Molecular Analysis of the Contributions of Human Immunodeficiency Virus Type-1 Integrase in Post Entry Steps of Early Stage Virus Replication

By

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Abstract

Human immunodeficiency virus type 1 (HIV-1) infection causes general loss of immune response in humans. Presently, an estimated 34 million (31.4-35.9 million) people worldwide are HIV-1 positive and many more are being newly infected. In the absence of a definitive cure, anti-HIV-1 drug therapy helps to manage the infection by suppressing virus replication. However, extensive drug resistance against most of existing drugs demands alternative anti-HIV-1 strategies. The proper knowledge about HIV-1 replication is essential to guide the development of new anti-HIV-1 strategies. The research presented in this thesis aims to understand the role of HIV-1 Integrase (IN) and cellular co-factors interactions in the early stage virus replication.

In the cytoplasm, HIV-1 cDNA exists as a high molecular weight nucleoprotein complex called pre-integration complex (PIC). The cDNA enters the nucleus as a part of PIC by active nuclear import and integrates into the host genome. HIV-1 Integrase (IN) protein has been recognized as a primary viral factor for HIV-1 nuclear import, but the key contributing cellular factor(s) is unknown. We have examined the requirement of different Importinα (Impα) isoforms for HIV-1 replication and identified the requirement of Impα3 for HIV-1 replication in HeLa cells, C8166T cells, and human macrophages. Further investigations showed the specific requirement of Impα3 for HIV-1 nuclear import. By analyzing the Impα3 interaction with HIV-1 proteins, we detected the IN interaction with Impα3 and C-terminal domain (CTD) of IN was essential for Impα3 interaction. These data led to the conclusion that Impα3 is required for HIV-1 nuclear import and interacts with IN. The IN-CTD consists of conserved basic amino acid rich

motifs (^{211}KELQKQITK, ^{236}KGPAKLLWK, and ^{262}RRKAK) that closely resemble the consensus classical nuclear localization signal (NLS) for Impα interaction. By substitution mutation and interaction analysis, ^{211}KELQKQITK and ^{262}RRKAK motifs in IN were identified as required for Impα3 interaction, IN nuclear localization, and HIV-1 nuclear import. Together, these data were useful in explaining the molecular mechanism of IN and Impα3 interaction and its requirement for HIV-1 nuclear import.

Retrograde transportation of macromolecules in the cytoplasm is one of the prerequisites for their nuclear import. Although an earlier study implicated the dynein complex in retrograde transport of HIV-1, cellular and viral factors that are involved in this process are unknown. In this study, we have elucidated the HIV-1 IN interaction with the dynein light chain 1 (DYNLL1) in 293T cells, *in vitro*, and in HIV-1 infected cells. DYNLL1 is one of the adapter proteins that mediate the cargo recruitment to dynein complex. However, our data suggested that the IN and DYNLL1 interaction is essential for proper HIV-1 uncoating and cDNA synthesis but not for nuclear import. Surprisingly, DYNLL1 interaction of IN was dispensable for HIV-1 recruitment to dynein complex. These data led to the conclusion that the IN and DYNLL1 interaction is essential for proper HIV-1 uncoating and cDNA synthesis but not required for HIV-1 recruitment to the dynein complex or for retrograde transport.

In summary, this study advances our knowledge on the role of IN and cellular factors interactions in different early steps of HIV-1 replication and offers potential contributions in the development of future anti-HIV-1 strategies.

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Dedication

I dedicate this thesis to my mother, father, and teachers. It is their support and motivation allowed me to pursue my research interests.

Table of Contents

Abstract I

Acknowledgements III

Dedication V

List of Tables 1

List of Figures 1

Copyright Materials 3

Abbreviations 4

Chapter 1. 9

Introduction 9

1.1. The General Description of HIV-1 9

1.2. The Epidemiology of HIV Infection 10

1.3. HIV Virology 11

- 1.3.1 HIV Classification 11
- 1.3.2 HIV Genome 11
- 1.3.3 HIV Virion Structure 12
- 1.3.4 HIV Tropism and AIDS Pathogenesis 20
- 1.3.5 HIV Replication Cycle 22

1.4. HIV Nuclear Import 26

- 1.4.1 The Nuclear Import of Cellular Proteins 26
- 1.4.2 HIV Nuclear Import and Virus Replication 31
- 1.4.3 Viral and Cellular Factors Associated With the HIV Nuclear Import 33
- 1.4.4 HIV Nuclear Import and Establishment of Infection 41

1.5. The Intracytoplasmic Transport of HIV RTC/PIC 43
1.5.1 Retrograde and Anti-retrograde Transportation 43
1.5.2 Molecular Mechanism of Retrograde Transportation 44
1.5.3 Functional Relationship Between Retrograde Transportation and Nuclear Import 47
1.5.4 Retrograde Transport of HIV Replication Complex in the Cytoplasm 48
1.5.5 The Importance of RTC/PIC Retrograde Transport for HIV Replication 48
1.6. HIV Uncoating and Early Stage HIV Replication 50
1.7. Anti-HIV Drug Development 52
1.7.1 HIV IN Inhibitors 53
1.7.2 Protein-Protein Interactions Inhibitors 53
1.8. Study Rationale, Central Hypothesis, and Outline 56
Chapter 2. 58
Materials and Methods 58
2.1 General Reagents 58
2.1.1 Cell Lines and Transfection 58
2.1.2 Plasmids 58
2.1.2.1 HIV IN and Other Viral Protein Expression Plasmids 58
2.1.2.2 Provirus and Virus Related Plasmids 60
2.1.2.3 Cellular Protein Expression Vectors 61
2.1.3 Chemicals and Antibodies 63
2.2 General Methods 63

2.2.1 Co-Immunoprecipitation (Co-IP) and Chemiluminescent Co-IP Assays 63

2.2.2 *In Vitro* Protein Interaction Assay 65

2.2.3 Immunofluorescence Staining 66

2.2.4 The shRNA Mediated Knockdown of Cellular Gene Expression 66

2.2.5 WST-1 Cell Proliferation Assay 67

2.2.6 Virus Production and Infections 68

2.2.7 Subcellular Protein Fractionation 70

2.2.8 qPCR Analysis for HIV Replication 71

2.2.9 Fate of Capsid Assay 73

2.2.10 Statistical Analysis 74

Chapter 3. 75

Investigation of the role of Impα Isoforms in HIV Nuclear Import and the mechanism of Impα recruitment by HIV during replication 75

3.1 Rationale 75

3.2 Hypothesis 77

3.3 Objectives 77

3.4 Results 78

3.4.1 The Effect of Impα1, Impα3, Impα5, or Impα7 KD on HIV Replication in HeLa Cells 78

3.4.2 Wild Type HIV replication was Impaired in Impα3-KD C8166T Cells 79

3.4.3 HIV Nuclear Import was Impaired in Impα3-KD Cells 84

3.4.4 HIV IN Interacts with Impα3 84

3.4.5 The IN-CTD is Involved in Impα3 Interaction 88

3.4.6 Impα3 is Essential for HIV Replication in Human Primary Macrophages 90

3.4.7 The Conserved Basic Amino Acid Rich Motifs in IN-CTD are Required for Impα3 Interaction 93

3.4.8 The Major and Minor NLS Binding Grooves of Impα3 are Involved in IN Interaction 98

3.4.9 Impα3 Interaction Defective $IN_{K215A/K219A/R263A/K264A}$ Mutant is Defective for Nuclear Localization 100

3.4.10 Impα3 Interaction Defective $HIV\text{-}IN_{K215A/K219A/R263A/K264A}$ Mutant is Defective for Replication at or Prior to Integration Step 101

3.4.11 $HIV\text{-}IN_{K215A/K219A/R263A/K264A}$ Mutant Virus is Defective for Nuclear Import in Dividing and Non-Dividing Cells 103

3.5 Discussion 107

Chapter 4. 119

The IN and DYNLL1 Interaction and Its Role in Early Stage HIV Replication 119

4.1 Rationale 119

4.2 Hypothesis 121

4.3 Objective 121

4.4 Results 121

4.4.1 Analysis of Interaction of IN, CA, or MA Protein with the DYNLL1 121

4.4.2 DYNLL1 is Required for Early Stage HIV Replication 122

4.4.3 DYNLL1 is Required for HIV cDNA Synthesis 128

4.4.4 Conserved Motifs in N- and C-terminal Domains of IN are Required for DYNLL1 Interaction 130

4.4.5 The Loss of IN and DYNLL1 Interaction has Impaired HIV cDNA Synthesis 136

4.4.6 The Defective cDNA Synthesis in DYNLL1-KD Cells or HIV-$IN_{Q53A/Q252A}$ Mutant Virus Infection is Attributed to Impaired Virus Uncoating 137

4.4.7 IN and DYNLL1 Interaction is Dispensable for HIV Recruitment to Dynein Complex 146

4.5 Discussion 150

Chapter 5. 158

Major Findings, General Discussion, and Future Directions 158

5.1 Major Findings 158

5.1.1 Investigation of the Role of Impα Isoforms in HIV Nuclear Import and the Mechanism of Impα Recruitment by HIV during Replication 159

5.1.2 The IN and DYNLL1 Interaction and Its Role in Early Stage HIV Replication 162

5.2 General Discussion 164

5.2.1 Role of IN in Post Entry Steps of Early Stage HIV Replication 164

5.2.2 Requirement of Impα3 for HIV Nuclear Import and Its Interaction with IN 165

5.2.3 IN and DYNLL1 Interaction and Its Role In Proper Uncoating of HIV 171

5.3 Potential Applications 173

5.4 Future Directions 174

5.5 Concluding Remark 175

Chapter 6. References 177

Chapter 7. Publications 205

List of Table

Table 1. Nucleotide sequences of primers used in the construction of expression vectors. 62

List of Figures

Figure 1. HIV genome organization and viral protein synthesis. 13

Figure 2. The cross sectional cut out diagram of HIV. 15

Figure 3. Organization and functions of HIV IN protein. 18

Figure 4. HIV replication cycle. 23

Figure 5. Molecular mechanism of cellular protein nuclear import. 28

Figure 6. Molecular structure of dynein complex. 45

Figure 7. The effect of Impα1, Impα3, Impα5, or Impα7 KD on HIV replication in HeLa cells. 80

Figure 8. Impα1, Impα3, or Impα5-KD in $CD4^+$ C8166 T cells and VSV-G-pseudotyped HIV infection. 83

Figure 9. Infection of wild type HIV was impaired in Impα3-KD $CD4^+$ C8166T cells. 85

Figure 10. HIV nuclear Import was impaired in Impα3-KD cells. 86

Figure 11. HIV IN interacts with Impα3. 89

Figure 12. The IN-CTD is involved in Impα3 interaction. 91

Figure 13. Impα3 is required for HIV replication in human primary macrophages. 94

Figure 14. The conserved basic amino acid rich motifs in IN-CTD are required for Impα3 interaction. 96

Figure 15. $IN_{K215A/K219A/R263A/K264A}$ mutant interacts with INI1 and TNPO3. 97

Figure 16. The major and minor NLS binding grooves of Impα3 are involved in IN interaction. 99

Figure 17. YFP-$IN_{K215A/K219A/R263A/K264A}$ mutant fusion protein is defective

for nuclear localization. 102

Figure 18. HIV-IN $_{K215A/K219A/R263A/K264A}$ mutant virus is defective for replication at or prior to integration step. 104

Figure 19. HIV-IN$_{K215A/K219A/R263A/K264A}$ mutant virus is defective for nuclear import in dividing and non-dividing cells. 106

Figure 20. HIV-IN$_{K215A/K219A/R263A/K264A}$ is impaired for nuclear import by cell fractionation method. 108

Figure 21. HIV IN interacts with DYNLL1. 123

Figure 22. DYNLL1 is required for early stage HIV replication. 126

Figure 23. DYNLL1-KD affects MMLV replication. 129

Figure 24. DYNLL1-KD affects HIV cDNA synthesis. 131

Figure 25. DYNLL1 do not interact with HIV RT. 132

Figure 26. Consensus motifs of N- and C-terminal domain of IN are required for DYNLL1 interaction. 134

Figure 27. DYNLL1 interaction defective IN mutant HIV is impaired for cDNA synthesis and replication. 138

Figure 28. IN$_{Q53A/Q252A}$ mutant protein interacts with RT. 139

Figure 29. DYNLL1 interaction is not required for IN multimerization. 141

Figure 30. Fate of capsid assay optimization. 143

Figure 31. The defective HIV cDNA synthesis in DYNLL1-KD cells or HIV-IN$_{Q53A/Q252A}$ mutant virus infection is attributed to impaired virus uncoating. 145

Figure 32. The model depicting the potential mechanism by which the interaction of DYNLL1 and IN contributes to HIV-1 uncoating and cDNA synthesis. 147

Figure 33. The IN and DYNLL1 interaction is dispensable for the recruitment of the HIV complex to dynein. 149

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1. Christian Hoffmann, MD, PhD and Jürgen K. Rockstroh, MD, PhD. HIV Book 2012. Copyright © by Medizin Fokus Verlag, Hamburg. Used with permission. All Rights Reserved. doi:10.1128/JVI.02017-12.

Figure 1. HIV genome organization and viral protein synthesis

Figure 2. The cross sectional cut out diagram of HIV

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Figure 4. HIV replication cycle

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Figure 7. Effect of Impα1, Impα3, Impα5, or Impα7 KD on HIV replication in HeLa cells

Figure 8. Impα1, Impα3, or Impα5-KD in CD4+ C8166T cells and VSV-G-pseudotyped HIV infection

Figure 9. Impα3 KD affected the infection of wild-type HIV in CD4+ C8166T cells

Figure 10. Effect of Impα3 KD on HIV total viral, 2-LTR circle, and integrated provirus DNA synthesis

Figure 11. HIV IN interacts with Impα3

Figure 12. C-terminal domain of HIV IN is involved in Impα3 interaction

Figure 13. Impα3 is required for HIV replication in human primary macrophages

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Figure 14. The conserved basic amino acid rich motifs of IN-CTD are required for Impα3 interaction

Figure 15. $IN_{K215A/K219A/R263A/K264A}$ mutant interacts with INI1 and TNPO3

Figure 16. The major and minor NLS binding grooves of Impα3 are required for IN interaction

Figure 17. YFP-$IN_{K215A/K219A/R263A/K264A}$ mutant fusion protein is defective for nuclear localization

Figure 18. HIV $IN_{K215A/K219A/R263A/K264A}$ mutant virus is defective for replication at or prior to integration step

Figure 19. $HIVIN_{K215A/K219A/R263A/K264A}$ mutant virus is defective for nuclear import in dividing and non-dividing cells

Figure 20. $HIVIN_{K215A/K219A/R263A/K264A}$ is impaired for nuclear import by cell fractionation methods

Abbreviations

Aa	Amino acid
AIDS	Acquired immunodeficiency syndrome
ALV	Avian leukosis virus
APOBEC3G	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
ARM	Armadillo
ARV	Antiretroviral
BIV	Bovine immunodeficiency virus
BSA	Bovine serum albumin
CA	Capsid
CCD	Catalytic core domain
cDNA	Complementary deoxyribonucleic acid
Co-IP	Coimmunoprecipitation
cPPT	Central polypurine tract
CPSF6	Cleavage-and-polyadenylation factor 6
CTD	C-terminal domain
CypA	Cyclphilin A
DHC	Dynein heavy chain
DIC1/2	Dynein intermediate chain1/2
DLIC	Dynein light intermediate chains
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide

DYNLL1	Dynein light chain 1
DYNLT1	Dynein light chain Tctex1
Dyn2p	Dynein light chain protein
ECL	Enhanced chemiluminescence
Env or *env*	Envelope
EDTA	Ethylenediaminetetraacetic acid
eEF1	Eukaryotic elongation factor 1
FACS	Fluorescence-activated cell sorting
FAM	Carboxyfluorescein
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HAART	Highly active antiretroviral therapy
Hsp70	Heat shock protein 70
HFV	Human foamy virus
HRP	Horseradish peroxidase
HTLV	Human T-cell leukemia virus
IBB	Importinβ binding
IBD	IN binding domain
INI1	Integrase interactor 1
INSTIs	Integrase strand transfer inhibitors

Impα1	Importinα1
Impα3	Importinα3
Impα5	Importinα5
Impα7	Importinα7
Imp7	Importin 7
Impβ	Importin β
INA	Inactivated virus control
kbp	Kilo basepair
kDa	Kilodalton
KD	Knockdown
LEDGF/p75	Lens epithelium-derived growth factor
LTR	Long terminal repeat
LVPs	Lentiviral vector particles
LAV	Lymphadenopathy-associated virus
Luc/*luc*	Luciferase
MA	Matrix
MDa	Megadalton
MDMs	Monocyte derived macrophages
MMLV	Meloney murine leukemia virus
MOI	Multiplicity of infection
MT	Microtubule
MTOC	Microtubule organizing center
NC	Nucleocapsid

Nef	Negative factor
NLS	Nuclear localization signal
NNRTIs	Non-nucleoside reverse-transcriptase inhibitors
NPC	Nuclear pore complex
NRTIs	Nucleotide reverse transcriptase inhibitors
NTD	N-terminal domain
Nups	Nucleoporins
PBS	Phosphate buffered saline
PIC	Preintegration complex
PL	ProLabel
Pol or *pol*	Polymerase
PPIs	Protein -protein interactions
qPCR	Real time quantitative polymerase chain reaction
RLU	Relative light unit
RRE	Rev response element
RSV	Rous sarcoma virus
RTC	Reverse transcription complex
RT	Reverse transcriptase
RLU	Relative light unit
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus

shRNA	Short hairpin RNA
TAMRA	Carboxytetramethylrhodamine
TAR	Trans-activation response
TNPO3	Transportin 3
VPR	Viral protein R
VSV-G	Vesicular stomatitis virus G glycoprotein
WB	Western blot
WST	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt

Chapter 1.

Introduction

1.1 The General Description of HIV-1

HIV-1 is an enveloped, positive sense, single stranded RNA virus. It belongs to the family *Retroviridae* and the genus *Lentivirus*. The family *Retroviridae* consists of viruses that are highly diverse and most primitive. As early as 1908, a Danish veterinary team showed that the chicken leukosis is caused by a virus, which was subsequently identified as avian leukosis virus (ALV). Later in 1911, Peyton Rous identified another type of virus that caused sarcoma in chickens, which was called Rous sarcoma virus (RSV) [2]. In the next five decades, several members of *Retroviridae* family were identified in various different species, including mouse, cat, cattle, and primates. In 1980, the first human retrovirus, human T-cell leukaemia virus (HTLV-1), that causes cancer in human was identified [3, 4]. The isolation of HTLV-1 was coincided with acquired immunodeficiency syndrome (AIDS) epidemic in several parts of the world. Then came the discovery of the most complex and extremely unusual retrovirus that causes AIDS in affected individuals, and this virus was called by different names, the lymphadenopathy-associated virus (LAV), HTLV-3, or AIDS-related virus (ARV) [5-7]. The end stage of HIV infection with severe loss of immune response is called AIDS. Soon HTLV-3 was renamed as HIV. Two sub-types of HIV have been described; HIV type 1 (HIV-1) and HIV type 2 (HIV-2). Although HIV-1 and HIV-2 are related, HIV-2 is more closely related to SIV that infects Sooty mangabeys. Meanwhile, the human infections of HIV-2 are restricted only to some of the western African countries. On the contrary, HIV-1 infection is commonly seen all over the world. From here onwards, unless specified

otherwise, HIV refers to HIV-1. HIV is mainly acquired during sexual intercourse with an infected individual. Use of contaminated injection needles and mother to child transmission are also known to serve as important modes of HIV acquisition. HIV is unequivocally linked to causation of AIDS. AIDS is attributed to the general loss of immune response due to HIV-mediated depletion of $CD4^+$ T lymphocytes. Therefore, end stage of AIDS is associated with various opportunistic infections and death. At this time, neither the protective vaccine nor curative treatment is available for HIV infection control. The extreme human suffering caused by the continued spread of HIV has been one of the key concerns for the research community and health professionals. At the moment, the most important priority for the research community is to evolve new and effective anti-HIV strategies that help to control the infection.

1.2 The Epidemiology of HIV Infection

HIV pandemic is undoubtedly one of the world's most serious public health crises of recent history. Beyond just health consequences, HIV infection inflicts serious negative consequences on social and economic well being of people. According to UNAIDS global report, 2012, an estimated 34 million (31.4-35.9 million) people worldwide are living with HIV infection. About 0.8% of the global adult population, between 15-49 years of age, is estimated to be HIV positive. HIV prevalence is variable across countries. Sub-Saharan Africa is the most affected, with nearly 4.9% of its population being HIV positive. This accounts for 69% of global HIV prevalence. While HIV incidence is declining globally, Eastern Europe, Central Asia, and the Middle East and Northern Africa are experiencing relatively higher levels of HIV incidence. An estimated 2.5

million (2.2-2.8 million) people worldwide were newly infected with HIV in the year 2011 alone and 280,000-390,000 of them are children. In 2011, approximately 1.7 million (1.5-1.9 million) people died of AIDS-related causes. Noticeably, introduction of anti-HIV drug prophylaxis has helped to prevent 409,000 children from getting HIV infection in middle and low-income countries. Given the fact that there are no curative therapies or adequate preventive measures, HIV will remain a daunting challenge to people worldwide.

1.3 HIV Virology

1.3.1 HIV Classification

HIV belongs to the family *Retroviridae* and the genus *Lentivirus*. HIV is comprised of four different lineages, termed as group M, N, O, and P. Each of these lineages resulted from independent cross species transfer events. It is now clear that most HIV lineages are the result of chimpanzees to human cross species transmission [8]. Among all the known lineages, group M represents the pandemic form of HIV and is virtually found in every country. Group M is further divided into 11 major clades based on genetic diversity, named as A1, A2, B, C, D, F1, F2, G, H, J, and K. The clade B circulates in North America [9]. HIV genetic diversity is further complicated by "circulating recombinant forms" that are derived from genetic recombination between viruses of different clades.

1.3.2 HIV Genome

HIV genome is single stranded positive sense RNA of approximately 9.8 kilobase pairs (kbp) in size. Each HIV virion contains two copies of genomic RNA [10, 11]. The

genomic RNA not only carries information for viral protein synthesis but also contains unique internal structures that are fundamental for viral replication. The primer binding site, trans-activation response (TAR) hairpin, the dimerization site, the packaging signal, and rev response element (RRE) are some of the functionally important structures in HIV genomic RNA. These structures play key roles in different steps of HIV replication such as initiation of reverse transcription, transcription activation, genomic RNA dimerization, genomic RNA virion packaging, and RNA nuclear export [12-14]. HIV genome consists of nine open reading frames, which together encode fifteen viral proteins (reviewed in [15]) (**Figure 1**). The *envelope* (*env*) and *gag* genes encode structural proteins that constitute viral coat and inner core, respectively. The *gag* gene encodes Gag precursor polyprotein, which is synthesized and proteolytically cleaved into Matrix (MA), Capsid (CA), Nucleocapsid (NC), and p6 proteins. The *gag* and *pol* gene together encode a Gag-Pol polyprotein by translational frameshifting and individual Pol proteins, Protease (PR), Reverse transcriptase (RT), and Integrase (IN), are synthesized from proteolytic cleavage of Gag-Pol polyprotein. The remaining six genes code for two regulatory (Tat and Rev) and four accessory [Negative factor (Nef), Viral protein R (Vpr), Vpu, and Viral infectivity factor (Vif)] proteins (depicted in **Figure 1**). Among these, Vif, Vpr, Nef, IN, MA, NC, RT, and PR are incorporated into progeny virus. Tat and Rev are synthesized early in the infection and contribute to the establishment of HIV infection. Vif, Vpr, Vpu, and Nef are involved in HIV replication and AIDS pathogenesis.

1.3.3 HIV Virion Structure

HIV is an enveloped virus with an average diameter of 145±25 nm [16]. The lipid bilayer

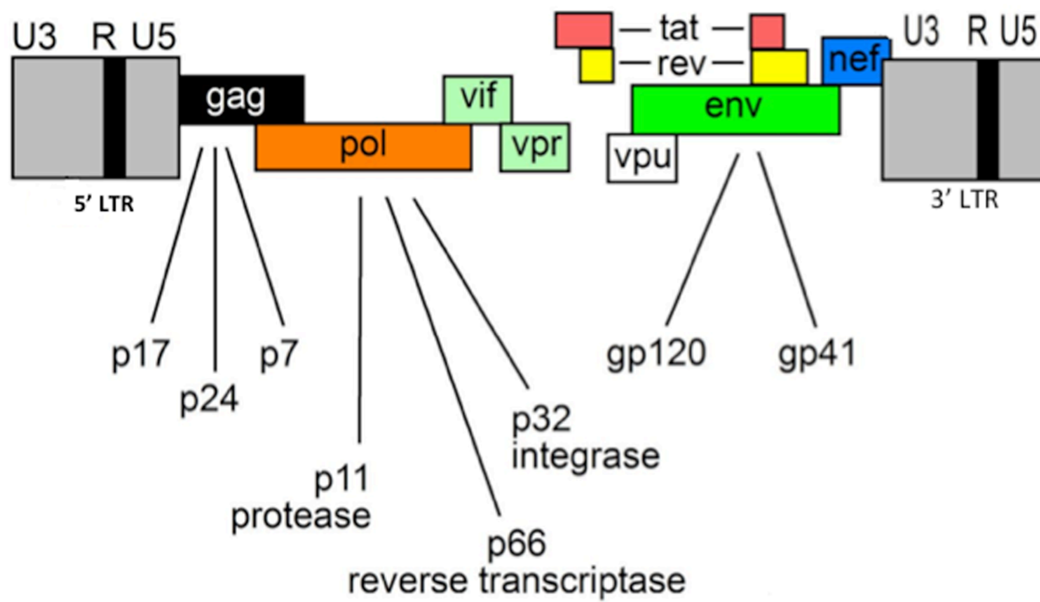

Figure 1. HIV genome organization and viral protein synthesis: The schematic diagram showing HIV genome organization and different viral protein synthesis.

of the HIV envelope is derived from the cell membrane during virus budding. HIV Env glycoproteins are inserted into lipid bilayer and protrude outside the surface. HIV envelope glycoprotein is comprised of two proteins: the surface protein (SU) gp120 and the transmembrane protein (TM) gp41. The gp120 binds to CD4 receptor on the cell surface during infection. MA protein forms a lining just underneath the envelope. Inside the MA layer, two copies of HIV genomic RNA are encapsidated in the viral core. The viral core is made up of CA protein. The HIV core also contains several accessary and regulatory proteins such as IN, RT, PR, NC, and Vpr (see **Figure 2** for HIV structure). In addition to viral proteins, several cellular proteins are also incorporated into the HIV virion. Some of those HIV incorporated cellular proteins are integrase interactor 1 (INI1) [17, 18], cyclphilin A (CypA) [19], heat shock protein 70 (Hsp70) [20], ubiquitin [19, 21], staufen [22], and Ku70 [23]. Each of these HIV proteins has specific roles in virus replication and/or disease pathogenesis.

Structural Proteins of HIV (Env-gp120/gp41, MA, CA, and NC)

The gp120 binds to the cell surface receptor and mediates the virus entry [24]. The variable region of gp120 protein is an important target for neutralizing antibody development. However, constant mutations at the variable region allow virus to escape immune recognition and give rise to new strains. The transmembrane glycoprotein gp41 facilitates the virus and cell membrane fusion during virus attachment [25]. MA protein, due to its presence beneath the lipid bilayer, provides structural support to the envelope. A small amount of MA is also found inside the virus core and becomes a component of HIV replication complex after virus entry into the cell [26]. MA is important for both

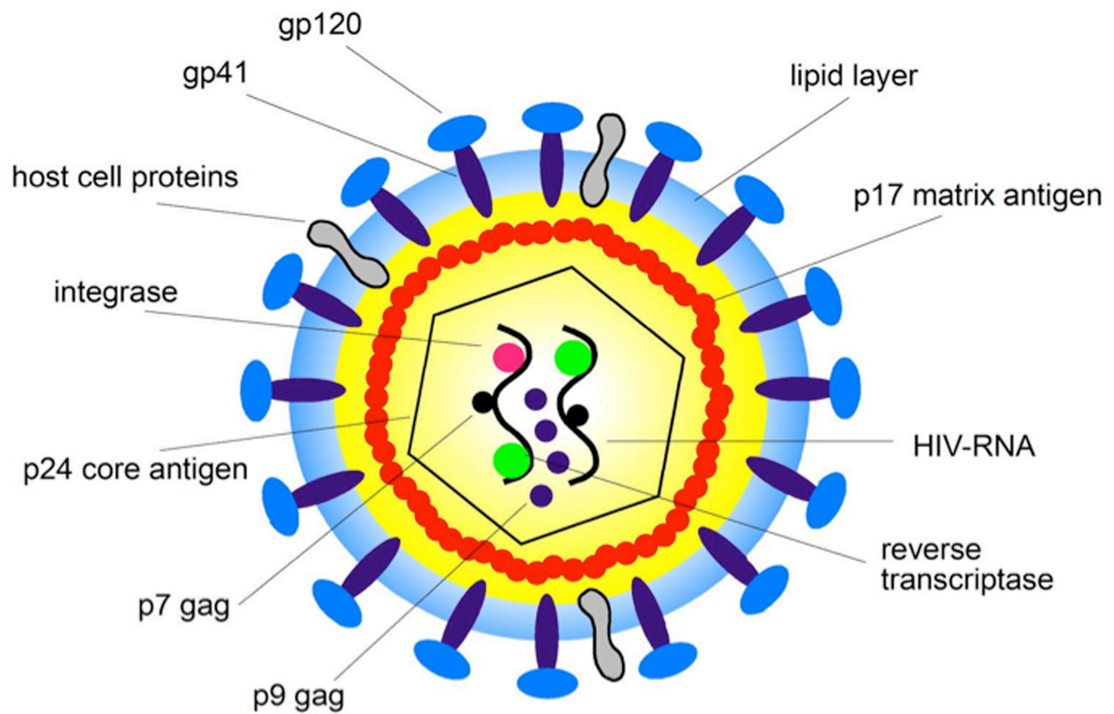

Figure 2. The cross sectional cut out diagram of HIV: HIV envelope consists of viral envelope proteins (gp120 and gp41), cellular proteins, and cell membrane derived lipid bilayer. Underneath the envelope, there is a matrix protein layer. Inside the matrix layer, the capsid protein forms a core, which contains two copies of HIV genomic RNA and associated proteins.

early and late stage of HIV replication. During late stage replication, MA mediates the plasma membrane targeting of Gag and Gag-Pol polyproteins and contributes to the virus incorporation of Env protein [12, 27-30]. As a component of PIC, MA has been implicated in nuclear transportation of PIC, which allows HIV to infect non-dividing cells [31, 32]. In addition, MA also shows immunoregulatory function during HIV infection by interacting with the cell surface receptor p17R [33, 34]. The interleukin(IL)-2 stimulation induces the p17R expression on peripheral $CD4^+$ or $CD8^+$ T lymphocytes. Studies have shown that MA protein can synergize the IL-2 induced T cell proliferation, cytokine release, and immune cells chemotaxis [33-36]. The extracellular MA protein has been implicated in this immunomodulatory role. The abundant amount of extracellular MA has been detected in lymph nodes of HIV infected individuals[37]. It is suggested that this immunomodulatory function of MA protein help creating a favorable environment for HIV replication. CA protein has been traditionally known to give structural support to HIV core and protect HIV genomic RNA and associated proteins in the core. In addition, CA is also involved in various functional aspects of HIV replication (reviewed in [38]). By the process of uncoating, HIV sheds off CA protein during early infection. Although the mechanism is not clear, recent studies have highlighted the role of CA in HIV nuclear import and integration (reviewed in [38]). NC is found associated with HIV genomic RNA. The primary function of NC is to bind the packaging signal (Ψ) of genomic RNA and deliver them into assembling progeny virus [12, 39].

Enzymatic Proteins of HIV (RT, IN, and PR)

RT, IN, and PR are indispensable for HIV replication. RT is a heterodimer, consisting of

a 66 kilodalton (kDa) subunit (p66) and 51kDa subunit (p51) [40, 41]. RT catalyzes the reverse transcription of HIV genomic RNA into a cDNA. The $tRNA^{Lys}$ binds to the RNA binding site at the 5' end of HIV genomic RNA and serves as a primer for HIV reverse transcription (reviewed in [42-44]). The reverse transcription starts from the 3' end of $tRNA^{Lys}$. As both RT and $tRNA^{Lys}$ are incorporated into virion, reverse transcription often is initiated within the virus and a small stretch of cDNA is synthesized in mature virion [45, 46]. IN is an important enzymatic protein of HIV. IN is a 288 amino acid (Aa) protein and functions as homotetramer [47]. IN has three functionally distinct domains; N-terminal domain (NTD) (Aa1-50), catalytic core domain (CCD) (Aa51-212), and C-terminal domain (CTD) (Aa213-288) [48]. Each of these domains has specific roles in HIV replication (**Figure 3**). NTD contains a highly conserved zinc finger like motif, which gives stability to IN structure and enhances IN catalytic activity [49]. CCD has a highly conserved triad of acidic residues (D64, D116, and E152) called the DDE motif, which catalyzes the HIV cDNA integration reaction [50-54]. CTD is a relatively less conserved region of IN. CTD along with CCD is implicated in HIV nuclear import [55-58]. PR mediates Gag, Gag-Pol polyprotein processing in progeny virus and contributes to maturation of HIV [12, 59, 60].

HIV Accessary Proteins (Vpr, Vif, Nef, Vpu, Tat, and Rev)

Accessary proteins play an important role in HIV replication and AIDS pathogenesis (reviewed in [61-64]). Vpr is a 96 Aa protein (14kDa) and is implicated in HIV nuclear import [65-73], cell cycle arrest [74-78], apoptosis (reviewed in [79]), and protection from natural killer (NK) cell lysis. The cell cycle arrest during HIV infection may

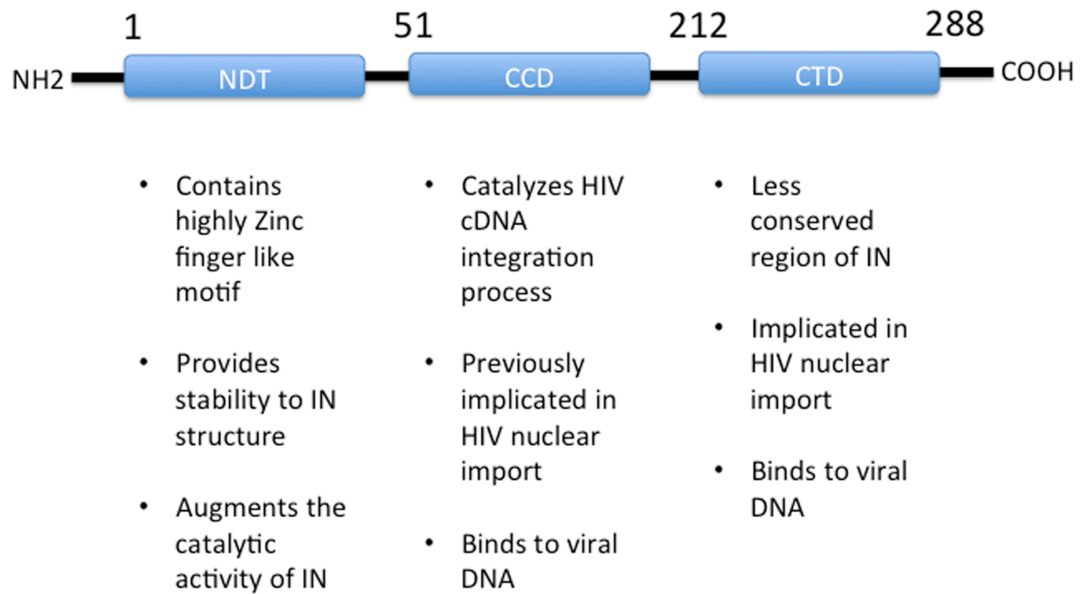

Figure 3. Organization and functions of HIV IN protein: Diagram showing a full-length IN protein, different domain of IN, and functions of individual domains. IN is a 288 amino acid protein, which is divided into three functionally distinct domains: NTD (Aa1-50), CCD (Aa51-212), and CTD (Aa212-288). The list of different functions of individual domains is included.

promote optimal transcription from long terminal repeat (LTR), which favors increased virus production. Vpr mediated apoptosis of $CD4^+$ T lymphocyte contributes to immune suppression and AIDS pathogenesis in infected individuals. The recent studies have suggested a role for Vpr in protection of HIV infected cells from NK cell mediated lysis (reviewed in[80]). Vpr up-regulates the expression of ligand for NK cell receptor NKG2D called NKG2D-L on the surface of infected cells, which prevents the infected cells from being lysed by NK cells [81]. The up-regulation of NKG2D-L expression is associated with Vpr interaction

Vif is a 192 amino acid protein and is important for HIV infectivity (reviewed in [82]). The presence of Vif in virus producing cells enhances infectivity of progeny virus by preventing virus incorporation of APOBEC3G, an antiviral factor. APOBEC3G induces the deamination of deoxycytidine to deoxyuridine during the viral DNA minus strand synthesis. As a result of deamination, adenosine residues are added in the place of guanosine residues during the plus strand viral DNA synthesis, resulting in guanosine-to-adenosine hypermutation (reviewed in [83]). Strikingly, APOBEC3G mediated guanosine- to- adenosine hypermutation of viral DNA is associated with increased expression of ligand for NK cell receptor NKG2D called NKG2D-L on the surface of HIV infected cells [84], which promotes the NK cells mediated lysis of infected cells. The recent studies have suggested that the Vpr is essential for NKG2D-L expression during HIV replication (reviewed in [80]). Therefore, Vif plays important role in HIV replication by not only protecting virus from APOBEC3G mediated degradation but also preventing infected cells from being lysed by NK cells. Nef is a 206Aa protein and is

known to play an important role in AIDS pathogenesis [85-87]. Nef down regulates the expression of MHC class I molecules, which may help to protect infected cells from cytotoxic T cell mediated killing [86, 87]. Nef also down regulates CD4 receptor on T cells, which promotes virus release and possibly alters $CD4^+$ T-cell signaling pathways [85]. A small amount of Nef is also incorporated into virion. Although the mechanism is not clear, virus incorporated Nef can influence reverse transcription in target cells [88, 89]. Similar to Nef, Vpu also induces CD4 receptor degradation, down regulation of MHC class I expression, and contributes to Env proteins incorporation into assembling virus and progeny virus release [90-92]. In addition, Vpu also down regulates the cell surface expression of BST2/Tetherin, which is known as an antiviral factor by interfering with the release of progeny viruses from cell membrane (reviewed in [93]). Tat and Rev are synthesized early in the infection and contribute to HIV transcription and nuclear export of unspliced viral mRNAs, respectively (reviewed in [12]).

1.3.4 HIV Tropism and AIDS Pathogenesis

HIV accesses the body through mucosal or direct contact with blood. The entry at vaginal or rectal mucosa during sexual intercourse is the most common mode of HIV acquisition. Nevertheless, initial steps of HIV acquisition at vaginal or rectal mucosa are not well characterized, partly due to the lack of appropriate models. $CD4^+$ T lymphocytes, macrophages, and DCs serve as early targets of HIV infection in vaginal or rectal sub mucosa [94-96]. In the sub mucosa, HIV either directly infects $CD4^+$ T lymphocytes or DCs may facilitate the transmission of HIV to $CD4^+$ T lymphocytes by DC-T lymphocyte conjugation [97-102]. HIV is believed to infect DCs by interacting with DC-SIGN

receptor [103-105]. Although productive replication of HIV in DCs is contradictory, HIV virions in DCs are capable of infecting T lymphocyte. Following infection, DCs can also migrate to nearby lymph nodes and transmit infection to lymph node associated T-lymphocyte. Following initial replication at the site of infection, HIV spreads to the general circulation and infects large numbers of circulating and tissue associated lymphocytes and macrophages. Acute HIV infection is usually associated with a febrile illness and general symptoms overlap with systemic spread of virus to lymphoid tissue, central nervous system, and other sites. The initial peak in HIV replication during acute infection is followed by a gradual attenuation of replication. The appearance of virus specific $CD8^+$ cytotoxic T cells is likely to temporarily curtail HIV load by sustained lysis of infected cells [106, 107]. In the absence of therapeutic intervention, the steady state level of HIV replication will be gradually established over a period of time and remain relatively stable over a long period of time. The steady state of HIV replication in any given individual is determined by several factors. Some of the factors that determine viral load are host immune defenses, viral replicative capacity, and cellular co-factors. During the course of HIV propagation, virus establishes a reservoir in latently infected quiescent $CD4^+$ T lymphocytes and macrophages. As the nuclear membrane of macrophages and quiescent T lymphocytes is always intact due to lack of mitosis in these cells, the nuclear import of PIC is mandatory for HIV replication in these cells. In addition, as a large proportion of activated T lymphocytes at any given point of time are in the interphase at which the nuclear membrane is intact, the transportation of HIV complex across the intact nuclear membrane contributes to enormous replication potential of HIV and possibly contributes to early onset of AIDS symptoms [108, 109]. The

establishment of a long lasting viral reservoir and the extreme potential for replication are some of formidable challenges to curing HIV infection. The uncontrolled virus propagation over a long period of time will lead to gradual depletion of $CD4^+$ T lymphocytes and eventual loss of natural defenses against infections. It appears that depletion of $CD4^+$ T lymphocytes in HIV infection is not just due to the cytopathic effect of the virus itself but several other factors, such as dysregulated activation, diminished production, and lymph node sequestration of CD4+ T lymphocytes, also play a role (reviewed in [110]).

1.3.5 HIV Replication Cycle

HIV replication cycle includes the following nine steps; receptor binding and entry, reverse transcription, uncoating, nuclear import, provirus integration, virus transcription and viral RNA nuclear export, viral protein synthesis, virus assembly and budding, and progeny virus maturation (reviewed in [111]). The steps of HIV replication are broadly grouped into early and late stage virus replication (depicted in **Figure 4**). The early stage replication includes steps from virus entry to integration. Late stage replication includes post integration steps of HIV replication. Like any other intracellular pathogen, HIV uses several cellular proteins during the course of replication. For detailed information on HIV replication and contribution of viral and/or cellular factors, please refer to the following reviews [111-114]. HIV entry into the cell is initiated by the interaction of gp120 with CD4 receptor and CCR5 or CXCR4 co-receptor at the cell surface [24]. The interaction of gp120 with the CD4 receptor changes the conformation of gp120 such that it is able to establish contact with the co-receptor. Following gp120 interaction with CD4 and

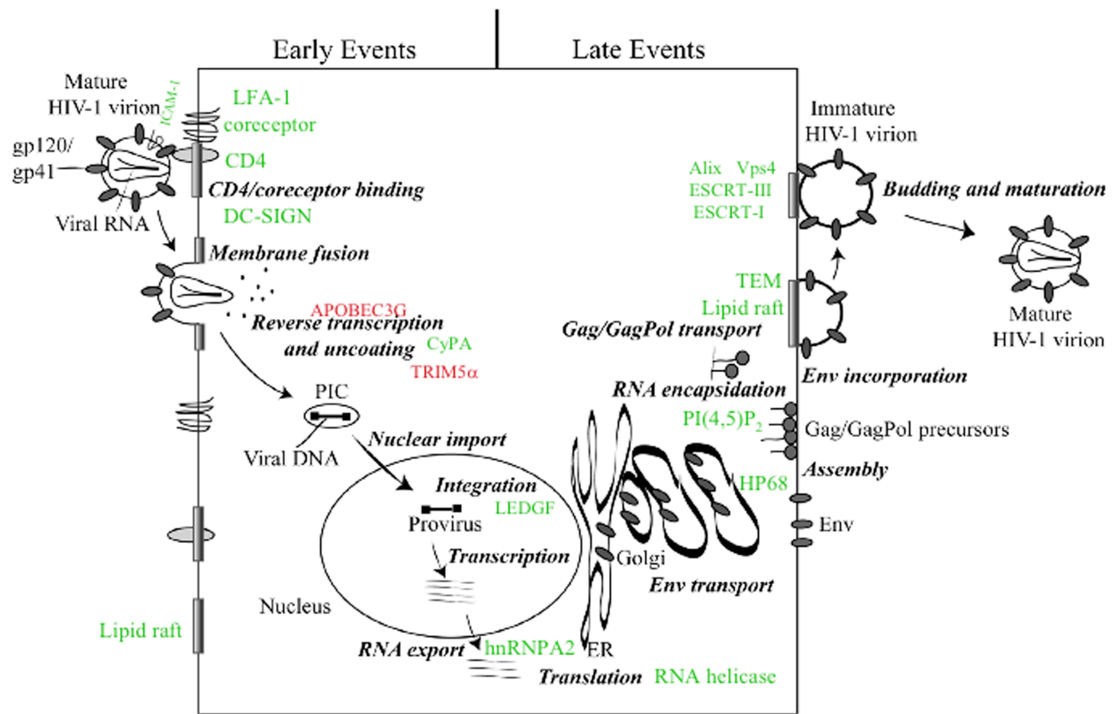

Figure 4. HIV replication cycle: Diagram showing the different steps of HIV replication cycle. The HIV replication steps are divided into early and late stage replication. The steps of early stage replication are as follows: Receptor mediated HIV entry to the cell, reverse transcription, uncoating, cDNA nuclear import, and integration. The late stage HIV replication includes the following steps: HIV genome transcription, RNA nuclear export, viral protein synthesis, HIV assembly and release, and HIV maturation.

co-receptor, the lipid bilayer of the viral envelope and the cell membrane will fuse and the viral core will be released into the cytoplasm. Following entry, uncoating of the core occurs in the cytoplasm. Uncoating is a process where CA protein is released from the core, which coincides with reorganization of the genomic RNA and associated proteins. HIV genomic RNA with its associated protein complex is called reverse transcription complex (RTC). Accumulated studies suggested that the intravirion phosphorylation of CA by virus incorporated extracellular signal-regulated kinases 2 (ERK2) induces the disruption of CA-CA protein interactions followed by dissociation of CA (i.e., uncoating) from the HIV core[115, 116]. The process is assisted by cellular cofactors [117, 118]. Within RTC, viral RNA is reverse transcribed into a double stranded cDNA with the help of RT. This process is called reverse transcription. Reverse transcription occurs mainly in the cytoplasm. However, reverse transcription may begin within the free virion and occasionally persist even after the translocation of RTC into the nucleus [119, 120]. With the completion of reverse transcription, RTC is termed as pre-integration complex (PIC), which is a capable of integration both *in vivo* as well as *in vitro* [121, 122]. PIC is a high molecular weight nucleoprotein complex, with protein components including both viral and cellular proteins. The viral proteins of PIC are IN, MA, Vpr, NC, and CA [123, 124]. Some of the known cellular proteins in PIC are Ku70, HSP70, high mobility group AT-hook 1 (HMGA1), and lens epithelium-derived growth factor (LEDGF/p75) [125-127]. High molecular mass of PIC precludes its passive diffusion into the nucleus. PIC enters the nucleus through active nuclear import by engaging cellular nuclear import machinery. Following nuclear import, HIV cDNA integrates into the cellular genome by a mechanism called integration. The late stage HIV replication begins at the transcription

step. The integrated HIV proviral DNA provides the template for transcription. HIV LTR serves as the site for RNA polymerase II binding and transcription initiation. The basal transcription from HIV LTR is very slow, but the transcription is greatly enhanced by Tat protein interaction with TAR region of nascent viral RNA [128-130]. HIV transcription generates a large number of viral mRNAs. HIV mRNAs fall into the following three major classes; a) Unspliced RNAs, which serve as the template for Gag and Gag-Pol polyprotein synthesis and the genomic RNA for progeny virus incorporation. b) Partially spliced mRNAs (approximately 5kbp), which encode for Env, Vif, Vpu, and Vpr proteins. c) Small multiply spliced mRNAs, which encode Rev, Tat, and Nef proteins. As mRNAs are fully spliced before transport to the cytoplasm, HIV uses Rev protein to overcome this problem [131]. The unspliced or partially spliced HIV mRNAs contain a cis-element called rev response element (RRE) to which Rev protein binds. The Rev protein binding leads to accelerated nuclear export of viral mRNAs, which leaves little time for splicing of newly transcribed viral mRNAs in the nucleus and results in export of unspliced or partially spliced mRNAs to the cytoplasm. Following nuclear export of mRNAs, viral protein will be synthesized in the cytoplasm. The virus particle assembly process coincides with the synthesis of all viral proteins. The $Pr55^{Gag}$ polyprotein is essential for the progeny virus assembly. HIV assembly is initiated by the association of $Pr55^{Gag}$ at the cell membrane. $Pr55^{Gag}$ encapsidates HIV genomic RNA and other viral proteins and induces virus budding. Shortly after the progeny virus release from the plasma membrane, virus maturation takes place with PR mediated cleavage of Gag and Gag-Pol polyproteins into precursor Gag and Pol proteins within the virion. The progeny virus maturation plays important role in HIV infectivity.

