard itself against restriction by human TRIM5α, when CA–CypA interaction is disrupted. Surprisingly, the mutation occurs at the CypA-binding loop region of the CA, implying that human TRIM5α likely binds to CA at a site proximal to the CypA-binding site. If so, this may indicate that CA–CypA interaction sterically blocks HIV-1 recognition by human TRIM5α, as suggested in Chapters II and III. Alternatively, the conformation made by combination of the cis-formed P90 residue and the A92 residue may be required for human TRIM5α binding to the HIV-1 CA, which would be disrupted by the peptidyl prolyl isomerase activity of CypA. A report showing the CA-A92E resistance to TRIM5α in Jurkat and primary CD4+ T cells has been recently published (Selyutina et al. 2020). Our data support this study by providing results from direct examinations of the relationship between the CA-A92E mutation and restriction by TRIM5α in the disruption of CA–CypA interaction in a variety of cell types.


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