1.4 HIV Nuclear Import

1.4.1 The Nuclear Import of Cellular Proteins

Eukaryotic cells consist of distinct cytoplasm and nuclear compartments that are physically separated by a nuclear membrane. The cytoplasm and nuclear compartmentalization has a clear advantage for eukaryotic cells. The confinement of genome to a specialized organelle may provide genetic stability and help eukaryotic cells in handling huge genetic information. Compartmentalization can also favor selective gene expression in eukaryotic cells. Additionally, compartmentalization allows splicing of primary mRNA transcripts in the nucleus before they are exported to the cytoplasm for translation, which favors normal cellular function, by reducing the chances of accidental production of abnormal proteins. However, the separation of cytoplasm and nucleus also requires a specialized mechanism to allow the exchange of molecules between compartments. The nuclear membrane is penetrated by specialized protein channels called nuclear pore complexes (NPCs). NPC is a large 125 MDa protein complex and is made up of a group of proteins called nucleoporins (Nups) [132, 133]. NPCs extend into the nucleus and cytoplasm through nuclear membrane. NPC has an inner pore size of 25nm diameter and a 9nm water channel through which molecules are transported [134, 135]. Water molecules, electrolytes, and small molecular weight proteins ($\leq$ 9kDa) can freely pass through the NPC. However, large proteins and/or macromolecules are only selectively transported into the nucleus through NPC by an active nuclear import mechanism. The nuclear import of macromolecules is a highly complex process and involves approximately 100-200 different regulatory and/or adapter proteins. For more information on nuclear import, please refer to the following reviews [136-138].

The protein nuclear import in eukaryotic cells is classified under two distinct pathways: the classical and non-classical nuclear import pathways. Proteins are targeted to a particular nuclear import pathway through their interaction with specific soluble nuclear import receptors and these receptors are called karyopherins/importins (Imps). There are more than 20 different Imps in mammalian cells. The classical nuclear import constitutes a major pathway for protein nuclear import in eukaryotic cells. One study estimated that about 57% of nuclear proteins are expected to use classical nuclear import pathway, whereas the remaining 43% of proteins may use non-classical nuclear import (reviewed in [139]). The classical nuclear import pathway involves the initial recognition of cargo proteins by an adapter protein called Impα in the cytoplasm. The cargo protein bound Impα in turn recruits a soluble import receptor called Impβ in the cytoplasm. Impβ docks this nuclear import complex to NPC through interaction with Nups and initiates the nuclear translocation of nuclear import complex [136]. There are six different Impα isoforms in human cells; Impα1 [140, 141], Impα3 [142, 143], Impα4 [142, 144], Impα5 [145], Impα6 [142], and Impα7 [146]. In non-classical nuclear import, cargo proteins are imported to the nucleus by interaction with Imps other than Impβ and without the involvement of Impα adapter proteins.

The molecular mechanism of nuclear import is more or less the same in both classical and non-classical nuclear import. In either case, nuclear import cycle consists of following four major general steps; assembly of cargo-import receptor complex, transportation through NPC, disassembly of cargo-import receptor complex, and recycling of import receptor complex (shown in **Figure 5**). Proteins recognize import receptors through a

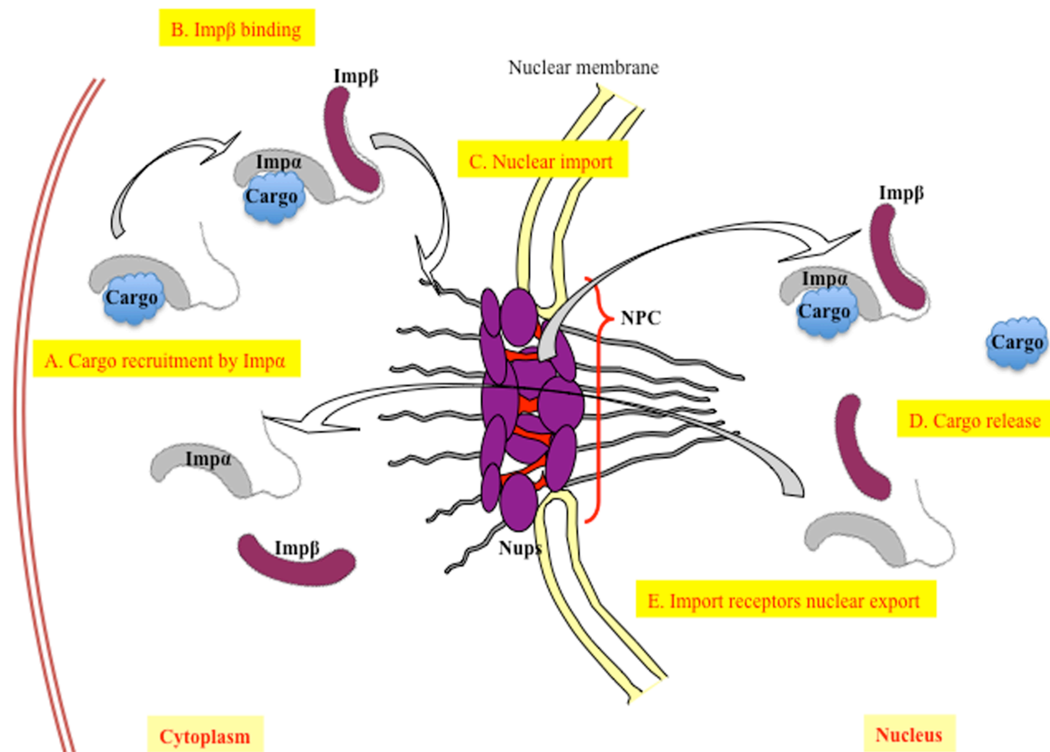

Figure 5. Molecular mechanism of cellular protein nuclear import: Diagram showing the molecular mechanism of cellular protein nuclear import by classical nuclear import pathway. On cytoplasmic side of nuclear membrane, Impα (gray colored bean shape) binds with NLS-cargo (blue colored structure) and this complex will in turn recruit Impβ (maroon colored bean shape) through Impα interaction. This trimeric nuclear import complex will pass through NPC (direction of flow is shown by a arrow) into nucleus. In the nucleus, nuclear import complex dissociates and free Impα and Impβ are re-circulated to the cytoplasm by the nuclear export mechanism. The RanGDP to RanGTP gradient plays important role in Impα-cargo recruitment in the cytoplasm and dissociation in the nucleus (not shown in figure).

consensus basic amino acid rich motif called nuclear localization signal (NLS). NLSs for classical nuclear import are well characterized. NLS for classical nuclear import is a cluster of 4-5 highly conserved basic amino acids and is called monopartite NLS. It is referred to as bipartite NLS when a monopartite NLS is connected with another monopartite NLS by a stretch of ~10–12 linking amino acids. Impα is made up of tandem series of 10 armadillo (ARM) repeats that together form a banana-shaped molecule. ARM2-4 and ARM6-8 form shallow groves called major and minor NLS binding grooves, respectively [147-149]. NLSs of cargo bind to specific sites (major and/or minor NLS binding grooves) on Impα and this process is crucial for the formation of cargo-import receptor complex [147, 149, 150]. Additionally, N-terminal region of Impα contains cluster of basic amino acid rich regions similar to NLS called Importinβ binding (IBB) domain. The IBB domain facilitates Impα binding to Impβ [151-154]. Unlike the recognition of import receptor by cargo, the transportation of import complex through the NPC is poorly understood. A subset of Nups that contain characteristic tandem repeats of Phenylalanine-Glycine (FG) amino acids sequence [155-157] are suspected to mediate the movement of import complex through NPC [158-163]. Consistent with this observation, Impβ mutant that lacks interaction with FG-nucleophorin was unable to enter the nucleus [164]. The exclusion of macromolecules or selective transportation of import complex through NPC is explained by two models: the molecular crowding and sieve- like gel models. The molecular crowding model is explained by the fact that concentration of FG repeats in NPC is high, and combined with native unfolded conformation of Nups will prevent the passive diffusion of macromolecules of certain sizes. Alternatively, a sieve-like gel model is proposed based on the mess like

arrangement of FG repeats between different Nups in NPC. FG repeats sieve will possibly allow passive diffusion of molecules with an average molecular weight of less than 40 kDa. Unfortunately, none of these models have been proven or accepted unequivocally.

Inside the nucleus, RanGTP binds to Impα of nuclear import complex, which facilitates the dissociation of import complex. Then, RanGTP bound Impα complex will be recycled back to the cytoplasm by nuclear export. In the cytoplasm, RanGTPase activating proteins (i.e., RanBP1, RanBP2, and RanGAP) induce the hydrolysis of RanGTP to RanGDP, which facilitates the release of free Impα for subsequent round of nuclear import. Therefore, RanGTP-GDP gradient is considered as one of the key regulators of protein nuclear import in cells [165-167]. Additionally, IBB domain is also implicated in the release of cargo from Impα in the nucleus. Although IBB domain resembles NLS, IBB domain is no longer able to compete with NLS of cargo when it is bound to Impβ in the cytoplasm. Therefore, it is hypothesized that when Impβ is released from Impα-cargo complex in the nucleus, IBB domain will compete with NLS of cargo for binding to Impα and contributes to cargo release in the nucleus [154]. Consistent with this hypothesis, the Impα-IBB domain deletion mutant (ΔIBB-Impα) but not the full-length Impα showed substantially higher affinity for NLS [136, 168, 169]. These findings indicate the possible molecular dynamics of Impα and cargo proteins interactions or release during nuclear import.

1.4.2 HIV Nuclear Import and Virus Replication

The nuclear import is one of the requirements for successful completion of replication by several viruses that uses the nucleus as their site for replication. The free entry of viruses into the nucleus is hindered by the large size of the virus and the selective permeability of the nuclear membrane. Different viruses have evolved different strategies to overcome this restriction. For example, adenovirus docks its partially disassembled core to the cytoplasmic side of NPC and enters the nucleus by exploiting host nuclear import machinery [170-172]. Similarly, the herpes simplex virus (HSV) docks its core to the cytoplasmic side of NPC and releases viral genome directly into NPC or very close to NPC [173-177]. For successful replication, HIV cDNA as a part of PIC should enter the nucleus and integrate into the host cell genome. However, the large size of PIC precludes its passive diffusion through NPC. Studies have indicated that PIC enters the nucleus by active nuclear import with the help of host nuclear import machinery [178-183]. The nuclear import allows HIV to replicate in the cell cycle arrested cells [183] and metabolically active non-dividing cells such as macrophages [184-187] and DCs [188, 189]. On the contrary, oncoretroviruses such as meloney murine leukemia virus (MMLV) that lack nuclear import will have to rely on nuclear membrane dissolution during mitosis to access the host cell genome [190, 191]. Therefore, MMLV is only able to replicate in actively dividing cells. The lack of nuclear import by oncoretroviruses makes the transduction of non-dividing cells by oncoretrovirus based vectors unsuccessful [192, 193]. Contrary to metabolically active non-dividing cells (i.e., macrophages and DCs), HIV replication in metabolically inactive non-dividing monocytes and quiescent T lymphocytes is inefficient [194-196]. The inefficient HIV replication in metabolically

inactive non-dividing cells is not simply due to the presence of an intact nuclear membrane, but due to defective reverse transcription, nuclear import, and integration [196-199]. Studies have shown that quiescent T lymphocytes contain very small nucleotide pool in their cytoplasm, which is suspected to cause the impaired de novo HIV reverse transcription [198] and cDNA elongation in these cells [196, 200]. The specific cause for impaired HIV nuclear import or integration in quiescent T lymphocytes is not very clear. The incomplete cDNA synthesis or lack of availability of certain cellular co-factors might have attributed to defective HIV nuclear import or integration in quiescent T cells. Although it was earlier assumed that HIV nuclear import is only essential for non-dividing cell infection, recent studies have provided evidence for the key contribution of HIV nuclear import in dividing cell infection [201-203]. It is known that only a small proportion of T-lymphocytes in the body are actively proliferating at any given point of time. The majority cells are in the interphase of the cell cycle in which the nuclear membrane is intact. Therefore, the ability of HIV to enter the cell nucleus during interphase would greatly facilitate viral replication and possibly accounts for the high replication rate observed in infected individuals [108, 109]. Moreover, recent studies have suggested yet another interesting concept; the nuclear import coupled integration. It is now quite evident that the nuclear import is not simply a mechanism for HIV to access the nucleus but rather plays a distinct role in genomic integration [204, 205]. Therefore, nuclear import is not only important for HIV infection in non-dividing cells but also essential for virus replication in dividing and non-dividing cells. The ability of HIV to successfully infect non-dividing and dividing cells plays a crucial role in the establishment of virus reservoir and disease progression [206-208].

1.4.3 Viral and Cellular Factors Associated With the HIV Nuclear Import

HIV nuclear import has been extensively studied during last two decades. After the initial demonstration of HIV infection in mononuclear phagocytes from tissue samples of infected individuals and terminally differentiated macrophages in *in vitro* [184], the active nuclear import of PIC was elucidated [181]. It led to the conclusion that HIV uses nuclear import machinery to deliver PIC into the nucleus. In subsequent studies, several viral and cellular factors were implicated in HIV nuclear import. In the following section, I will discuss the role of some of key viral and cellular factors in HIV nuclear import.

MA Protein: MA protein was the first viral factor implicated in HIV nuclear import. In a seminal study, Burkinsky *et al.,* have identified a classical NLS in MA protein and demonstrated that this NLS was able to induce the nuclear localization of heterologous protein upon conjugation [209]. In addition, substitution mutations in this NLS have impaired HIV replication in cell cycle arrested cell [209]. A subsequent study showed the loss of HIV nuclear import in the presence of excess of NLS peptide of SV40 large T antigen that closely resembled NLS of MA protein [65]. Although these findings highlighted the significance of MA protein for HIV nuclear import, a more conflicting picture emerged in later studies. The presence of NLS in MA protein itself was proved non-essential for HIV nuclear import and/or replication in non-dividing cells [27, 210-212]. Nevertheless, a novel NLS was reported in MA by a later study [213]; a mutant HIV that lacks entire MA protein except N-terminal myristoylation signal was still able to replicate in dividing and non-dividing cells, albeit at reduced level [214]. Thus, even though it was difficult to entirely rule out the contribution of the MA protein, studies

have failed to suggest the MA protein as a key mediator of HIV nuclear import.

Vpr Protein: Vpr is another viral protein implicated in HIV nuclear import. Vpr is relatively a small protein of 14 kDa and is incorporated into HIV virion [215, 216]. Vpr consists of a negatively charged flexible N-terminal domain, a hydrophobic core with three alpha helices (αH1, αH2 and αH3), and an arginine rich C-terminal domain [217]. Vpr will localize to the nucleus following transient expression in cells and the nuclear localization was equally evident when expressed as alone or as a fusion protein with green fluorescent protein (GFP), β-galactosidase, or bovine serum albumin (BSA) [73, 218-221]. Surprisingly, Vpr lacks any of the known classical NLSs. However, two non-conventional nuclear targeting signals, each in N and C- terminal domains, have been identified in Vpr [73, 222]. Consistently, only nuclear localization of MA protein but not Vpr was impaired in the presence of excess of the peptide corresponding to SV40 large T antigen NLS [65]. However, neither the precise role nor the molecular mechanism of Vpr in HIV nuclear import is clearly understood. Nevertheless, various explanations have been put forward. Vpr interacts with Impα [65] and the nuclear localization of Vpr in *in vitro* transport assay was enhanced in presence of Impα [66]. Furthermore, Impα1 interaction defective Vpr mutant, "αLA/N17C7", was unable to localize to the nucleus [67]. These findings have highlighted the widely accepted hypothesis that Vpr undergoes nuclear import through the classical nuclear import pathway. However, conflicting results do exist. Interestingly, the presence of Vpr increases the binding of classical NLS peptides to Impα, including NLSs of MA protein [68]. Coinciding with this finding, Nitahara *et al.,* showed that the nuclear localization of Vpr does not require Impβ [67].

On the contrary, the presence of excess of Impβ prevented the nuclear import of Vpr, which in other words suggests that Vpr could potentially function as Impβ. Indeed, interaction of Impβ to Impα facilitates the cargo protein binding to Impα by relieving the auto inhibitory control of Impα (Reviewed in [136]). In support of this assumption, recent studies have found the Vpr interaction with various Nups [69, 70, 223, 224] as well as the localization of Vpr to the nuclear envelope following the transient expression in cells [69, 70]. Additionally, some unconventional roles of Vpr were also suggested. A study suggested that Vpr induces the herniation of the nuclear membrane and facilitate the direct entry of PIC into the nucleus based on the evidence of local disruptions in the nuclear membrane of cells expressing Vpr [182]. However, the underlying mechanism of Vpr mediated local disruption of nuclear membrane or whether PIC is able to access the nucleus by this mechanism is not clear. Surprisingly, although Vpr has been implicated in HIV nuclear import, the replication of mutant HIV that lacks Vpr is almost unaffected in $CD4^{+}$ T lymphocytes [70, 225]. However, only in macrophages, the replication of Vpr deleted HIV or HIV with specific mutations in Vpr was moderately impaired [70, 71, 225, 226]. Therefore, the present view is that Vpr can only act as an accessory factor by enhancing overall nuclear import of HIV [71].

CA Protein: Interestingly, some of recent studies have also implicated CA protein in HIV nuclear import. HIV/MMLV chimera virus in which HIV CA coding region was replaced with MMLV CA was defective for nuclear import [227]. Based on this observation, authors have proposed CA protein as a primary contributor of HIV nuclear import. However, this argument needs to be considered with caution as exactly how CA

contributes to HIV nuclear import is still unknown or at best is inconclusive. Unlike HIV, MMLV exhibits delayed uncoating and MMLV PIC contains higher amounts of CA protein at any given point in time [228-230], which is suggested for lack of nuclear import by MMLV. The proper uncoating may help to expose viral proteins of PIC to the nuclear import machinery or the lack of uncoating would make PIC too large to be able to pass through NPC during nuclear import.

IN Protein: IN has been implicated in HIV nuclear import [210, 231-233]. The pioneering work by Gallay *et al.,* provided first convincing evidence that IN plays a key role in HIV nuclear import [210]. In this paper, authors have shown that the mutant HIV ($MA_{\Delta NLS}$ ΔVpr) that lacks NLS of MA and entire Vpr protein was equally infectious as a wild type virus in cell cycle arrested P4 cells. In contrast, triple mutant HIV that lacks IN protein in addition to NLS of MA and Vpr failed to enter the nucleus. From these findings, authors have claimed that IN is a key mediator of HIV nuclear import. IN is incorporated into the virus and has been tightly associated with PIC during nuclear import and integration [124]. IN localizes to the nucleus when it is expressed as alone or as a fusion protein with GFP [56, 234], FLAG [235], pyruvate kinase [203], or glutathione S-transferase (GST) [210], and this strong nucleophilic property of IN is believed to be essential for HIV nuclear import [56]. However, some studies argued that IN nuclear localization is brought about by its passive diffusion into the nucleus followed by retention in the nucleus due to non-specific DNA binding [236, 237]. But these arguments simply fail to reconcile with temperature and energy dependent nuclear accumulation of IN [238]. IN contains several putative NLSs (^{186}KRK, ^{211}KELQKQITK,

^{236}KGPAKLLWK, and ^{262}RRKAK) and some of these NLSs have been implicated in HIV nuclear import by earlier studies [56, 210]. Gallay *et al*., proposed a non-conventional bi-partite NLS (^{186}KRK and ^{215}KELQKQITK) based on the finding that IN mutants, IN_{K186Q} and $IN_{Q214/216L}$, have lost their nuclear accumulation and interaction with Impα1 in *in vitro* [210] . However, a subsequent study by Bouyac *et al.,* has suggested an additional atypical NLS (^{161}IIGQVRDQAEHLK^{173}) in IN-CCD for HIV nuclear import [203]. $IN_{161IIGQVRDQAEHLK173}$ peptide interacted with Impα1 and induced the nuclear localization of BSA when expressed as a fusion protein. Surprisingly, in follow-up studies to reassess these findings, authors have failed to confirm these results [239, 240]. Coincidentally, the same group who proposed "^{161}IIGQVRDQAEHLK^{173}" has claimed that their findings were just a misinterpretation of results due to a technical nuance [240]. Therefore, "^{161}IIGQVRDQAEHLK^{173}" has been discarded as an NLS of IN. Recently, IN interaction with additional nuclear import receptors such as Imp7 [57] and Transportin 3 (TNPO3) [241] has been demonstrated. Imp7 and TNPO3 are members of non-classical nuclear import pathway. Fassati *et al.,* made an interesting observation that purified RTCs were underwent nuclear import in an *in vitro* nuclear import assay following incubation with Imp7 protein [242]. Based on this finding, authors have implicated Imp7 in HIV nuclear import. Subsequently, a study from our lab demonstrated the IN interaction with Imp7. However, in the same study, we found that Imp7 interaction defective IN mutant HIV was only moderately impaired for nuclear import, but by using Imp7 knockdown (KD), we found that Imp7 was primarily required for late stage HIV replication [57], even though the mechanism is not known. Coincidentally, a subsequent study by Zielske *et al*., failed to demonstrate the requirement of Imp7 for HIV nuclear

import in MDMs [243]. It was later shown that Imp7 plays a supportive role by enhancing HIV nuclear import [244]. The requirement of TNPO3 for HIV replication was first appreciated in two large-scale RNAi screening studies [205, 245]. Later, Christ *et al.,* demonstrated the interaction of TNPO3 with IN and its requirement for HIV nuclear import [241]. Moreover, a study showed that the expression of a peptide aptamer ("WQCLTLTHRGFVLLTITVL") that interacted with IN [246] and disrupted IN and TNPO3 interaction led to impaired HIV nuclear import [247]. Furthermore, the over expression of either cargo-binding domain mutants ($TNPO3_{F918A/F922A}$ or $TNPO3_{L967A/L968A}$) or TNPO3Δcargo-binding domain in TNPO3-KD cells was unable to rescue HIV replication, underscoring the importance of TNPO3 for HIV nuclear import [248]. It is important to note that TNPO3 interacts cargo proteins through serine-arginine (SR) amino acids rich repeats [249, 250] and no such SR motifs are found in IN. Therefore, it is quite difficult to explain the mechanism of IN and TNPO3 interaction and its contribution in HIV nuclear import. Meanwhile, recent studies have suggested CA protein as the determinant for requirement of TNPO3 during HIV replication. By using HIV/MMLV chimeric virus, the group from Engelman's lab showed that CA but not IN is the viral determinant for TNPO3 requirement in HIV infection [227]. HIV/MMLV chimeric virus was also insensitive to TNPO3 KD [251]. Interestingly, a recent study showed that TNPO3-KD induced cytoplasmic accumulation of CA-binding protein, cleavage-and-polyadenylation factor 6 (CPSF6), and delayed HIV uncoating. This suggests that TNPO3 may indirectly influence HIV nuclear import [252]. In fact, CPSF6 is known to impair HIV infection by delaying virus uncoating [253, 254]. Therefore, it is now widely perceived that TNPO3 is not a primary contributor for HIV nuclear import.

In addition to nuclear import receptors, Nups are also implicated in HIV nuclear import. In a recent study, Woodward *et al.,* demonstrated the IN interaction with Nup153 in an *in vitro* interaction assay [255]. They also showed that the overexpression of Nup153-CTD alone disrupted IN and Nup153 interaction and HIV nuclear import. Moreover, two more studies have confirmed the requirement of Nup153 for HIV replication by the gene KD approach [253, 256]. Additionally, Nup98 was also implicated in HIV nuclear import based on the finding that inhibition of Nup98 by transduction of VSV matrix protein led to impaired HIV nuclear import [257]. VSV matrix protein binds and masks the availability of FG repeat region of Nup98 [257]. However, a recent study contradicted the requirement of Nup98 for HIV nuclear import [205]. Therefore, it is still not clear whether Nup98 directly mediates the translocation of PIC to the nucleus or acts as an intermediate docking site during HIV nuclear import. Although the role of Nups in HIV nuclear import is not clear, above reports do suggest the existence of alternative or redundant pathways for HIV nuclear import. Clearly, among all the known viral factors, IN emerged as a key contributor for HIV nuclear import. While studies have highlighted the interaction of IN with different components of cellular nuclear import machinery, only the requirement Impα1 for HIV nuclear import remained undisputed. However, about six Impα isoforms have been identified in human cells and all of them are capable of mediating the protein nuclear import. One study suggested that nuclear import efficiencies of Impα isoforms towards a particular cargo protein would considerably vary when presented along with different cellular proteins [146]. However, although Impα isoforms exhibit high level of similarity at amino acid level (50-80%) [146], they often differ in their substrate recognition[146]. Nevertheless, the classical nuclear import

pathway is the single most common route for protein nuclear import in eukaryotic cells (reviewed in [139]). Therefore, further investigation is essential to verify the contributions of individual impα isoforms for HIV nuclear import.

Accessary Viral and Cellular Factors: A small number of viral and cellular factors are also known to influence nuclear import by poorly defined mechanisms. The genomic cDNA of all lentiviruses has a small stretch of triple strand DNA called central polypurine tract (cPPT) [258]. Either specific mutations in cPPT or deletion of cPPT affect HIV replication and nuclear import [1, 259, 260]. However, a recent study showed that HIVΔcPPT is defective for uncoating, which might have indirectly affected the nuclear import [261]. In another study, an unusual link between the cellular tRNA and HIV nuclear import was observed [262]. Authors in this paper showed that a fraction of cytosolic extract that lacks most of the soluble proteins was able to support HIV RTC nuclear import in *in vitro* and demonstrated that this particular fraction was enriched with tRNAs. Moreover, the tRNAs that supported RTC nuclear import were incorporated into HIV virion. Although tRNAs can promote HIV nuclear import, the mechanism by which these tRNAs drive HIV nuclear import is so far a big mystery and there have been no follow up studies to support the role of tRNA in HIV nuclear import. While the bulk of studies put forward IN as a key viral factor involved in HIV nuclear import, the contribution of other known viral factors has been found to be either non-significant or contradictory. In the case of cellular factors, only Impα1 has remained undisputed for its role in HIV nuclear import and thus can be considered as a likely contributor of HIV nuclear import.

1.4.4 HIV Nuclear Import and Establishment of Infection

The ability of HIV to replicate in non-dividing cells such as macrophages, quiescent T cells, and DCs plays an important role in the establishment of infection, the dissemination of the virus, and the disease progression (reviewed in [263]). The active nuclear import of HIV PIC is an important requirement for its replication in the non-dividing cells. The primary targets for HIV at the vaginal or rectal mucosa are macrophages, DCs, and $CD4^{+}$T cells (reviewed in [264]). The productive HIV replication in these cells gives an excellent opportunity for initial establishment of infection. In addition, HIV infection of perivascular macrophages that are highly migratory and infiltrate various organs allows quick dissemination of the virus to different organs in the body. As macrophages are usually resistant to the HIV induced cytotoxic effect [184] and at the same time capable of colonizing the immune privileged organs such as the brain, macrophages serve as a perfect reservoir for HIV infection. The life span of macrophages can be very long. Depending on the type of macrophage and the anatomical location, the life span of macrophages can vary as long as two months for alveolar macrophages to about a decade for microglial cells [265]. In addition, as antiretroviral drugs are either ineffective in macrophages or exhibit poor tissue penetration, HIV infection of macrophages contributes to incomplete elimination of infection in patients undergoing highly active antiretroviral therapy (HAART) [266, 267]. On the other hand, HIV infection of quiescent T cells helps to establish a long lasting latent reservoir, which is probably one of the daunting reasons why HIV infection is almost impossible to cure. Undoubtedly, productive infection of HIV in macrophage and quiescent T lymphocytes plays an important role in the establishment of infection and disease progression. As discussed in

the above sections, HIV nuclear import also plays an important role in the infection of dividing cells and possibly contributes to tremendous replication potential of HIV. In fact, the extreme potential for replication helps HIV to take over the host and quickly establish infection. Apart from the known contributions of HIV nuclear import in the clinical course of infection, recent studies have suggested the existence of functional link between PIC nuclear import and successful integration of HIV proviral DNA. The existence of a functional link between PIC nuclear import and proviral DNA integration is a key requirement for successful replication of HIV [205]. It is worth noting that lentiviral integration in dividing cells occurs mainly at the “S” phase of the cell cycle, prior to mitosis [268, 269]. Katz *et al.,* made another interesting observation. Following infection of G1 synchronized HeLa cells with single cycle replication competent HIV at a very low MOI (<0.05 to 0.1) that could theoretically generate just one copy of integration per cell, both daughter cells acquired one copy of integrated HIV DNA following cell division [202]. This finding by Katz *et al.,* suggested that HIV integration has occurred prior to cell mitosis. On the contrary, if cells were infected at the time of mitosis, only one daughter cell should have contained integrated HIV DNA. Moreover, authors in this paper also showed that when cells were similarly infected just prior to mitosis, HIV integration was suspended until the following interphase. These findings clearly indicate that HIV integration specifically occurs prior to mitosis. In other words, although this study stops short of presenting a direct link between HIV nuclear import and integration, it does suggest a potential functional association between HIV nuclear import and integration. In agreement with this earlier report on possible link between HIV nuclear import and integrtion, a recent study showed that HIV integration in RanBP2 or TNPO3-

KD cells was favored towards less gene dense regions of the chromosome, whereas HIV integration in control cells occurred at the G/C rich and the highly gene expressing region of the chromosome [204]. It is also known that depletion of TNPO3 or RanBP2 affects the transportation of PIC to the nucleus. Therefore, it is possible to suggest, based on the above findings, that trafficking of PIC through NPC is not only essential for HIV to access nucleus but may also play essential role in proper HIV integration. Therefore, HIV nuclear import may be functionally linked to proper HIV integration and could play a important role in the establishment of infection.

1.5 The Intracytoplasmic Transport of HIV RTC/PIC

The cytoplasm is a viscous aqueous environment and densely packed cellular components. The macromolecules movement by passive diffusion is not feasible in the cytoplasm due to extensive steric hindrance and frequent interactions with other cytoplasmic components [270]. For example, the diffusion of a 2000bp DNA fragment is about 100 times slower in the cytoplasm than in water [271]. Therefore, macromolecules such as RTC/PIC have to be transported in the cytoplasm by an active mechanism.

1.5.1 Retrograde and Anti-Retrograde Transportation

Macromolecules are actively transported in the cytoplasm along cytoskeleton filaments, with the help of specialized motor protein complexes called myosin, dynein, or kinesin (reviewed in [272-276]. The cytoskeleton is a dynamic three-dimensional cellular structure found in most eukaryotic cells and consists of heterogeneous filaments that are subdivided into actin, microtubule (MT), and intermediate filaments. While intermediate

filaments provide mechanical stability to the cell, both actin and MT filaments are engaged in intracellular trafficking. The myosin is associated with actin filament, and dynein and kinesin are associated with MT. Dynein and myosin-V mediate the active transportation of macromolecules towards nucleus, whereas kinesin mediates movement of macromolecules away from the nucleus. The transportation of macromolecules from the cytoplasmic periphery towards the nucleus is called retrograde transportation and transportation of macromolecules away from the nucleus is called anti-retrograde transportation. Myosin-V mediates retrograde transportation of macromolecules for a very short distance. On the contrary, dynein and kinesin supports the retrograde and anti-retrograde transportation of macromolecules over long distances, respectively. Retrograde transportation of RTC/PIC plays a key role in early stage HIV replication. The dynein dependent active retrograde transportation was previously observed for many viruses including HSV-1 [277], Adenovirus [278], Vaccinia virus [279, 280], and Canine parvo virus [281]. Recently, the dynein dependent retrograde migration of HIV complex in the cytoplasm has been elucidated [282].

1.5.2 Molecular Mechanism of Retrograde Transportation

Dynein was initially identified as a force-generating ATPase in *Tetrahymena cilia* [283], and it was later discovered as a mediator of retrograde transportation of macromolecules in nonciliated cells [284]. Dynein is known to mediate the transportation of a wide variety of cargoes including mRNA, endosomes, proteins, and viruses. Dynein is a massive multi-subunit complex with an overall molecular weight of approximately 1.2MDa (**Figure 6**). It consists of dynein heavy chain (DHC), dynein light intermediate

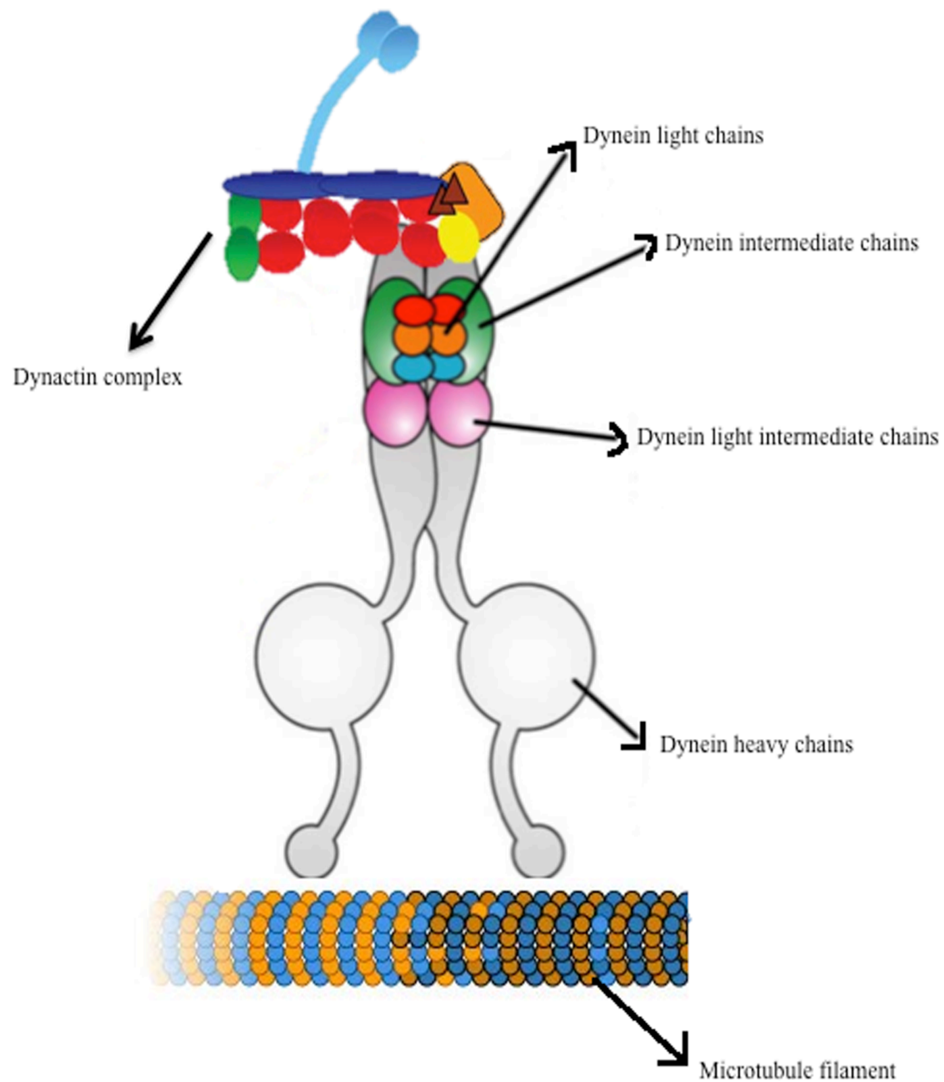

Figure 6. Molecular structure of dynein complex: Diagram showing the different components of dynein complex. The main components of dynein complex include two DHC (white colored club like structures), two DLIC (pink circles), two DIC (green circles), three light chains (blue, orange, and red circles on intermediate chain), and dynactin complex (shown as a complex attached to dynein complex from outside). DHCs attach the dynein complex to MT filaments.

chain (DLIC), and dynein light chain1/2 (DIC1/2). DIC1/2 provides a site for the interaction of three different dynein light chains; dynein light chain 1 (DYNLL1), dynein light chain Tctex1 (DYNLT1), and roadblock light chain. DIC1/2 is also associated with an additional multiprotein protein complex called dynactin. Dynactin consists of eleven different subunits, including p150^{glued} and actin related protein 1 (ARP1). DHC, DLIC, and DICs are the main structural proteins in dynein complex. They interact with each other to provide a structural framework for dynein complex. Roadblock light chain and some components of dynactin constitute regulators that control the functions of dynein (reviewed in [285]). DYNLT1, DYNLL1, and p150^{Glued} act as adapter proteins for cargo recruitment to dynein complex. DHC head has a 13nm long MT-binding stalk that connects dynein to MT filament. In addition, DHC head has several globular domains that are arranged in ring like conformation around the central cavity. Four of these globular domains bind ATP and generate the mechanical force for dynein complex movement along MT. Although the recruitment of cargo by dynein complex has been extensively studied, still it is the least understood. Although DYNLL1, DYNLT1, and p150^{Glued} have been suggested to mediate the recruitment of several cellular and viral cargoes to dynein complex, very few studies have unambiguously demonstrated the recruitment of cargoes to dynein by these adapter proteins. In addition, the structural and thermodynamic studies have expressed doubt about the ability of DYNLL1 and DYNLT1 to recruit cargo to dynein complex [286]. Also, DYNLL1 and DYNLT1 interact with various cellular proteins outside the dynein complex as well as are involved in various cellular processes [287-293]. Therefore, more investigation is required to clearly define the molecular mechanism of cargo recruitment by dynein complex.

1.5.3 Functional Relationship Between Retrograde Transportation and Nuclear Import

Retrograde transportation of macromolecules is one of the prerequisites for successful nuclear import. Although no specific evidence is available to establish direct association between cytoplasmic transportation and nuclear import, a study demonstrated the impaired nuclear accumulation of proteins following disruption of MT filaments or introduction of specific mutations into the proteins that impair their association with MT [294, 295]. Additionally, stabilization of MT filament by chemical treatment enhanced the nuclear accumulation for certain cellular proteins [294, 296]. It was also evident that competitive inhibition of dynein mediated retrograde transportation of tumor suppressor protein Rb by over expression of dynamitin (p50), a component of dynactin complex, reduced the rate of nuclear accumulation of tumor suppressor protein Rb [294]. Another study showed that nuclear accumulation was significantly enhanced for proteins that are modified to contain dynein light chain association sequence of rabies virus P-protein and this phenomenon was dependent on intact MT and association with dynein light chains [297]. Similar findings were also observed for Rb protein and parathyroid hormone proteins when specific mutations that impair the association of these proteins with MT were introduced [295]. However, exceptions do exist. In a large scale investigation involving several different cellular proteins, Roth *et al.,* showed that not all the cellular proteins nuclear import is affected by MT depolymerization, contradicting the universal requirement of MT for nuclear import [295]. Nevertheless, accumulated information clearly suggests the presence of a functional relationship between retrograde transportation and successful nuclear import. However, it is not clear how retrograde

migration and nuclear import are linked. There is no evidence to suggest the physical association between components of dynein and nuclear import machineries; it even may be difficult to rule out the association of retrograde migration and nuclear import as just related sequential events.

1.5.4 Retrograde Transport of HIV Replication Complex in the Cytoplasm

Given the fact that HIV replication complex (RTC/PIC) is a high molecular weight nucleoprotein complex with approximately 56 nm in size, the passive diffusion of RTC/PIC in the cytoplasm is extremely unlikely. Moreover, as passive diffusion could exhibit a random movement, the migration of RTC/PIC to the perinuclear compartment may not always be guaranteed by passive diffusion. On the contrary, active intracytoplasmic transportation would facilitate the specific localization of RTC/PIC to the perinuclear space.

1.5.5 The Importance of RTC/PIC Retrograde Transport for HIV Replication

Although recent studies have demonstrated the dynein dependent retrograde transportation of RTC/PIC in the cytoplasm, the cellular and viral factors that mediate the recruitment of RTC/PIC to dynein complex or retrograde migration are unknown. An earlier study showed the moderate reduction of luciferase reporter HIV replication in cells treated with MT deploymerizing agent (Nocodazole, 10mM) [298], implicating the requirement of MT for early stage HIV replication. Later, another study demonstrated the co-localization of HIV RTC/PIC with cytoplasmic MT and subsequent concentration of RTC/PIC at microtubule organizing center (MTOC), near the nuclear periphery [282].

However, microinjection of target cells with anti-DIC antibody has abolished HIV RTC/PIC localization at MTOC [282]. These findings clearly indicated that HIV utilizes dynein molecular motors for retrograde migration in cytoplasm. Adapter proteins recruit cargo proteins to dynein through direct protein-protein interaction. Dynein adapter proteins such as DYNLL1 [299-301], DYNLT1 [302], and p150^{Glued} [277, 279] have been implicated in recruitment of viruses to dynein or retrograde transportation. However, none of these dynein adapter proteins have been examined for HIV recruitment to dynein complex or early stage replication.

DYNLL1 is shown to interact with CA protein of bovine immunodeficiency virus (BIV) and facilitate BIV retrograde transport [299]. Similarly, human foamy virus (HFV) Gag protein interacts with DYNLL1 and localizes to the nuclear periphery [303]. In a yeast two hybrid screening, de Soultrait *et al.,* found the HIV IN interaction with Dyn2p, a yeast homolog of mammalian DYNLL1 [304]. Later, Desfarges *et al.,* elucidated the MT dependent localization of IN-GFP fusion protein to perinuclear space in *S. cerevisiae* (yeast) [305]. However, nuclear localization of IN-GFP was lost in ΔDyn2p mutant strain [305], suggesting the potential involvement of Dyn2p in retrograde transportation of IN-GFP. Further, this finding helped to make the assumption that DYNLL1 could be a most likely candidate adapter protein for linking HIV to dynein complex. However, it is important to note that although Dyn2p is a yeast homolog of human DYNLL1, Dyn2p and DYNLL1 show extensive diversity at amino acid level (~ 25% amino acid diversity). Therefore, the findings made by using the yeast system may not necessarily represent the similar mechanism in mammalian cells. Meanwhile, as explained in the previous section,

DYNLL1 is also associated with several different cellular functions outside the dynein complex. Indeed, DYNLL1 has been known to facilitate rabies virus replication by mediating virus transcription but not retrograde transport [306-308]. Therefore, involvement of DYNLL1 in step(s) of HIV replication other than RTC/PIC recruitment to dynein complex or retrograde transportation cannot be ruled out. In order to recruit cargoes to dynein complex, DYNLL1 has to simultaneously interact with both DIC1/2 in dynein complex and cargo. However, as mentioned in an earlier section, a recent study by using structural and thermodynamic analysis questioned whether DYNLL1 is able to simultaneously interact with cargo and DIC1/2 [286]. Thus, even the very ability of DYNLL1 to mediate the recruitment of cargoes to dynein complex is not completely ascertained. Hence, it justifies the need for a detailed investigation on the role of DYNLL1 in HIV recruitment of dynein complex or in other steps of HIV replication. HIV RTC/PIC contains several important viral proteins such as IN, MA, CA, NC, RT, and Vpr [26, 124]. In particular, IN, MA, and CA proteins have been implicated in early stage HIV replication, including nuclear import (reviewed in [309]). However, it is unknown whether these viral proteins can interact with DYNLL1 or mediate RTC/PIC recruitment to dynein complex. It is possible that IN, MA, or CA proteins may tether RTC/PIC to dynein complex through interaction with DYNLL1.

1.6 HIV Uncoating and Early Stage HIV Replication

Uncoating is an early post entry step of HIV replication and is functionally linked to reverse transcription, nuclear import, and integration. HIV CA is primary viral protein involved in the process of uncoating. However, a recent report suggests that HIV IN is

also essential for proper uncoating [310]. Although the role of IN in HIV uncoating is not clearly defined, lack of IN resulted in defective viral incorporation of cyclphilin A (CypA) protein and accelerated uncoating of HIV [310]. Although it is believe that HIV uncoating occurs after virus entry and prior to nuclear import, the field is very much divided on precise location and time of uncoating. The earlier assumption was in favor of uncoating occurring just after virus entry. The absence of significant amount of CA in intracellular RTC/PICs and failure to detect CA in cytoplasm of HIV infected cells by transmission electron microscopy lead to the conclusion that uncoating occurs immediately after virus entry [124, 197, 226, 311-313]. However, the contemporary view is that uncoating is a gradual process and it occurs in response to exposure of HIV RTC/PIC to multiple successive changes in the cytoplasmic environment, sequential interaction with various cellular factors, and molecular rearrangement of RTC that coincides with reverse transcription. This view is supported by the fact that RTC/PIC exhibits in different sizes and shapes in the cytoplasm, and this transformation of RTC/PIC is accompanied by reverse transcription and nuclear import (reviewed in [120]). Studies have shown that both premature and delayed uncoating is accompanied by low levels of HIV cDNA synthesis [310, 314, 315]. Interestingly, suppression of reverse transcription by drug treatment or modification of the central DNA flap delayed the uncoating [316, 317]. Thus, sequential events that facilitate the uncoating or reverse transcription are not clear. Nevertheless, there is clear evidence for the functional association between uncoating and reverse transcription steps. Proper uncoating of HIV is also essential for nuclear import, which is evident by the fact that HIV/MMLV chimera virus in which HIV CA coding region was replaced with MMLV CA was defective for

nuclear import [227]. MMLV shows delayed uncoating [228], which is suspected for lack of nuclear import by MMLV. Therefore, uncoating may be essential for the successful passage of HIV complex through NPC, as the lack of uncoating resulted in accumulation of HIV complexes at the cytoplasmic face of nuclear membrane [317]. Alternatively, lack of uncoating may mask viral components to come in contact with HIV nuclear import machinery, of which no evidence is yet available.

1.7 Anti-HIV Drug Development

Since the introduction of highly active antiretroviral therapy (HAART) in 1996, antiretroviral (ARVs) development has witnessed huge progress and has significantly contributed in delaying the AIDS progression and prolonging the life span of infected individuals. Presently, six major classes of food and drug administration (FDA) approved ARV drugs are available for HIV treatment; non-nucleoside reverse-transcriptase inhibitors (NNRTIs), nucleotide reverse transcriptase inhibitors (NRTIs), PR inhibitors, IN inhibitor (Raltegravir), chemokine receptor 5 antagonist (Maraviroc), and fusion inhibitor [Enfuvirtide (T-20)] (reviewed in [318]). Despite the availability of ARVs, these drugs present several drawbacks such as extensive drug resistance, drug toxicities, drug-drug interactions, and high cost. In particular, all presently available ARVs exhibit antiviral property by targeting HIV proteins, which makes them prone to easy development of drug resistance. Thus, there is a real need to develop new and more effective ARVs for future anti-HIV strategies. Specifically, we need to develop ARVs that are safer and less vulnerable to development of drug resistance.

1.7.1 HIV IN Inhibitors

HAART generally includes a combination of two RT inhibitors and a PR inhibitor or a NNRTI. However, drug resistance impedes the long term institution of HAART. To overcome this, new classes of ARVs are being periodically added to HAART combinations. The periodic addition of new classes of ARV limit the emergence of drug resistant strains in infected individuals. Since the newly identified anti-IN inhibitors showed fewer tendencies for drug resistance, they were considered second line drugs in HAART. Most IN inhibitors identified so far are IN strand transfer inhibitors (INSTIs). INSTIs bind to IN and specifically inhibit the strand transfer step of HIV cDNA integration [319, 320]. The first generation INSTI, Raltegravir and Elvitegravir, have shown to be effective against HIV [321-323]. Raltegravir has been approved by FDA for clinical use. However, drug resistance against first generation INSTI has already been observed in cell culture and infected individuals [324-326]. Recently, second generation INSTIs have been developed. Dolutegravir and MK2048 are recently identified second generation INSTIs [327, 328]. Although second generation INSTIs were thought less likely to develop drug resistance, the drug resistance against second generation INSTIs has been demonstrated in cell culture [329]. Therefore, continued search for new classes of ARVs become inevitable in order to keep up the fight against HIV infection.

1.7.2 Protein-Protein Interaction Inhibitors

Fundamental processes in cells are regulated by proteins, frequently acting in accordance with other cellular proteins through protein-protein interactions (PPIs). Virus replication also depends on viral and cellular PPIs. Lack of viral and cellular protein interaction

would lead to aborted replication. Usually, motifs in viral proteins for cellular protein interaction are highly conserved and mutations in these motifs are often detrimental to viral replication. The competitive inhibition of PPIs with the help of selectively binding small peptides has been used to study the interactions. However, small peptides per se are not feasible as drugs due to their lack of inherent ability to freely distribute in the body or diffuse into cells and establish therapeutically effective concentrations at the site of function. On the contrary, small molecule PPI inhibitors would freely diffuse into cells and easily reach therapeutically effective concentrations. The small molecule inhibitors may pose cytotoxic side effects, which requires careful selection of molecules followed by extensive *in vitro* and *in vivo* evaluation for toxicity.

Selective, small molecule mediated inhibition of PPIs is therefore a very promising anti-viral strategy (reviewed in [330-332]). One of the daunting challenges in earlier days for small molecule based inhibition of PPIs was that protein interaction interfaces were often difficult to access because of the large and discontinued nature of PPI interfaces. This difficulty was further compounded by the lack of high throughput screening approaches and limited availability of essential small molecules that exhibit the necessary size and functionality to modulate protein-protein interactions. Thus, small molecule based PPIs inhibition was previously perceived as a non-viable option. However, recently, several reports have challenged the earlier perceived idea that the disruption of PPIs by small molecule inhibitors is an unattainable task. Disruption of PPIs by small molecule inhibitor was made easy in recent years because of the identification of “PPI hot spots”. “PPI hot spots” represent small numbers of highly conserved amino acids within the

interaction interface that are crucial for PPI, and thus are feasible targets for PPI small molecule inhibitors (reviewed in [333]). Small molecule PPI inhibitors can also serve as novel drugs for HIV treatment. The proof of concept for IN and LEDGF interaction inhibition as anti-HIV strategy has been demonstrated in some recent studies [334, 335]. For example, the overexpression of IN binding domain (IBD) of LEDGF/p75 was able to compete with endogenous LEDGF/p75 for IN interaction and inhibited HIV replication [334]. Similarly, small peptides derived from LEDGF/p75 loops (LEDGF/p75 Aa353-378, Aa361-370, and Aa402-411) were able to bind IN in *in vitro* and inhibit HIV integration in cells [335]. In the following study, a more detailed investigation confirmed the inhibitory effect of LEDGF/p75-Aa361-370 on HIV replication in a mouse model [336]. Following successful inhibition of HIV replication by disruption of IN and LEDGF/p75 interaction, a structure based and computer aided drug development study was able to identify two small molecules, 4-[1-(4-fluorobenzyl)-4- hydroxy-1H-indol-3-yl]-2-hydroxy-4-oxobut-2-enoic acid (CHI-1043) and a benzoic acid derivative (D77), that were capable of impairing IN and LEDGF/p75 interaction and HIV replication [337]. In the mean time, by screening 200,000 commercially available compounds, several 2-(quinolin-3-yl)acetic acid derivatives (LEDGINs) that fit LEDGF/p75 binding pocket of IN and inhibit LEDGF/p75 interaction and HIV replication in cells were also identified [338]. However, surprisingly, the LEDGIN resistant HIV IN mutant, IN_{A128T}, has been found in cell culture infections [338], which undermines the clinical usefulness of LEDGIN.

The interaction of IN with several new cellular proteins has been found in several recent

studies. Theoretically, any viral and cellular protein interactions can serve as attractive targets for the anti-HIV drug development. However, careful evaluation of viral and cellular factor interaction for HIV replication followed by crystallographic or NMR characterization of interaction is essential for successful drug development. The successful development of small molecule inhibitors that bind to IN or other viral proteins and inhibit their interaction with cellular proteins will surely benefit future anti-HIV strategies. This concept of drug development can also be easily applied against other infectious agents or general ailments.

1.8 Study Rationale, Central Hypothesis, and Outline

HIV is an obligatory intracellular parasite and relies heavily on different cellular factors for successful completion of its replication cycle. IN is a key enzymatic protein of HIV and its enzymatic function is required for the process of HIV cDNA genomic integration. The process of HIV cDNA integration involves two steps; 3' processing and strand transfer reactions. IN, by virtue of its enzymatic property, removes two nucleotides from 3' end of HIV cDNA, creating CA_{OH} overhanging ends. In the next step, the 3' processed HIV cDNA is inserted into the host cell genome by IN through strand transfer reaction (reviewed in [339]). In addition to its role in integration reaction, IN contributes to different steps of early stage HIV replication. As discussed in the above sections, although IN has been implicated in HIV nuclear import and RTC/PIC recruitment to dynein complex, the molecular mechanism by which IN contributes to HIV nuclear import or RTC/PIC recruitment to dynein complex is unknown. Nevertheless, IN is known to interact with components of nuclear import machinery and a dynein adapter

protein (Dyn2p) of *S. cerevisiae*. In the past, IN and cellular factor interactions have been known to contribute to different steps of HIV replication, including some of the steps of integration. IN is also likely to function in other steps of HIV replication by interacting with specific cellular co-factors.

Therefore, the central hypothesis of this thesis is that HIV IN will facilitate the completion of the post entry steps of early stage HIV replication by interacting with specific cellular co-factors.

The results of experiments aimed at testing the above hypothesis are presented in chapter 3 and 4 of this thesis. In chapter 3, we have examined the requirement of different Impα isoforms for HIV nuclear import and their interaction with IN. We have elucidated the critical motifs in IN for Impα3 interaction and HIV nuclear import. In chapter 4, we have explored the IN and DYNLL1 interaction and its requirement for steps of early stage HIV replication and RTC/PIC recruitment to dynein complex. Under each chapter, specific rationale, hypothesis, and objectives are stated.

Chapter 2.

Materials and Methods

2.1 General Reagents

2.1.1 Cell Lines and Transfections

African green monkey kidney COS-7, Human embryonic kidney 293T, and HeLa cell lines were maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% fetal calf serum (FCS). Human $CD4^+$ C8166T cell line was maintained in RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% FCS. 293T cells, COS-7, or HeLa cells were transfected with the standard calcium phosphate precipitation technique, as previously described [73]. PBMCs were isolated from whole blood of healthy human volunteers by sedimentation on a Ficoll gradient and plated in 12-well plates at a desired density. Macrophages were obtained from PBMCs by culturing in DMEM with 10% FCS and 10ng/ml of macrophage colony-stimulating factor (M-CSF; R&D Systems) for 1 week.

2.1.2 Plasmids

2.1.2.1 HIV IN and Other Viral Protein Expression Plasmids

pAcGFP-IN was constructed by cloning cDNA fragment for full-length HIV IN into CMV-AcGFP-C1 plasmid (Clontec) at *BglII* and *BamHI* restriction enzyme sites, in frame with GFP coding region. The cDNA for HIV IN was obtained by digesting YFP-IN expressor [57] with *BglII* and *BamHI* restriction enzymes. IN deletion mutant pAcGFP-IN_{1-212}, pAcGFP-IN_{1-250}, pAcGFP-IN_{1-270}, pAcGFP-$IN_{206-288}$, pAcGFP-$IN_{117-288}$, pAcGFP-$IN_{180-230}$, and pAcGFP-IN_{50-288} expressors were constructed by cloning

corresponding cDNAs into AcGFP-C1 at *HindIII* and *BamHI* restriction enzyme sites, in frame with GFP coding region. The cDNAs for different IN deletion mutants were PCR amplified from AcGFP-IN plasmid. MA-YFP expressor has been described earlier [57]. CMV-ProLabel (PL)-IN has been previously described [340]. PL-$IN_{Q53A/Q252A}$ and PL-$IN_{K186A/R187A}$ expressors were generated by cloning cDNA for IN with corresponding mutations into CMV-PL (Clontec) plasmid at *SalI* and *BamHI* restriction enzyme sites, in frame with PL coding region. The cDNAs having desired mutations were obtained by digesting AcGFP-$IN_{Q53A/Q252A}$ and AcGFP-$IN_{K186A/R187A}$ with *SalI* and *BamHI* restriction enzymes. AcGFP-$IN_{R186A/K187A}$ has been described earlier [23]. AcGFP-CA expressor was generated by cloning cDNA for *CA* gene into CMV-AcGFP-C1 plasmid at *BamHI* and *XbaI* restriction enzyme sites, in frame with GFP coding region. *CA* gene was PCR amplified from HIV Bru. AcGFP-IN (MMLV) expressor was generated by cloning cDNA for MMLV *IN* into CMV-AcGFP-C1 plasmid at *BamHI* and *XbaI* restriction sites, in frame with GFP coding region. The cDNA for MMLV *IN* was PCR amplified from pVPack-GP (MMLV gag-pol expressor) (Stratagene). Construction of T7-RT and T7-IN fusion protein expressors has been previously described [23]. IN point mutant expressor AcGFP-$IN_{K215A/K219A}$, AcGFP-$IN_{K240A/K244A}$, and AcGFP-$IN_{R263A/K264A}$ were generated by two-step PCR method [341]. The IN mutant cDNAs were generated by PCR using a forward primer having *BglII* restriction enzyme site and a reverse primer having *BamHI* restriction enzyme site and complementary primers with intended mutations. The IN mutant cDNAs were sub-cloned into AcGFP1-C vector (Clontech) at *BamHI* restriction site, and mutations were confirmed by sequencing. SvCMVin-YFP-$IN_{K215A/K219A/R263A/K264A}$ was generated by PCR mutagenesis. The cDNA was generated by

using a forward primer having *BglII*, a reverse primer having *XhoI* restriction enzyme site, and complementary primers with intended mutations. Then, $IN_{K215A/K219A/R263A/K264A}$ mutant cDNA was cloned into pEYFP-C1 vector (ClonTech) at the corresponding sites. AcGFP-IN_{Q53A}, AcGFP-IN_{Q252A}, AcGFP-IN_{Q209A}, and AcGFP-$IN_{Q53A/Q252A}$ expressors were generated by cloning cDNA for IN with corresponding mutations into CMV-AcGFP-C plasmid at *HindIII* and *BamHI* restriction enzyme sites, in frame with GFP coding region. The cDNAs having desired mutations were generated by two step PCR by using a forward primer having *HindIII* restriction enzyme site, reverse primer having *BamHI* restriction enzyme site, and complementary primers having intended mutations. The Vpr-YFP expressor was constructed by cloning HxBru*Vpr* cDNA into pCMV-YFP-N1 vector [57]. The nucleotide sequences for forward and reverse primers used in the construction of above-mentioned expression vectors are provided in **table 1**.

2.1.2.2 Provirus and Virus Related Plasmids

HIV provirus pNL4.3-Nef^{+}/GFP^{+} (pNL4.3-GFP) and pNL-BruΔBgl/Luc^{+} have been previously described [57, 342]. pNL-BruΔBgl/Luc^{+}/R^{-} provirus was generated by replacing *ApaI-SalI* region in pNL-BruΔBgl/Luc^{+} with the same fragment from HIV provirus, HxBru-R^{-} [343]. The MMLV-based vector, pFB-Luc, was obtained from Stratagene Corp. SvCMVin-R-RT-$IN_{K215A/K219A/R263A/K264A}$ mutant fusion protein expressor was generated by introducing $IN_{R263A/K264A}$ mutation into SvCMVin-R-RT-$IN_{K215A/K219A}$ plasmid [56]. SvCMVin-Vpr-RT-IN_{Q53A}, SvCMVin-Vpr-RT-IN_{Q252A}, SvCMVin-Vpr-RT-$IN_{Q53A/Q252A}$ mutant fusion protein expressors were obtained by introducing individual mutations into SvCMVin-Vpr-RT-IN plasmid by two step PCR

method. Following primers were used in the construction of SvCMVin-R-RT-$IN_{K215A/K219A}$ expressor; forward primer 5′-GCAGCTAGCAGGGAGACTAA-3′ targeting RT gene at natural *NheI* restriction enzyme site and a reverse primer 5′-CTGTTCCTGCAGCTAATCCTCATCCTG-3′ with *PstI* restriction enzyme site, targeting 3′ end of IN and complementary primers having intended mutations. $HIVHxBruR^{-}/\Delta RI/E^{+}$ proviral DNA clone has been described previously [1].

2.1.2.3 Cellular Protein Expression Vectors

SvCMVin-T7-Impα3 was generated by cloning cDNA for Impα3 into SvCMV-T7 vector at *BamHI* and *NotI* restriction enzyme sites, in frame with T7 tag coding region. The cDNA for Impα3 was PCR amplified from pCMV6 Entry Impα3-myc (OriGene Technologies). pCAGGS-PL-Impα3 was constructed by cloning PL-Impα3 cDNA into pCAGGS vector [344] at *ClaI* and *XhoI* restriction enzyme sites. The PL-Impα3 cDNA was PCR amplified from CMV-PL-Impα3 plasmid. Similarly, pCAGGS PL-TNPO3 was constructed by cloning PL-TNPO3 into pCAGGS vector at *ClaI* and *XhoI* restriction enzyme sites. The cDNA for PL-TNPO3 was PCR amplified from CMV-PL-TNPO3. CMV-PL-Impα3 and CMV-PL-TNPO3 were constructed by cloning cDNA encoding Impα3 or TNPO3 from pCMV6-Entry-Impα3-myc (OriGene) and pCMV6-Entry-TNPO3-myc (OriGene) into a CMV-PL vector (Clontech) at *SalI* and *NotI* restriction enzyme sites. pCAGGS-PL-Impα$3_{W179A/N183A}$ and pCAGGS PL-Impα$3_{W348A/N352A}$ mutant expressors were generated by by two-step PCR method. The cDNAs for PL-Impα$3_{W179A/N183A}$ and PL-Impα$3_{W348A/N352A}$ were generated by two-step PCR by using a forward primer having *ClaI* restriction enzyme site, reverse primer having *XhoI*

restriction enzyme site, and complementary primers having intended mutations. The cDNAs were cloned into the pCAGGS vector at *ClaI* and *XhoI* restriction enzyme sites. pCGN-HA-INI1 plasmid has been described earlier [17]. CMV-PL-DYNLL1 plasmid was generated by subcloning cDNA for DYNLL1 gene into CMV-PL-Ku70 plasmid at *BamHI* and *NotI* restriction enzyme sites, by replacing *Ku70* gene. The cDNA for DYNLL1 gene was obtained by digesting CMV-T7-DYNLL1 plasmid with *BamHI* and *NotI* restriction enzymes. CMV-T7-DYNLL1 plasmid was obtained from Adgene, MA, USA. CMV-PL-Ku70 was constructed by cloning cDNA for *Ku70* gene into CMV-PL plasmid (Clontec) at *BamHI* and *NotI* restriction enzyme sites, in frame with PL tag coding region. All the substitution mutations were confirmed by sequencing. The nucleotide sequences for forward and reverse primers used in the construction of above-mentioned expression vectors are provided in **table 1.**

Plasmid	Primer type	The nucleotide sequences of primers
AcGFP-CA	Forward	5’ GCCAGGTCGGATCCCCTATAGTGCAG 3’
	Reverse	5’ TTGTTACGCGGCCGCTCTAGATTACAAAACTCTTGC 3’
AcGFP-IN (MMLV)	Forward	5’CTCGGATCCGAGAATTCATCACCCTA3’
	Reverse	5’GCAGCTAGCTTAGGGAGCTTCGCGGTTAACCT3’
AcGFP-IN$_{K215A/K219A, K240A/K244A, and R263A/K264A}$	Forward	5′-GCCAGATCTTTCTTAGATGGAATAGATAAG-3′
	Reverse	5′-CTAAACGGATCCATGTTCTAA-3′
SvCMVin-YFP-IN$_{K215A/K219A/R263A/K264A}$	Forward	5′-GCCAGATCTTTCTTAGATGGAATAGATAAG-3′
	Reverse	5′-GTTCCTCGAGCTAATCCTCATC-3′
AcGFP-IN$_{Q53A, Q252A, Q209A, and Q53A/Q252A}$	Forward	5’- GCGCTCGAGAAGCTTGGCTTTTTAGATGGAATAG-3’
	Reverse	5’-CTAAACGGATCCATGTTCTAA-3’
SvCMVin-T7-Impα3	Forward	5’-ATAGGATCCGTCGA CGCGGACAACGAGAAACTGG-3’
	Reverse	5’-CTGCGGATCCAGC GGCCGCGTACGCGT-3’.
pCAGGS-PL-Impα3	Forward	5′-GCGCTAGAATTCATCGA TATGAGCTCCAATTCA-3′
	Reverse	5′-TCCGGTCTCGAGGCGGCC GCCTAAAACTG-3′.
pCAGGS PL-TNPO3	Forward	5′-GCGCTAGAATTCATCGATATGAGCTCCAATTCA-3′
	Reverse	5′- TTTCTGCTCGAGCGGCCGCGTACGCTAATTAA-3′.
pCAGGS-PL-Impα3$_{W179A/N183A and W348A/N352A}$	Forward	5′-GCGCTAGAATTCATCGATATGAGCTCCAATTCA-3’
	Reverse	5′-TCCGGTCTCGAGGCGGCCGCCTAAAACTG-3′

Table 1. Nucleotide sequences of primers used in the construction of expression vectors

2.1.3 Chemicals and Antibodies

Chemicals and antibodies used in this study are as follows. The Western blot (WB) detection ECL kit was obtained from Perkin-Elmer Life Science (Boston, MA). NP-40 Alternative and Puromycin were purchased from Calbiochem. ProLabel^{TM} Detection Kit II was purchased from Clontech. The Aphidicolin was purchased from Sigma Inc. The purified recombinant GST-IN (catalog no. 9420), GST-MA, and HIV pNL4.3 IN (catalog no. 9420) proteins were acquired through AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. DYNLL1 recombinant protein was purchased from ProsPec Corporation. The rabbit anti-GFP polyclonal antibody, mouse anti-Impα3 antibody, and mouse anti-T7 monoclonal antibody were obtained from Molecular Probes, Abcam, Inc, and Novagen, respectively. The rabbit anti-hemagglutinin (HA) antibody was purchased from Sigma. The human anti-CD4 monoclonal and mouse anti-HIV p24 antibodies have been described earlier [56, 343]. Horseradish peroxidase (HRP)-conjugated anti-GFP and anti-HA antibodies were obtained from Miltenyi Biotec. HRP-conjugated anti-mouse IgG and anti-rabbit IgG were procured from Amersham Biosciences. An anti-GST polyclonal antibody was purchased from Amersham Biosciences. Rabbit anti-DYNLL1 antibody and anti-α-tubulin were obtained from Abcam, Sigma, respectively. The rabbit anti-IN antibody (catalog no. 757) was procured from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.2 General Methods

2.2.1 Co-immunoprecipitation (Co-IP) or Chemiluminescent Co-IP Assays

The viral and cellular proteins interactions were analyzed by Co-IP or chemiluminescent

Co-IP assays. The viral protein expressor AcGFP-IN, MA-YFP, or AcGFP-Vpr was cotransfected with cellular protein expressor T7-Impα3 in 293T cells. Similarly, AcGFP-$IN_{Wt/Mt}$ expressor was co-transfected with PL-Impα3, PL-DYNLL1, or T7-INI1. To detect the viral proteins interaction with endogenous DYNLL1, AcGFP-IN, MA-YFP, or AcGFP-CA were transfected to 293T cells. After 48h of transfection, cells were lysed in 0.12%-0.2% NP-40 lysis buffer with protease inhibitor cocktail. Lysates were clarified and supernatants were subjected Co-IP with anti-GFP antibody. Immunoprecipitates were resolved in 10-12% SDS-PAGE and co-precipitation of T7-Impα3, T7-INI1, or endogenous DYNLL1 was detected by probing the WB with corresponding antibodies. The co-precipitation of PL-Impα$3_{Wt/Mt}$ or PL-TNPO3 was detected by measuring the PL activity in immunoprecipitates, by following manufacturer's instruction. Briefly, PL is a fragment of split®-galacosidase enzyme and therefore, PL-fusion protein alone lacks enzymatic activity. PL-tag forms active enzyme complex when provided with missing galactosidase fragment and this complex induces the cleavage of chemiluminescent substrate, which generates chemiluminescence. The galactosidase enzyme fragment and chemiluminescent substrate are provided with the kit in separate vials. To detect the PL activity, the immunoprecipitates were first mixed with lysis/ER (3:1) buffer provided with the kit and to each sample, a substrate mix containing 6 μl of Emerals-IITM solution, 1.2 μl of Galacton-Star® substrate, and 22.8 μl of CL substrate diluent was added. Then, samples were transferred to 96 well format assay plate (Corning, New York) and PL activity (i.e., chemiluminescence) was measured using a POLARstar OPTIMA microplate reader (BMG Labtech, Germany). The PL activity was expressed as relative light units (RLU). To detect the HIV IN interaction with endogenous cellular

proteins in virus infected cells, 10^7 C8166T cells were infected with equal amount of HIVHxBru or HxBru-IN-HA viruses in 75 cm^2 cell culture flask containing 500 ml of RPMI medium (at 100 ng of virus-associated $p24^{Gag}$). Cells were washed at 4h post infection with fresh medium and cultured in fresh RPMI medium. At 72h of infection, cells were lysed in 0.12%-0.2% NP40 lysis buffer and lysates were immunoprecipitated with an anti-HA antibody. Immunoprecipitates were resolved in 10-12% SDS-PAGE and co-precipitation of cellular proteins (Impα3 or DYNLT1) was detected by probing the WB with corresponding antibodies.

2.2.2 *In Vitro* Protein Interaction Assay

To detect IN interaction with Impα3 in *in vitro*, GST and Impα3-GST proteins were purified from *Escherichia coli* JM101, as previously described [345]. Equal amounts of GST and GST-Impα3 were incubated with AcGFP-C or AcGFP-IN expressing 293T cell lysates at 4°C for 4h. 100 μl of glutathione-Sepharose 4 beads (Amersham Biosciences) was added to the mixtures and incubated at 4°C for another 2h. Glutathione-Sepharose 4 beads were eluted with SDS-gel loading buffer and elutes were resolved in 12% SDS-PAGE. The coprecipitation of AcGFP-C and AcGFP-IN with GST-Impα3 was detected by probing the WB with mouse anti-GFP antibody. To detect the IN interaction with DYNLL1 in *in vitro*, 0.2 μg of purified recombinant DYNLL1 protein was incubated with equal amount of purified GST, GST-IN, or GST-MA recombinant protein in 0.12% NP40 lysis buffer. 100 μl of glutathione-Sepharose 4 beads was added to each sample and incubated at 4°C for another 2h. Then, beads were eluted with SDS gel loading buffer and elutes were resolved in 12% SDS-PAGE. The coprecipitation of DYNLL1

with GST, GST-IN, or GST-MA was detected by probing the WB with anti-DYNLL1 antibody. Immunoprecipitation of GST, GST-IN, and GST-MA was detected by probing the WB with anti-GST antibody.

2.2.3 Immunofluorescence Staining

The nuclear localization of AcGFP-IN_{Wt} or various IN deletion or point mutant fusion proteins was examined by immunofluorescence staining. Immunofluorescence staining was carried out as previously described [346]. Briefly, HeLa or COS-7 cells were cultured on 12mm^2 glass coverslips in 24 well formats. AcGFP-$IN_{Wt/Mt}$ expressors were transfected to cells by using lipofectamine 2000 transfection reagent (Invitrogen). After 36h of transfection, cells were fixed with methanol/acetone (1:1 ratio) for 30 minutes at room temperature. Cells were incubated with rabbit anti-GFP primary antibody (1:500) followed by FITC-conjugated anti-rabbit secondary antibody (1:500). The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) (at 10 μg/ml). The immunostaining was examined using fluorescent microscope (AxiovertTM 200; Carl Zeiss), under a 63_Xmagnification objective lens with oil immersion.

2.2.4 The shRNA Mediated Knockdown of Cellular Gene Expression

To KD different Impα isoforms or DYNLL1, lentiviral vectors containing short hairpin RNA (shRNA) for Impα1, Impα3, Impα5, Impα7, and DYNLL1 were obtained from Open Biosystems. Sense oligonucleotide sequences of shRNA for Impα1, Impα3, Impα5, Impα7, and DYNLL1 are as follows: 5'-CTACCTCTGAAGGCTACACT T-3', 5'-GCCCTCTCTTACCTTACTGAT-3', 5'-GCAGTTATTCAAGCGGAGAAA-3', 5'-

GCTGCCATGTTCGATAGTCTT-3’, and 5’-GCGCTGGAGAAATACAACATA-3’, respectively. The VSV-G-pseudotyped, lentiviral vector particles (LVPs) containing individual shRNA were produced in 293T cells by trans-complementation method. Briefly, 293T cells were co-transfected with individual pLKO.1 shRNA, an HIV packaging proteins expressor plasmid (CMVΔ8.2), and a VSV-G expression plasmid. The pLKO.1 vector expressing scramble shRNA or no shRNA was used to produce control LVPs. At 48h of transfection, LVPs were concentrated from the supernatants by ultracentrifugation (126,000xg for 1.5h at 4°C) and LVPs titer was determined by HIVp24^{Gag} ELISA (NCI Fredrick AIDS Vaccine Program). HeLa or C8166T cells were transduced with control or Impα1, Impα3, Impα5, or Impα7 LVPs. After 48h of transduction, cells were cultured in complete DMEM/RPMI medium containing Puromycin (0.5 to 2 μg/ml). After 6 to 7 days of selection, different Impα isoforms KD was examined by individual Impα protein expression in WB, using corresponding antibodies. To KD Impα3 in macrophages, primary human MDMs were transduced with control or Impα3 LVPs twice in 24h time interval. After 4 days of transduction, Impα3 KD was examined in WB, using anti-Impα3 antibody. To KD DYNLL1, C8166T or 293T cells were transduced with LVPs for control or DYNLL1. At 3rd day of transduction, DYNLL1 KD was examined in WB by using anti-DYNLL1 antibody.

2.2.5 WST-1 Cell Proliferation Assay

The WST-1 cell proliferation assay is used to estimate the viable cell content. The stable tetrazolium salt WST-1 is biodegrated into a soluble formazan at the cell surface by a cellular mechanism, which involves the glycolytic production of NAD(P)H in viable

cells. Therefore, the extent of soluble formazan formed in the culture is directly proportional to the number of metabolically active cells in the culutre. The WST-1 assay (Roche) was used to measure the proliferation of C8166T cells transduced with different LVPs. Briefly, control, different Impα isoform, or DYNLL1 LVP-transduced C8166T cells were cultured at a density of $20x10^3$ cells/well in a 96-well format and maintained at 37°C. On different days, WST-1 reagent was added to the cultures at 10 µl/well, the cultures were incubated at 37°C for 4 h and the absorbance was measured at 490 nm using a microplate reader.

2.2.6 Virus Production and Infections

VSV-G-pseudotyped, single cycle replication competent pNL4.3BruΔBgl/Luc^+/R^- and HIVpNL4.3-GFP were produced in 293T cells, as previously described [56, 342]. The production of single cycle replication competent HIV_{Wt}, HIV-$IN_{K215A/K219A/R263A/K264A}$, HIV-$IN_{D64E,}$ HIV-$IN_{Q53A,}$ HIV-$IN_{Q252A,}$ or HIV-$IN_{Q53A/Q252A}$ is previously described [1]. Briefly, HxBruR^-/ΔRI/E^+ provirus was co-transfected with CMV-Vpr-RT-INwt, CMV-Vpr-RT-$IN_{K215/K219A/R263A/K264A}$, CMV-Vpr-RT-$IN_{D64E}$, CMV-Vpr-RT-$IN_{Q53A}$, CMV-Vpr-RT-$IN_{Q252A}$, or CMV-Vpr-RT-$IN_{Q53A/Q252A}$ plasmids. At 48h post transfection, viruses were concentrated from supernatants by ultracentrifugation (126,000xg for 1.5h at 4^0C). The single-cycle-replication competent, luciferase reporter HIVpNL4.3/R^-/Luc^+ (HIV-Luc) was produced in 293T cells by co-transfecting HIVpNL4.3/R^-/E^-/Luc^+ and pLET-Lai. pLET-Lai is a X4 trophic HIV envelope expressor (gifted by Dr. Vicente Planelles, the university of Utah). After 48h of transfection, viruses were concentrated from the supernatant by ultracentrifugation (126,000xg for 1.5h at 4°C). The virus titers were

estimated by HIVp24^{Gag} ELISA (NCI Fredrick AIDS Vaccine Program). VSV-G-pseudotyped MMLV vector particles (MMLV-Luc) were produced by cotransfecting a MMLV based retroviral plasmid (pFB-Luc; purchased from Stratagene) with VSV-G plasmid into MMLV packaging phoenix cell line. After 48h, supernatants containing MMLV particles were filtered using a 0.45 μm-pore size filter.

In this thesis, unless specified otherwise, all virus infections were performed in a 12 well plate format having a growth area of 3.8cm^2 and the cells were incubated with the viruses in a final volume of 500 μl cell culture medium. We have used several different mutant HIV viruses in this study and most of these viruses are defective for replication at specific post entry step(s), which made it not possible to determine the virus titer in terms of TCID50. Therefore, unless specified otherwise, all the virus inoculums were normalized by using virus-associated HIVp24^{Gag} protein content. To study the effect of different Impα subtype KD on HIV replication, 0.5x10^6 control or Impα1, Impα3, Impα5, Impα7-KD HeLa or C8166T cells were infected with equal amounts of VSV-G-pseudotyped, single cycle replication competent pNL4.3BruΔBgl/Luc$^+$/R$^-$ (5-10 ng of p24^{Gag}). At 2h post infection, cells were washed twice and subcultured in complete RPMI at 37^{0}C for 48h. 10^6 cells from each panel were lysed in 50 μl of luciferase (Luc) lysis buffer (Promega) and equal amount lysates (adjusted by protein concentration) were used for Luc assay. The Luc activity was quantified by using POLARstar OPTIMA microplate reader (BMG Labtech, Germany). Luc activity was interpreted as relative light unit (RLU). To determine the effect of Impα3-KD on HIV infection in macrophages, the control or Impα3-KD primary human macrophages were infected with VSV-G-

pseudotyped, single cycle replication competent pNL4.3-Bru-ΔBgl/Luc^+/R^- (at 30 ng of $p24^{Gag}$). At 12h post infection, cells were washed and cultured in complete DMEM at 37°C. At different time intervals, cells were harvested and subjected to Luc assay. To analyze the replication kinetics of HIV in Impα3-KD C8166T cells, control or Impα3-KD C8166T-cells were infected with equal amount of HIVpNL4.3-GFP virus (at 0.02 MOI). At different time intervals, HIV replication was examined by quantifying $HIVp24^{Gag}$ content from supernatants by using HIV $p24^{Gag}$ ELISA. Meanwhile, at 4th day of infection, HIV replication was examined by observing GFP expression under fluorescent microscope or through detection of intracellular $p24^{Gag}$ protein by WB. To determine the infectivity of HIV_{Wt}, HIV-$IN_{K215A/K219A/R263A/K264A}$, HIV-$IN_{D64E,}$ HIV-$IN_{Q53A,}$ HIV-$IN_{Q252A,}$ or HIV-$IN_{Q53A/Q252A}$ virus, 0.5×10^6 C8166T cells were infected with equal amounts of wild type or mutant viruses (5 ng of $p24^{Gag}$). At 2h post infection, cells were washed thrice with medium and cultured in complete RPMI. At different time intervals, $HIVp24^{Gag}$ protein contents from the supernatants were determined by $HIVp24^{Gag}$ ELISA. To test the effect of DYNLL1 KD on HIV replication, $0.5x10^6$ control or DYNLL1-KD C8166T cells were infected with different concentrations of HIV-Luc (3.3 to 30 ng of $p24^{gag}$). Cells were harvested at 24h post infection and subjected to Luc assay. To examine MMLV infection, control, Impα3-KD, or DYNLL1-KD C8166T cells were infected with equal amount of MMLV-Luc and MMLV replication was examined by measuring Luc activity at different time points post infection.

2.2.7 Subcellular Protein Fractionation

The subcellular fractionation of 293T cells was performed using ProteoJETTM

Cytoplasmic and Nuclear Protein Extraction Kit, as described by the manufacturer. Briefly, AcGFP-C or AcGFP-$IN_{Wt/Mt}$ expressors were transfected into 293T cells. At 36h post transfection, cells were harvested by treatment with phosphate buffered saline (PBS)– ethylenediaminetetraacetic acid (EDTA) and cytoplasm and nuclear contents were extracted by treatment with cytoplasmic extraction buffer. The cytoplasm and nuclear lysates were clarified by centrifugation (at 14,000xg for 20min) and both the lysates were resolved in 10% SDS-PAGE. AcGFP-C or AcGFP-$IN_{Wt/Mt}$ protein content in the cytoplasm or nucleus fractions was examined by probing WB with anti-GFP antibody. The effective fractionation of cytoplasm and nuclear contents was determined by probing γ- tubulin protein from each fraction in WB, using anti- γ- tubulin antibody.

2.2.8 qPCR Analysis of HIV Replication

The qPCR analysis was carried out to determine the total viral DNA, 2-LTR circle DNA, and integrated HIV DNA contents from infected cells. Total viral, 2-LTR circle, and integrated HIV DNA represent standard marker for HIV reverse transcription, nuclear import, and integration, respectively [347]. To determine nuclear import defect in Impα3-KD cells, control or Impα3-KD C8166T cells were infected with equal amount of HIVpNL4.3-GFP virus (at 10 ng of virus-associated $p24^{Gag}$). HIVpNL4.3-GFP virus stock was treated with DNAse to remove carryover plasmid DNA contamination. A heat inactivated pNL4.3-GFP^{+} virus (pretreated to $70^{0}C$ for 30 min) infection was included as negative control to monitor carryover plasmid DNA contamination. At 2h post infection, cells were washed twice and cultured in fresh complete RPMI medium. To limit virus replication to single cycle replication, Zidovudine (AZT) (1 μg/ml) was added to the

culture at 12h post infection. At 12 and 24h of infection, 10^6 cells were harvested and genomic DNA was isolated by using QIAamp blood DNA minikit (Qiagen). The total viral, 2-LTR circle, and integrated DNA contents in genomic DNA were determined by Mx3000P real-time PCR system (Stratagene, CA). qPCR for total viral DNA was performed by using primers that amplify the region of HIV *gag* gene not found in PLKO.1 shRNA (Open Biosystems). The PCR reaction was performed with 1xFastStart DNA Master SYBR green I (Roche Diagnostics, Germany) and 0.2 μM of sense (TD-Gag Fr; 5'-ATCAAGCAGCCATGCAAATG-3') and antisense (TD-Gag-Rv; 5'-CTGAAGGGTACTAGTAGTTCC-3') primers. 2-LTR DNA was quantified by using 0.2 μM of MH535 (5'-AACTAGGGAACCCACTGCTTAAG-3' and MH536 (5'-TCCACAGATCAAGGATATCTTGTC-3') primers and the 2-LTR probe FAM-ACACTACTTGAAGCACTCAAGGCAAGCTTT-TAMRA-5'), as previously described [348]. The integrated HIV DNA was measured by an Alu-LTR-nested PCR approach, as previously described [349], with minor modifications. Briefly, the first PCR was done using primers that targeted *Alu* region in human genomic DNA (Alu-Fr; 5'-TCCCAGCTACTCGGGAGGCTGAGG-3') and *gag* gene of HIV cDNA (Int-Gag; 5'-GTCCAGAATGCTGGTAGGGCTATACA-3'). The *gag* region targeted in first PCR is not found PLKO.1 shRNA vector. The second PCR was carried out by using primers for total viral DNA; TD-Gag Fr and TD-Gag Rv. A first PCR without Taq DNA polymerase enzyme was included to track the background amplification from unintegrated HIV DNA. Total viral, 2-LTR circle, and integrated DNA were estimated as copy numbers per cell, with DNA template normalized by *β-globin* gene amplification. The *β-globin* gene was amplified by using 1xFastStart DNA Master SYBR green mix and 0.2 μM each of

Bglo1 (5’-CAACTTCATCACGTTCACC-3’) and glob2 (5’-GAAGAGCCAAGGACA GGTAC-3’) primers.

The qPCR analysis for Impα3 interaction defective IN mutant virus infection was performed as described above. Briefly, $1.5x10^6$ C8166T cells were infected with HIV-$IN_{D64E,}$ or HIV-$IN_{K215A/K219A/R263A/K264A}$ (at 10 ng of virus-associated $p24^{Gag}$). For qPCR analysis of DYNLL1-KD cell infection, $1.5x10^6$ control or DYNLL1-KD C8166T-cells were infected with HIV-Luc (at 10 ng of virus-associated $p24^{Gag}$). For qPCR analysis of DYNLL1 interaction defective IN mutant virus infection, $1.5x10^6$ C8166T cells were infected with HIV_{Wt}, HIV-$IN_{Q53A/Q252A,}$ or HIV-ΔIN (at 50 ng of virus-associated $p24^{Gag}$). A heat inactivated virus control (pretreated to 70°C for 30 min) for each virus stock was included to monitor carryover plasmid DNA contamination. All the virus stocks were treated with DNAseI (340IU/ml) to remove the residual plasmid DNA. At different time interval, cells were harvested and genomic DNA was isolated by using QIAamp blood DNA minikit (Qiagen). The total viral and 2-LTR circle DNA were quantified by Mx3000P real-time PCR system (Stratagene, CA). Total viral DNA was quantified by using the following primer and probe set: TD-Gag-Fr-5′ (5′-ATCAAGCAGCCATGCAAATG-3′), TD-Gag-Rv-3′ (5′- CTGAAGGGTACTAGTAG TTCC-3′), and TD-Gag probe [3′ FAM-ATCAATGAGGAAGCTGCAGAATGGGA-6-TAMRA5′]. 2-LTR DNA was quantified essentially as described above.

2.2.9 Fate of Capsid Assay

The fate of capsid assay was used to examine the HIV uncoating. The fate of capsid assay

was performed as previously described [350]. Briefly, 10^7 control or DYNLL1-KD C8166T cells were infected with equal amount of HIV_{Wt}, HIV-$IN_{Q53A/Q252A,}$ or HIV-ΔIN. In the beginning, cells were incubated on ice (at 4°C) for 20min to mediate the uniform virus attachment, followed by cells were incubated at 37°C to facilitate the infection. At 4h post infection, cells were washed with fresh cell culture medium for five times and cultured in fresh complete RPMI. An additional 10^7 C8166T cells were similarly infected with HIV_{Wt} on ice for 20 min. and harvested as a negative control. At different time intervals, cell were harvested and incubated with 1.5 ml hypotonic lysis buffer (10mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM EDTA) on ice for 15min. Then, cells were separated by centrifugation (800xg for 2min) and supernatants were transferred to fresh tubes. Glass beads were added to each cell pellet and vortexed for 3x10 sec to lyse the cells. The cell lysis was confirmed by observing under microscope. Then, lysates were clarified by centrifugation (3min at 2,000xg) and supernatants were harvested. Supernatants were overlaid onto a 7ml of 50% sucrose cushion (prepared using 1xPBS) and subjected to ultracentrifugation (in Beckman SW41 rotor at 125,000xg for 2h at 4°C). After centrifugation, 100 μl of lysate from topmost layer and pellet fraction dissolved in 100 μl hypotonic lysis buffer were collected from each sample. $HIVp24^{Gag}$ (CA) content from both supernatant and pellet was quantified by using $HIVp24^{Gag}$ ELISA.

2.2.10 Statistical Analyses

The Student's t-test or ANOVA was used to calculate the statistical significance between the samples, and a P value of ≤ 0.05 was considered significant.

Chapter 3.

Investigation of the role of Impα Isoforms in HIV Nuclear Import and the mechanism of Impα recruitment by HIV during replication

3.1 Rationale

As discussed in chapter 1, HIV productively replicates in terminally differentiated non-dividing cells such as macrophages, DCs, and quiescent $CD4^+$ T lymphocytes [184-189]. Active nuclear import of HIV is one of the key requirements for its replication in non-dividing cells. In addition, HIV nuclear import is also important for its replication in dividing cells [201-203]. The successful transport of HIV PIC into the nucleus during the interphase would enhance virus production in dividing cell infection. Interphase is the resting phase of cell cycle at which the nuclear membrane is intact. Thus, nuclear import could be one of the important contributing factors for very high replication rate observed in infected individuals [108, 109]. Additionally, by facilitating HIV replication in macrophages, DCs, and quiescent $CD4^+$ T cells, the nuclear import could also contribute to the efficient HIV transmission at the site of infection and the establishment of an HIV reservoir.

The newly synthesized HIV cDNA enters the nucleus as a component of PIC by active nuclear import and stably integrates into the host cell genome. Previous studies have implicated the components of HIV such as MA [65, 209], Vpr [67, 69, 73, 182, 218-222], IN [56, 57, 203, 210, 231-235, 241, 255], CA [227], and central DNA flap [1, 259, 260] in HIV nuclear import. But some of later studies have contradicted the role of Vpr, MA, and central DNA flap in HIV nuclear import [27, 210-212, 214]. Whether and how CA

contributes to HIV nuclear import is still contradictory (discussed in chapter 1). So far, the accumulated evidences suggest that IN is a key viral factor involved in HIV nuclear import [56, 124, 203, 210, 234, 235]. Although IN was considered as a key viral factor involved in HIV nuclear import, the key contributing cellular factor(s) in HIV nuclear import are not clearly defined. To date, at least three nuclear import factors (Impα1, Imp7, and TNPO3) and one nucleoporin protein (Nup153) have been shown to interact with IN and engage in HIV nuclear import [57, 210, 245, 255]. The role of Imp7 and TNPO3 in HIV nuclear import has been questioned by several recent studies (discussed in chapter 1). In an initial study, Gallay *et al.,* demonstrated the IN interaction with Impα1 and also showed that, by introducing Impα1 interaction defective IN_{K186Q} and $IN_{Q214L/Q216L}$ mutations into virus, disruption of IN and Impα1 interaction affected HIV replication in non-dividing cells [210]. The IN interaction with Impα1 and the Impα1 dependent nuclear accumulation of IN in an *in vitro* nuclear import assay was later confirmed by Hearps and Jans [238]. However, relevance of IN and Impα1 for HIV nuclear import in cells still needs to be verified. Moreover, human cells express six different Impα isoforms; Impα1/Rch1 [140], Impα3/Qip1 [142, 143], Impα4 [142], Impα5 [141, 145, 152], Impα6 [142], and Impα7 [146]. However, it is not known whether Impα isoforms can substitute for one another *in vivo*. Nevertheless, Impα isoforms show specificity for substrates during nuclear import [351-356]. So far, the role of different Impα isoforms in HIV nuclear import and their interaction with IN are not investigated.

In an earlier study, Limon *et al.,* generated a mutant HIV that lacks IN-CTD (HIVΔIN-CTD) and showed that 2-LTR circle but not total viral DNA synthesis was impaired in

HIVΔIN-CTD virus infection, providing a clear evidence for the involvement of IN-CTD in HIV nuclear import [357]. Noticeably, IN-CTD contains highly conserved basic amino acid rich motifs (^{211}KELQKQITK, ^{236}KGPAKLLWK, and ^{262}RRKAK) that closely resemble the classical NLS. Also, IN-YFP$_{1-212}$ fusion protein, an IN-CTD deletion mutant, was defective for nuclear localization by immunostaining [56]. Earlier, a study from our lab showed that the introduction of lysine to alanine substitution mutations into ^{211}KELQKQITK and ^{236}KGPAKLLWK motifs of IN-CTD resulted in reduced HIV nuclear import [56]. Recently, another study from our laboratory showed the requirement of ^{236}KGPAKLLWK and ^{262}RRKAK motifs in IN for Imp7 interaction [57]. However, Imp7 do not paly a significant role in HIV nuclear import [57]. Therefore, the mechanism by which ^{211}KELQKQITK, ^{236}KGPAKLLWK, and/or ^{262}RRKAK motifs of IN engaged in HIV nuclear import is still an open question. It is possible that these putative NLSs in IN-CTD would mediate the Impα interaction and contribute to the HIV nuclear import.

3.2 Hypothesis

Based on the above discussion, we make the following hypotheses. HIV nuclear import is mediated by specific Impα isoform(s) and Impα isoforms are involved in HIV nuclear import by interacting with IN. The putative NLSs of IN-CTD (^{211}KELQKQITK, ^{236}KGPAKLLWK, and/or ^{262}RRKAK) will mediate Impα interaction and HIV nuclear import.

3.3 Objectives

3.3.1 Identification of functionally significant Impα isoform(s) for HIV replication

3.3.2 Analysis of the requirement of Impα isoform(s) for HIV nuclear import

3.3.3 Investigation of the viral protein(s) interaction with Impα isoform(s)

3.3.4 Characterization of IN and Impα interaction by substitution mutagenesis and -proteins interaction analyses

3.3.5 Analysis of the requirement of Impα interaction motif/s in IN for HIV replica -tion and nuclear import

3.4 Results

3.4.1 Effect of Impα1, Impα3, Impα5, or Impα7 KD on HIV Replication in HeLa Cells

Impα isoforms KD and infection analysis was carried out to identify the functionally significant Impα isoform for HIV replication. Impα1, Impα3, Impα5, or Impα7 was KD in HeLa cells by using shRNA lentiviral vector system (pLKO1 lentiviral vector). Briefly, LVPs containing shRNA for Impα1, Impα3, Impα5, or Impα7 were produced in 293T cells, as described in the Materials and Methods. $0.2x10^6$ HeLa cells were transduced with equal amount of LVPs. LVPs containing Sc shRNA was included as control. After 48 h of transduction, cells were cultured in complete DMEM supplemented with Puromycin (2 µg/ml). After 6 days of selection, KD for individual Impα isoform was examined by WB using the corresponding antibodies. Results showed about 90-95% KD for Impα1, Impα3, or Impα5 and about 70% KD for Impα7 compared to control (scramble) **(Figure 7A).** It was not possible to compare the relative expression levels between Impα isoforms due to differences in antibody affinities. Subsequently, control or Impα-KD cell lines were infected with equal amount of VSV-G-pseudotyped luciferase

reporter HIV (HIVpNL-BruΔBgl/Luc^{+}) (at 10 ng of virus-associated p24^{Gag} antigen). After 48h of infection, HIV infection was examined by measuring Luc activity, as described in the Materials and Methods. We observed about 50% reduction in HIV replication in Impα1 and Impα5-KD cells compared to Sc control cells, whereas about 3- to 3.5-fold reduced HIV replication was evident in Impα3-KD cells **(Figure 7B).** On the contrary, HIV replication in Impα7-KD cells showed no change **(Figure 7B).** Since *luc* gene was replaced with *nef* gene in HIV pNL-BruΔBgl/Luc^{+} virus, the above results suggest that Impα1, Impα3, or Impα5 may be involved in the steps of early stage HIV replication at or prior to gene expression. Since Impα3-KD showed a relatively higher reduction in HIV replication, we further explored the role of Impα3 in HIV replication. First, HIV transcription and gene expression were examined in Impα3-KD cells. Briefly, control or Impα3-KD HeLa cells were transfected with HIVpNL-BruΔBgl/Luc^{+} provirus DNA. In parallel, Impα3-KD HeLa cells were transfected with a MMLV vector plasmid (pBpSTR-Luc^{+}) in which *luc* gene expression is driven by a cytomegalovirus (CMV) promoter. After 48h of transfection, Luc activity was measured. In HIVpNL-BruΔBgl/Luc^{+} or pBpSTR-Luc^{+} transfected cells, we found no difference in Luc activity between Impα3-KD and Sc control cells **(Figure 7C),** indicating unimpaired HIV transcription and/or gene expression in Impα3-KD cells. Together, these results suggested that Impα3 is essential for steps of early stage HIV replication such as reverse transcription, nuclear import, and/or integration.

3.4.2 Wild Type HIV Infection was Impaired in Impα3-KD CD4^{+} C8166T Cells

In this part, the requirement of Impα subtypes for HIV replication was examined in CD4^{+}

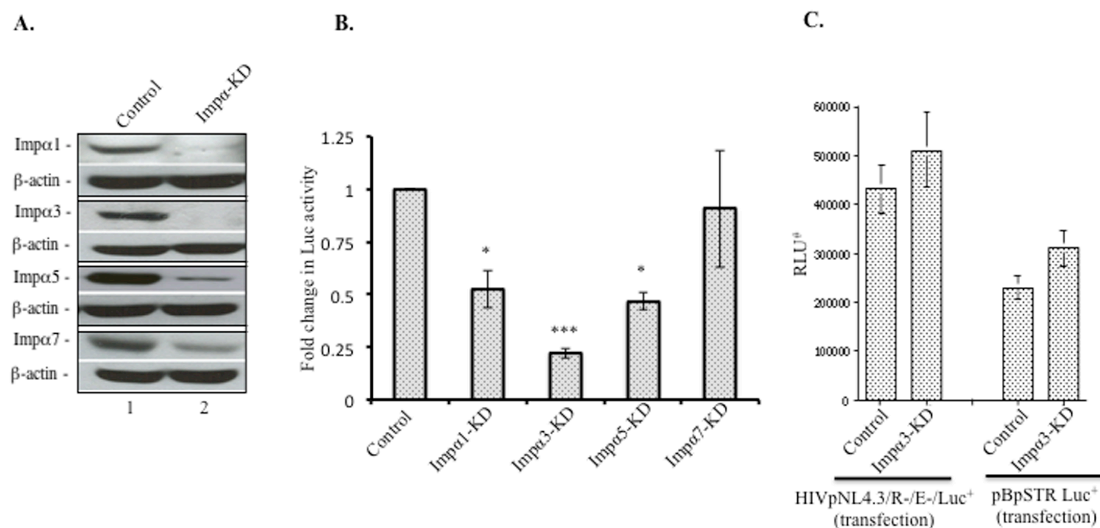

Figure 7. Effect of Impα1, Impα3, Impα5, or Impα7-KD on HIV replication in HeLa cells: (A) HeLa cells were transduced with LVPs that express shRNA for Impα1, Impα3, Impα5, Impα7, or scramble (control). Two days after transduction, cells were selected with Puromycin (2 μg/ml) for 5 days and the extent of KD for Impα1, Impα3, Impα5, or Impα7 was examined by WB using corresponding antibodies. The β-actin protein was detected by WB as an internal control. (B) $0.2x10^6$ control or different Impα isoform-KD HeLa cells were incubated with VSV-G-pseudotyped luciferase-reporter HIV (pNL-BruΔBgl/R^-/Luc^+) (at 10 ng of virus-associated $p24^{Gag}$ antigen). At 48h post infection, cells were subjected to Luc activity analysis. The data shown are fold change in Luc activity as compared to control, the sample means and standard errors were drawn from three independent experiments. The one way ANOVA analysis was performed to determine the statistical significance. *P<0.05, ***P<0.001. (C) Control or Impα3-KD HeLa cells were transfected with the pNL-BruΔBgl/E^-/Luc^+ provirus or a MMLV expression plasmid having a *luc* reporter gene (pBp-STR Luc^+). At 48h post transfection, cells were subjected to Luc activity analysis. Data shown are means and standard errors and are representative of the results for triplicate samples from two independent experiments. # Relative light unit.

C8166T-cells. C8166T-cells were transduced with LVPs for Sc control, Impα1, Impα3, or Impα5. After 1 week of Puromycin selection, we observed a KD efficiency of about 90% for different Impα subtypes **(Figure 8A)**. Impα subtypes KD was examined by probing WB with corresponding antibodies. Importantly, specific KD of any one Impα subtype did not affect the expression of other Impα subtypes **(Figure 8A)**. However, stable KD for Impα subtypes only lasted 3 to 4 weeks, even under continuous Puromycin selection. Therefore, all experimental analyses in Impα KD cells were carried out between 1 to 3 weeks of selection. First, the effect of Impα subtypes KD on proliferation of C8166T cells was examined by using WST-1 assay (**Figure 8B)**, as described in the Materials and Methods. Results showed that Impα5-KD C8166T cells proliferated similar to that of control. However, the proliferation of Impα1 or Impα3-KD C8166T cells was moderately reduced, even though Impα1 or Impα3-KD was not lethal to cells. Next, to examine the requirement of Impα1, Impα3, or Impα5 for HIV replication, Impα-KD C8166T cells were infected with equal amount of VSV-G-pseudotyped luciferase reporter HIV (pNL-BruΔBgl/Luc^{+}) (at 10 ng of virus-associated p24^{Gag} antigen). Results concluded that Impα3-KD resulted in relatively higher reduction in HIV replication (4-fold), while only 50-60% reduction in HIV replication was evident in Impα1 or Impα5-KD cells **(Figure 8C).** Clearly, these results are consistent with the data obtained in Impα-KD HeLa cells. To further examine whether Impα3 requirement is only restricted to HIV replication, MMLV replication analysis was carried out in Impα3-KD C8166T cells. Briefly, control or Impα3-KD C8166T cells were transduced with VSV-G-pseudotyped luciferase reporter MMLV particles. At 48 and 72h post transduction, MMLV replication was examined by measuring Luc activity. Interestingly, unlike in HIV

infection, MMLV replication was only reduced by 40-50% in Impα3-KD cells when compared to control cells (**Figure 8D**). It is possible that relatively slower proliferation of Impα3-KD cells might have caused the moderate reduction in MMLV replication, as MMLV lacks active nuclear import capacity and requires cell division to access nuclear compartment (discussed in chapter 1). Therefore, based on the above data, we conclude that Impα3 is required for HIV replication in proliferating $CD4^+$ C8166T cells.

In order to examine the multiple round HIV replications in Impα3-KD cells, $0.5x10^6$ Impα3-KD or control C8166T cells were infected with wild type HIV ($pNL4.3\text{-}GFP^+$) (at 0.02 MOI). At different time intervals of post infection, HIV replication was monitored by measuring HIV $p24^{Gag}$ production in supernatants using $HIVp24^{Gag}$ ELISA. In control cells, HIV production progressed quickly and reached peak at day 4-post infection, which coincided with the rapid killing of cells due to syncytium formation and cytopathic effect **(Figure 9A)**. In contrast, viral infection was significantly attenuated in Impα3-KD cells. Impα3-KD cell infection showed no $p24^{Gag}$ protein production in the first 3 days of infection and only low level of $p24^{Gag}$ was detected at 4th and 5th day of infection by ELISA (**Figure 9A**) and by WB (**Figure 9B, right side**). Similar results were also obtained by examining GFP expression under fluorescence microscopy (**Figure 9B, left side**). However, at 6 days post infection, viral production was detected in Impα3-KD cells, which may be attributed to the build-up of higher virus load in the culture. In order to exclude the possibility of altered CD4 receptor expression for defective HIV replication in Impα3-KD cells, CD4 receptor expression on the surface of Impα3-KD and Sc control C8166T cells were measured by FACS analysis. CD4 receptor expression was

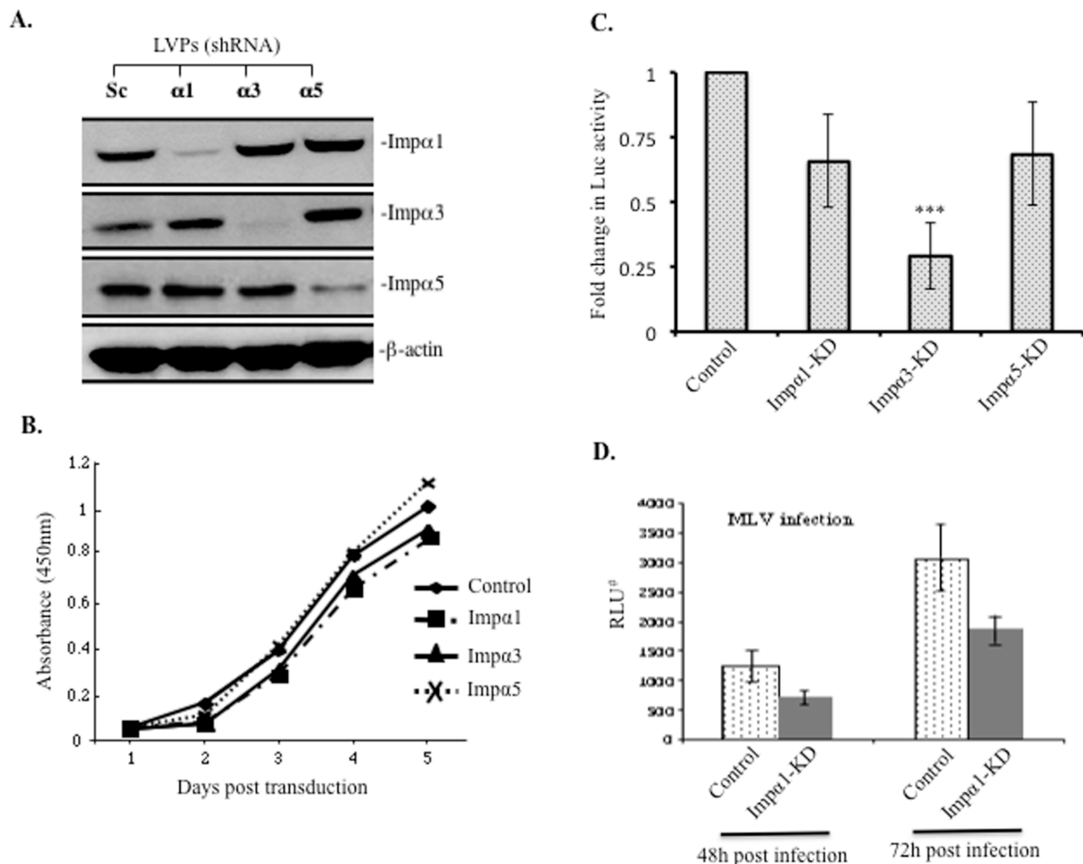

Figure 8. Impα1, Impα3, or Impα5-KD in CD4^{+} C8166T cells and VSV-G-pseudotyped HIV infection: (A) C8166T cells were transduced with LVPs that express shRNA for Impα1, Impα3, Impα5, or control. After 2 days of transduction, cells were selected with Puromycin (0.5 µg/ml) for 5 days and KD for Impα1, Impα3, or Impα5 was examined by WB, using corresponding antibodies. The β-actin protein was detected by WB as an internal control. (B) A WST-1 assay was performed to determine the proliferation of Impα1, Impα3, or Impα5-KD cells at different time points as indicated. (C) 0.5 x106 Impα1, Impα3, Impα5, or control-KD C8166T cells were infected with VSV-G-pseudotyped, Luc-reporter HIV (pNL-Bru-Luc+/E-) (at 10 ng of virus-associated p24Gag antigen). After 48h of infection, equal amounts of the cell lysates were subjected to Luc activity analysis. The data shown are fold change in Luc activity as compared to control, the sample means and standard errors were drawn from four independent experiments. The one way ANOVA analysis was performed to determine the statistical significance, ***$P<0.001$. (D) 0.5 x10^{6} control or Impα3-KD C8166T cells were infected with a VSV-G-pseudotyped, luciferase reporter MMLV vector particles and Luc activity was analyzed at 48h and 72h post infection. Data shown are means and standard errors and are representative of the results of triplicate samples from a typical experiment, which were confirmed in two other independent experiments. # Relative light unit.

not altered in control or Impα3-KD cells (**Figure 9C**). Taken together, data suggested that HIV replication and spread was impaired in Impα3-KD in cells.

3.4.3 HIV Nuclear Import was Impaired in Impα3-KD Cells

In order to identify the HIV replication step(s) that are impaired in Impα3-KD cells, we examined the synthesis of HIV total viral DNA, 2-LTR circle DNA, and integrated DNA in Impα3-KD cells by qPCR. HIV total viral DNA, 2-LTR circle DNA, and integrated DNA are standard markers for HIV reverse transcription, nuclear import, and integration, respectively [347]. Briefly, control or Impα3-KD C8166T cells were infected with HIVpNL4.3-GFP^{+} virus (at 10 ng of virus-associated p24^{Gag} antigen), as described in the Materials and Methods. At 12h and 24h of infection, cells were subjected to qPCR analysis, as described in the Materials and Methods. Interestingly, while a similar level of total viral DNA was detected in control and Impα3-KD cells **(Figure 10A, top and bottom panels)**, a 3-5-fold reduced 2-LTR circle DNA was detected in Impα3-KD cells at both 12h and 24h post infection **(Figure 10B, top and bottom panels).** Also, about 7-fold reduced integrated DNA was detected in Impα3-KD cells at 24h post infection **(Figure 10C)**, but no integrated DNA was detectable in either control or Impα3-KD cells at 12h post infection. This reduced 2-LTR circle and integrated DNA are well correlated with the level of attenuated HIV replication in Impα3-KD C8166T cells. Together, these data helped to conclude that Impα3 is specifically required for HIV nuclear import.

3.4.4 HIV IN Interacts with Impα3

Although above results clearly suggested that Impα3 is involved in HIV nuclear import,

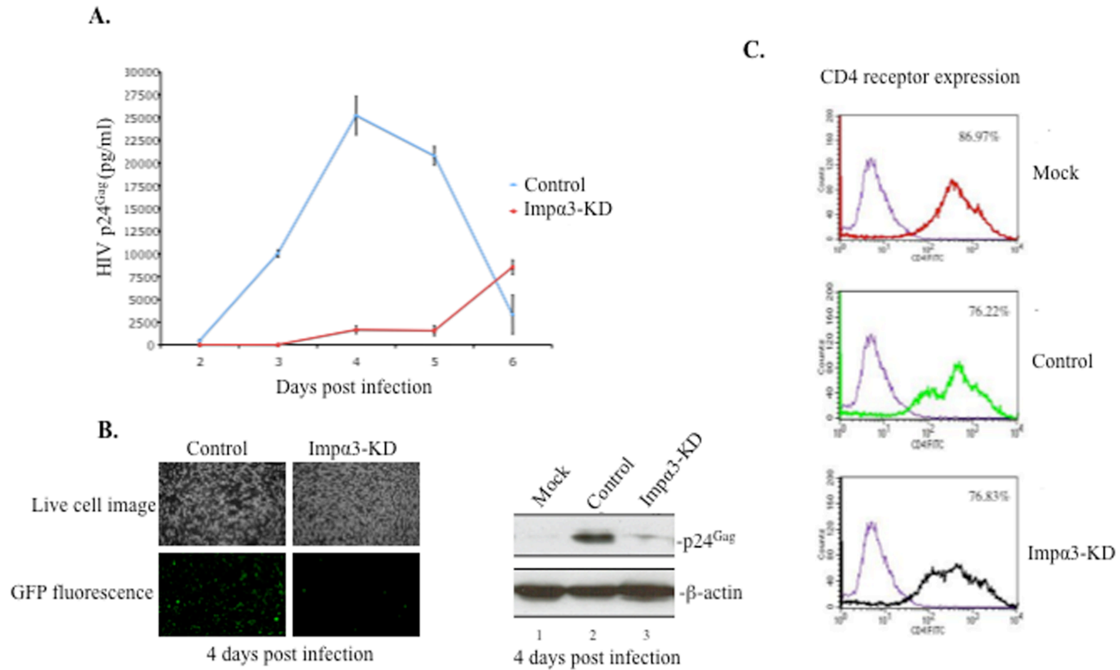

Figure 9. Infection of wild type HIV was impaired in Impα3-KD CD4⁺ C8166T cells. (A) $0.5x10^6$ control or Impα3-KD C8166T cells were infected with HIVpNL4.3-GFP at a MOI of 0.02. At various days post infection (*x* axis), supernatants were collected and $HIVp24^{Gag}$ protein concentrations from supernatants were measured by $HIVp24^{Gag}$ ELISA. (B) Control or Impα3-KD C8166T cells were similarly infected with HIVpNL4.3-GFP and GFP fluorescence was examined under fluorescent microscope (left side figure) or $p24^{Gag}$ protein was detected by WB (right side figure), at 4 days post infection. (C) The surface expression of CD4 receptor in mock, Impα3, or control LVP transduced C8166T cells was determined by anti-CD4 staining and FACS analysis.

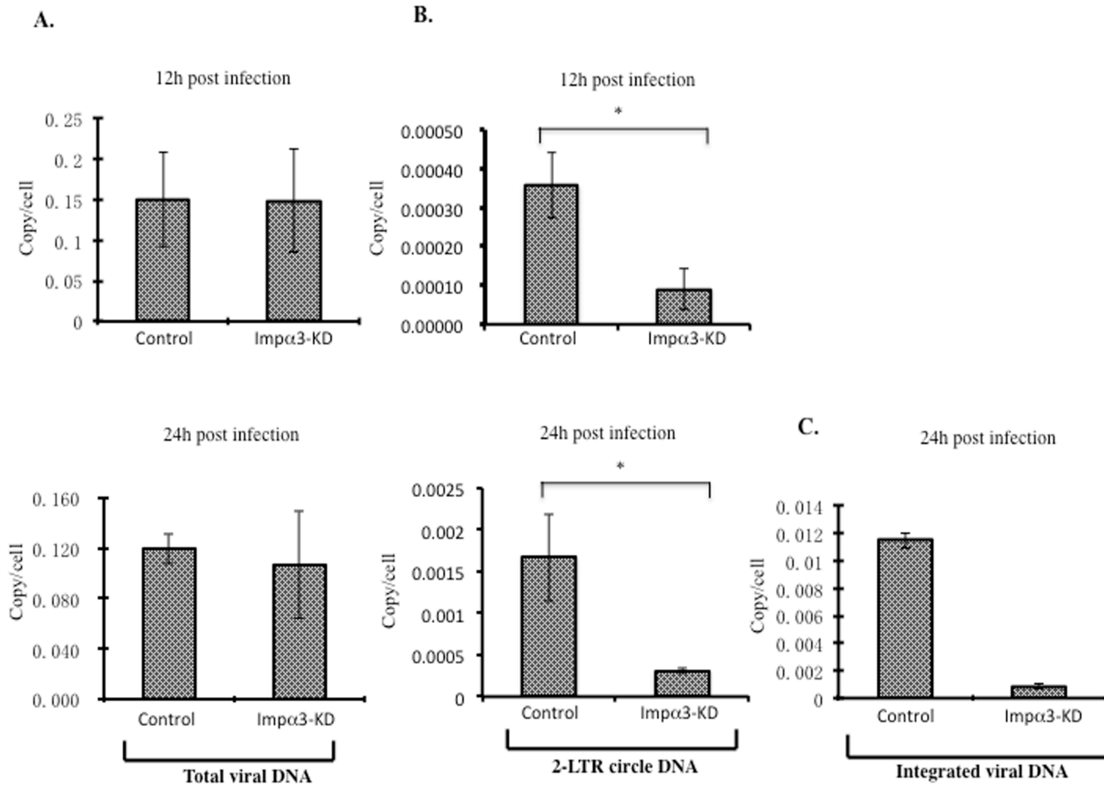

Figure 10. HIV nuclear import was impaired in Impα3-KD cells: Control or Impα3-KD C8166T cells were infected with HIVpNL4.3-GFP (at 10 ng of virus-associated $p24^{Gag}$ antigen). At 12h and 24h post infection, cells were harvested and HIV total viral DNA (A, upper and lower panels), 2-LTR circles DNA (B, upper and lower panels), and integrated DNA (C) were quantified by qPCR, as described in the Materials and Methods. Data shown are means and standard errors and are representative of the results for triplicate samples from a typical experiment. The data was confirmed in three independent experiments. Statistical significance was determined by Student's t-test, *$P<0.05$ (N=3).

how HIV recruits Impα3 was still an open question. Predictably, nucleophilic viral proteins of PIC could interact with Impα3 and recruit Impα3 for HIV nuclear import. Since IN is viewed as a primary mediator for HIV nuclear import (discussed in rationale section), we focused on IN interaction with Impα3. First, the cell lysate prepared from AcGFP-IN expressing 293T cells was incubated with Impα3-GST or GST alone. The AcGFP-IN interaction with Impα3-GST or GST alone was examined by *in vitro* pull down assay, as described in the Materials and Methods. Results showed that recombinant Impα3-GST fusion protein but not GST alone was interacted with AcGFP-IN **(Figure 11A, left side)**. To demonstrate direct protein-protein interaction between IN and Impα3, the interaction of Impα3-GST or GST alone with recombinant IN protein was examined, as described in the Materials and Methods. Results showed that purified IN protein was able to co-precipitate with Impα3-GST but not GST alone **(Figure 11A, right side)**. To confirm IN and Impα3 interaction in cells, the interaction between AcGFP-IN and T7-Impα3 was examined in 293T cells by Co-IP, as described in the Materials and Methods. In parallel, T7- Impα3 interaction with other nucleophilic viral proteins such as MA and Vpr was analyzed. Results showed that T7-Impα3 co-precipitated with AcGFP-IN and Vpr-YFP but not with AcGFP or MA-YFP **(Figure 11B).** These data indicated that both IN and Vpr can interact with Impα3. Then, intracellular localization of these fusion proteins was examined in HeLa cells by immunofluorescence staining, as described in the Materials and Methods. While AcGFP-IN and Vpr-YFP were localized predominately in cell nucleus, MA-YFP was only located outside the nucleus **(Figure 11C)**, confirming an earlier observation [232]. To further test IN and Impα3 interaction in HIV infected cells, 10^7 C8166T cells were infected with HxBru or HxBru-IN-HA virus and Impα3

interaction with IN-HA was examined as described in the Materials and Methods. Results showed that endogenous Impα3 was successfully co-precipitated with IN-HA in HxBru-IN-HA but not in HxBru virus infected cells **(Figure 11D, top and middle rows, compare lanes 1 and 2).** A similar level of infection was detected by probing the direct cell lysate for $HIVp24^{Gag}$ in WB **(Figure 11D, bottom row, compare lanes 1 and 2).** These results help to confirm the interaction between IN and Impα3 in HIV infected $CD4^{+}$ T cells. Together, the above data suggests that Impα3 is a novel cellular co-factor for IN interaction.

3.4.5 The IN-CTD is Involved in Impα3 Interaction

Following successful identification of IN interaction with Impα3, the next logical step was to define the minimum region in IN for Impα3 interaction. The identification of the minimum region in IN for Impα3 interaction was also essential for further characterization of the IN and Impα3 interaction and its requirement for HIV nuclear import. To delineate the minimum region in IN for Impα3 interaction, AcGFP-IN, GFP-IN_{50-288} (NTD deletion) or GFP-IN_{1-212} (CTD deletion) expressors were co-transfected with T7-Impα3 expressor in 293T cells and their interaction was examined by anti-GFP immunoprecipitation and Impα3 interaction analysis, as described in the Materials and Methods. Interestingly, AcGFP-IN_{50-288} showed wild type like interaction with T7-Impα3 **(Figure 12A, compare lanes 2 and 4).** However, CTD deletion mutant, AcGFP-IN_{1-212}, lost the interaction with T7-Impα3 **(Figure 12A, lane 3),** suggesting the requirement of Impα3 interaction, T7-Impα3 interaction analysis with two CTD deletion mutants, GFP-IN_{1-250} and GFP-IN_{1-270}, was performed. Interestingly, both GFP-IN_{1-212} and GFP-IN_{1-250}

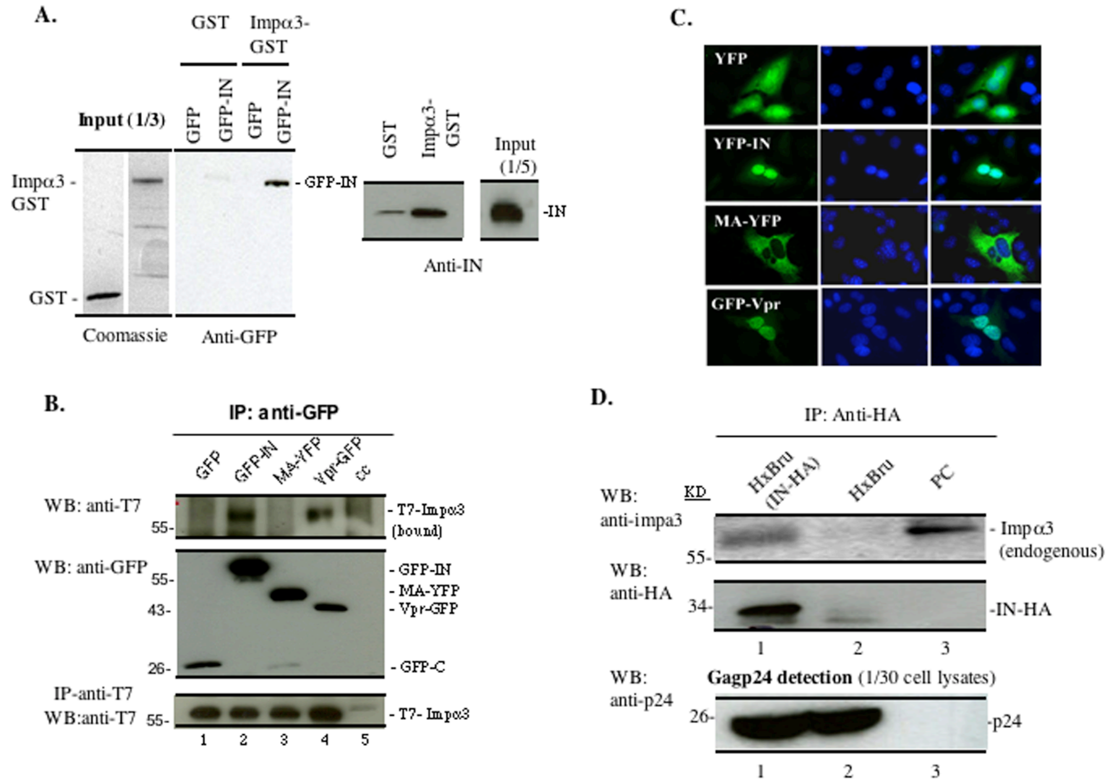

Figure 11. HIV IN interacts with Impα3: (A; left side) 293T cells expressing AcGFP or AcGFP-IN were lysed and the lysates were incubated with GST or GST-Impα3 recombinant protein and samples were subjected to GST pull-down assay. The co-precipitation of AcGFP or AcGFP-IN protein was detected by WB using an anti-GFP antibody. (A; right side) Equal amount of GST or GST-Impα3 was incubated with purified recombinant HIV IN and samples were subjected to GST pull-down analysis. The co-precipitation of IN was detected by WB using an anti-IN antibody. (B) T7-Impα3 expresser was co-transfected with AcGFP, AcGFP-IN, MA-YFP, or Vpr-YFP expressor into 293T cells. After 48h of transfection, cells were subjected to immunoprecipitation with anti-GFP antibody. The co-precipitation of T7-Impα3 was detected by WB using an anti-T7 antibody (top row). AcGFP, AcGFP-IN, MA-YFP, or Vpr-YFP in the immunoprecipitates was detected by WB using an anti-GFP antibody (middle row). T7-Impα3 expression in the total cell lysates was detected by WB using an anti-T7 antibody (bottom row). (C) HeLa cells were transfected with AcGFP, AcGFP-IN, MA-YFP, or Vpr-YFP expressors. After 48h of transfection, cells were fixed and immunostained with anti-GFP antibody followed by FITC conjugated anti-rabbit antibody. The cell nucleus was stained with DAPI. Immunostaining was analyzed under the fluorescence microscope (60x objective lens with oil immersion). (D) 10^7 C8166T cells were infected with an HxBru or HxBru-IN-HA virus. After 72h of infection, cells were subjected to immunoprecipitation using an anti-HA antibody and the co-precipitation of Impα3 was detected by WB using an anti-Impα3 antibody (top row). The uninfected C8166T cell lysate was used as a positive control (PC) (top row, lane 3). The immunoprecipitation of IN-HA was detected by WB using an anti-HA antibody (middle row). HIV $p24^{Gag}$ protein in the total cell lysates was detected by WB using an anti-$p24^{Gag}$ antibody (bottom row).

mutants were unable to interact with T7-Impα3, whereas GFP-IN_{1-270} mutant showed wild IN-CTD for Impα3 interaction. To further define minimum region(s) in IN-CTD for type of interaction with T7-Impα3 **(Figure 12B, top row, lane 2-5)**. These data suggested that Aa212-270 region of IN is required for Impα3 interaction. Following the delineation of minimum region of IN for Impα3 interaction, the intracellular localization of Impα3 interaction defective IN deletion mutants was examined by immunofluorescence staining. Briefly, AcGFP-IN_{Wt} or various IN deletion mutant expressors were transfected to HeLa cells. After 48h of transfection, cells were immunostained for anti-GFP antibody followed by FITC-conjugated anti-rabbit secondary antibody. Interestingly, both AcGFP-IN_{1-212} and AcGFP-IN_{1-250} failed to localize in the nucleus, whereas AcGFP-IN_{1-270} and AcGFP-IN_{Wt} were localized in the nucleus **(Figure 12C).** Thus, these data support our claim that Aa212-270 region of IN is involved in Impα3 interaction and IN nuclear localization.

3.4.6 Impα3 is Essential for HIV Replication in Human Primary Macrophages

The productive infection of HIV in non-dividing cells such as macrophages requires active nuclear import of PIC. As above data showed the involvement of Impα3 in HIV nuclear import in proliferating T cells, it is interesting to know whether Impα3 is also essential for HIV replication in macrophages. Monocyte derived macrophages (MDMs) were obtained from healthy human volunteers, as described in the Materials and Methods. MDMs were transduced with equal amount of control or Impα3 LVPs. After 4 days of transduction, Impα3 KD was examined by WB analysis. Impα3 LVPs transduced MDMs showed 75-80% KD for Impα3 when compared to Sc LVPs transduced MDMs

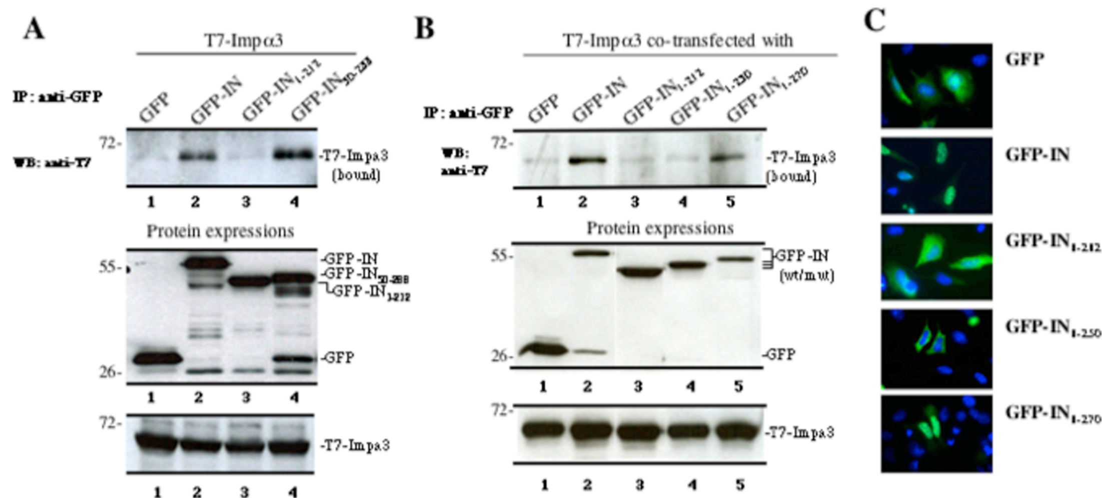

Figure 12. The IN-CTD is Involved in Impα3 Interaction: (A) AcGFP, AcGFP-IN, AcGFP-IN_{1-212}, or AcGFP-IN_{50-288} expressor was co-transfected with the T7-Impα3 expressor in 293T cells. At 48h of post transfection, cells were subjected to anti-GFP immunoprecipitation and the co-precipitation of T7-Impα3 was detected by WB using an anti-T7-antibody (top row). The immunoprecipitation of AcGFP, AcGFP-INwt, or various AcGFP-IN deletion mutants was detected by WB using an anti-GFP antibody (middle row). The expression of T7-Impα3 in the total cell lysates was detected by WB using an anti-T7 antibody (bottom row). (B) AcGFP, AcGFP-IN_{Wt}, AcGFP-IN_{1-212}, AcGFP-IN_{1-250} or AcGFP-IN_{1-270} were co-transfected with T7-Impα3 expressor in 293T cells and cells were subjected to immunoprecipitation using an anti-GFP antibody. The co-precipitation of T7-Impα3 was detected by WB using an anti-T7 antibody (top row). The AcGFP, AcGFP-INwt, or AcGFP-$IN_{deletion\ mutant}$ proteins in immunoprecipitates were detected by WB using an anti-GFP antibody (middle row). The expression of T7-Impα3 in the total cell lysates was detected by WB using an anti-T7 antibody (bottom row). (C). HeLa cells were transfected with AcGFP, AcGFP-IN, AcGFP-IN_{1-212}, AcGFP-IN_{1-250}, or AcGFP-IN_{1-270} expressor. After 48h of transfection, cells were fixed and immunostained using an anti-GFP antibody followed by FITC conjugated anti-rabbit antibody. The cell nucleus was stained with DAPI. The immunostaining was analyzed under fluorescent microscope (60x objective lens with oil immersion).

(Figure 13A, top row). At the same time, cell morphology for both LVP transduced and non-transduced MDMs was observed under microscope. No visible change in morphology was evident between control and Impα3 LVPs transduced MDMs **(Figure 13A, bottom panel).** Control or Impα3-KD MDMs from two independent donors were infected with VSV-G-pseudotyped, luciferase reporter HIV (pNL-BruΔBgl/R^-/Luc^+) (at 30 ng of virus-associated $p24^{Gag}$ antigen). At different time points (3, 5, 7, and 9 days for donor 1; day 7 for donor 2) post infection, HIV replication was examined by measuring Luc activity **(Figure 13B and 13C).** In control of donor 1, HIV replication reached peak at day 7 after infection. In contrast, HIV was unable to replicate in Impα3-KD MDMs **(Figure 13B).** At peak virus replication, a 10-fold difference in HIV replication was evident between control and Impα3-KD MDMs. A similar difference in HIV replication was also seen in the infection donor 2 MDMs **(Figure 13C)**. These data clearly indicate that Impα3 is required for productive HIV replication in macrophages. As Vpr also interacted with Impα3, the possible role of Vpr in the requirement of Impα3 for HIV replication was also examined. Control or Impα3-KD MDMs were infected with VSV-G-pseudotyped, Vpr^+ or Vpr^- HIVpNL-BruΔBgl/Luc^+ viruses (at 30 ng of virus-associated $p24^{Gag}$ antigen) and the Luc activity was measured after 7 days of infection. In agreement with previous reports, requirement of Vpr for productive replication in MDM was evident, as the overall replication of Vpr^- virus was 4- to 5-fold reduced than Vpr^+ virus in control MDM **(Figure 13D, compare bar 3 with bar 1)**. However, interestingly, results showed similar levels of attenuated virus replication were detected in Impα3-KD cells infected with Vpr^+ or Vpr^- HIV. In Vpr^+ or Vpr^- HIV infection, HIV replication in Impα3-KD cells was reduced by about 7.6 and 6.6-folds, respectively **(Figure 13D,**

compare bar 2 with bar 4). These data clearly suggested that the attenuated HIV replication in Impα3-KD macrophages was Vpr independent. Even though there is a possibility of donor dependent variation in HIV susceptibility, there was a similar level of attenuated HIV replication in Impα3-KD MDMs from different donors, and these results are in agreement with the data obtained from HeLa or C8166T cell line infections. Based on the above findings, we concluded that Impα3 is functionally significant nuclear import receptor for HIV replication and/or nuclear import and HIV would recruit Impα3 for nuclear import through interaction with its IN protein.

3.4.7 The Conserved Basic Amino Acid Rich Motifs in IN-CTD are Required for Impα3 Interaction

In order to gain more insight into the requirement for IN and Impα3 interaction in HIV nuclear import, we proposed to identify Impα3 interaction motif(s) in IN. Proteins interact Impα through a stretch of highly conserved basic amino acid rich motif called classical NLS [358-360]. As IN-CTD is involved in Impα3 interaction, we chose to examine the requirement of putative NLSs of IN-CTD (^{211}KELQKQITK, ^{236}KGPAKLLWK, or ^{262}RRKAK) for Impα3 interaction. Lysine (K) or Arginine (R) to alanine (A) mutations were introduced into each of these putative NLSs (AcGFP-$IN_{K215A/K219A}$, AcGFP-$IN_{K240A/K244A}$, and AcGFP-$IN_{R263A/K264A}$) **(Figure 14A)** and their interactions with PL-Impα3 were studied by chemiluminascent Co-IP assay, as described in the Materials and Methods. Interestingly, we found a specific but moderate reduction in PL-Impα3 interaction with AcGFP-$IN_{K215A/K219A}$ and AcGFP-$IN_{R263A/K264A}$, but not with AcGFP-$IN_{K240A/K244A}$ **(Figure 14B, upper panel).** PL-Impα3 or IN mutants showed

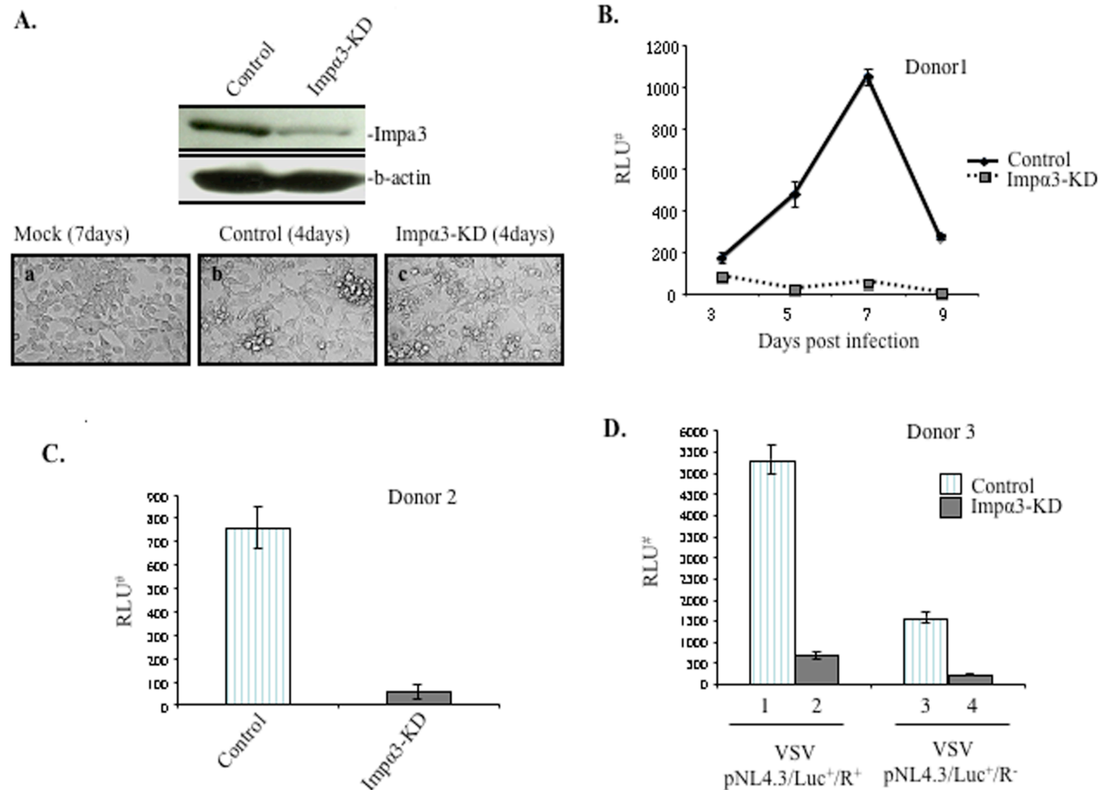

Figure 13. Impα3 is required for HIV replication in human primary macrophages: (A) MDMs from different donors were transduced with equal amounts of LVPs for control or Impα3. At day 4 post-transduction, Impα3 expression was detected by WB, using an anti-Impα3 antibody (top panel). β-actin protein was detected as an internal control. The morphology of non-transduced (a), control LVP transduced (b), and Impα3 LVP transduced (c) MDMs were observed under a microscope with a 20x magnification (bottom panel). (B and C) Control or Impα3-KD MDMs from donor 1 or donor 2 were infected with VSV-G-pseudotyped luciferase reporter HIVpNL-Bru-Luc^{+}/R^{-}. At various days post infection (donor 1) or at day 7 of post infection (donor 2), cells were subjected for Luc activity analysis. (D) Control or Impα3-KD MDMs from donor 3 were infected with a VSV-G-pseudotyped luciferase reporter HIVpNL-Bru-Luc^{+}/R^{-} or VSV-G-pseudotyped, luciferase reporter HIVpNL-Bru-Luc^{+}/R^{+} viruses. After 7 days of infection, cells were collected and equal amount of the cell lysates were analyzed for Luc activity. # Relative light unit.

similar level expression **(Figure 14B, lower panel; data not shown for IN mutant expression**), ruling out the difference in interaction due to variation in protein expression. From the above data, it was evident that ^{211}KELQKQITK and ^{262}RRKAK regions in IN serve as NLSs for Impα3 interaction. Since ^{211}KELQKQITK and ^{262}RRKAK regions in IN are separated by relatively long stretch of linker amino acids (approximately 40 amino acids), these regions may constitute a non-conventional bipartite NLS for Impα3 interaction. To gain more insight into the mechanism of IN and Impα3 interaction, we generated YFP-$IN_{K215A/K219A/R263A/K264A}$ mutant expressor and probed its interaction with PL-Impα3 by chemiluminescent Co-IP assay. YFP-$IN_{K215A/K219A/R263A/K264A}$ showed a severely attenuated interaction with PL-Impα3 as compared to AcGFP-$IN_{K215A/K219A}$ or AcGFP-$IN_{R263A/K264A}$ protein alone **(Figure 14C, top panel).** To rule out the non-specific or general negative effects of mutations on IN interaction with other proteins, we examined YFP-$IN_{K215A/K219A/R263A/K264A}$ interaction with some of the known IN interacting proteins such as PL-TNPO3 and HA-Integrase interactor 1 (INI1/hSNF5) by Co-IP, as described in the Materials and Methods. INI1/hSNF5 is a component of chromatin remodeling switch/sucrose non-fermentable complex and was identified as a cellular co-factor for IN interaction and early stage HIV replication [361, 362]. Interestingly, both AcGFP-IN_{Wt} and YFP-$IN_{K215A/K219A/R263A/K264A}$ similarly interacted with PL-TNPO3 or HA-INI1 **(Figure 15A and 15B)**. These results indicated that YFP-$IN_{K215A/K219A/R263A/K264A}$ is specifically impaired for the interaction with Impα3. Together, above data unraveled the requirement of ^{211}KELQKQITK and 262RRKAK motifs of IN-CTD for Impα3 interaction and therefore, these motifs can serve as a non-conventional bipartite NLS for Impα3.

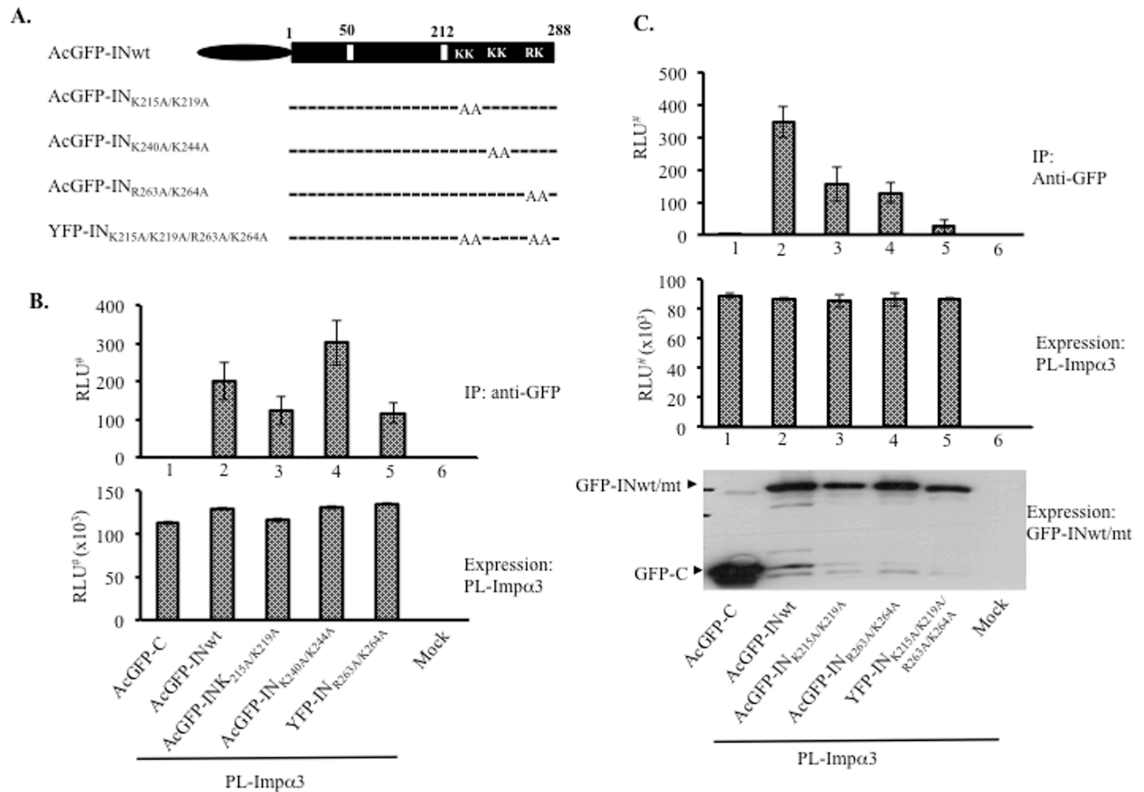

Figure. 14. The conserved basic amino acid rich motifs in IN-CTD are required for Impα3 interaction: (A) Diagrammatic depiction of AcGFP/YFP-$IN_{Wt/Mt}$ proteins. (B) AcGFP, AcGFP-IN_{Wt}, or various AcGFP-IN_{Mt} expressors were co-transfected with PL-Impα3 expressor in 293T cells. After 48h of transfection, cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation of PL-Impα3 was detected by measuring PL activity (upper panel). PL-Impα3 expression in the total cell lysates was detected from by measuring PL activity (bottom panel). (C) AcGFP, AcGFP-IN_{Wt}, or various AcGFP-IN_{Mt} expressors were co-transfected with PL-Impα3 expressor in 293T cells. After 48h of transfection, cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation of PL-Impα3 was detected by measuring PL activity (top panel). PL-Impα3 expression in the total cell lysates was detected by measuring PL activity (middle panel). GFP/YFP-$IN_{Wt/Mt}$ expression in the total cell lysates was detected by WB using an anti-GFP antibody (bottom panel). All the results were confirmed in three independent experiments. # Relative light unit.

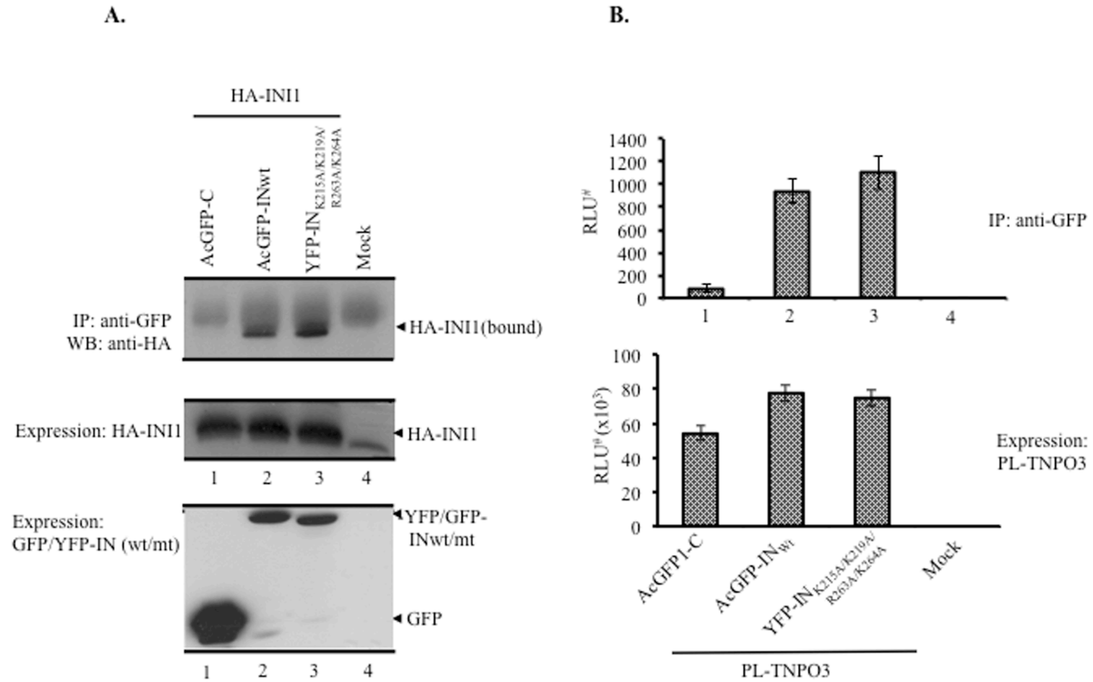

Figure 15. $IN_{K215A/K219A/R263A/K264A}$ mutant interacts with INI1 and TNPO3: (A) AcGFP or AcGFP/YFP-$IN_{Wt/Mt}$ expressor was co-transfected with HA-INI1 expressor in 293T cells. 48h post transfection, cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation of HA-INI1 was detected by WB using an anti-HA antibody (top row). AcGFP/YFP-$IN_{Wt/Mt}$ in the immunoprecipitates was detected by WB using an anti-GFP antibody (bottom row). Expression of HA-INI1 in the total cell lysates was detected by WB using an anti-HA antibody (middle row). (B) AcGFP or AcGFP/YFP-$IN_{Wt/Mt}$ expressor was co-transfected with PL-TNPO3 expressor in 293T cells. At 48h post transfection, the cells were subjected to immunoprecipitation using an anti-GFP antibody and the co-precipitation of PL-TNPO3 was detected by measuring PL activity (top panel). PL-TNPO3 expression in the total cell lysates was detected by measuring PL activity (middle panel). The expression of AcGFP/YFP-$IN_{Wt/Mt}$ in the total cell lysates was detected by WB using an anti-GFP antibody (bottom panel). # Relative light unit.

3.4.8 The Major and Minor NLS Binding Grooves of Impα3 are Involved in IN Interaction

In this section, we explored the binding site of IN in Impα3. In the past, structural studies have found that the central ARM domains of Impα3 form two shallow regions for NLS binding; the major (ARM2-4) and minor (ARM6-8) NLS binding grooves (discussed in chapter 1) [147-149]. Each of ARM repeats in NLS binding grooves contains highly conserved Tryptophan (W)–Asparagine (N) amino acid pairs. These amino acid pairs facilitate cargo proteins binding at major or minor NLS binding groves of Impα3 by interaction with basic amino acids in NLS [149]. Consistently, Melen *et al*., showed that the substitution mutation of W-N amino acid pairs to alanine in ARM3 of Impα3 impaired Impα3 interaction with SV40 large T antigen and similar mutations in ARM8 impaired Impα3 interaction with influenza A virus nucleoprotein [353]. Therefore, in order to know whether NLS binding groves of Impα3 are involved in IN interaction, we introduced W179A/N183A (ARM3) or W348A/N352A (ARM7) mutations into PL-Impα3 and probed their interaction with AcGFP-IN by chemiluminascent Co-IP (**Figure 16A**), as described in the Materials and Methods. Interestingly, both Impα3-major and -minor NLS binding groove mutants showed a attenuated interaction with IN (**Figure 16B**), which suggested that both major and minor NLS binding grooves of Impα3 are involved in IN interaction. Moreover, this finding also suggests a typical cargo protein and import receptor type of interaction between IN and Impα3. Studies have earlier showed that a bipartite NLS will simultaneously bind to both major and minor NLS binding grooves of Impα [147, 149]. Hence, in addition to clarifying the mechanism of Impα3 interaction with IN, above data further justify our claim that ^{211}KELQKQITK and

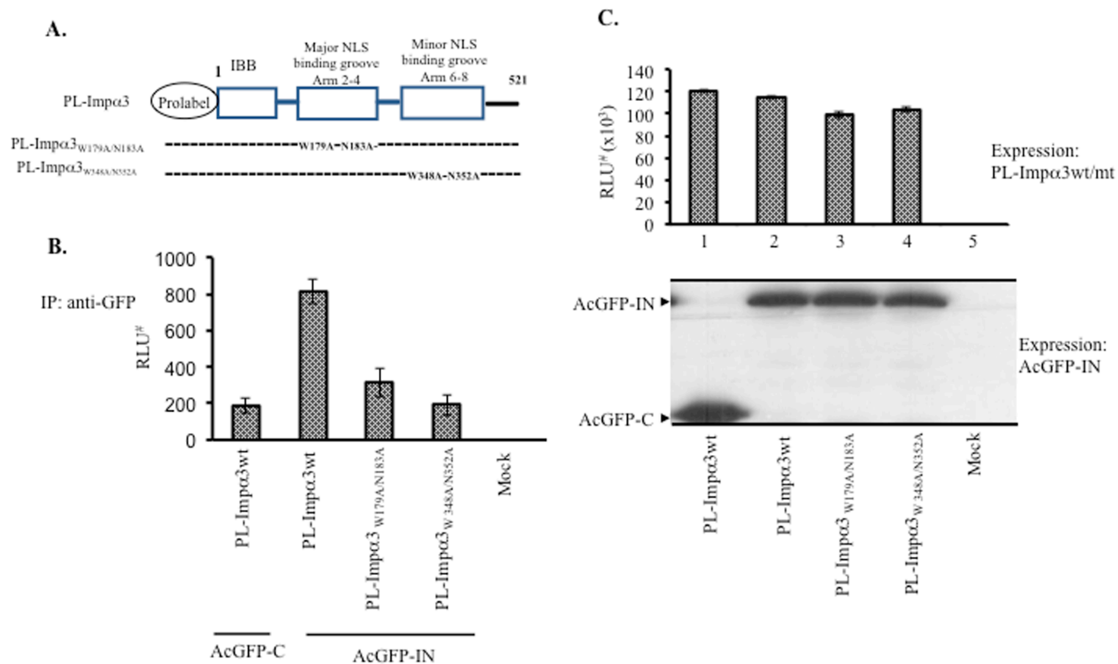

Figure 16. The major and minor NLS binding grooves on Impα3 are involved in IN interaction: (A) Diagrammatic representation of PL-Impα3-NLS binding groove mutant protein expressors. (B) AcGFP or AcGFP-IN expressors were co-transfected with PL-Impα3$_{Wt}$ or NLS binding grooves mutants in 293T cells. After 48h of transfection, 8/10th of cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation of PL-Impα3$_{Wt}$ or NLS binding grooves mutants was detected by measuring PL activity. (C) The expression of PL-Impα3$_{Wt}$ or NLS binding grooves mutants in the total cell lysates was examined by measuring PL activity (upper panel) and AcGFP-IN in the total cell lysates was detected by WB using an anti-GFP antibody (lower panel). # Relative light unit.

^{262}RRKAK motifs of IN acts as a non-conventional bipartite NLS for Impα3 interaction.

3.4.9 Impα3 Interaction Defective $IN_{K215A/K219A/R263A/K264A}$ Mutant is Defective for Nuclear Localization

HIV IN undergoes active nuclear import and localizes exclusively to the nucleus when expressed in cells (discussed in chapter 1). To test whether Impα3 interaction is required for IN nuclear import, we have examined the nuclear localization of Impα3 interaction defective IN mutant, YFP-$IN_{K215A/K219A/R263A/K264A,}$ by immunostaining. Briefly, YFP-$IN_{K215A/K219A/R263A/K264A}$, AcGFP-$IN_{Wt}$, AcGFP-$IN_{1-212}$ (IN-CTD deletion mutant expressors), or AcGFP-$IN_{206-288}$, (IN-CTD alone expressor) was transfected into COS-7 cells. At 36h post transfection, cells were fixed and immunostained for GFP, as described in the Materials and Methods. The results showed that YFP-$IN_{K215A/K219A/R263A/K264A}$ failed to localize to the nucleus **(Figure 17A)**, whereas AcGFP-IN_{Wt} and AcGFP-$IN_{206-288}$ were exclusively localized in the nucleus and AcGFP-IN_{1-212} lost the nuclear localization (**Figure 17A**), which is consistent with our observation in an earlier study [56] as well as in this study. We also confirmed the nuclear localization of YFP-$IN_{K215A/K219A/R263A/K264A}$ by cytoplasm and nucleus fractionation method. Briefly, AcGFP-IN_{Wt} or YFP-$IN_{K215A/K219A/R263A/K264A}$ were expressed in 293T cells and the cells were subjected to subcellular fractionation, as described in the Materials and Methods. AcGFP-IN_{Wt} or YFP-$IN_{K215A/K219A/R263A/K264A}$ protein in the cytoplasm and nuclear fractions was detected by WB using an anti-GFP-HRP antibody. By analyzing WB band densities, we found that about 73% of YFP-$IN_{K215A/K219A/R263A/K264A}$ was retained in the cytoplasmic fraction (**Figure 17B, the second row from the top**), where as about 82% of AcGFP-IN_{Wt} was

found in the nuclear fraction (**Figure 17B**, **the top row**). The successful fractionation of the cytoplasm and the nucleus was determined by probing γ-tubulin protein from each fraction by WB using an anti-γ-tubulin antibody. As expected, γ-tubulin was detected only in the cytoplasmic fraction (**Figure 17B**, **bottom row**). The above data clearly suggests that the karyophilic property of IN is impaired by its lack of interaction with Impα3, which in turn suggests that Impα3 interaction is required for IN nuclear import.

3.4.10 Impα3 Interaction Defective HIV-$IN_{K215A/K219A/R263A/K264A}$ Mutant Virus is Defective for Replication at or Prior to Integration Step

Following the identification of motifs in IN for Impα3 interaction and their requirement for IN nuclear localization, we asked whether IN and Impα3 interaction is required for HIV replication. To test the requirement of IN and Impα3 interaction for HIV replication, $IN_{K215A/K219A/R263A/K264A}$ mutations were introduced into a previously described HIV single-cycle replication system [1] and its replication was examined. Briefly, $IN_{K215A/K219A/R263A/K264A}$ mutation was introduced into a Vpr-RT-IN fusion protein expressor and Vpr-RT-IN_{Wt} or Vpr-RT-$IN_{K215A/K219A/R263A/K264A}$ was co-transfected with RT/IN-deleted $HxBruR^{-}$/ΔRI provirus (shown in **Figure 18A**) in 293T cells. After 48h of transfection, HIV_{Wt} and HIV-$IN_{K215A/K219A/R263A/K264A}$ mutant viruses were concentrated from supernatant by ultracentrifugation. IN protein incorporation in wild-type and mutant viruses was detected by WB using an anti-IN antibody. As expected, both wild type and mutant viruses showed similar level of IN incorporation (**Figure 18B**). Then, $0.5x10^{6}$ C8166T cells were infected with an equal amount of HIV_{Wt} or HIV-$IN_{K215A/K219A/R263A/K264A}$ (at 5 ng of virus-associated $p24^{Gag}$). At different time intervals after infection,

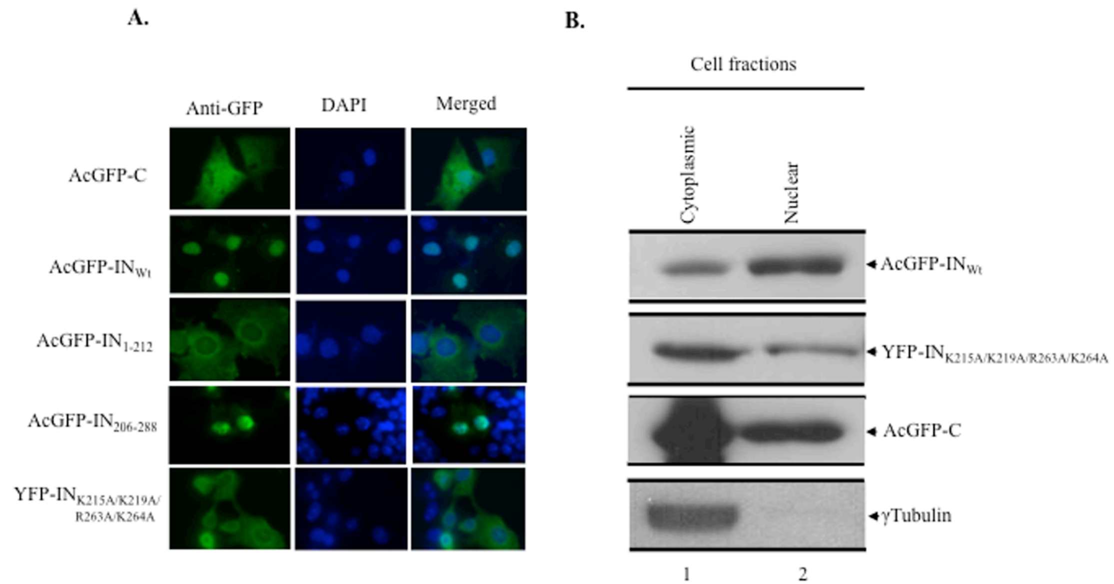

Figure 17. YFP-IN$_{K215A/K219A/R263A/K264A}$ mutant fusion protein is defective for nuclear localization: (A) COS-7 cells were transfected with AcGFP-IN$_{Wt}$, AcGFP-IN$_{1-212}$, AcGFP-IN$_{206-288}$, or YFP-IN$_{K215A/K219A/R263A/K264A}$ expressor. After 36h of transfection, cells were immunostained with the rabbit anti-GFP antibody followed by FITC-conjugated anti-rabbit IgG secondary antibody. The cell nucleus was stained with DAPI. The immunostaining was analyzed under fluorescence microscope (AxiovertTM 200; Carl Zeiss, at 63x magnification with oil immersion). (B) 292T cells were transfected with expressors for AcGFP, AcGFP-IN$_{Wt}$, or YFP-IN$_{K215A/K219A/R263A/K264A}$. After 36h of transfection, the cells were subjected to cytoplasm/nuclear fractionation. AcGFP (third row from the top), AcGFP-IN$_{Wt}$ (top row), or YFP-IN$_{K215A/K219A/R263A/K264A}$ protein content (second row from the top) in the cytoplasm (lane 1) and the nuclear (lane 2) fractions was determined by WB using an anti-GFP antibody. The γ-tubulin was detected by WB using an anti-γ-tubulin antibody (bottom row).

virus replication was examined by measuring HIVp24^{Gag} protein concentration from supernatants, using HIVp24^{Gag} ELISA. Results showed that the high level of replication was evident in wild type virus infection, but very little or no virus replication was observed in mutant virus infection (**Figure 18C**). To assess the early stage HIV replication, the genomic integration of HIV$_{Wt}$ and HIV-IN$_{K215A/K219A/R263A/K264A}$ viruses was probed by qPCR, as described in the Materials and Methods. Although wild type virus showed normal genomic integration, the integration of mutant virus was undetectable at 24h of infection (**Figure 18D**). These data led to the interpretation that HIV-IN$_{K215A/K219A/R263A/K264A}$ virus is replication defective and this replication defect is in early-stage HIV replication at, or prior to, the integration step.

3.4.11 HIV-IN$_{K215A/K219A/R263A/K264A}$ Mutant Virus is Defective for Nuclear Import in Dividing and Non-Dividing Cells

In this section, we examined the early HIV replication steps such as reverse transcription and nuclear import in HIV-IN$_{K215A/K219A/R263A/K264A}$ infection. The lack of integration causes higher levels of 2-LTR circle DNA in HIV infection, provided reverse transcription and nuclear import are not affected [205]. As HIV-IN$_{K215A/K219A/R263A/K264A}$ lacks integration, a integration defective HIV-IN$_{D64E}$ virus was used to compare the results. HIV-IN$_{D64E}$ is a class I catalytic mutant and is impaired for genomic integration, but has normal reverse transcription and nuclear import functions [51, 56, 363]. The infection was carried in dividing and cell cycle arrested cells (i.e., non-dividing cells). The cell cycle arrest was induced by treatment with Aphidicolin. Briefly, $0.5x10^6$ C8166T cells were treated with or without Aphidicolin (1.3 μg/ml) and infected with

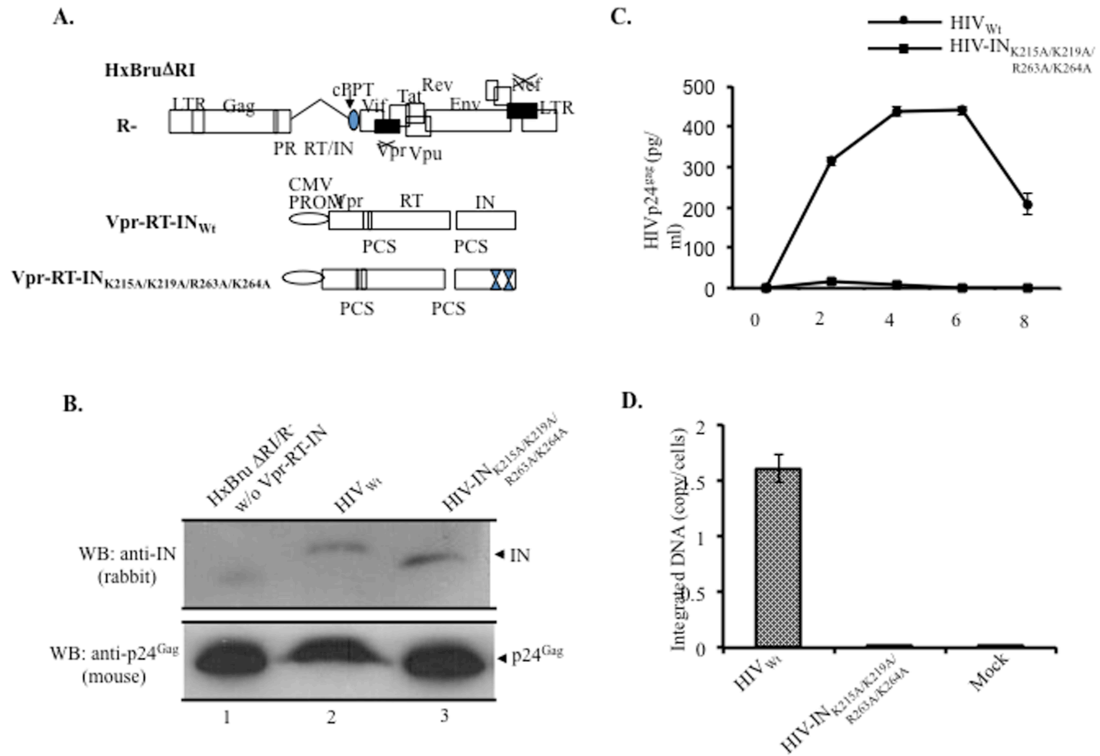

Figure 18. HIV-IN$_{K215A/K219A/R263A/K264A}$ mutant virus is defective for replication at or prior to integration step: (A) Schematic representation of HIV provirus having mutation and/or deletion in *pol* gene and expressor for Vpr-RT-IN$_{Wt/Mt}$ fusion proteins. This figure is adopted from a previous study with minor modifications[1]. (B) The virus incorporated IN (upper row) and p24^{Gag} (lower row) proteins in HIV$_{Wt/Mt}$ were detected by WB, using the anti-IN and the anti-p24^{Gag} antibody, respectively (C) 0.5×10^{6} C8166T cells were infected with HIV$_{Wt}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ (at 5 ng of virus-associated p24^{Gag} antigen). At different days post infection, the virus replication was monitored by measuring HIVp24^{Gag} levels in the supernatants by p24^{Gag} ELISA. (D) 0.5×10^{6} C8166T cells were infected with HIV$_{Wt}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ (at 5 ng of virus-associated p24^{Gag} antigen) and integrated proviral DNA was detected by *Alu-LTR*-based nested qPCR, as described in the Materials and Methods.

HIV-IN$_{D64E}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ viruses (at 10 ng of virus-associated p24^{Gag}). At 12h and 24h of infection, the total viral DNA and 2-LTR circle DNA were quantified by qPCR. We observed a 40–60% reduction in total viral DNA synthesis in HIV-IN$_{K215A/K219A/R263A/K264A}$ mutant virus infection as compared to control (HIV-IN$_{D64E}$) **(Figure 19A and 19B, top panels)**. However, a more pronounced reduction in 2-LTR circle DNA synthesis was observed in HIV-IN$_{K215A/K219A/R263A/K264A}$ infection as compared to HIV-IN$_{D64E}$ infection in both dividing and non-dividing cells, at 12h and 24h of infection (**Figure 19A and 19B, middle panels**). No integrated viral DNA was detected in HIV-IN$_{K215A/K219A/R263A/K264A}$ or HIV-IN$_{D64E}$ infection. These results indicated that HIV-IN$_{K215A/K219A/R263A/K264A}$ is defective for nuclear import. For a better interpretation of the results, we calculated the percentage of 2-LTR circle DNA to total viral DNA copies per cell (**Figure 19A and 19B, bottom panels**). In proliferating C8166T cells, we observed an average 4.5- and 10-fold reduced 2-LTR circle DNA at 12h and 24h post infection, respectively (**Figure 19A, bottom panel**). In HIV-IN$_{K215A/K219A/R263A/K264A}$ infection of cell cycle arrested C8166T cells, we observed an average 8.5- and 13.5-fold lowered 2-LTR circle DNA compared to HIV-IN$_{D64E}$ control, at 12h and 24h post infection, respectively (**Figure 19B, bottom panel**). Further, the nuclear import was assessed by cell fractionation method. Briefly, C8166T cells were infected with HIV-IN$_{D64E}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ viruses (10 ng of virus-associated p24^{Gag}). At 24h post infection, the cytoplasm and the nucleus were fractionated as described in the Materials and Methods. The total viral DNA was quantified from the cytoplasm and nuclear fractions by qPCR. As expected, a major proportion of viral DNA in HIV-IN$_{D64E}$ infected cells was exclusively found in the

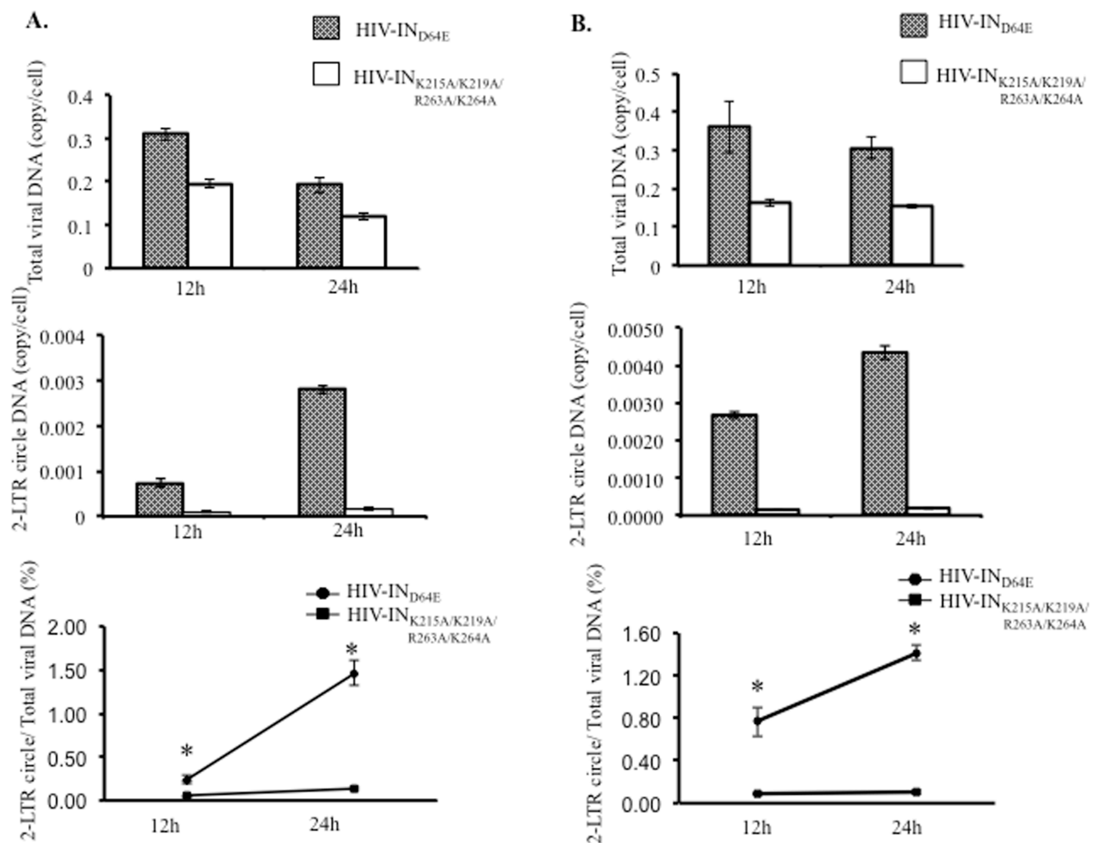

Figure 19. HIV-IN$_{K215A/K219A/R263A/K264A}$ mutant virus is defective for nuclear import: (A) C8166T cells were infected with HIV-IN$_{D64E}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ virus (at 10 ng of virus-associated p24^{Gag}). At 12h and 24h of infection, total viral DNA (top panel on left side) and 2-LTR circle DNA (middle panel on left side) were quantified by qPCR. The percentage of 2-LTR circle DNA in total viral DNA was estimated (bottom panel on left side). (B) The cell cycle arrested C8166T cells were infected with HIV-IN$_{D64E}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ virus (at 10 ng of virus-associated p24^{Gag}). At 12h and 24h of infection, the total viral DNA (top panel on right side) and 2-LTR circle DNA (middle panel on right side) were quantified by qPCR, and the percentage of 2-LTR circle DNA in total viral DNA was estimated (bottom panel on right side). Data shown are means and standard errors and are representative of the results for triplicate samples from two independent experiments. The statistical significance was determined by a Student t-test, $\star P<0.05$ (N=3).

nucleus (**Figure 20B**). However, on the contrary, a significantly higher proportion of viral DNA in HIV-$IN_{K215A/K219A/R263A/K264A}$ infected cells was found in the cytoplasmic fraction **(Figure 20A)**, confirming a defective nuclear import in HIV-$IN_{K215A/K219A/R263A/K264A}$ infection. The cumulative viral DNA from the cytoplasm and nuclear fractions of HIV-IN_{D64E} or HIV-$IN_{K215A/K219A/R263A/K264A}$ infection **(Figure 20C)** was comparable to the viral DNA obtained from total cell lysates in the above experiments **(Figure 19A and 19B, top panels).** The cytoplasm and nucleus fractionation was examined by detecting γ-tubulin protein by WB. As expected, γ-tubulin was only detected in cytoplasmic fractions but not in nuclear fractions (**Figure 20D**). The above data led to the conclusion that HIV-$IN_{K215A/K219A/R263A/K264A}$ is impaired for nuclear import in both dividing and non-dividing cells.

3.5 Discussion

Early stage HIV replication includes virus entry, uncoating, reverse transcription, nuclear import, and integration steps. Following reverse transcription, the cDNA enters nucleus as a part of PIC by nuclear import and integrates into the cellular genome. The significance of nuclear import for HIV replication in both non-dividing and dividing cells is well known (discussed in chapter 1). However, the mechanism of HIV nuclear import is not well understood. In this chapter, we elucidated the functionally important Impα isoform (i.e., Impα3) for HIV nuclear import and the mechanism by which Impα3 is engaged in HIV nuclear import. Earlier reports have implicated Imp7 and TNPO3 in HIV nuclear import [241, 242, 245, 247]. However, recent reports have undermined or contradicted the role of Imp7 or TNPO3 in HIV nuclear import [57, 243, 244, 252]

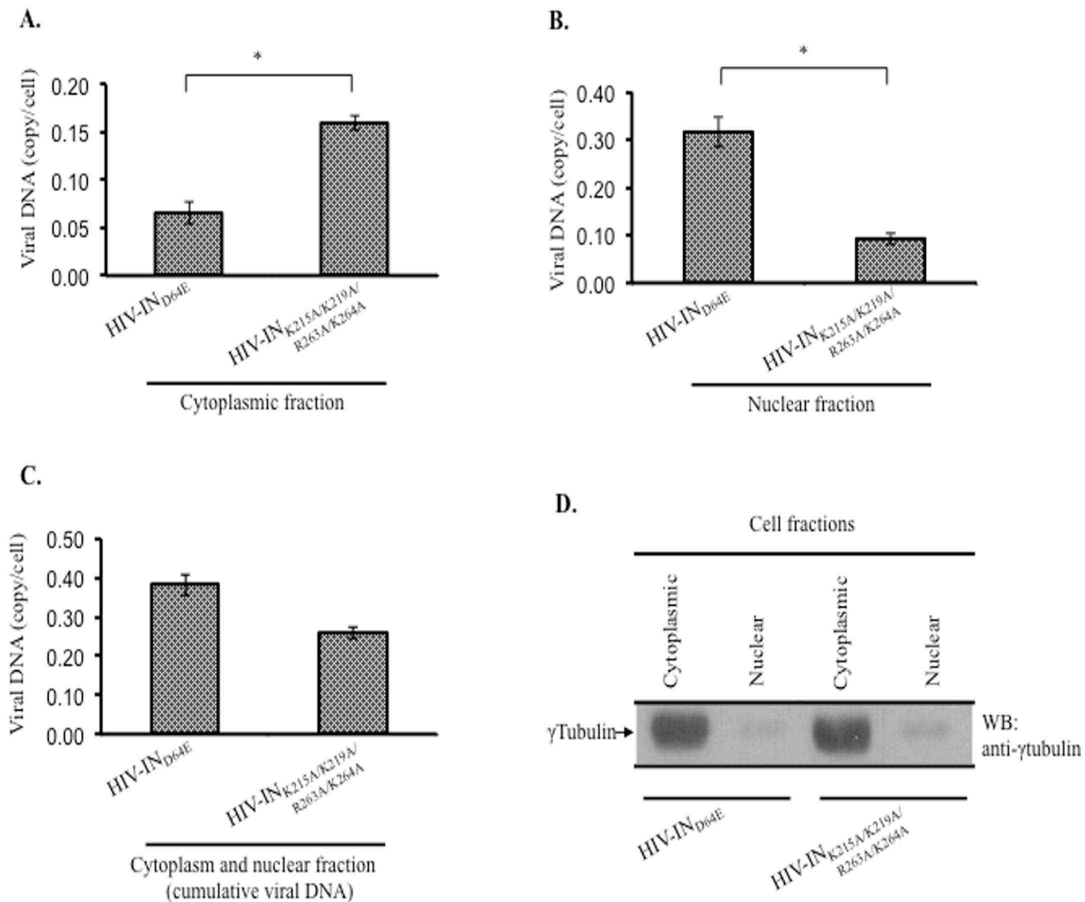

Figure 20. HIV-IN$_{K215A/K219A/R263A/K264A}$ is impaired for nuclear import by cell fractionation method: C8166T cells were infected with HIV-IN$_{D64E}$ or HIV-IN$_{K215A/K219A/R263A/K264A}$ (at 10 ng of virus-associated p24^{Gag}). At 24h post infection, the cytoplasm and nucleus was fractionated. The total viral DNA from 9/10th of each fraction was quantified by qPCR. (A) The viral DNA in cytoplasmic fractions of HIV-IN$_{D64E}$ and HIV-IN$_{K215A/K219A/R263A/K264A}$ infected cells. (B) The viral DNA in nuclear fractions of HIV-IN$_{D64E}$ and HIV-IN$_{K215A/K219A/R263A/K264A}$ infected cells. (C) Cumulative total viral DNA content from both cytoplasm and nuclear fractions of HIV-IN$_{D64E}$ and HIV-IN$_{K215A/K219A/R263A/K264A}$ infected cells (D) The γ tubulin protein was detected in 1/10th of cytoplasm or nuclear faction by WB using an anti-γ tubulin antibody. Data shown are means and standard errors and are representative of the results for triplicate samples of a typical experiment. Data was confirmed by two independent experiments. The statistical significance was determined by Student's t-test, ⁎P<0.05 (N=3).

(discussed in chapter 1). Impα1 was a first nuclear import receptor implicated in HIV nuclear import [210] and was perceived as an important cellular factor for HIV nuclear import. Impα/Impβ-mediated classical nuclear import pathway is well characterized for nuclear import of large numbers of cellular macromolecules (reviewed in [136, 139, 358, 364]). There are six different Impα isoforms in human cells and all these isoforms are known to mediate macromolecule nuclear import. These Impα isoforms are grouped into three subfamilies and there is about 50% and 80% amino acid sequence identity between subfamilies and within subfamilies, respectively. In this study, the key Impα isoforms (Impα1, Impα3, Impα5, and Impα7) from each subfamily were examined for HIV replication by gene KD approach. Different Impα subtypes were stably knocked down in HeLa or C8166T cells by shRNA approach. While similar level of KD was obtained for different isoforms except Impα7, the replication of HIV showed significant variation. There was about 3- to 4-fold reduction in HIV replication in Impα3-KD cells, where as only 50-60% reduction in HIV replication was evident in Impα1 or Impα5-KD cells **(Figure 7B and Figure 8C).** Impα7 KD did not show any effect on HIV replication. Since siRNA mediated down regulation of Impα1, Impα3, Impα5, or Impα7 expression resulted in slow proliferation of HeLa cells in an earlier study [354], cell proliferation of different Impα isoform-KD C8166T cells was examined by WST-1 assay. Results showed that the proliferation of Impα1 and Impα3-KD cells was slightly reduced (about 30% reduced), where as no proliferation difference was evident in Impα5 or Impα7-KD cells (**Figure 8B; data not shown for Impα7**). We have also obtained similar proliferation results by Tryphan blue dye exclusion assay. The specific reason(s) for reduced proliferation of Impα1 or Impα3-KD cells is not known. It is possible to

speculate that the nuclear localization of some cell proliferation specific proteins would be affected by Impα1 or Impα3 depletion in cells. However, attenuation of HIV replication in Impα3-KD cells appears to be specific as both Impα1 and Impα3 KD cells showed a similar level proliferation but HIV replication defect was relatively more pronounced in Impα3-KD cells than in Impα1-KD cells. Moreover, HIV gene expression was unaffected in Impα3-KD cells (**Figure 7C**). Based on these data, we suggested that Impα3 is likely to play an important role in the steps of early stage HIV replication. In addition, as Impα1 or Impα5 KD also led to approximately 50% reduction in HIV replication, it is difficult to exclude the contribution of these proteins in efficient HIV replication and/or nuclear import. Interestingly, studies showed the cell type specific differential expression of Impα isoforms, which is often dependent on the metabolic state of cell and cell differentiation [365-367]. Therefore, Impα1 or Impα5 may contribute to efficient nuclear import of HIV or possibly in other steps of early stage HIV replication in a cell type and/or cell state specific manner, which requires further investigation.

To identify the specific early stage HIV replication step(s) that are impaired in Impα3-KD cells, the synthesis of HIV total viral DNA, 2-LTR circle DNA, and integrated-DNA was analyzed in Impα3-KD C8166T cells by qPCR. Interestingly, synthesis of 2-LTR circle DNA was specifically impaired in Impα3-KD cells **(Figure 10B).** Coincidentally, in contrast to HIV replication, MMLV replication in Impα3-KD C8166T cells was reduced by only 40 to 50% (**Figure 8D**). Since MMLV lacks active nuclear import, its replication is dependent on cell mitosis. Therefore, slight reduction of MMLV replication could be attributed to slower proliferation of Impα3-KD cells, as shown in **Figure 8B**.

Nevertheless, since HIV replication but not MMLV replication is greatly reduced in Impα3-KD cells, it justifies the specific requirement of Impα3 for HIV nuclear import.

The possible mechanism by which Impα3 is recruited by HIV was explored in this study. HIV PIC contains viral karyophilic proteins such as IN, MA, and Vpr and these proteins have been implicated in HIV nuclear import by several different studies (discussed in chapter 1 and the rationale section of this chapter). As IN is considered as the most promising viral factor involved in HIV nuclear import, we first examined IN and Impα3 interaction. Interestingly, IN interacted with Impα3 in *in vitro* interaction assay **(Figure 11A)**, confirming the direct protein-protein interaction between IN and Impα3. Subsequently, the interaction of Impα3 with IN was also evident in 293T cells by cell based Co-IP. At this time, MA and Vpr were also included in Impα3 interaction analysis. Interestingly, Impα3 interacted with IN as well as Vpr (**Figure 11B)**. Noticeably, IN and Impα3 were also found interacting in HIV infected C8166T cells **(Figure 11D)**. These data clearly suggested that Impα3 is a novel cellular co-factor for IN interaction, which in turn also justifies the involvement of Impα3 in HIV nuclear import.

In addition to IN, Vpr protein also interacted with Impα3 **(Fig. 11B).** Consistent with our data, a recent study also demonstrated Vpr interaction with Impα1, Impα3, and Impα5 in an *in vitro* binding assay [67]. The significance for Impα3 interaction with Vpr is not very clear. As discussed in chapter 1, Vpr does not play a significant role in HIV nuclear import. In this study, our data tends to rule out the possible contribution of Vpr and Impα3 interaction in HIV nuclear import. Firstly, Impα3-KD significantly impaired HIV

nuclear import and replication in dividing HeLa and C8166T cell lines, while Vpr is dispensable for HIV replication in dividing cells (discussed in chapter 1). Secondly, both HIV-Vpr$^-$ and and HIV-Vpr$^+$ viruses exhibited similar level of attenuated replication in Impα3-KD human MDMs **(Figure 13D).** These observations clearly suggested that the impaired HIV replication in the Impα3-KD cells is Vpr independent. However, in agreement with the previous reports [71, 225, 226, 368], our results also exhibited lower levels of replication of Vpr$^-$-HIV compared to Vpr$^+$-HIV in primary human macrophages. This suggests that Vpr may facilitate HIV replication in macrophages, but possibly through alternative mechanism(s). In fact, studies have suggested that Vpr binds to nuclear import factors, including Impα1 and hCG1, and facilitates HIV nuclear import and replication in macrophages [66-68, 71, 224, 225, 369].

While characterizing the IN interaction with Impα3, we found that GFP-IN_{1-212} and GFP-IN_{1-250} deletion mutant fusion proteins failed to interact with Impα3, but GFP-IN_{1-270} retained Impα3 interaction (**Figure 12B)**. These data clearly indicated that IN-CTD is involved in Impα3 interaction. Apart from defining the interaction site, this finding also shows the specificity of IN interaction with Impα3 as most of the known putative NLSs of IN are found in CTD. Consistently, by immunostaining, the Impα3-binding-deficient mutants, AcGFP-IN_{1-212} and GFP-IN_{1-250}, also failed to localize in the nucleus, whereas AcGFP-IN_{1-270} and AcGFP-IN_{Wt} retained localized in the nucleus **(Figure 12C).** It is worth mentioning that earlier studies showed the requirement for IN-CTD in HIV nuclear import (discussed in the rationale part of this chapter). Gallay *et al.,* suggested a bipartite NLS that spans between CCD (^{186}KRK) and CTD (^{211}KELQKQITK) of IN for HIV

nuclear import [210]. Subsequently, an earlier study from our own laboratory showed that HIV-IN$_{K115A/K219A}$ and HIV-IN$_{K240A/K244E}$ mutant viruses are defective for nuclear import in the infection experiments, underscoring the involvement of IN-CTD in HIV nuclear import [56]. In the current study, we have provided evidence for the involvement of the Aa212-270 region of IN in Impα3 interaction and IN nuclear localization. Together these findings further support the earlier suggestion that IN-CTD plays a key role in HIV nuclear import by binding with cellular proteins. Our previous study showed that substitution mutations (IN$_{K240A/K244A/R263A/K264A}$) in putative NLSs (^{211}KELQKQITK and ^{236}KGPAKLLWK) of IN-CTD resulted in loss of binding to Imp7 [57] but this IN and Imp7 interaction was found not playing a significant role in HIV nuclear import [57]. Since these motifs are located within the minimum region of IN (Aa212-270) that is involved in Impα3 interaction, it suggested that these motifs may be engaged in the IN interaction with Impα3. However, it raises an important question about how IN co-ordinates interaction with two different import receptors, even though they interact IN through the same motifs. By comparing the results of this study with that of other reports, only Impα3 constitute major contributor for HIV nuclear import, as previous studies have ruled out the significant contribution of Imp7 for HIV nuclear import [57, 243].

HIV nuclear import is essential for productive infection of both non-dividing and dividing cells. The importance of IN for HIV nuclear import in both dividing and non-dividing cells has been suggested in earlier reports [57, 241]. Consistently, Katz *et al.,* [202, 370] and Riviere *et al.,* [371] have indicated that nuclear entry of RSV or HIV can occur at the interphase of cycling cells where the nuclear membrane is intact. In the

present study, we have observed that HIV 2-LTR circle DNA synthesis was impaired in Imp3-KD dividing C8166T cells **(Figure 10B),** indicating the requirement of Impα3 for HIV nuclear import in dividing cells. Meanwhile, we have demonstrated the impaired HIV replication in Impα3-KD primary macrophages **(Figure 13),** indicating the requirement of Impα3 for HIV replication in non-dividing cells. Thus, we suggest that Impα3 is required for HIV replication and/or nuclear import in both dividing and non-dividing cells.

In this study, we presented data on the molecular mechanism of IN and Impα3 interaction and its requirement for HIV replication and nuclear import. As an earlier study from our laboratory identified the putative NLSs (^{211}KELQKQITK and ^{236}KGPAKLLWK, and ^{263}RRKAK) in IN-CTD, we examined the requirement of these motifs for Impα3 and HIV nuclear import. Our results showed that specific lysine to alanine substitution mutations in ^{211}KELQKQITK and ^{263}RRKAK regions of IN ($IN_{K215A/K219A}$ and $IN_{R263A/K264A}$) resulted in moderately attenuated interaction with Impα3 (**Figure 14B, upper panel**) and Impα3 interaction was severely impaired for interaction with $IN_{K215A/K219A/R263/K264A}$ (**Figure 14C, upper panel**). These findings have, for the first time, indicated the requirement of ^{211}KELQKQITK and ^{263}RRKAK regions of IN for Impα3 interaction. It is important to note that ^{211}KELQKQITK and ^{262}RRKAK regions in IN are separated by a relatively long stretch of amino acids (ie., approximately 40 amino acids), which is not in accordance with the linker region of approximately 10-12 amino acids in standard classical bipartite NLS. Therefore, we suggest that ^{211}KELQKQITK and ^{262}RRKAK regions may act as a non-conventional bipartite NLS for Impα3 interaction.

Indeed, such non-conventional bi-partite NLSs have been reported earlier (reviewed in [139]). Although $IN_{K215A/K219A/R263/K264A}$ has lost interaction with Impα3, it still interacted with INI1 and TNPO3, ruling out the possible nonspecific negative effect of mutations on IN interaction with other proteins. However, at this point, it is not clear whether $IN_{K215A/K219A/R263/K264A}$ is defective for Impα1 interaction. In the past, Impα1 was found interacting with IN and was implicated in HIV nuclear import. Nevertheless, in figure 7B and 8C, Impα1-KD showed only moderately impaired HIV replication. Interestingly, by crystallographic analysis of $IN_{50–288}$ [372], ^{211}KELQKQITK and ^{262}RRKAK regions were situated on the outer face of $IN_{50–288}$ dimer, which underscores their easy accessibility for protein interactions. A co-crystal structure for IN and Impα3 interaction would be helpful to ascertain the motifs engaged in the interaction and to allow rational design of small-molecule inhibitors against IN and Impα3 interaction as a novel anti-HIV drugs.

Impα3 protein is made up of 10 ARM repeats. ARM2–4 and ARM6–8 forms a major and minor NLS binding grooves, respectively [147-149] and these NLS binding grooves provide the site for cargo protein interaction. The W–N amino acid pairs in ARM repeats of NLS binding groves interact with lysine/arginine amino acids in NLS of cargo protein and mediate cargo protein binding [149]. Although it is not clear whether W–N pairs in different ARM repeats are equally important or differentially influence cargo protein interaction, a study showed that even a single mutation in any of W–N amino acid pairs of NLS binding groove is sufficient to disrupt cargo interaction [353]. The mutation of W-N pairs in any given binding groove will specifically affect cargo that bind to Impα through that particular NLS binding groove. It means, W-N pair mutation in minor NLS

binding groove of Impα will only affect cargoes that bind to Impα through minor NLS binding groove and vice versa. The binding site(s) (major or minor NLS binding grooves) for cargo has been defined based on the mutational analysis of W–N amino acid pairs in NLS binding grooves of Impα [353, 373]. In this study, we introduced W179A-N183A mutations in ARM3 of the major NLS binding groove or W348A-N352A mutation in ARM7 of the minor NLS binding groove of Impα3 and examined their interaction for IN. Interestingly, both Impα3 mutants showed reduced interaction with IN **(Figure 16B),** suggesting that major and minor NLS binding grooves of Impα3 are involved in IN interaction. Interestingly, crystallographic studies have shown that the bipartite NLS simultaneously establishes contact with both major and minor NLS binding grooves of Impα [147, 149]. Therefore, the data in this study justify our claim that ^{211}KELQKQITK and ^{262}RRKAK motifs of IN serve as a bipartite NLS for Impα3 interaction.

To know the requirement of Impα3 interaction for IN nuclear import, we examined the nuclear localization of YFP-$IN_{K215A/K219A/R263A/K264A}$ by immunostaining and cell fractionation methods. In both assays, YFP-$IN_{K215A/K219A/R263A/K264A}$ was failed to localize in the nucleus. On the contrary, AcGFP-$IN_{206-288}$ (IN-CTD fusion protein) was exclusively localized to the nucleus by immunostaining. However, at this point, it would be difficult to rule out the passive diffusion of AcGFP-$IN_{206-288}$ into the nucleus followed by nonspecific binding to genomic DNA. Nevertheless, motifs required for the nonspecific DNA interaction have been mapped to both CCD [374] and CTD of IN [375], which necessitates the intact CTD and CCD in IN for nonspecific DNA binding.

Moreover, relatively high molecular weight of AcGFP-$IN_{206-288}$ fusion protein (approximately 35 kDa) makes it less likely to be able to passively diffuse into the nucleus. Therefore, these results suggest that Impα3 interaction defective IN mutant is defective for nuclear import.

In the last two sections of this chapter, we have asked whether IN and Impα3 is required for HIV replication and nuclear import. To study this, we introduced $IN_{K215A/K219A/R263A/K264A}$ mutations into our previously described HIV single cycle replication system (**Figure 18A**) [1, 56], which allows the introduction of different mutations into IN gene without differentially affecting viral morphogenesis or functions of the central DNA flap. We found that HIV-$IN_{K215A/K219A/R263A/K264A}$ virus is replication defective in an infection analysis and by analyzing integrated HIV DNA, HIV-$IN_{K215A/K219A/R263A/K264A}$ replication was found impaired at or prior to genomic DNA integration (**Figure 18D**). Although HIV-$IN_{K215A/K219A/R263A/K264A}$ showed moderately reduced reverse transcription (40–60% reduced total viral DNA synthesis) (**Figure 19A and 19B, top panels**), it was greatly impaired for 2-LTR circle DNA synthesis (**Figure 19A and 19B, middle and lower panels**), indicating a defective HIV nuclear import. The 2-LTR circle DNA synthesis defect of HIV-$IN_{K215A/K219A/R263A/K264A}$ was more pronounced in cell cycle arrested than in dividing cells, which gives a clear indication that HIV-$IN_{K215A/K219A/R263A/K264A}$ is impaired for nuclear import as HIV nuclear import is mandatory for its replication in non-dividing cells. Additionally, by cytoplasm and nuclear fractionation and qPCR analysis, we ascertained the nuclear import defect of HIV-$IN_{K215A/K219A/R263A/K264A}$ virus (**Figure 20**). However, in contrast to 2-LTR circle

DNA synthesis analysis that exhibited a substantial impairment of nuclear import in HIV-$IN_{K215A/K219A/R263A/K264A}$ virus infection, the cell fractionation and viral DNA quantification showed a relatively less pronounced nuclear import defect in HIV-$IN_{K215A/K219A/R263A/K264A}$ virus infection. We would attribute this variation in results to technical limitations. As we know that the non integrated HIV cDNA or PIC in the cytoplasm could be subject to degradation, or PICs that stick to the cytoplasmic side of NPC or nuclear membrane could still be a part of nucleus fraction, thus quantitative estimation of HIV nuclear import by cell fractionation may not be very precise. Nevertheless, HIV-$IN_{K215A/K219A/R263A/K264A}$ is consistently defective for nuclear import by both 2-LTR circle DNA analysis and cell fractionation method, even though the precise extent of the nuclear import defect may be debatable. Overall, findings of this study provided clear evidence for the requirement of ^{211}KELQKQITK and ^{262}RRKAK motifs in IN for Impα3 interaction and HIV nuclear import. It can be said that, ^{211}KELQKQITK and ^{262}RRKAK motifs of IN act as a non-conventional bi-partite NLS for Impα3 interaction and HIV nuclear import. In conclusion, this study provides first time evidence for IN interaction with Impα3 and essential role of this viral and cellular factors interaction in HIV nuclear import.

Chapter 4

The IN and DYNLL1 Interaction and Its Role in Early Stage HIV Replication

4.1 Rationale

In chapter 3, we have elucidated the IN and Impα3 interaction, the molecular mechanism of IN and Impα3 interaction, and its requirement for HIV nuclear import [201, 376]. However, prior to nuclear import, HIV RTC/PIC has to pass through the cytoplasm and reach perinuclear space. The cytoplasm is a highly dense environment and passive diffusion of macromolecules such as RTC/PIC is not possible due to extensive steric hindrance [270]. Studies have shown that macromolecules are actively transported in the cytoplasm with the help of MT associated motor protein complexes called dynein and kinesin (reviewed in [272-276]. The dynein complex mediates the retrograde transportation of macromolecules in cytoplasm along MT (discussed in chapter 1). In an earlier study, HIV replication was moderately impaired in cells that are treated with MT depolymerizing agent Nocodazole (10mM) [298]. A subsequent study showed the co-localization of HIV replication complexes (RTC/PIC) with cytoplasmic MT followed by concentration of RTC/PIC at MTOC, near the nuclear periphery [282]. However, RTC/PIC localization at MTOC was impaired in cells that were microinjected with anti-DIC antibody [282], implicating dynein complex in HIV retrograde transportation. Consistently, another study also demonstrated the MT dependent retrograde transportation of HIV complex in cytoplasm [377]. These findings led to the conclusion that HIV utilizes dynein complex for retrograde migration in the cytoplasm. However, whether and how HIV targets dynein complex for retrograde transportation and its significance for early replication steps remains elusive.

The dynein adapter proteins such as DYNLL1 [299-301], DYNLT1 [302], and p150^{Glued} [277, 279] mediate cargo recruitment to dynein complex by simultaneous interaction with cargo and DIC1/2 (reviewed in[285]). By yeast two hybrid screening, de Soultrait *et al.,* found HIV IN interaction with Dyn2p, a yeast homolog of human DYNLL1 [304]. Later, Desfarges *et al.,* showed that HIV IN-GFP fusion protein is transported to the perinuclear space of *S. cerevisiae* (yeast) in MT dependent manner [305]. However, IN-GFP localization at the perinuclear space was lost in ΔDyn2p mutant *S. cerevisiae* strain [305]. These findings suggest that DYNLL1 could mediate the recruitment of HIV RTC/PIC to dynein complex. Recently, Su *et al*., reported the DYNLL1 interaction with BIV CA protein and its involvement in retrograde transportation of BIV [299]. Similarly, HFV Gag protein also interacted with DYNLL1 and localized to MTOC [303]. However, it should be noted that although DYNLL1 is a human homolog of Dyn2p, DYNLL1 and Dyn2p show extensive diversity at the amino acid level (ie., approximately 25% amino acid diversity). Therefore, the findings made in yeast system may not necessarily be true for mammalian cells. Moreover, by structure and thermodynamic analysis of DYNLL1, Williams *et al*., recently questioned the ability of DYNLL1 to mediate the recruitment of cargo to dynein complex [286]. Moreover, DYNLL1 is also known to be associated with several different cellular functions outside the dynein complex [287, 301, 378-380]. In fact, DYNLL1 is known to facilitate the gene expression step of Rabies virus replication but has no known role in retrograde transportation of this virus [306, 307]. In addition to IN, RTC/PIC also contains several other important viral proteins such as MA, CA, NC, RT, and Vpr [26, 124]. Notably, IN, MA, and CA are involved in early stage HIV replication, including nuclear import (reviewed in[309]). However, it is not known

whether any of these viral proteins mediate the recruitment of RTC/PIC to dynein complex or retrograde transportation. IN, MA, or CA proteins can possibly facilitate the recruitment of RTC/PIC to dynein complex through interaction with DYNLL1.

4.2 Hypothesis

From the above discussion, we hypothesized that IN, MA, and/or CA proteins will interact with DYNLL1 and this viral and cellular proteins interaction will facilitate the association of HIV with the dynein complex and retrograde transportation or other early steps of HIV replication.

4.3 Objectives

4.3.1 The investigation of IN, MA, or CA protein interaction with DYNLL1

4.3.2 The analysis of requirement of DYNLL1 for steps of early stage HIV replication

4.3.3 The characterization of IN interaction with DYNLL1

4.3.4 The investigation of requirement of IN and DYNLL1 interaction for RTC/PIC recruitment to dynein complex or other steps of early stage HIV replication

4.4 Results

4.4.1 Analysis of the Interaction of IN, CA, or MA Protein with DYNLL1

In this section, we examined the interaction of HIV IN, CA, or MA protein with endogenous DYNLL1 in 293T cells by Co-IP. Briefly, AcGFP-IN, YFP-MA, and AcGFP-CA proteins were expressed in 293T cells and their interaction was examined with endogenous DYNLL1 by anti-GFP immunoprecipitation and DYNLL1 interaction

analysis, as described in the Materials and Methods. We found a specific interaction of DYNLL1 with AcGFP-IN **(Figure 21A, top row, lane 2)** but not with MA-YFP or AcGFP-CA. As DYNLL1 is a component of cellular protein complexes, including dynein complex (reviewed in [287]), IN and DYNLL1 interaction in 293T cells may not necessarily suggest direct protein-protein interaction. Therefore, to determine the direct interaction between IN and DYNLL1, we carried out an *in vitro* interaction analysis for IN and DYNLL1 recombinant proteins, as described in the Materials and Methods. The results showed a specific co-precipitation of DYNLL1 with GST-IN but not with GST-MA or GST alone **(Figure 21B, top row).** The immunoprecipitation of GST, GST-IN, and GST-MA was detected by probing elutes in WB using anti-GST antibody **(Figure 21B, bottom row)** and presence of DYNLL1 in supernatants was detected by probing WB with anti-DYNLL1 antibody. Next, we examined IN and DYNLL1 interaction in an actual HIV infection, as described in the Materials and Methods. The results showed specific co-precipitation of DYNLL1 with IN-HA in HxBru-IN-HA infected but not in HxBru infected cells **(Figure 21C, top row).** Both HxBru-IN-HA and HxBru infected samples showed similar level of infection **(Figure 21C, bottom row)**. Together, these results led the conclusion that DYNLL1 is a novel cellular co-factor for IN interaction.

4.4.2 DYNLL1 is Required for Early Stage HIV Replication

In this section, we asked whether DYNLL1 is required for early stage HIV replication. As we speculated that DYNLL1 would function in the recruitment of HIV to dynein complex, DYNLL1 would most likely be involved in early stage HIV replication. In order to examine the requirement of DYNLL1 for HIV replication, HIV replication

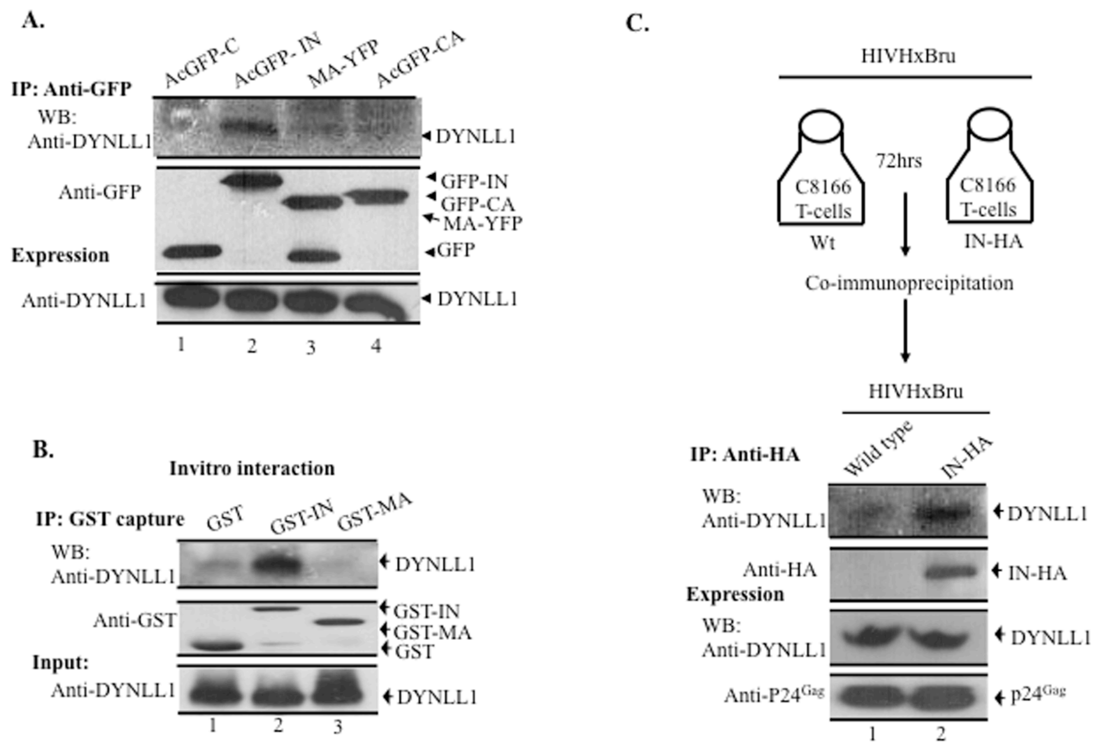

Figure 21. HIV IN interacts with DYNLL1: (A) AcGFP, AcGFP-IN, MA-YFP, or AcGFP-CA expressors were transfected into 293T cells. After 48h of transfection, cells were subjected to immunoprecipitation using an anti-GFP antibody and the co-precipitation of DYNLL1 was detected by WB using an anti-DYNLL1 antibody (top row). The DYNLL1 expression in the total cell lysates was detected by WB using an anti-DYNLL1 antibody (bottom row). Immunoprecipitation of AcGFP, AcGFP-IN, MA-YFP, and AcGFP-CA was detected by WB using an anti-GFP antibody (middle row). (B) Equal amount of GST, GST-IN, or GST-MA was incubated with DYNLL1 recombinant protein (at 0.2 µg of each protein). Samples were subjected to GST pull-down assay, as described in the Materials and Methods. The co-precipitation of DYNLL1 was detected by WB with an anti-DYNLL1 antibody (top row). GST, GST-IN, or GST-MA protein in the elutes was detected by WB using an anti-GST antibody (middle row). DYNLL1 protein in the supernatants was detected by WB using an anti-DYNLL1 antibody (bottom row). (C) $15x10^6$ C8166T cells were infected with HxBru or HxBru-IN-HA virus (at 10 ng of virus-associated $p24^{Gag}$ antigen). After 72h of infection, cells were subjected to immunoprecipitation using an anti-HA antibody and the co-precipitation of DYNLL1 was detected by WB using an anti-DYNLL1 antibody (top row). The IN-HA in the immunoprecipitates was detected by WB using an anti-HA antibody (second row from the top). The expression of DYNLL1 and equal amount of HIV infection was determined by detecting DYNLL1 and HIV $p24^{Gag}$ proteins in the total cell lysates by WB (3rd and 4th row from the top, respectively).

analysis was performed in DYNLL1-KD cells. DYNLL1 was KD using shRNA approach, as described in the Materials and Methods. Briefly, LVPs for different clones of DYNLL1 shRNA (Clone #1, #2, and #3) or no shRNA (control) were produced in 293T cells. $2.5x10^6$ C8166T cells were transduced with different clones of DYNLL1 or control LVPs (400 ng of $p24^{Gag}/2.5x10^6$ cells in 12 well plate format). At day 3 of the post transduction, KD efficiency was determined by detecting DYNLL1 protein expression by WB using anti-DYNLL1 antibody. Among all the different clones, the KD was relatively better in cells transduced with LVPs for DYNLL1 shRNA cone#2 (**Figure 22A, upper row**). Therefore, LVP for clone#2 was used for DYNLL1-KD experiments in this study. Prior to performing the infection studies, we analyzed the viability of DYNLL1-KD cells by WST-1 cell proliferation assay, as described in the Materials and Methods. Both DYNLL1 and control-LVPs transduced cells showed similar level of proliferation until five days after transduction. However, after 5 days of transduction, the proliferation of DYNLL1-KD cells was moderately reduced (**Figure 22B).** Therefore, we carried out all infection experiments within 5 days of transduction. To determine the requirement of DYNLL1 for HIV replication, $0.5x10^6$ control or DYNLL1-KD C8166T cells were infected with wild type HIVpNL4.3-GFP^+. At 48h of infection, cells were lysed using 0.5% NP40 lysis buffer and $p24^{Gag}$ protein in cell lysates was detected in WB by using anti-$p24^{Gag}$ antibody. The $p24^{Gag}$ production in DYNLL1-KD cells was greatly reduced **(Figure 22C, upper row)**, suggesting defective HIV replication in DYNLL1-KD cells. Next, we explored the requirement of DYNLL1 for early stage HIV replication. To examine early stage HIV replication, $0.5x10^6$ control or DYNLL1-KD C8166T cells were infected with different concentrations of single cycle replication competent Luc

reporter HIV (HIV-Luc). At 24h post-infection, virus replication was detected by measuring the Luc activity. In general, HIV luciferase reporter virus infection analysis will help to study the virus replication steps until gene expression. In all the investigated infection doses, we found an average 5- to 7-fold reduced Luc activity in DYNLL1-KD cells compared to the control **(Figure 22D).** To rule out the involvement of DYNLL1 in HIV gene expression or viral protein synthesis steps, we transfected HIVpNL4.3/R^-/E^-/Luc^+ or HIVpNL4.3/R^-/E^-/GFP^+ proviral DNAs into control and DYNLL1-KD 293T cells and examined Luc activity or GFP fluorescence. Results showed no differences in Luc activity **(Figure 22E)** or GFP fluorescence **(Figure 22F)** between control and DYNLL1-KD cells. In the next step, HIV entry into DYNLL1-KD cells was examined by quantifying viral genomic RNA. Briefly, $3x10^6$ control or DYNLL1-KD C8166T cells were cultured in presence of a reverse transcription inhibitor AZT (1 μg/ml) and infected with HIV-Luc virus (at 200 ng of virus-associated $p24^{Gag}$). At 3h post infection, RNA from cells was isolated using RNeasy Mini Kit (Qiagen), by following manufacturer's instructions. The cDNA was prepared from the RNA samples and HIV genomic cDNA (corresponds to HIV genomic RNA) was quantified by qPCR, as described in the Materials and Methods. We found no significant differences in HIV genomic RNA quantities between control and DYNLL1-KD cells **(Figure 22G).** In order to know whether DYNLL1 is required for replication of other retroviruses, we examined the MMLV-Luc replication in DYNLL-KD cells. MMLV is a member of the *Retroviridae* family. MMLV-Luc replication in DYNLL1-KD cells was reduced by approximately 4-fold (**Figure 23A**), indicating the DYNLL1 requirement for MMLV replication. Consistent with this observation, we were also able to detect the MMLV IN interaction

with

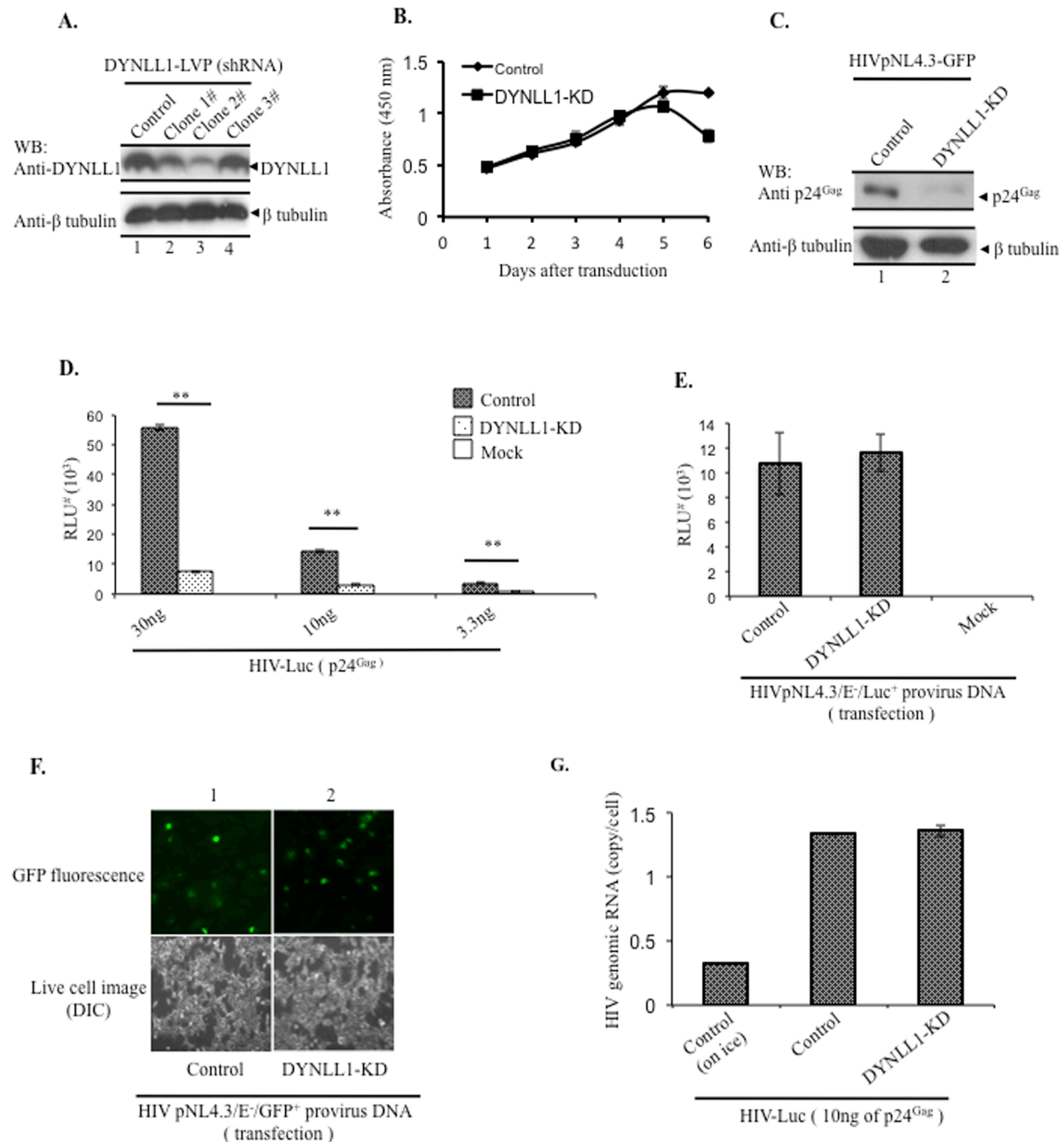

Figure 22. DYNLL1 is required for early stage HIV replication: (A) $3x10^6$ C8166T cells were transduced with LVPs that express shRNA for DYNLL1 (clone 1#, 2#, or 3#) or no shRNA (control). At 72h post transduction, the extent of DYNLL1 KD was determined by WB, using the anti-DYNLL1 antibody (top row). The β tubulin protein expression was detected as a loading control (bottom row). (B) A WST-1 assay was performed to determine the proliferation of control or DYNLL1 LVP transduced C8166T cells, at different time points as indicated. (C) Control or DYNLL1-KD C8166T cells were infected with HIVpNL4.3-GFP (at 5 ng of virus-associated $p24^{Gag}$ antigen)

At 48h of post-infection, p24^{Gag} protein in the total cell lysates was detected by WB using an anti-p24^{Gag} antibody (upper row). The β tubulin protein expression was detected as a loading control (lower row). (D) 0.5x10^{6} control and DYNLL1-KD C8166T cells were infected with different doses of HIV-Luc. At 24h post infection, virus replication was examined by measuring Luc activity. The statistical significance for differences in infections between control and DYNLL1-KD cells was determined by Student t-test. **P<0.01. (E and F) Control or DYNLL1-KD 293T cells were transfected with HIVpNL4.3/E^{-}/Luc^{+} or HIVpNL4.3/E^{-}/GFP^{+} provirus DNA (1.5 μg of DNA/well, in 6 well plate) and Luc activity was measured (Fig E) or GFP fluorescence was observed under fluorescence microscope (AxiovertTM 200; Carl Zeiss, at 20x magnification) (Fig F). (G) 3x10^{6} control or DYNLL1-KD C8166T cells were infected with HIV-Luc (at 200ng of virus-associated p24^{Gag}) and HIV genomic RNA at 3h post infection was quantified by qPCR, as described in the Materials and Methods. # Relative light unit.

DYNLL1 **(Figure 23B),** justifying the requirement of DYNLL1 for MMLV replication. To further define the specificity of DYNLL1 requirement for only retroviruses, we carried out replication analysis for Adenovirus and VSV. Surprisingly, Adenovirus replication was enhanced, where as VSV virus replication was severely impaired in DYNLL1-KD cells. However, due to the lack of availability of proper information on the role of DYNLL1 in Adenovirus or VSV infection, we were not able to clearly interpret these latter findings. Nevertheless, the above data clearly suggest that DYNLL1 is required for post entry steps of early stage HIV replication such as reverse transcription, nuclear import, and/or integration.

4.4.3 DYNLL1 is Required for HIV cDNA Synthesis

In this section, we examined the requirement of DYNLL1 for specific step(s) of early stage HIV replication. As DYNLL1 is one of the adapter proteins that mediate cargo recruitment by dynein complex [299-301], we speculated that DYNLL1 might be contributing to nuclear import or reverse transcription steps of early stage HIV replication. Therefore, we examined HIV total viral DNA and 2-LTR circle DNA synthesis in DYNLL1-KD cells by qPCR. Briefly, 1.5×10^6 control and DYNLL1-KD C8166T cells were infected with HIV-Luc (at 10 ng of virus-associated $p24^{Gag}$). At 12 and 24h of infection, total viral DNA and 2-LTR circle DNA were quantified by qPCR. Interestingly, we found an average 3- to 4-fold reduced total viral DNA synthesis in DYNLL1-KD cells (**Figure 24A**) and a similar reduction in 2-LTR circle DNA synthesis (**Figure 24B**). This suggested that DYNLL1 is specifically involved in the HIV cDNA

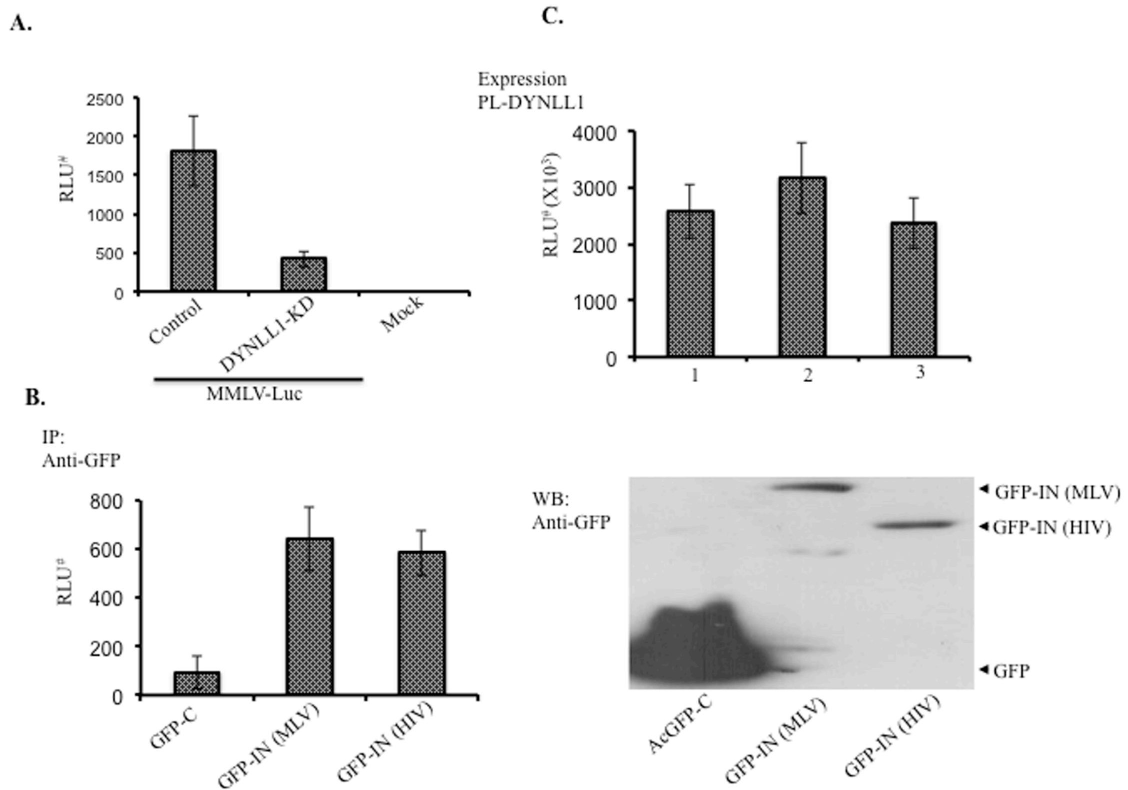

Figure 23. DYNLL1-KD affects MMLV replication: (A) $0.5x10^6$ control or DYNLL1-KD cells were infected with equal amount MMLV-Luc. At 24h of infection, MMLV replication was examined by measuring Luc activity. (B) AcGFP, AcGFP-IN (MMLV), or AcGFP-IN (HIV) were co-transfected with PL-DYNLL1 in 293T cells. After 48h of transfection, cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation of PL-DYNLL1 was detected by measuring PL activity. (C) PL-DYNLL1 expression in the total cell lysates was detected by measuring PL activity (upper panel). AcGFP, AcGFP-IN (MMLV), or AcGFP-IN (HIV) expression in the total cell lysates was detected by WB using an anti-GFP antibody (lower panel). # Relative light unit.

synthesis step. Next, as DYNLL1 interacted with IN, we asked whether the loss of HIV cDNA synthesis in DYNLL1-KD cells is IN dependent. The control and DYNLL1-KD C8166T cells were infected with HIV_{Wt} or HIV-ΔIN virus (at 50 ng of virus-associated $p24^{Gag}$). At 12h of infection, total viral DNA was quantified by qPCR. Although total viral DNA synthesis was reduced in DYNLL1-KD cells infected with HIV_{Wt}, no difference was evident in cells infected with HIV-ΔIN virus **(Figure 24D)**. Above data indicated that DYNLL1 requirement for HIV cDNA synthesis is IN dependent. Alternatively, DYNLL1 could also contribute to HIV cDNA synthesis by interacting with RT protein. RT is a key viral enzymatic protein for HIV reverse transcription (reviewed in [12] and discussed in chapter 1) and cellular factors have been known to play a role in RT mediated HIV reverse transcription. To detect RT and DYNLL1 interaction, T7-RT or T7-IN was co-transfected with PL-DYNLL1 in 293T cells. After 48h of transfection, the interaction of T7-RT or T7-IN with PL-DYNLL1 was examined by Co-IP using an anti-T7 antibody followed by detection of PL activity from the immunoprecipitates. However, we failed to detect the interaction between DYNLL1 and RT, even though there was a positive interaction between IN and DYNLL1 **(Figure 25B).** Together, the above data led to the conclusion that DYNLL1 is specifically required for HIV cDNA synthesis and the DYNLL1 requirement for HIV cDNA synthesis is IN dependent.

4.4.4 Conserved Motifs in IN N- and C-terminal Domains are Required for DYNLL1 Interaction

Proteins bind to DYNLL1 with the help of consensus motifs. DYNLL1 interaction motifs are broadly grouped into three classes based on the sequence similarity; K*X*TQT*X*,

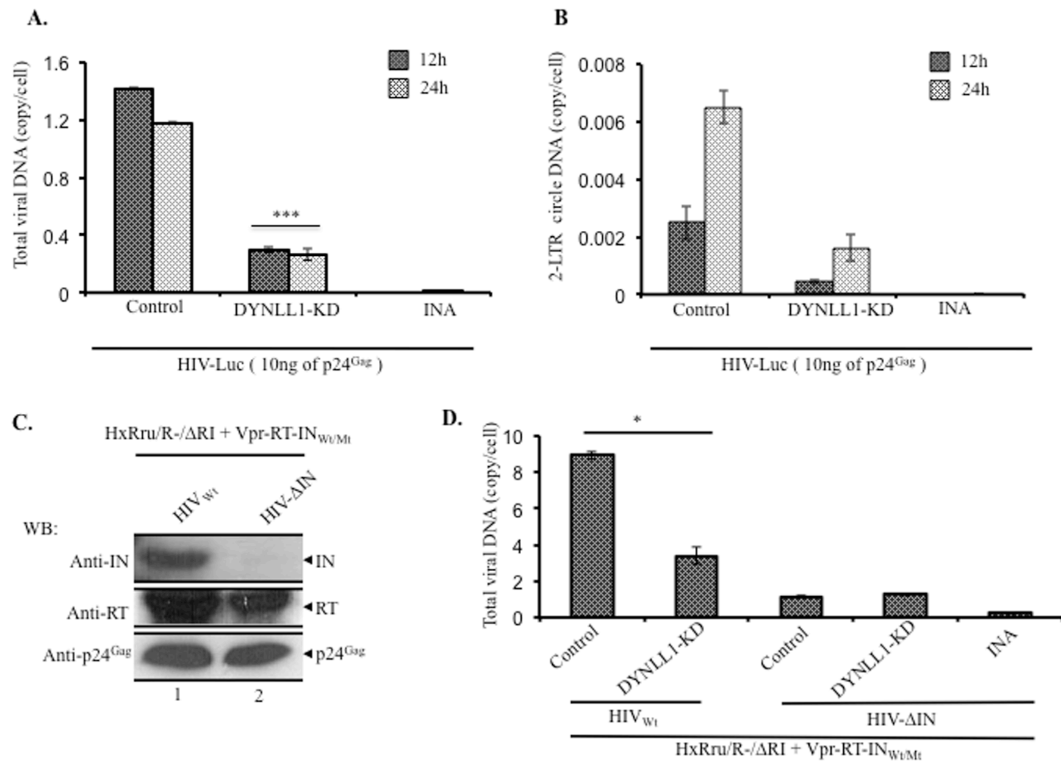

Figure 24. DYNLL1-KD affects HIV cDNA synthesis: (A) and (B) 1.5×10^6 control or DYNLL1-KD C8166T cells were incubated with HIV-Luc (at 10 ng of virus-associated $p24^{Gag}$ antigen). At 12 and 24h post infection, total viral DNA (Fig. A) and 2-LTR circle DNA (Fig. B) were quantified by qPCR. Data shown are means and standard errors and are representative of the results for triplicate samples of a typical experiment. Data was confirmed in three independent experiments. A two way ANOVA analysis was performed to determine the statistical significance between control and DYNLL1-KD cell infection, ***$P<0.001$. (C) An equal amount of HIV_{Wt} or HIV-ΔIN virus (at 30 ng of virus-associated $p24^{Gag}$) was lysed in 0.5% NP40 lysis buffer and the virus incorporated RT (top row), IN (middle row), and $p24^{Gag}$ (bottom row) proteins were detected by WB using corresponding antibodies. (D) 1.5×10^6 control and DYNLL1-KD C8166T cells were infected with HIV_{Wt} or HIV-ΔIN virus (at 50 ng of virus-associated $p24^{Gag}$ antigen). At 12h post infection, total viral DNA was quantified by qPCR. Data shown are means and standard errors and are representative of the results from a typical experiment. Data were confirmed in two independent experiments. The statistical significance was determined by Student's t-test analysis, *$P<0.05$ (N=3).

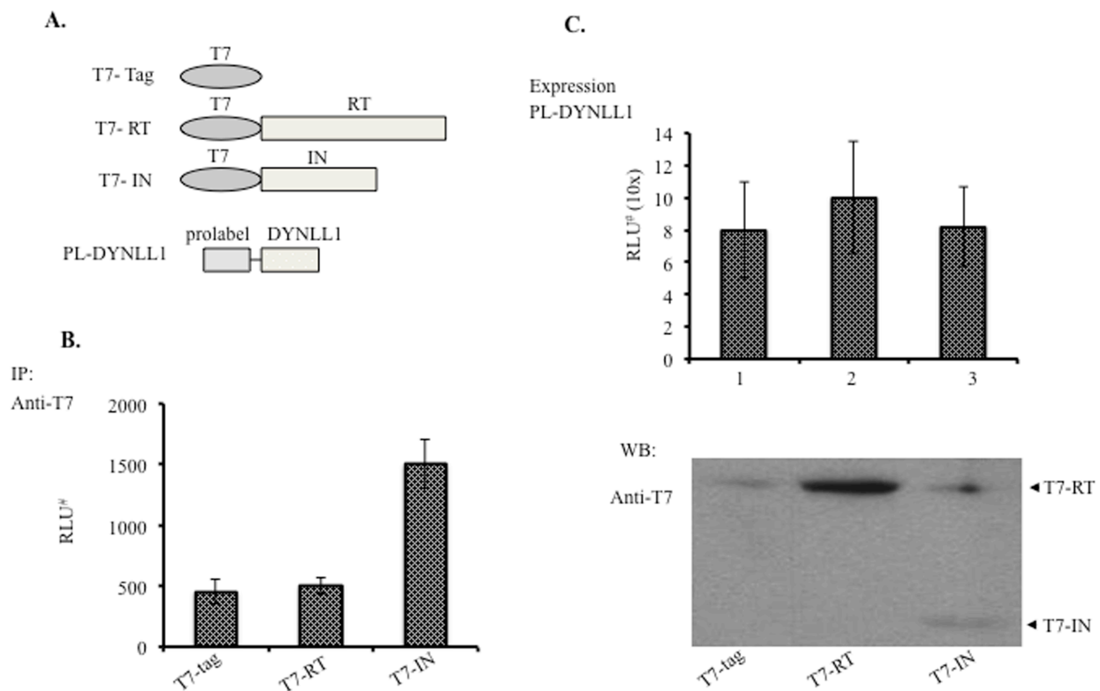

Figure 25. DYNLL1 do not interact with HIV RT: (A) Diagram showing different fusion protein expressors used in this experiments. (B) T7-tag, T7-RT, or T7-IN expressors were co-transfected with PL-DYNLL1. After 48h of transfection, 8/10th of cells were subjected to immunoprecipitation using anti-T7 antibody and the co-precipitation of PL-DYNLL1 was detected by measuring PL activity. (C) 1/10th of cells were subjected to PL-DYNLL1 expression analysis, by detecting PL activity (upper panel). 1/10th of cells were subjected to T7-RT or T7-IN expression analysis in WB by using an anti-T7 antibody (lower panel). # Relative light unit.

*X*G(I/V)QVD, and non-canonical [381-383]. To identify the DYNLL1 interaction motif(s) in IN, we examined the DYNLL1 interaction with various IN deletion mutants. Briefly, AcGFP-C, AcGFP-IN_{Wt}, or AcGFP-IN deletion mutant (Aa50-288, Aa117-288, Aa180-230, Aa1-212, and Aa1-230) expressors **(Figure 26A)** were co-transfected with PL-DYNLL1 in 293T cells and PL-DYNLL1 interaction with AcGFP-$IN_{Wt/Mt}$ was examined by chemiluminescent Co-IP using an anti-GFP antibody. We found that IN deletion mutants that do not contain residues Aa1-117 ($IN_{117-288}$ mutant) or Aa250-288 ($IN_{1-212,}$ $IN_{1-230,\ and}$ IN_{1-250} mutants) were impaired for the interaction with DYNLL1 **(Figure 26B).** The $IN_{180-230}$ deletion mutant that lacks both Aa1-117 and Aa250-288 residues was severely attenuated for interact with DYNLL1 **(Figure 26B).** These data led to the conclusion that Aa50-117 and Aa250-288 residues in IN are required for DYNLL1 interaction. By analyzing IN amino acid sequence, we found three motifs ("^{52}GQVD", ^{207}DIQT, and "^{250}VIQD") in IN (**shown in Figure 26C**) that closely resembled the consensus sequence for DYNLL1 interaction. We introduced Gln(Q) to Ala(A) substitution mutation into these IN motifs ($IN_{Q53A,}$ INQ_{209A}, IN_{Q252A}) and probed their interaction with DYNLL1. Results showed that, while IN_{Q209A} showed a wild type of interaction, IN_{Q53A} and IN_{Q252A} exhibited attenuated interaction with DYNLL1 **(Figure 26D, top panel**). Then, we introduced Q53A/Q252A double mutations into IN ($IN_{Q53A/Q252A}$) and probed its interaction with DYNLL1. Our data showed that the PL-DYNLL1 was more impaired for interaction with the AcGFP-$IN_{Q53A/Q252A}$ double mutant than with the AcGFP-IN_{Q53A} or AcGFP-IN_{Q252A} single mutant (Figure 26E, top panel). From these data, we indicate that the "^{52}GQVD" and "^{250}VIQD" motifs in IN are essential for interaction with DYNLL1.

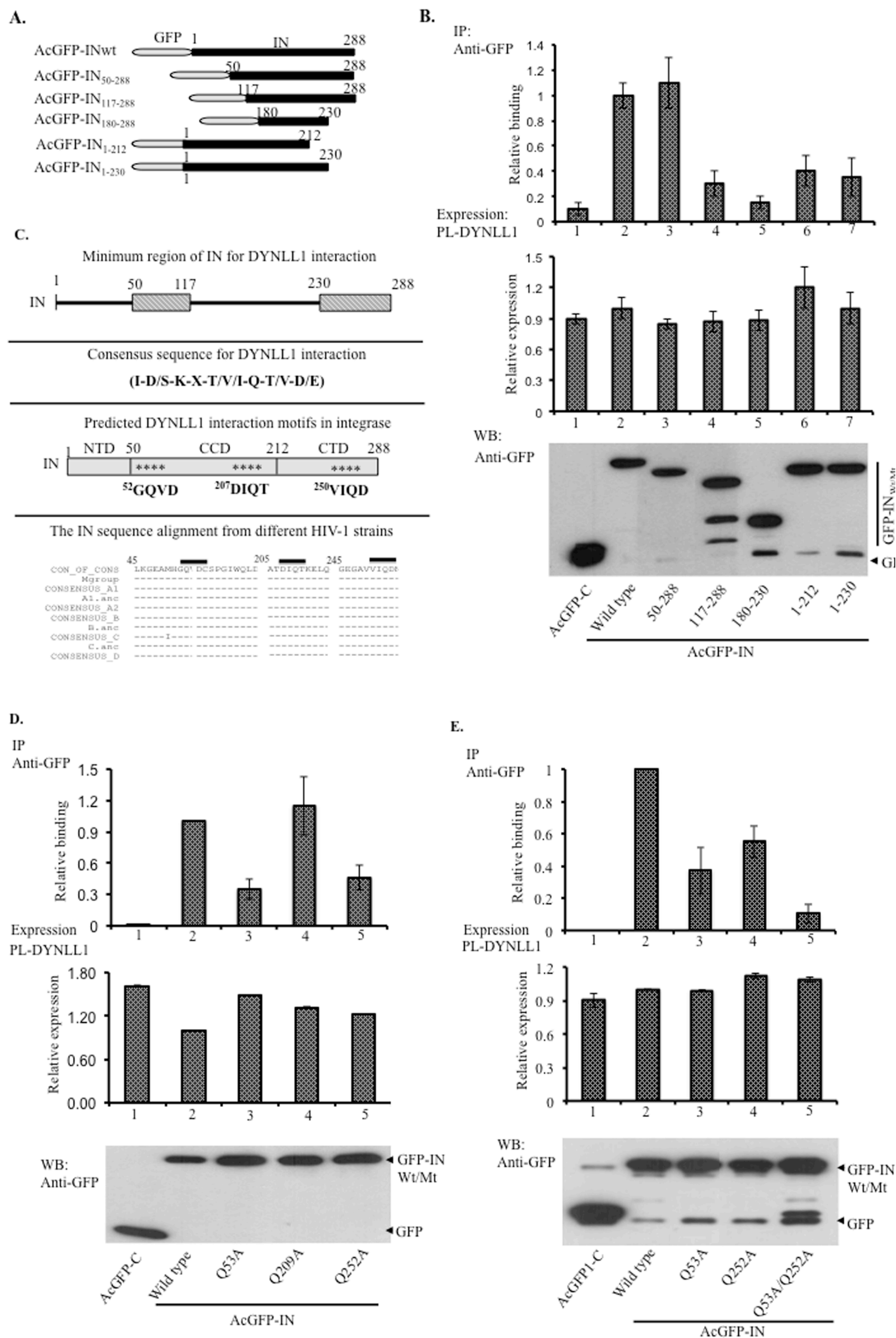
A.
AcGFP-INwt
AcGFP-IN50-288
AcGFP-IN117-288
AcGFP-IN180-288
AcGFP-IN1-212
AcGFP-IN1-230
GFP
IN
B.
IP:
Anti-GFP
Relative binding
Expression:
PL-DYNLL1
Relative expression
WB:
Anti-GFP
AcGFP-C
Wild type
50-288
117-288
180-230
1-212
1-230
AcGFP-IN
C.
Minimum region of IN for DYNLL1 interaction
Consensus sequence for DYNLL1 interaction
(I-D/S-K-X-T/V/I-Q-T/V-D/E)
Predicted DYNLL1 interaction motifs in integrase
NTD
CCD
CTD
52GQVD
207DIQT
250VIQD
The IN sequence alignment from different HIV-1 strains
D.
IP
Anti-GFP
Relative binding
Expression
PL-DYNLL1
Relative expression
WB:
Anti-GFP
GFP-IN
Wt/Mt
GFP
AcGFP-C
Wild type
Q53A
Q209A
Q252A
AcGFP-IN
E.
IP
Anti-GFP
Relative binding
Expression
PL-DYNLL1
Relative expression
WB:
Anti-GFP
GFP-IN
Wt/Mt
GFP
AcGFP1-C
Wild type
Q53A
Q252A
Q53A/Q252A
AcGFP-IN

Figure 26. Consensus motifs of IN N- and C-terminal domains are required for DYNLL1 interaction: (A) Schematic diagram showing various AcGFP-IN deletion mutants expressors used in this experiment. (B) AcGFP or AcGFP-IN_{Wt}/deletion mutant expressors were co-transfected with PL-DYNLL1 expressor in 293T cells. After 48h of transfection, cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation PL-DYNLL1 was detected by measuring PL activity (upper panel). PL-DYNLL1 expression in total the cell lysates was detected by measuring PL activity (middle panel) and AcGFP-IN_{Wt} or various deletion mutant proteins expression in the total cell lysates was detected by WB using an anti-GFP antibody (lower panel). (C) Diagram showing the minimum region in IN for DYNLL1 interaction, consensus DYNLL1 interaction sequence, predicted DYNLL1 interaction motifs in HIV IN, and amino acid sequence alignment for IN sequence from representative HIV strains. (D) AcGFP-IN_{Wt}, AcGFP-IN_{Q53A}, AcGFP-IN_{Q209A}, or AcGFP-IN_{Q252A} expressor was co-transfected with PL-DYNLL1 expressor in 293T cells. At 48h of post-transfection, cells were subjected to immunoprecipitation using an anti-GFP antibody and the co-precipitation of PL-DYNLL1 was detected by measuring PL activity (top panels). The expression of PL-DYNLL1 and AcGFP-IN_{Wt} or various mutants was examined by measuring PL activity (middle panel) and WB using the anti-GFP antibody (bottom panel), respectively. (E) AcGFP-IN_{Wt}, AcGFP-IN_{Q53A}, AcGFP-$IN_{Q252A,}$ or AcGFP-$IN_{Q53A/Q252A}$ expressor was co-transfected with PL-DYNLL1 expressor in 293T cells. At 48h of post-transfection, cells were subjected to immunoprecipitation using an anti-GFP antibody and the co-precipitation of PL-DYNLL1 was detected by measuring PL activity (top panels). The expression of PL-DYNLL1 and AcGFP-IN_{Wt} or various mutants was examined by measuring PL activity (middle panel) and WB using the anti-GFP antibody (bottom panel), respectively. Data shown here is the average values from three independent experiments. The data presented for expression of GFP-$IN_{Wt/Mt}$ is from a typical experiment.

4.4.5 The Loss of IN and DYNLL1 Interaction has Impaired HIV cDNA Synthesis

To gain more insight into the requirement of IN and DYNLL1 interaction for HIV replication and cDNA synthesis, we introduced DYNLL1 interaction defective IN mutations ($IN_{Q53A,}$ IN_{Q252A}, or $IN_{Q53A/Q252A}$) into a previously described HIV single-cycle replication system [1] and examined their replication and cDNA synthesis. Briefly, various HIV_{Mt} viruses were produced by co-transfecting HxBruR^{-}/ΔRI provirus DNA with Vpr-RT-IN_{Q53A}, Vpr-RT-IN_{Q252A}, or Vpr-RT-$IN_{Q53A/Q252A}$ mutant fusion proteins expressors, respectively **(shown in Figure 27A).** In parallel, IN negative HIV mutant (HIV-ΔIN) was also included as a negative control. Prior to infection analysis, virus incorporated IN, RT, and $p24^{Gag}$ proteins were detected by probing equal amounts of virus lysates in WB using anti-IN, anti-RT, and anti-$p24^{Gag}$ antibodies, respectively. Results showed equal amount of RT, IN, and $p24^{Gag}$ proteins incorporation into HIV_{Wt} or HIV_{Mt} viruses **(Figure 27B).** Then, $0.5x10^{6}$ C8166T cells were infected with equal amount of HIV_{Wt}, HIV_{Mt}, or HIV-ΔIN viruses and virus replication was analyzed at 72h post infection by detecting $p24^{Gag}$ content from supernatants using $HIVp24^{Gag}$ ELISA. A moderately reduced virus replication was evident in HIV-IN_{Q53A} and HIV-IN_{Q252A} mutant virus infection, whereas virus production was severely impaired in HIV-$IN_{Q53A/Q252A}$ double mutant and HIVΔIN virus infection (approximately, 5- to 7-fold reduced) (**Figure 27C**). Next, we examined total viral DNA synthesis from control and DYNLL1-KD C8166T cells that were infected with wild type or mutant viruses. Briefly, $1.5x10^{6}$ control and DYNLL1-KD C8166T cells were infected with equal amount HIV_{Wt}, HIV-$IN_{Q53A/Q252A}$, or HIV-ΔIN (at 50 ng of virus-associated $p24^{Gag}$ each) and total viral DNA was quantified by qPCR at 12h post infection, as described in the Materials and Methods.

Results showed a significantly reduced cDNA synthesis in HIV-$IN_{Q53A/Q252A}$ infected control cells and was comparable with cDNA synthesis in HIV-ΔIN virus infected control cells (**Figure 27D**). On the contrary, no obvious cDNA synthesis difference was evident between HIV_{Wt} and HIV-$IN_{Q53A/Q252A}$ infected DYNLL1-KD cells **(Figure 27D).** As IN and RT interaction was implicated in HIV reverse transcription by earlier studies [369, 384-388], we examined the interaction between $IN_{Q53A/Q252A}$ and RT proteins. However, our data showed no change in interaction of RT with IN_{Wt} or $IN_{Q53A/Q252A}$ **(Figure 28, top row).** From the above data, we conclude that IN and DYNLL1 interaction is required for HIV cDNA synthesis.

4.4.6 The Defective cDNA Synthesis in DYNLL1-KD Cells or HIV-$IN_{Q53A/Q252A}$ Mutant Virus Infection is Attributed to Impaired Virus Uncoating

In this section, we explored the cause(s) for defective HIV cDNA synthesis in DYNLL1-KD cells or DYNLL1 interaction defective HIV-IN mutant virus infection. Although importance of DYNLL1 interaction for proteins is not completely clear, accumulated evidences suggest that proteins undergo conformational change and exhibit higher α helical content following DYNLL1 binding [389-391]. Therefore, we speculated that DYNLL1 interaction would stabilize the coiled-coils and create additional sites for secondary interactions in IN. IN functions as a tetramer. During tetramer formation, IN monomers assemble into a homodimer and one of the IN monomers of a homodimer binds with IN monomer from another homodimer and establishes IN tetramer [392]. However, IN monomer interactions in tetramer are not stable and the binding of IN with cellular proteins has been suggested for stabilization of these interactions [393].

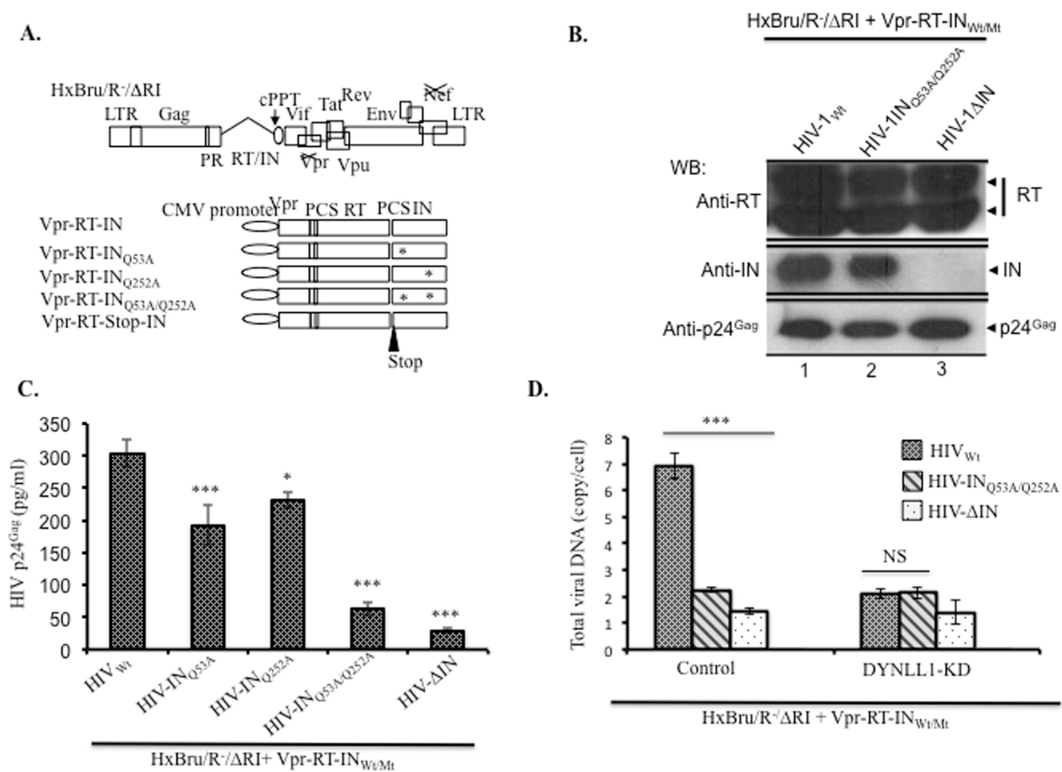

Figure 27. DYNLL1 interaction defective IN mutant HIV is impaired for cDNA synthesis and replication: (A) Diagrammatic representation of HxBru/R^-/ΔRI provirus and Vpr-RT-IN_{Wt} or various IN mutant fusion protein expressors. (B) The virus incorporation of RT (top row), IN (middle row), and $p24^{Gag}$ (bottom row) proteins in HIV_{Wt}, HIV-$IN_{Q53A/Q252A}$, or HIV-ΔIN virus (at 30 ng of virus-associated $p24^{Gag}$ antigen) were detected by WB using corresponding antibodies. (C) $0.5x10^6$ C8166T cells were infected with HIV_{Wt}, HIV-IN_{Q53A}, HIV-IN_{Q252A}, HIV-$IN_{Q53A/Q252A}$, or HIV-ΔIN virus (at 10 ng of virus-associated $p24^{Gag}$ antigen). The supernatants were harvested at 48h and $p24^{Gag}$ concentrations were estimated by HIV$p24^{Gag}$ ELISA. Data shown are means and standard errors and are representative of the results for triplicate sample from two independent experiments. The statistical significance for differences between wild type and mutant virus infections was determined by a one way ANOVA, ⁎P<0.05 (N=3), ***P<0.001 (N=3). (D) $1.5x10^6$ control or DYNLL1-KD C8166T cells were infected with HIV_{Wt}, HIV-$IN_{Q53A/Q252A,}$ or HIV-ΔIN virus (at 50 ng of virus-associated $p24^{Gag}$ antigen). At 12h post infection, total viral DNA was quantified by qPCR. Data shown are means and standard errors and are representative of the results for triplicate samples from two independent experiments. The statistical significance for differences between wild type and mutant virus infections was determined by a one way ANOVA, ***P<0.001 (N=3).

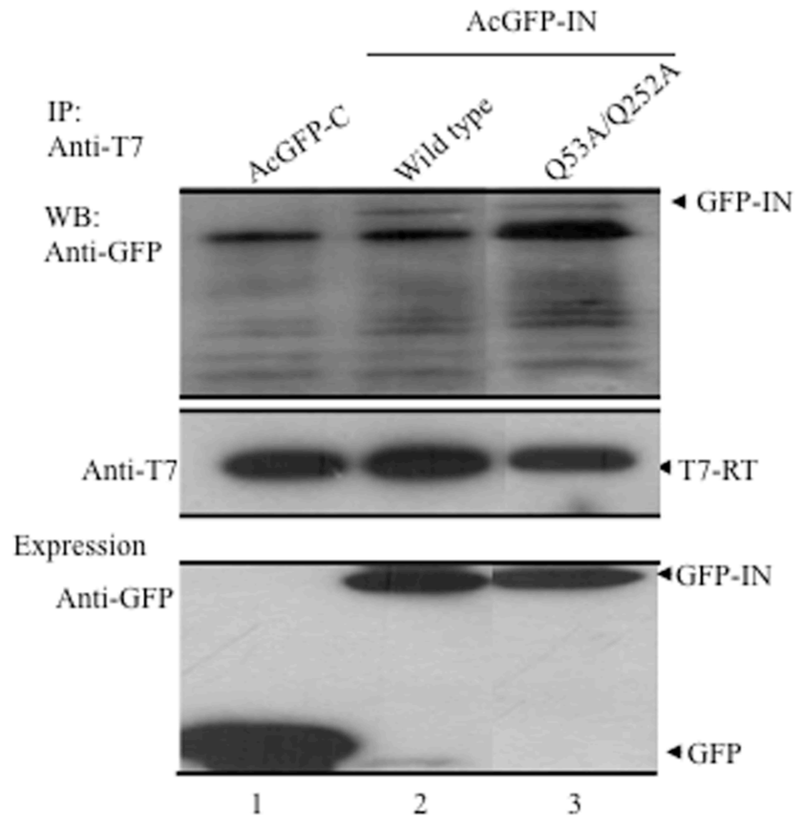

Figure 28. $IN_{Q53A/Q252A}$ mutant protein interacts with RT: AcGFPc, AcGFP-IN, or AcGFP-$IN_{Q53A/Q252A}$ was co-transfected with T7-RT in 293T cells and cells were subjected to immunoprecipitation using the anti-T7 antibody. The co-precipitation of AcGFP-$IN_{Wt/Mt}$ with T7-RT was detected by WB using an anti-GFP antibody (top row), and immunoprecipitation of T7-RT was detected by WB using an anti-T7 antibody (middle row). The expression of AcGFPc, AcGFP-IN_{Wt}, or $AcGFP_{Q53A/Q252A}$ was detected by WB using an anti-GFP antibody (bottom row).

Therefore, it is possible that DYNLL1 interaction may contribute to a stable IN tetramer formation and promote IN tetramer interaction to additional proteins in cells. As studies also suggested that IN and RT interaction facilitates reverse transcription [369, 384-388], we presumed that IN tetramerization would contribute to stable IN and RT complex formation and facilitate reverse transcription. To test this hypothesis, we examined the requirement of DYNLL1 interaction for IN-IN association in cells. Briefly, 293T cells were transfected with different protein expressors in the following order: AcGFPc with PL-IN_{Wt} (**Figure 29, lane1**), AcGFP-IN_{Wt} with PL-IN_{Wt} (**Figure 29, lane2**), AcGFP-$IN_{Q53A/Q252A}$ with PL-$IN_{Q53A/Q252A}$ (**Figure 29, lane3),** or AcGFP-$IN_{K186A/R187A}$ with PL-$IN_{K186A/R187A}$ (**Figure 29, lane4)** expressors. After 48h of transfection, cells were lysed in 0.2% NP40 lysis buffer and immunoprecipitated with an anti-GFP antibody. GFP-$IN_{Wt/Mt}$ bound PL-$IN_{Wt/Mt}$ were detected by measuring PL activity from the immunoprecipitates. Results showed no difference in interactions between AcGFP-IN_{Wt} and PL-IN_{Wt} (**Figure 29, lane 2**) or AcGFP-$IN_{Q53A/Q252A}$ and PL-$IN_{Q53A/Q252A}$ (**Figure 29, lane 3**). However, as expected, interaction between GFP-$IN_{K186A/R187A}$ and PL-$IN_{K186A/R187A}$ was reduced (**Figure 29, lane 4**). $IN_{K186A/R187A}$ mutant is known for defective multimerization [394]. From these data, it was clear that DYNLL1/IN interaction is unlikely to influence IN multimerization. After ruling out the role of DYNLL1 in IN tetramerization, we decided to examine the possible involvement of IN and DYNLL1 interaction in HIV uncoating. It is known that HIV cDNA synthesis is tightly coupled with virus uncoating. Either accelerated or delayed uncoating can impair the cDNA synthesis [310, 314, 315]. Interestingly, HIV-ΔIN showed accelerated uncoating and reduced cDNA production in an infection analysis [310]. Since DYNLL1 is required for HIV cDNA synthesis

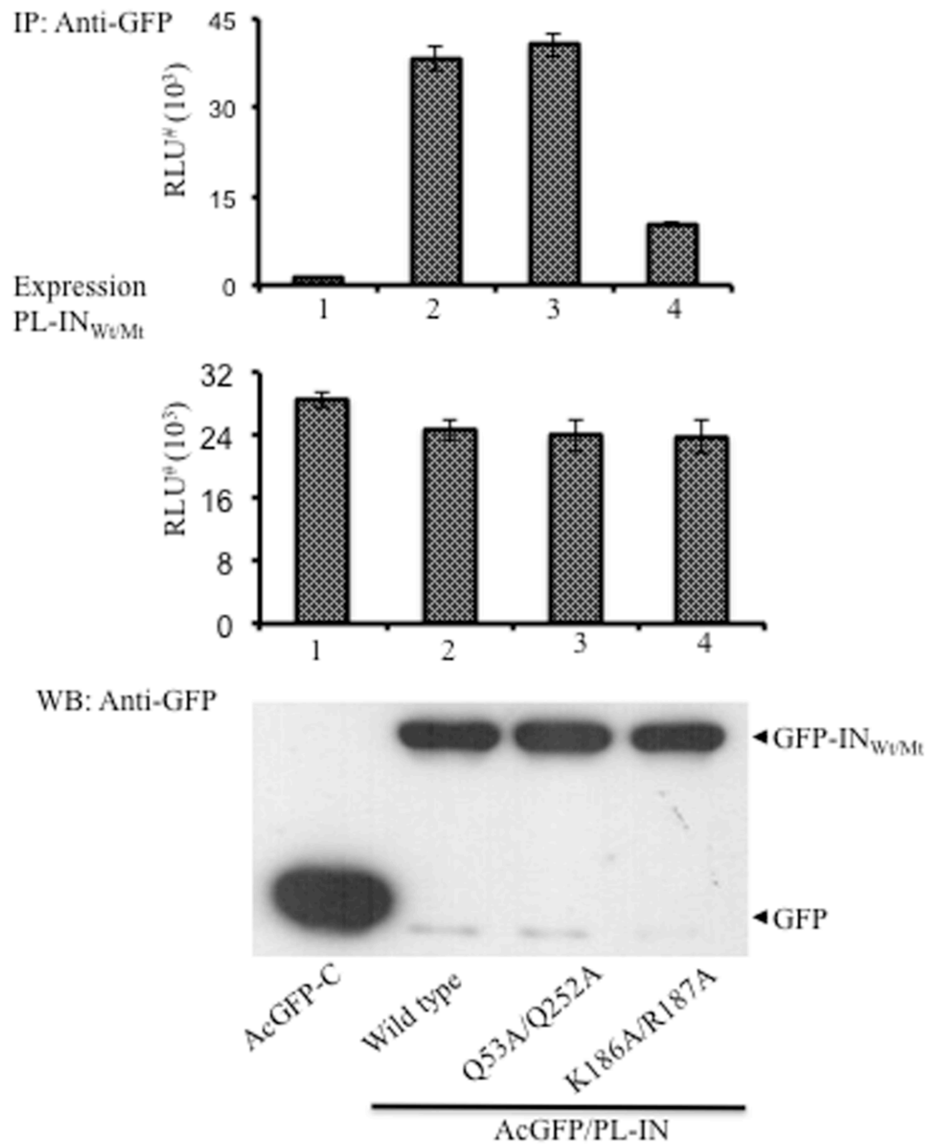

Figure 29. DYNLL1 interaction is not required for IN multimerization: 293T cells were co-transfected with AcGFP/PL-$IN_{Wt/Mt}$ fusion protein expressors as follows: AcGFPc with PL-IN_{Wt} (**lane1**), AcGFP-IN_{Wt} with PL-IN_{Wt} (**lane2**), AcGFP-$IN_{Q53A/Q252A}$ with PL-$IN_{Q53A/Q252A}$ (**lane3),** or AcGFP-$IN_{K186A/R187A}$ with PL-$IN_{K186A/R187A}$ (**lane4)**. After 48h of transfection, cells were subjected to immunoprecipitation using the anti-GFP antibody and the co-precipitation of PL-$IN_{Wt/Mt}$ was examined by measuring PL activity (top panel). The expression of PL-$IN_{Wt/Mt}$ in the total cell lysates was detected by measuring PL-activity (middle panels) and expression of AcGFP-$IN_{Wt/Mt}$ protein was detected by WB using an anti-GFP antibody (bottom panel). # Relative light unit.

and interacts with IN, we have investigated the requirement of DYNLL1 or IN and DYNLL1 interaction for HIV uncoating. To examine DYNLL1 requirement for HIV uncoating, the "fate of capsid assay" approach was used, as described in the Materials and Methods **(Figure 30A)**. Prior to performing uncoating analysis, the feasibility of fate of capsid assay in our laboratory condition was examined. Briefly, AcGFP-CA expressing 293T cells or HIV infected C8166T cells were subjected to fate of capsid assay. Supernatant and pellet fractions were resolved in 12% SDS-PAGE and WB was probed for AcGFP-CA, HIVp24^{Gag}, or IN proteins, using corresponding antibodies. In AcGFP-CA transfected cells, AcGFP-CA was found only in supernatant fraction but not in pellet fraction **(Figure 30B, top row, lane1 & 2).** In HIV infected cells, IN was found only in pellet fraction but not in supernatant fraction (**Figure 30B, bottom row, lane 3 & 4**) and p24^{Gag} protein was found in both fractions, which is expected **(Figure 30B, middle row, lane 3 & 4)**. This indicates that soluble CA (AcGFP-CA in transfected cells or free p24^{Gag} in HIV infected cells) is only found in supernatant fraction, where as PIC (IN) and PIC associated CA is found in pellet fraction. Then, $3x10^7$ control or DYNLL1-KD C8166T cells were infected with HIV_{Wt}. An additional panel of control cells were similarly infected on ice and included as a negative control. At 4h post infection, cells were washed with fresh RPMI medium for five times and 1/20th of cells were subjected to p24^{Gag} ELISA to detect the virus entry. HIV infection was detected only in cells infected at 37°C but not on ice **(Figure 30C).** The remaining cells were cultured in fresh medium. At 4h, 8h, and 12h post infection, 10^7 cells were harvested and subjected to fate of capsid assay. In all different time intervals of harvest, CA protein content in supernatant

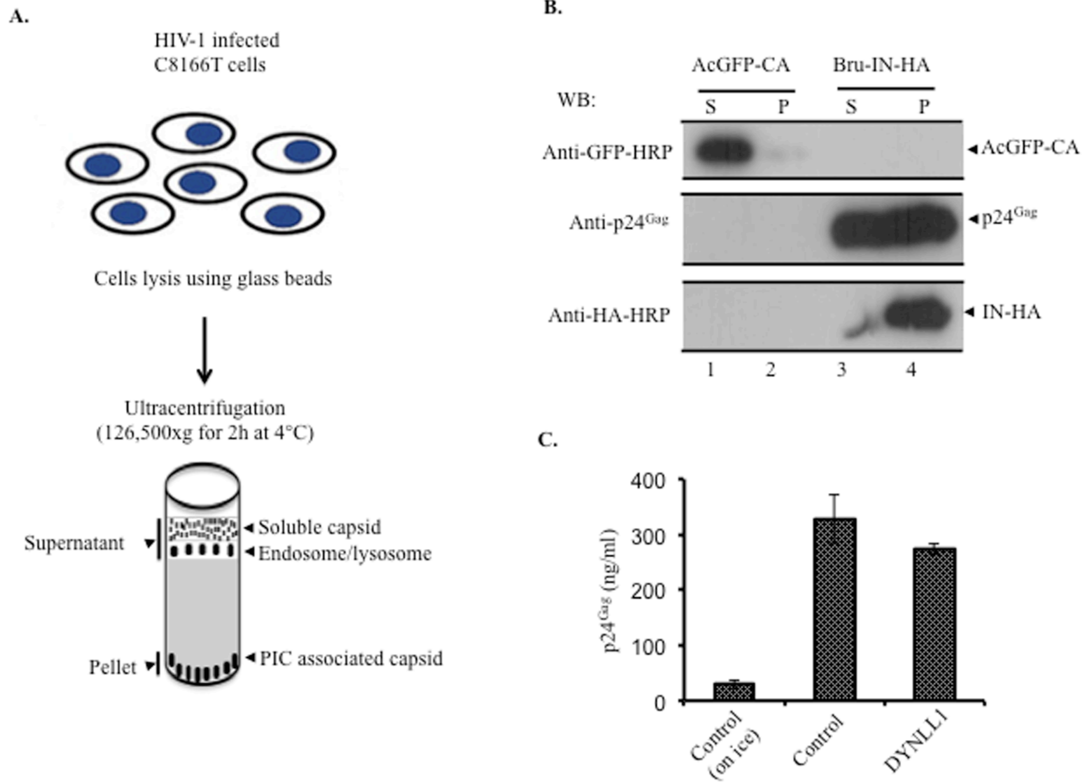

Figure 30. Fate of capsid assay optimization: (A) Diagram showing different steps of fate of capsid assay, as performed in this study. (B) AcGFP-CA expressor transfected 293T cells or HIVHxBru-IN-HA virus infected C8166T cells were subjected to fate of capsid assay, as described in the Materials and Methods. Supernatant and pallet fractions were resolved in 12% SDS PAGE and WB was probed with anti-GFP (top row), anti-$p24^{Gag}$ (middle row), and anti-HA antibodies (bottom row). (C) $1/50^{th}$ of control C8166T cells infected on ice or control and DYNLL-KD C8166T cells infected at 37^0C were subjected to HIV$p24^{Gag}$ ELISA and HIV infection was examined.

fractions of DYNLL1-KD cell infections was significantly higher than that of control cell infections **(Figure 31A),** suggesting the accelerated HIV uncoating in DYNLL1-KD cells. Subsequently, we examined the uncoating of HIV_{Wt} and $HIV\text{-}IN_{Q53A/Q252A}$ mutant viruses. HIV-ΔIN was included as a control. Briefly, $3x10^7$ C8166T cells were infected with equal amount of HIV_{Wt}, $HIV\text{-}IN_{Q53A/Q252A}$, or HIV-ΔIN. At 4h, 8h, and 12h post infection, 10^7 cells were harvested and subjected to fate of capsid assay. Interestingly, the supernatant fractions of $HIV\text{-}IN_{Q53A/Q252A}$ or HIV-ΔIN infection samples contained significantly higher proportion of CA as compared to HIV_{Wt} **(Figure 31B),** confirming the accelerated uncoating in $HIV\text{-}IN_{Q53A/Q252A}$ and HIV-ΔIN infections**.** Next, we examined the uncoating of HIV_{Wt} and $HIV\text{-}IN_{Q53A/Q252A}$ mutant viruses in DYNLL1-KD cells. Briefly, 10^7 control and DYNLL1-KD C8166T cells were infected with equal amount of HIV_{Wt} or $HIV\text{-}IN_{Q53A/Q252A}$. At 4h post infection, cells were processed for fate of capsid assay. Interestingly, although $HIV\text{-}IN_{Q53A/Q252A}$ showed accelerated uncoating in control cell infection, the uncoating difference between HIV_{Wt} and $HIV\text{-}IN_{Q53A/Q252A}$ in DYNLL1-KD cells was absent **(Figure 31C)**. This data clearly suggests that accelerated uncoating of $HIV\text{-}IN_{Q53A/Q252A}$ is DYNLL1 dependent. As an earlier study linked accelerated uncoating of HIV-ΔIN to lack of CypA incorporation into virus [310], we probed the CypA incorporation in HIV_{Wt} and $HIV\text{-}IN_{Q53A/Q252A}$ mutant viruses. We found no difference in CypA incorporation between wild type and mutant viruses **(Figure 31D, upper row).** Based on these data, we suggest that DYNLL1 and IN interaction is required for the proper uncoating of HIV. Further, from the above data, we speculate that accelerated uncoating in the absence of IN and DYNLL1 interaction may lead to the formation of unstable RTC/PIC, which would indirectly contribute to reduced cDNA

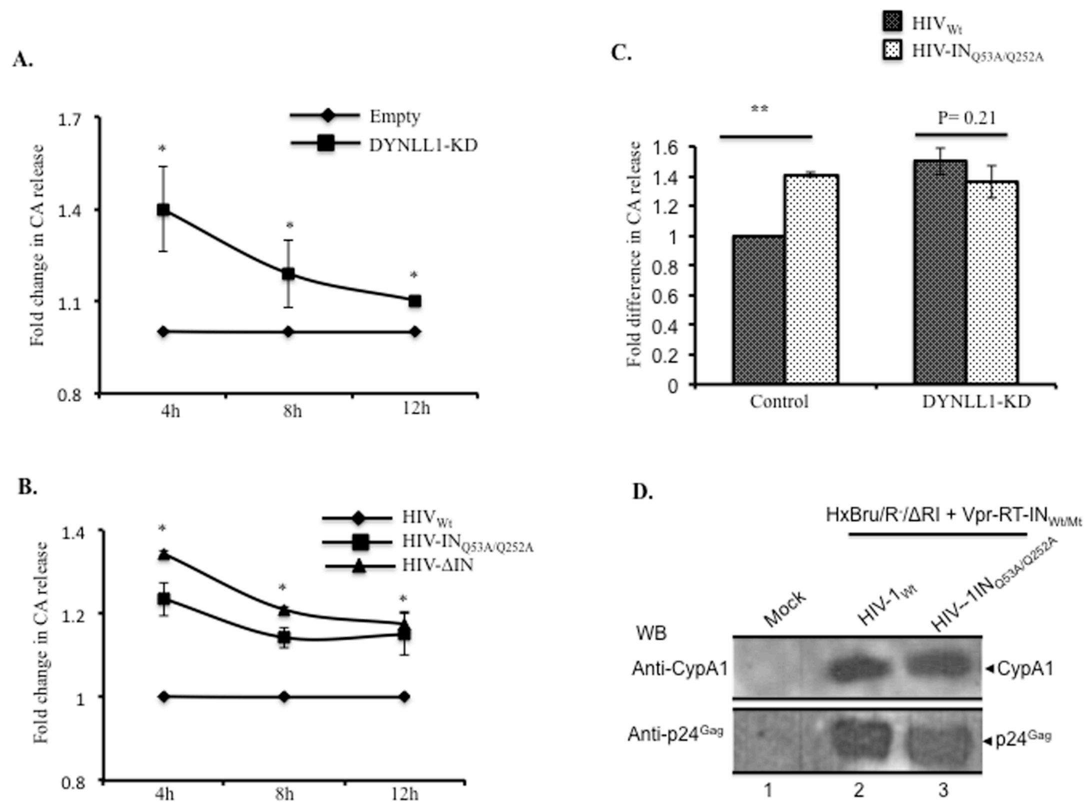

Figure 31. The defective HIV cDNA synthesis in DYNLL1-KD cells or HIV-$IN_{Q53A/Q252A}$ mutant virus infection is attributed to impaired virus uncoating: (A) Control or DYNLL1-KD C8166T cells were infected with equal amount of HIV_{Wt} virus. At 4h, 8h, and 12h post infection, cells were subjected to fate of capsid assay analysis. The data is interpreted as fold differences in CA release (fold difference in soluble CA protein) between control and DYNLL1-KD cell infections. Data shown are means and standard errors and are representative of the results for triplicate samples from two independent experiments. (B) C8166T cells were infected with equal amount of HIV_{Wt}, HIV-$IN_{Q53/252A}$, or HIV-ΔIN virus. At 4h, 8h, and 12h post infection, cells were subjected to fate of capsid assay analysis and data was interpreted as fold differences in CA release between $HIV_{Wt/Mt/\Delta IN}$ infected cells. Data shown are means and standard errors and are representative of the results for triplicate samples from two independent experiments. The statistical significance (Fig. A & B) for differences between infections was determined by a one way ANOVA, $\star P<0.05$ (N=3). (C) Control and DYNLL1-KD C8166T cells were infected with equal amount of HIV_{Wt} or HIV-$IN_{Q53A/Q252A}$ virus. At 4h post infection, cells were subjected to fate of capsid assay and data was interpreted as extent of CA release in wild type and mutant virus infected DYNLL-KD or control cells. Data shown are means and standard errors and are representative of the results for triplicate samples from two independent experiments. The statistical significance for differences between wild type and mutant virus infection was determined by Student t-test, $\star P<0.05$ (N=3). (D) The virus incorporated CypA (upper row) and $p24^{Gag}$ (lower row) proteins in HIV_{Wt} or HIV-$IN_{Q53/252A}$ virus.

synthesis (**depicted in Figure 32**). A detailed investigation on how IN and DYNLL1 interaction will contribute to proper uncoating of HIV and its mechanistic importance for cDNA synthesis is strongly encouraged.

4.4.7 IN and DYNLL1 Interaction is Dispensable for HIV Recruitment to Dynein Complex

One of the functions of DYNLL1 is to mediate the cargo recruitment to dynein complex [299-301]. Meanwhile, DYNLL1 also has several functions outside the dynein complex and/or retrograde transportation (reviewed in [287]). Therefore, we examined the role of IN and DYNLL1 interaction in possible recruitment of HIV RTC/PIC to dynein complex. Since adapter proteins recruit cargo to dynein complex through interaction with DIC1/2 (as depicted in **Figure 33A**), we performed DYNLL1 interaction defective $IN_{Q53A/Q252A}$ mutant interaction with DIC1 in 293T cells. Briefly, 293T cells were transfected with AcGFPc, AcGFP-IN_{Wt}, AcGFP-$IN_{Q53A/Q252A}$, or AcGFP-CA. At 48h of transfection, cells were subjected to immunoprecipitation with anti-GFP antibody and interaction analysis, as described in the Materials and Methods. Interestingly, AcGFP-INwt and AcGFP-$IN_{Q53A/Q252A}$ showed similar level of interaction with DIC1 (**Figure 33B, top row, lane 2 and 3**), whereas AcGFPc or AcGFP-CA showed no interaction with DIC1 (**Figure 33B, top row, lane1 and 4**), ruling out nonspecific binding of $IN_{Wt/Mt}$ with DIC. Also, we observed no differences in the AcGFP-IN and DIC1 interaction in the presence and absence of excess T7-DYNLL1 expression (**Figure 33C, top row, lane 2 and 3**). The above data clearly suggests that DYNLL1 interaction is dispensable for DIC interaction and therefore, IN and DYNLL1 interaction may not be essential for HIV recruitment to

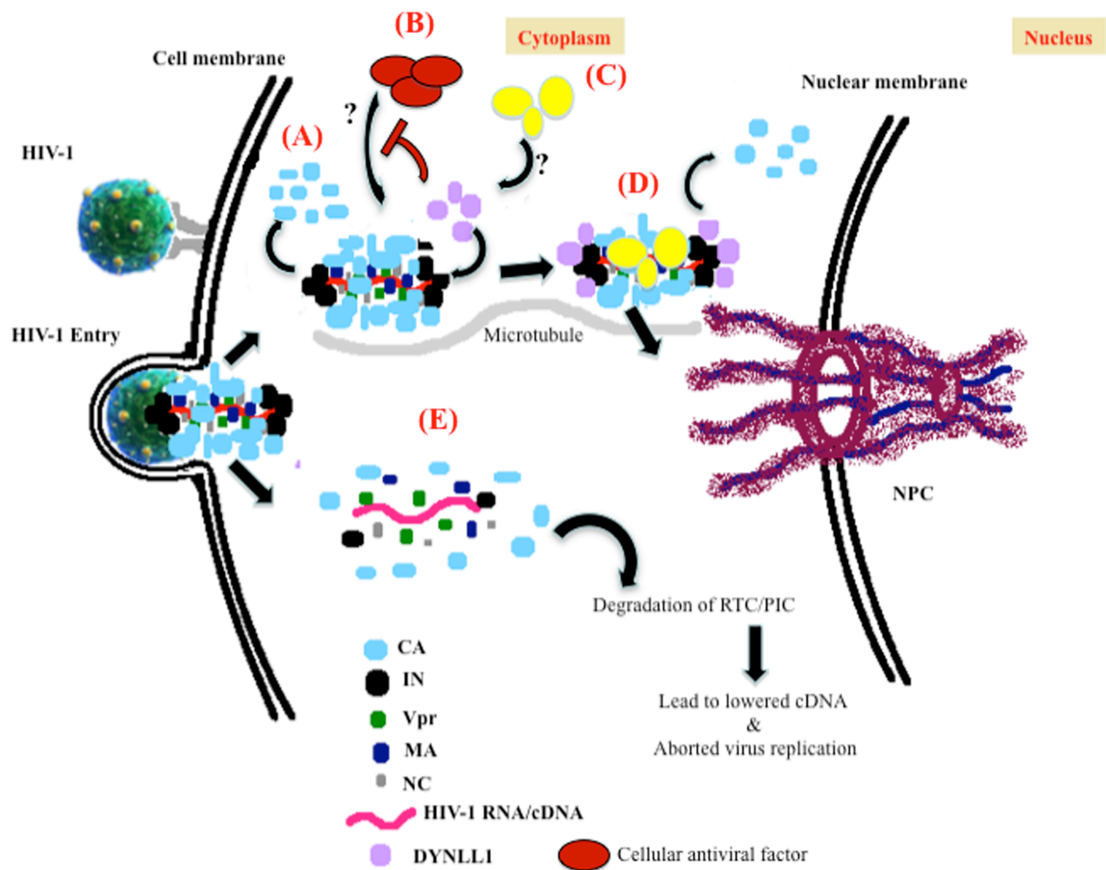

Figure 32. The model depicting the potential mechanism by which the interaction of DYNLL1 and IN contributes to HIV uncoating and cDNA synthesis. HIV entry is followed by the reorganization of the RTC, uncoating, and reverse transcription **(A)**. The normal uncoating of HIV following entry into the cytoplasm. **(B)** The cellular antiviral factor hypothesis: the recruitment of DYNLL1 to RTC/PIC via IN interaction will protect HIV from the cellular antiviral factors (known or unknown antiviral factors). **(C)** The cellular co-factor hypothesis: DYNLL1 may help to recruit previously known or unknown cellular factors to the RTC/PIC through simultaneous interactions with cellular factors and HIV IN, which could contribute to the stabilization and/or the re-organization of the RTC/PIC after virus entry into the cytoplasm. (D) The stabilization of the RTC/PIC that occurs due to the interaction between IN and DYNLL1 would favor the proper uncoating and reverse transcription of HIV (E). In the absence of DYNLL1 and IN interaction, the RTC/PIC would become unstable due to faster uncoating and undergoes degradation, which would result in low levels of HIV cDNA synthesis.

dynein complex. As intact MT is essential for dynein complex mediated retrograde migration, we asked whether intact MT network is essential for HIV reverse transcription or nuclear import. In order to address this question, HIV replication analysis was carried out in MT depolymerizing agent Nocodazole treated cells. Briefly, $0.5x10^6$ C8166T cells were cultured in presence of 10mM of Nocodazole or DMSO vehicle alone for 1h followed by infection with HIV-Luc (at 10 ng of virus-associated $p24^{Gag}$). At 12h post infection, Nocodazole was removed from culture by washing and cells were sub cultured in fresh medium without Nocodazole. At 48h post infection, HIV replication was examined by measuring Luc activity. HIV replication was substantially reduced in Nocodazole treated cells **(Figure 33D)**. Subsequently, $1.5x10^6$ C8166T cells were similarly treated with 10mM of Nocodazole or DMSO vehicle alone and infected with HIV-Luc (at 10ng of virus-associated $p24^{Gag}$). At 12h post infection, cells were harvested and total viral DNA and 2-LTR circle DNA synthesis was examined by qPCR. Interestingly, the total viral DNA synthesis showed no variation between Nocodazole and vehicle alone control cell infection (**Figure 33E**), whereas a moderately reduced 2-LTR circle DNA synthesis was detected in Nocodazole treated cell infection (**Figure 33F**). This data suggests that MT would favor retrograde migration of RTC/PIC in the cytoplasm but not reverse transcription. Together, above data suggest that IN and DYNLL1 interaction is probably not essential for HIV retrograde migration, as IN and DYNLL1 interaction was not essential for nuclear import but was involved in HIV cDNA synthesis. Therefore, findings from this study led to conclusion that although IN and DYNLL1 interaction will mediate the proper HIV uncoating and cDNA synthesis, it is not essential for HIV recruitment to dynein complex or retrograde migration.

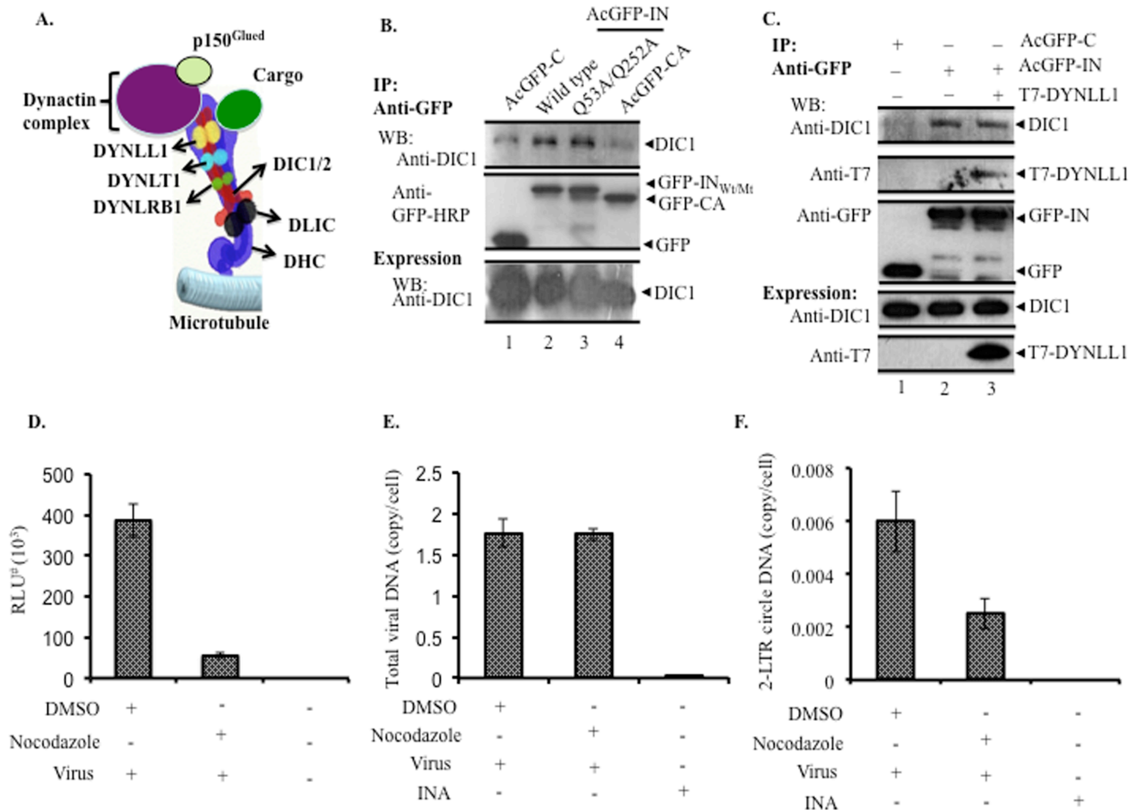

Figure 33. The IN and DYNLL1 interaction is dispensable for the recruitment of the HIV complex to dynein: (A) Figure showing different components of the dynein and the recruitment of cargo to the dynein complex through simultaneous interactions of DYNLL1 with DIC1/2 and cargo. (B) The AcGFP, AcGFP-IN_{Wt}, AcGFP-$IN_{Q53A/Q252A}$, and AcGFP-CA expressors were transfected in 293T cells and cells were subjected to immunoprecipitation with the anti-GFP antibody. The co-precipitation of endogenous DIC1 protein was detected by WB using an anti-DIC1 antibody (top row). The AcGFP, AcGFP-IN_{Wt}, AcGFP-$IN_{Q53A/Q252A}$, or AcGFP-CA was detected by WB using an anti-GFP antibody (middle row). DIC1 expression in the total cell lysates was detected by WB using an anti-DIC1 antibody (bottom row). (C) AcGFP or AcGFP-IN was co-expressed with or without T7-DYNLL1 in 293T cells. The co-precipitation of DIC1 (top row) and T7-DYNLL1 (second row from the top) with AcGFP or AcGFP-IN was detected by immunoprecipitation, as described above. The AcGFP or AcGFP-IN in the immunoprecipitates was detected by WB (third row from the top). The T7-DYNLL1 and DIC1 proteins expression in the total cell lysates was detected by WB using the corresponding antibodies (bottom two rows) (D) HIV-Luc replication in 10 mM Nocodazole or DMSO vehicle alone treated cells. (E & F) HIV-1 total viral DNA (Fig. E) and 2-LTR circle DNA (Fig. F) synthesis in 10 mM nocodazole or DMSO vehicle alone treated cells. The means and standard errors are representative of the results obtained for triplicate samples from a typical experiment and were confirmed in an additional independent experiment. # Relative light unit.

4.5 Discussion

Previously, HIV IN was found interacting with Dyn2p, a yeast homolog of mammalian DYNLL1 [304]. An earlier study demonstrated the HIV RTC/PIC co-localization with MT followed by concentration at MTOC [282]. However, microinjection of anti-DIC antibody resulted in loss of RTC/PIC co-localization with MT and concentration at MTOC [282], suggesting the involvement of dynein complex in HIV retrograde migration. However, viral and/or cellular factors that mediate the recruitment of RTC/PIC to dynein complex were unknown. A previous study elucidated the DYNLL1 interaction with BIV CA protein and its requirement for BIV retrograde transportation [299]. Similarly, another study demonstrated the DYNLL1 dependent transportation of HFV Gag protein in the cytoplasm [303]. Therefore, we wondered whether HIV IN, CA, or MA protein interacts with DYNLL1. In this study, we identified the specific interaction of IN with DYNLL1 in 293T cells, in *in vitro*, and in HIV infected cells. Thus, we were able to suggest that DYNLL1 is a novel cellular co-factor for IN interaction. The infection analysis showed that DYNLL1 is required for post entry steps of early stage HIV replication and, interestingly, DYNLL1 was essential for HIV cDNA synthesis but not nuclear import. By mutagenic analysis, ^{52}GQVD" and ^{250}VIQD motifs of IN were identified as essential for DYNLL1 interaction. Meanwhile, HIV-$IN_{Q53A/Q252A}$ mutant virus was impaired for cDNA synthesis. Further investigation showed that loss of IN and DYNLL1 interaction led to the accelerated uncoating of HIV but not compromised the HIV recruitment to dynein complex. Together these findings helped to conclude that IN and DYNLL1 interaction is essential for proper HIV uncoating and

cDNA synthesis but unlikely to play a role in HIV recruitment to dynein complex or retrograde migration.

Viruses have been known to target dynein complex for retrograde transportation (reviewed in [395]). Dynein is a MT associated protein complex that actively transports macromolecules towards the nucleus. Dynein complex consists of structural, regulatory, and adapter proteins. Adapter proteins mediate the recruitment of cargo to dynein complex by simultaneous interaction with cargo and DIC [285, 396]. So far, DYNLL1, DYNLT1, and, $p150^{Glued}$ are known to recruit macromolecules to dynein complex [277, 279, 299-302]. HIV RTC/PIC contains various viral proteins[26, 124], but only IN, MA, and CA are known to be essential for early stage HIV replication (Reviewed in [309]). Vpr was also found in RTC/PIC. However, Vpr is not mandatory for early stage HIV replication or HIV nuclear import [178, 210, 397]. Therefore, we excluded Vpr from our viral proteins interaction analyses with DYNLL1. We know that HIV IN interacts with Dyn2p, a yeast homolog of mammalian DYNLL1. Similarly, BIV CA also interacted with DYNLL1 and BIV retrograde transportation was dependent on DYNLL1. Even HFV Gag protein underwent retrograde migration in DYNLL1 dependent manner. These reports generated interest to explore whether HIV IN, CA, or MA protein interact with DYNLL1. In the interaction analysis, we found a specific interaction of IN with DYNLL1 in 293T cells and in *in vitro* **(Figure 21A and 21B)**. In order to know the relevance of these interaction results for HIV infection, we determined IN interaction with DYNLL1 in actual HIV infected cells (**Figure 21C**). However, at this moment, no direct evidence is available to show whether DYNLL1 is associated with RTC/PIC

during infection. Nevertheless, our protein interaction data clearly suggest for the first time that DYNLL1 is a novel cellular co-factor for IN interaction. This finding also helped us to focus our continued investigation on IN and DYNLL1 interaction and its role in early stage HIV replication.

As recruitment of PIC/RTC to dynein complex would most likely favor early stage HIV replication, we studied the requirement of DYNLL1 for early stage HIV replication. The gene KD approach was used to evaluate the requirement of DYNLL1 for HIV replication. First, we examined the viability of DYNLL1-KD cells by performing cell proliferation assay (WST-1 assay). DYNLL1-KD cells were able to proliferate equally well compared to control cells until five days post transduction (**Figure 22B**). However, DYNLL1-KD cells failed to survive beyond 7 days. Therefore, to avoid the possible non-specific effects of KD, we carried out all the experiments within five days of LVPs transduction. Using luciferase reporter HIV infection or HIV provirus DNA transfection, we were able to determine the specific requirement of DYNLL1 for early steps of HIV replication prior to HIV gene expression (**Figure 22D, 22E, and 22F**). As HIV entry was unaffected in DYNLL1-KD cells (**Figure 22G**), it was possible to further narrow down the requirement of DYNLL1 for post entry steps of early stage HIV replication such as reverse transcription, uncoating, nuclear import, and/or integration. The unimpaired entry or steps of late stage HIV replication in DYNLL1-KD cells further ruled out the possible nonspecific effects of gene KD. Subsequently, we performed MMLV infection in DYNLL1-KD cells. Interestingly, MMLV replication was also impaired in DYNLL1-KD cells (3- to 4-fold reduced) (**Figure 23A**). Consistent with infection data, MMLV IN also

interacted with DYNLL1 (**Figure 23B**). Therefore, it is evident that DYNLL1 is not only required for HIV but is also important for the replication of MMLV and possibly even other members of retroviruses. However, further investigation is essential to clearly define the requirement of DYNLL1 for replication of other retroviruses.

In order to identify which specific step(s) of early stage HIV replication that requires DYNLL1, we studied different post entry steps of early stage HIV replication in DYNLL1-KD cells. With the assumption that DYNLL1 will mediate the recruitment of HIV replication complex (RTC/PIC) to dynein complex, we initially assumed that HIV nuclear import would be delayed or impaired in DYNLL1-KD cells. In fact, protein retrograde transportation is essential for the efficient nuclear import of certain cellular proteins (discussed in chapter 1). Contrary to our assumption, HIV cDNA synthesis, but not nuclear import, was impaired in DYNLL1-KD cells (**Figure 24A and 24B**). In the subsequent step, we examined whether the loss of cDNA synthesis in DYNLL1-KD cells is IN dependent. Interestingly, HIV-ΔIN infection of control and DYNLL1-KD cells showed no difference in cDNA synthesis (**Figure 24D**), which in other words confirms the IN dependent requirement of DYNLL1 for HIV cDNA synthesis. Confounded with these surprising results, we examined the interaction between DYNLL1 and RT. As we know that RT is a key enzymatic protein involved in HIV reverse transcription (reviewed in [12]), it is possible that DYNLL1 could be able to contribute to HIV cDNA synthesis by interacting with RT. However, our data failed to show a positive interaction between DYNLL1 and RT **(Figure 25B),** thus ruling out any possible role for RT and DYNLL1 interaction in HIV cDNA synthesis.

In order to further clarify the requirement of IN and DYNLL1 interaction for HIV cDNA synthesis, we chose to identify DYNLL1 interaction motif/s in IN. Proteins interact DYNLL1 through consensus motifs. So far, DYNLL1 interaction motifs are grouped into three major classes based on sequence similarity; K*X*TQT*X*, *X*G(I/V)QVD, and non-canonical [381-383]. Interestingly, by sequence analysis, we found three motifs ("^{52}GQVD", ^{207}DIQT, and "^{250}VIQD") in IN that closely resemble the consensus motif for DYNLL1 interaction. All three motifs are highly conserved across different strains of HIV **(representative sequences are shown in Figure 26D).** Two of these motifs ("^{52}GQVD" and "^{250}VIQD") are within the minimum region of IN for DYNLL1 interaction and "Q" to "A" mutations in these motifs showed attenuated interaction with DYNLL1 **(Figure 26E, top panel, lane 3 and 4)**. However, similar mutations in "^{207}DIQT" motif of IN that found outside the minimum region of interaction had no effect on DYNLL1 binding **(Figure 26E, top panel, lane 4)**. It is worth noticing from an earlier crystal structure analysis of IN protein that the ^{207}DIQT motif is buried deep inside the interface of IN dimer [392], which probably makes this motif unlikely to come in contact with any of the external proteins in cells. By introducing $IN_{Q53A/Q252A}$ into HIV, it was evident that the DYNLL1 interaction defective HIV mutant was also defective for cDNA synthesis and replication (**Figure 27D and 27C**). Whereas the cDNA synthesis difference between $HIV_{Wt/Mt}$ was lost in DYNLL1-KD cell infection, this further emphasizes the specificity of IN and DYNLL1 interaction for HIV cDNA synthesis and argues against the possible pleotropic effects of IN mutations. Although we believe that demonstration of loss of cDNA synthesis by competitive inhibition of IN and DYNLL1 interaction would be more appropriate, disruption of IN and DYNLL1 interaction by

using IN small peptide may not be feasible. We make this presumption based on the fact that IN functions as a tetramer. It is likely that IN small peptides may fail to interact with DYNLL1 due to lack of appropriate secondary/tertiary structures. Nevertheless, we strongly believe that the above data is sufficient enough to support our claim that IN and DYNLL1 interaction is required for HIV cDNA synthesis.

The underlying mechanism by which IN and DYNLL1 interaction contributes to HIV cDNA synthesis was unknown. IN self assembles into a tetramer and tetramer is a functional form of IN in physiological condition [392]. A recent study showed that IN monomer interactions in the tetramer are highly dynamic and suggested that IN interaction with cellular proteins will stabilize IN monomer interactions in tetramer [393], leading to the formation of stable IN tetramer. Interestingly, one of the functions of DYNLL1 is to provide conformational stability to interacting proteins (reviewed in [287]). In addition, accumulated evidences suggest that proteins undergo conformational change and exhibit higher α helical content following DYNLL1 binding [389-391], which would facilitate secondary protein interaction and help in the formation of protein complexes. As IN and RT interaction is known to contribute to reverse transcription, we suspected that DYNLL1 interaction of IN would lead to stable tetramer formation, which would in turn lead to a stable IN/RT complex formation. However, our findings fail to suggest a role for DYNLL1 in IN multimerization **(Figure 29B).** Moreover, DYNLL1 interaction defective $IN_{Q53A/Q252A}$ mutant also efficiently interacted with RT in 293T cells **(Figure 28).** Together, these data suggested that DYNLL1 is unlikely to have a role in IN tetramerization or IN/RT complex formation. Since DYNLL1 fail to interact with RT, it

is also unlikely that DYNLL1 would be involved in de novo reverse transcription. Alternatively, given the fact that HIV reverse transcription is functionally linked to virus uncoating (reviewed in [315]), IN and DYNLL1 interaction could possibly influence cDNA synthesis by contributing to virus uncoating. In an earlier study, the addition of cell lysate in an *invitro* unocating asssay led to the efficient HIV uncoating and reverse transcription [117]. Indeed, the recruitment of cellular proteins by HIV prior to uncoating is clearly evident from the fact that RTC gains size soon after entry into cytoplasm and undergoes progressive or stepwise disassembly and conformational change [120]. In accordance with these reports, recent studies have uncovered some cellular factors that are associated with reverse transcription and/or uncoating. Hamamoto *et al.,* elucidated the cellular Gemin2 protein interaction with IN and its contribution to HIV cDNA synthesis by an unknown mechanism [398]. Similarly, Warren *et al.,* demonstrated the recruitment of eEF1 protein to RTC and its critical requirement for RTC stability and cDNA synthesis [399]. Although cellular factors have been suggested for reverse transcription or uncoating, how these cellular factors are recruited to RTC/PIC or mechanism by which they contribute to reverse transcription or uncoating is largely unknown. Briones *et al.,* implicated IN in HIV uncoating based on their finding that HIV-ΔIN exhibited accelerated uncoating and reduced cDNA synthesis [310]. Although lack of CypA incorporation was suggested for accelerated uncoating in HIV-ΔIN infection, this may not simply rule out the possible alternative mechanism/s. Even the role of virus incorporated CypA in uncoating is contradictory [400, 401]. Since DYNLL1 is required for HIV cDNA synthesis and interacts with IN, the role of DYNLL1 in HIV uncoating was rightly suspected. By employing fate of capsid assay, we detected the accelerated

uncoating of HIV in DYNLL1-KD cells, and similar results were also obtained in HIV-$IN_{Q53A/Q252A}$ mutant or HIV- ΔIN virus infections **(Figure 31A and 31B)**. Furthermore, the uncoating difference between HIV_{Wt} and HIV-$IN_{Q53A/Q252A}$ was lost in DYNLL1-KD cell infection **(Figure 31C)**, clearly implicating IN and DYNLL1 interaction in proper uncoating of HIV. Moreover, CypA incorporation into HIV-$IN_{Q53A/Q252A}$ was unaffected **(Figure 31D).** However, at this point, we do not know how IN and DYNLL1 interaction will contribute to HIV uncoating. Nevertheless, it is interesting to note that DYNLL1 interacts with several different cellular proteins and therefore, could act as a facilitator of proteins recruitment to RTC/PIC. Therefore, it is possible that the previously identified or unknown cellular proteins may be recruited to RTC/PIC through DYNLL1 and participate in stable RTC/PIC formation, reorganization, or uncoating. Alternatively, based on the recent assumption that CypA and CA interaction in target cells is possibly protecting HIV against the exaggerated uncoating induced by a cellular anti-HIV factor Ref1. DYNLL1 would be contributing to proper uncoating of HIV by promoting the CypA association with CA or stabilizing CypA and CA interaction in RTC/PICs within the target cells. Therefore, it is more likely that lack of IN and DYNLL1 interaction would make RTC/PIC unstable and subject to its degradation in the cell, leading to reduced levels of viral cDNA synthesis. Future investigations of these possibilities would be essential to define the precise role of IN and DYNLL1 interaction in HIV uncoating and cDNA synthesis.

Chapter 5

Major Findings, General Discussion, and Future Directions

5.1 Major Findings

HIV replication depends heavily on host factors. Viral and cellular factor interactions provide an attractive target for antiviral drug development. Although no such drug is yet available for clinical use, some of the viral and cellular factor interactions have been successfully targeted for anti-HIV drugs in recent studies (discussed in introduction). The available anti-HIV drugs for clinical use mostly target the enzymatic viral proteins that are prone to extensive mutation and development of drug resistance without seriously compromising viral fitness. On the contrary, the drug resistance against small molecule inhibitors that target the viral and cellular factor interactions is less likely. Therefore, identification of new targets for anti-HIV therapy should be a priority. My primary focus in this study was to elucidate the molecular mechanism of IN interaction with some key cellular factors and their contribution to post entry steps of early stage HIV replication. In earlier studies, IN has been implicated in HIV nuclear import and RTC/PIC recruitment to dynein complex. However, the molecular mechanism by which IN contributes to HIV nuclear import or RTC/PIC recruitment to dynein complex is unknown. IN is a key enzymatic protein of HIV and the enzymatic function of IN is necessary for the HIV cDNA integration process. Apart from the enzymatic function, IN is known to interact with several cellular proteins, including the components of nuclear import machinery and a dynein adapter protein (Dyn2p) of *S.cerevisiae*. In the past, IN and cellular factor interactions have been known to contribute to HIV replication steps, including some of the steps of HIV cDNA integration process. Thus, IN is most likely to function in steps of

early stage HIV replication by interacting with specific cellular co-factors. Therefore, **the central hypothesis of this thesis was that HIV IN will mediate the post entry steps of early stage HIV replication by interacting with specific cellular co-factors.** The findings in this study contribute to our knowledge of HIV replication and provide a proof-of-concept for the feasibility of inhibiting HIV replication by disrupting key interactions between IN and its cellular cofactors.

The study was carried out in two major parts. First (chapter 3), we have examined the requirement of different Impα isoforms for HIV replication and nuclear import, and identified the requirement of Impα3 for HIV nuclear import and its interaction with IN. We have also characterized the motifs in IN for Impα3 interaction and their requirement for HIV replication and nuclear import. Second (chapter 4), we have elucidated IN interaction with DYNLL1 and its requirement for HIV uncoating and cDNA synthesis.

5.1.1 Investigation of the Role of Impα Isoforms in HIV Nuclear Import and the Mechanism of Impα Recruitment by HIV during Replication

HIV cDNA as a part of PIC enters the nucleus by active nuclear import (discussed in chapter 1). The active nuclear import of HIV cDNA is essential for non-dividing and dividing cell infection [183-187]. From the accumulated evidence, IN was recognized as a primary viral mediator of HIV nuclear import (reviewed in [309]). However, the key cellular factor/s that facilitates HIV nuclear import is/are still not defined. Moreover, the molecular mechanism of how IN contributes to HIV nuclear import is also unknown. Imp7 and TNPO3 were initially implicated in HIV replication [241, 242, 247]. However,

subsequent studies either contradicted or undermined the significant role of Imp7 and TNPO3 in HIV nuclear import [57, 243, 244, 251, 252]. Gallay *et al*., showed the Impα1 interaction with IN and its requirement for HIV replication [210]. The role of IN and Impα interaction in HIV nuclear import has not been investigated extensively. Human cells express six different Impα subtypes and all of them are capable of mediating protein nuclear import (discussed in chapter 1 and 3). In chapter 3, we examined the requirement of Impα1, Impα3, Impα5, and Impα7 isoforms for HIV replication in HeLa (**Figure 7B**) and C8166T (**Figure 8C**) cells by gene KD approach. Although there was a similar level of KD for different Impα isoforms except Impα7, luciferase reporter HIV replication in different Impα isoform KD cells showed significant variation. There was about 3- to 4-fold reduction in HIV replication in Impα3-KD cells, but only 50-60% reduced HIV replication was observed Impα1 or Impα5-KD cells. HIV replication was not affected in Impα7-KD cells. These results provided convincing evidence for a major role of Impα3 in HIV replication. Further, HIV gene expression was unaffected in Impα3-KD cells **(Figure 7C**), suggesting the requirement of Impα3 for steps of early stage HIV replication. Upon analysis of HIV total viral DNA, 2-LTR circle DNA, and integrated DNA synthesis in Impα3-KD cells, we observed a substantial reduction in 2-LTR circle but not total viral DNA synthesis in Impα3-KD cells (**Figure 10A and 10B**). The loss of 2-LTR circle DNA synthesis in Impα3-KD cells clearly suggested the requirement of Impα3 for HIV nuclear import. In subsequent investigation, we explored the Impα3 interaction with HIV viral proteins. Impα3 interacted with IN in *in vitro*, in 293T cells, and in HIV infected C8166T cells (**Figure 11A, 11B, and 11D**). These findings have suggested for the first time that Impα3 is an IN interacting cellular co-factor.

Subsequently, we performed IN deletion mutation and Impα3 interaction analysis. The results showed the involvement of IN-CTD in Impα3 interaction (**Figure 12A and 12B**) and IN nuclear localization (**Figure 12C**). Next, we explored the functional relevance of Impα3 for HIV replication by performing HIV infection in Impα3-KD primary human macrophages. The experiment was performed in macrophages derived from two independent healthy donors. In both the infections, we observed about 10-fold reduction in HIV replication in Impα3-KD macrophages (**Figure 13B and 13C**). Interestingly, the extent of replication differences was unaltered between control and Impα3-KD macrophages infected with Vpr^+ or Vpr^- HIV (**Figure 13D**). This indicated that the requirement of Impα3 for HIV replication is not dependent on Vpr. Together, these results led to the conclusion that Impα3 interacts with IN and contributes to HIV nuclear import. In the following sections, we studied the molecular mechanism of IN and Impα3 interaction and its requirement for HIV nuclear import. In chapter 3, we provided evidence for the involvement of IN-CTD for Impα3 interaction. Indeed, IN-CTD has been implicated in HIV nuclear import by several other previous reports, including previous studies from our own lab [56-58, 240]. The nuclear localization of IN has been attributed to some of putative nuclear localization signals (NLSs) found in CTD (discussed in chapter 1 and 3), whereas the molecular mechanism(s) by which NLSs contribute to IN nuclear localization is not fully understood. Therefore, we examined the requirement for putative NLS of IN-CTD (^{211}KELQKQITK, ^{236}KGPAKLLWK, and ^{262}RRKAK) in Impα3 interaction by substitution mutation and interaction analysis. Results showed that $IN_{K215A/K219A}$ and $IN_{R263A/K264A}$ were moderately impaired for Impα3 interaction, whereas IN $_{K215A/K219A/R263A/K264A}$ mutant was severely impaired for Impα3

interaction (**Fig. 14**). These data indicated that ^{211}KELQKQITK and ^{262}RRKAK motifs of IN are required for Impα3 interaction. This also helped to suggest that IN interacts with Impα3 through a non-conventional bi-partite NLS. To gain more insight into the mechanism of IN interaction with Impα3, we examined the Impα3-NLS binding groove mutants (Impα3$_{W179A/N183A}$ and Impα3$_{W348A/N353A}$) interactions with IN. IN showed attenuated interaction with both of Impα3 mutants (**Figure 16B**), suggesting the involvement of major and minor NLS binding grooves of Impα3 in IN interaction. Indeed, these data also supported our claim that ^{211}KELQKQITK and ^{262}RRKAK motif of IN serve as a bipartite NLS for Impα3 interaction. In subsequent analysis, we found the defective nuclear localization of YFP-IN$_{K215A/K219A/R263A/K264A}$ fusion protein by immunostaining and cell fractionation methods (**Figure 17A and 17B**). The introduction of IN$_{K215A/K219A/R263A/K264A}$ mutations into HIV resulted in defective virus replication and viral cDNA nuclear import (**Figure 18**). The nuclear import defect of mutant virus was determined by quantifying the 2-LTR circle DNA as well as HIV DNA contents from cytoplasm and nuclear fractions by qPCR (**Figure 19 and 20**). Together, findings from this study led to the conclusion that ^{211}KELQKQITK and ^{262}RRKAK motifs of IN are required for Impα3 interaction and HIV cDNA nuclear import. Therefore, it is conceivable that IN and Impα3 interaction is required for HIV nuclear import.

5.1.2 The IN and DYNLL1 Interaction and Its Role in Early Stage HIV Replication

For successful nuclear import, HIV RTC/PIC has to pass through the cytoplasm. Due to the high molecular weight of RTC/PIC, passive diffusion of RTC/PIC is not permitted in

the cytoplasm. The earlier studies showed the dynein complex- or MT-dependent retrograde transportation of HIV complex in the cytoplasm [282, 377]. However, whether and how HIV targets dynein complex and its significance for early stage virus replication are unknown. Recently, HIV IN was found interacting with Dyn2p, a yeast homolog of mammalian DYNLL1 [305]. DYNLL1 was also known to interact with other viral proteins and contribute to viral replications [299, 303, 306, 307]. These reports favored DYNLL1 as a likely mediator for HIV RTC/PIC recruitment to dynein complex or for HIV replication. In chapter 4, we probed interaction of AcGFP-IN, AcGFP-CA, or MA-YFP fusion protein with endogenous DYNLL1 in 293T cells. The AcGFP-IN was specifically interacted with DYNLL1 (**Figure 21A**). Subsequently, we also found the IN interaction with DYNLL1 in *in vitro* and in HIV infected C8166T cells (**Figure 21B and C**). These data suggested for the first time that DYNLL1 is an IN interacting cellular cofactor. In the continued investigation, we examined the requirement of DYNLL1 for early stage HIV replication by gene KD and infection analysis. While replication of luciferase reporter HIV was substantially reduced in DYNLL1-KD C8166T cells (**Figure 22D**), HIV entry or gene expression was unaffected in DYNLL1-KD cells. These data led to the conclusion that DYNLL1 is required for post entry steps of early stage HIV replication such as reverse transcription, nuclear import, and/or integration. By qPCR analysis, we found that HIV cDNA synthesis but not nuclear import was impaired in DYNLL1-KD cells (**Figure 24**). To determine the requirement of IN and DYNLL1 interaction for HIV cDNA synthesis, we carried out IN deletion or substitution mutation and DYNLL1 interaction analysis. The data concluded that ^{52}GQVD” and ^{250}VIQD motifs of IN were required for DYNLL1 interaction (**Figure 26E**). The introduction of

$IN_{Q53A/Q252A}$ mutations into HIV showed the defective cDNA synthesis (**Figure 27D**). In further investigation, we found an abnormal HIV uncoating in DYNLL1-KD cells or HIV-$IN_{Q53A/Q252A}$ mutant virus infection (**Figure 31**). The differences in uncoating between HIV_{Wt} and HIV-$IN_{Q53A/Q252A}$ was absent in DYNLL1-KD cell infection, highlighting the requirement of IN and DYNLL1 interaction for proper HIV uncoating. At the end of this study, we asked whether IN and DYNLL1 interaction is required for HIV recruitment to dynein complex. To test this, we probed the DYNLL1 interaction defective $IN_{Q53A/Q252A}$ mutant interaction with DIC1. The result helped to conclude that IN and DYNLL1 interaction is dispensable for HIV recruitment to dynein complex (**Figure 32A**). Meanwhile, HIV cDNA synthesis was unaffected in MT depolymerizing agent Nocodazole treated cells (**Figure 32C**). Together, the findings of this study led to the conclusion that although IN and DYNLL1 interaction is required for proper HIV uncoating and cDNA synthesis, this viral and cellular proteins interaction is essential for HIV recruitment to dynein complex or retrograde transport.

5.2 General Discussion

5.2.1 Role of IN in Post Entry Steps of Early Stage HIV Replication

IN functions in different steps of early stage HIV replication. So far, IN has been implicated in HIV reverse transcription, uncoating, PIC nuclear import, and integration steps of early stage replication. IN is a key enzymatic protein of HIV and enzymatic function of IN plays a key role in HIV cDNA integration process. The genomic integration of HIV cDNA has been extensively studied and the role of IN and cellular cofactors in HIV cDNA integration has been well established. In addition, IN also

interacts with various cellular proteins and contributes to different steps of HIV replication, including some steps of HIV integration. However, the role of IN in other steps of early stage HIV replication remain poorly understood. HIV reverse transcription is mediated primarily by viral RT protein, and other viral and cellular proteins also contribute in this process. IN was identified as a requirement for efficient HIV reverse transcription by several different studies and the interaction of IN with RT has been implicated in efficient HIV reverse transcription [56, 369, 384-388]. However, the molecular mechanism with which IN contributes to reverse transcription is still missing. Recently, Briones *et al*., observed the accelerated uncoating of HIV-ΔIN virus and elucidated that the accelerated uncoating of HIV-ΔIN is due to lack of incorporation of CypA [310]. However, whether IN can also facilitate HIV uncoating by any other mechanisms was not discussed. The involvement of IN in HIV nuclear import is known for over a decade (discussed in chapter 1). Although extensive investigation has helped to unravel several key facts about the role of IN in HIV nuclear import, the general conclusion is still not achieved.

5.2.2 Requirement of Impα3 for HIV Nuclear Import and Its Interaction with IN

The research community has shown a great interest in understanding the molecular mechanism of HIV nuclear import and key viral and/or cellular factors involved in this process. In the past, several components of HIV have been implicated in nuclear import (discussed in chapter 1). MA protein was the first viral protein to be implicated in HIV nuclear import [209]. A canonical NLS was identified in MA protein and this NLS was shown to induce the nuclear localization of heterologous protein upon conjugation [209].

Meanwhile, MA protein was detected in PICs that are isolated from HIV infected cells. Although these initial reports claimed the involvement of MA protein in HIV nuclear import, the subsequent reports have failed to confirm these earlier findings. For example, the presence of NLS in MA protein itself was proved non-essential for HIV nuclear import and/or replication in non-dividing cells [27, 210-212]. Even the mutant HIV that lacks entire MA protein except N-terminal myristoylation signal was still able to replicate in dividing and non-dividing cells albeit at a lower level [214]. Later, the focus was shifted to the role Vpr in HIV nuclear import. Vpr has been suggested to mediate HIV nuclear import by several different mechanisms (reviewed in [309]). However, recent studies have found that Vpr is not essential for HIV replication in non-dividing or dividing cells. In chapter 3, we also made an observation that Vpr deletion did not overcome the requirement of Impα3 for HIV replication in primary human macrophages. Although the requirement of Vpr for HIV nuclear import is not completely ruled out, the present general consensus is that Vpr only serve as an accessory factor in HIV nuclear import. Recently, CA protein has been suggested for HIV nuclear import [227]. CA protein is associated with HIV uncoating, a replication step that is functionally linked to HIV nuclear import. Moreover, CA protein lacks any canonical NLSs or karyophilic property. Considering these facts, it is less likely that CA protein would directly mediate HIV nuclear import. Instead, CA may very well influence HIV nuclear import through uncoating. Gallay *et al.,* provided the first convincing evidence for the involvement of IN in HIV nuclear import and IN interaction with Impα1 [210]. This finding evoked tremendous interest in IN and its role in HIV nuclear import. Subsequently, the karyophilic property of IN and its significance for HIV nuclear import were evaluated in

several studies [56, 203, 210, 231-235]. IN has several putative NLSs in its CCD and CTD and some of these NLSs have been implicated in IN nuclear localization and interaction with various nuclear import receptors. In an earlier study from our lab, two putative NLSs in CTD were identified as required for HIV nuclear import [56]. However, how these NLSs contribute to HIV nuclear import was unknown. Fassati *et al.,* suggested the involvement of Imp7 in HIV nuclear import based on their finding that Imp7 induced the nuclear localization of PIC in an *in vitro* nuclear import assay [242]. In another study from our lab, although we were able to detect IN and Imp7 interaction, we found that Imp7 was not important for HIV nuclear import [57]. A similar conclusion was also made by other studies as well [243, 244]. Subsequently, Christ *et al.,* demonstrated the IN interaction with TNPO3 and the requirement of TNPO3 for HIV nuclear import [241]. But the role of TNPO3 in HIV replication is still inconclusive. TNPO3 is also required for nuclear import of CPSF6 [252], which is known to impair nuclear import of HIV through interaction with CA protein and affecting uncoating [253, 254]. Therefore, depletion of TNPO3 might have caused excessive accumulation of CPSF6 in the cytoplasm, which could have indirectly impaired HIV nuclear import. Alternatively, TNPO3 is also implicated in maturation of PIC in nucleus [402] and thus possibly contributing to HIV replication by additional unknown mechanisms. Nevertheless, the direct involvement of TNPO3 in HIV nuclear import is contradictory or at the best inconclusive. During the same time, some researchers have suspected that IN directly targets Nups without the involvement of importins and this IN and Nup interaction will facilitate HIV nuclear import. In this direction, Woodward *et al.,* demonstrated the IN and Nup153 interaction and its requirement for HIV nuclear import [255]. The

requirement of Nup153 for HIV nuclear import was also confirmed in other studies [253, 256]. However, Nup153 also interacts with CA protein [403] but the significance of this interaction is not fully understood. Nevertheless, whether Nups alone would significantly contribute to HIV nuclear import is something that needs to be verified. In all these years, even though Impα1 was first implicated in HIV nuclear import, no in-depth analysis on the role of Impα1 in HIV nuclear import has been carried out. As we know that Impα1 belongs to the classical nuclear import pathway, which accounts for about 57% of nuclear proteins entry into the nucleus in mammalian cells (reviewed in [139, 404]). Thus, Impα1 could potentially play a major role in HIV nuclear import. At the same time, it is important to note that Impα is also involved in the nuclear import of several important viruses (reviewed in [404, 405]). Therefore, knowledge gained through this study would also benefit other fields of investigation. There are about six Impα isoforms in human cells. However, requirement of different Impα isoforms for HIV replication is not known. In chapter 3, we examined the requirement of Impα1, Impα3, Impα5, and Impα7 isoforms for HIV replication by gene KD approach (**Figure 7B and 8C**). Among all the Impα isoforms that we have tested, Impα3 but not Impα1-KD induced a relatively higher reduction in HIV replication in different cell lines. These findings helped us to draw the conclusion that Impα3 is a functionally important Impα isoform for HIV replication. The Impα3-KD led to a reduction of 2-LTR circle DNA synthesis (**Figure 10B**) but not affected the total viral DNA synthesis (**Figure 10A**). These results showed the specific requirement of Impα3 for HIV nuclear import. Subsequently, we demonstrated the Impα3 interaction with IN and showed the requirement of IN-CTD for Impα3 interaction. Contrary to Impα3-KD, Impα1 or Impα5-KD showed a moderately reduced HIV

replication. However, significance of Impα1 or Impα5 for HIV replication is not clear. Studies have shown the cell type specific differential expression of Impα isoforms [365-367]. This could explain why HIV replication was differentially affected in different Impα isoforms KD cells. However, my preliminary analysis failed to suggest the differential expression of Impα1 or Impα3 in primary human CD4+T lymphocytes or macrophages. Alternatively, IN may differentially interact with different Impα isoforms. In fact, even though Impα isoforms show extensive similarity at amino acid sequence (50-60% similarity between subfamilies and 80% within in subfamilies), they often differ at substrate recognition and/or nuclear import. A detailed analysis of IN interaction with different Impα isoforms would help to explain the significance of individual Impα isoforms for HIV nuclear import. Nevertheless, this study provides conclusive evidence that Impα3 is IN interacting cellular co-factor and is essential for HIV nuclear import.

As discussed in chapter 1, cargo proteins bind to Impα with the help of basic amino acid rich motif called NLS. Several putative NLSs (^{186}KRK, ^{211}KELQKQITK, ^{236}KGPAKLLWK and ^{262}RRKAK) have been found in IN and they are highly conserved across different HIV strains. In the past, studies, including from our lab, have examined the requirement of these putative NLSs of IN for HIV nuclear import [56, 57, 210]. Gallay *et al.,* showed that IN_{K186Q} and $IN_{Q214/216L}$ mutants are defective for nuclear localization and interaction with Impα1. Based on these findings, authors have claimed that ^{186}KRK and ^{215}KELQKQITK motifs of IN serve as a non-conventional bi-partite NLS for HIV nuclear import [210]. However, later studies discredited this claim based on their finding that HIV-IN_{K186Q} and HIV-$IN_{Q214/216L}$ mutant viruses are defective for

reverse transcription, integration, or unknown post nuclear entry steps but not for nuclear import [55, 235, 406]. Subsequently, a study from our lab showed that $IN_{R215A/K219A}$ and $IN_{K240A/K244A}$ mutants were defective for nuclear localization and introduction of these mutations into HIV lead to defective cDNA nuclear import [56]. From these data, our lab claimed that ^{211}KELQKQITK and ^{236}KGPAKLLWK of IN can act as NLS for HIV nuclear import. In another study from our lab, ^{236}KGPAKLLWK and ^{262}RRKAK motifs of IN were identified as required for Imp7 interaction, but we found that Imp7 was not important for HIV nuclear import [57]. Therefore, how the putative NLSs of IN would engage in HIV nuclear import was an open question. Following our finding that Impα3 is required for HIV nuclear import, we examined the requirement of putative NLSs of IN-CTD (^{211}KELQKQITK, ^{236}KGPAKLLWK, and ^{262}RRKAK) for Impα3 interaction by mutagenic analysis. Results concluded that ^{211}KELQKQITK and ^{262}RRKAK were involved in Impα3 interaction and HIV nuclear import. The major and minor NLS binding grooves in Impα are involved in cargo binding. In the past, crystallographic analyses have uncovered that a bi-partite NLS peptide simultaneously binds to both major and minor NLS binding grooves in Impα [147, 149]. In this study, we made another interesting observation that major and minor NLS binding grooves of Impα3 are involved in the IN interaction. While this finding explains the molecular mechanism of the IN interaction with Impα3, it also supports our conclusion that ^{211}KELQKQITK and ^{262}RRKAK motifs of IN act as a bi-partite NLS for Impα3 interaction. Taken together, these data, in addition to clarifying the significance of NLSs of IN for HIV nuclear import, will explain the molecular mechanism of IN and Impα3 interaction.

5.2.3 IN and DYNLL1 Interaction and Its Role In Proper Uncoating of HIV

The cytoplasm is a highly crowded environment and this restricts passive diffusion of macromolecule in cytoplasm (reviewed in [309]). The macromolecules recruit to MT associated dynein or kinesin complexes and undergo active transportation in the cytoplasm. HIV is also known to target dynein complex for retrograde transportation in the cytoplasm. The migration of RTC/PIC in the cytoplasm is essential for successful completion of early stage HIV replication. However, mechanism by which HIV recruits to dynein complex and its contribution to early stage HIV replication were not known. By yeast two hybrid screening, de Soultrait *et al.,* found the HIV IN interaction with Dyn2p, a yeast homolog of human DYNLL1 [304]. Later, Desfarges *et al.,* demonstrated that HIV IN migration to perinuclear space in *S.cerevisiae* is Dyn2p dependent [305], which suggested that IN undergoes retrograde migration in cytoplasm with the help of Dyn2p. In chapter 4, we examined the role of IN and DYNLL1 interaction in HIV recruitment to dynein complex and retrograde transportation. First, we detected HIV IN interaction with DYNLL1 in 293T cells, in *in vitro*, and in HIV infected C8166T cells. Then, we showed that DYNLL1 is required for post entry steps of early stage HIV replication. However, our further analysis showed that IN and DYNLL1 interaction was essential for HIV cDNA synthesis (**Figure 27**) and proper uncoating of HIV (**Figure 31).** Although this finding was very interesting, it failed to support my initial assumption that DYNLL1 will facilitate the retrograde migration of HIV because lack of DYNLL1-KD did not affect HIV nuclear import. Surprisingly, DYNLL1 interaction was also not essential for IN interaction with DIC1 (**Figure 32**). However, we believe that IN is the key viral factor involved in the recruitment of the HIV RTC/PIC to the dynein complex, as IN but not CA

was able to interact with DIC1. Meanwhile, we found that MT depolymerization affected HIV nuclear import to a moderate extent but failed to affect reverse transcription. However, contradictory findings from a previous report failed to demonstrate an HIV replication defect in Nocodazole-treated cells [407]. We suspect that these differing results could be due to differences in cell type or to differences in Nocodazole treatment. It is important to note that MT can re-polymerize within 15 min after the removal of Nocodazole from the culture [408]. To obtain sustained MT depolymerization, we maintained the cells in the presence of Nocodazole for 12h after infection; this time point corresponds to the near completion of early-stage replication. Noticeably, the results obtained during infection of Nocodazole-treated cells are in contrast to those obtained during infection of DYNLL1-KD cells or during infection with the HIV-$IN_{Q53A/Q252A}$ mutant. These data indirectly suggest that the DYNLL1 and IN interaction is unlikely to be involved in HIV retrograde migration. The findings presented in chapter 4 also help to guide the future investigation on the role of DYNLT1 or $p150^{Glued}$ in HIV recruitment to dynein complex and/or retrograde migration. The "R/KK/KXXR/K" is a consensus motif for DYNLT1 binding and is found in most DYNLT1 binding partners [409, 410]. It is worth noticing that IN protein contains highly conserved motifs that closely resemble "R/KK/KXXR/K" and some of these IN motifs are important for HIV cDNA localization to nucleus (reviewed in [309]). Hence, DYNLT1 could be a likely mediator for HIV recruitment to dynein complex or retrograde transportation. However, it is also important to note that a study by structural and thermodynamic analyses of DYNLT1 questioned the ability of DYNLT1 to tether cargo to the dynein complex [286]. In addition, DYNLT1 also interacts with several cellular factors and involved in various cellular

functions outside the dynein complex [288-293]. Therefore, given the nature and functional diversity of these proteins, a careful examination of the roles of DYNLT1 and p150^{Glued} in mediating HIV recruitment to dynein and the contribution of this process to distinct step(s) of HIV replication is important. Further characterization of the roles of DYNLT1 and p150^{Glued} in HIV RTC/PIC-dynein targeting or retrograde migration would be necessary to clarify the mechanism by which HIV is recruited to the dynein complex and the role of this process in early-stage HIV replication.

5.3 Potential Applications

Most of the drugs used in the present anti-HIV therapy are known to bind viral enzymatic proteins and impair their functions. This enables HIV to easily develop drug resistance by introducing specific point mutations in its proteins. Thus, drug resistance is a common phenomenon in all known anti-HIV drug regimens. Even the recently approved IN inhibitors are not free from the development of drug resistance. Therefore, current effort in anti-HIV therapeutic development is to evolve mechanism(s) to overcome the drug resistance and simultaneously maintain safety and anti-viral property. In this direction, the viral and cellular protein interactions can serve as an attractive target for anti-HIV drug development. As discussed in chapter 1, the small molecule PPI inhibitors have been successfully tested against HIV and cellular protein interactions. The focus of my research was on virus and host protein interactions that facilitate steps of early stage HIV replication. The development of new IN inhibitors will be driven by an improved understanding of IN and cellular factor interactions and their role in HIV replication. In chapter 3, we elucidated critical requirement of Impα3 for HIV nuclear import and its

interaction with IN, and we identified critical motifs in IN for Impα3 interaction and their requirement for HIV nuclear import. In chapter 4, we elucidated the IN interaction with DYNLL1 and its requirement for proper HIV uncoating and cDNA synthesis. The future goal of our lab is to translate this basic knowledge into development of novel anti-HIV therapies, by developing inhibitors for host-HIV protein interactions.

5.4 Future directions

Based on the findings presented in this thesis, the following future directions were drawn. In chapter 3, we observed that Impα1 and Impα5-KD also resulted in approximately 50% reduction of HIV replication. Therefore, although Impα3 was found to have a significant role in HIV replication and nuclear import, Impα1 or Impα5 may also be playing a role in HIV replication and/or nuclear import. Studies have found the cell type specific differential expression of Impα subtypes, which is dependent on metabolic state of cell and cell differentiation. Therefore, future studies need to investigate the role of Impα1 and Impα5 in efficient HIV replication in different cell types and/or possible contributions in other steps of HIV replication. IN interaction with Impα3 was essential for the HIV nuclear import. Therefore, a detailed analysis of IN interaction with different Impα isoforms may be useful for explaining the differences in contributions of individual Impα isoforms for HIV nuclear import. In chapter 4, we have elucidated the requirement of IN and DYNLL1 interaction for proper uncoating of HIV but this interaction was not essential for HIV recruitment to dynein complex. However, it is not known how the IN interaction with DYNLL1 contributes to the proper uncoating of HIV. Therefore, the future studies should examine the mechanism(s) by which IN and DYNLL1 interaction

contributes to the proper uncoating of HIV. In addition, as DYNLT1 and p150^{Glued} are also known to mediate cargo recruitment to the dynein complex as well as involved in the cellular process outside the dynein complex, it is interesting to know whether DYNLT1 or p150^{Glued} is involved in the HIV recruitment to dynein complex or contributes to distinct step(s) of HIV replication. Further characterization of the roles of DYNLT1 and p150^{Glued} in HIV recruitment to dynein complex or retrograde migration would be necessary to clarify the mechanism by which HIV is recruited to the dynein complex and the role of this process in the early-stage HIV replication. In the end, the studies describing the co-crystal structure of IN interaction with the Impα3 and the DYNLL1 would be essential to ascertain the IN motifs involved in Impα3 and DYNLL1 interactions and to allow a rational design of small-molecule inhibitors against these viral and cellular proteins interactions as a novel anti-HIV drugs.

5.5 Concluding Remarks

HIV replication is heavily dependent on viral and cellular protein interactions. IN contributes to different steps of early stage HIV replication by interacting with several different cellular proteins. In this study, a detailed investigation was carried on IN interaction with specific cellular proteins and their contributions for steps of early stage HIV replication. Based on the data presented in chapter 3 and chapter 4, some of following conclusions are made. The Impα3 is required for HIV nuclear import and it interacts with IN. The interaction of IN with Impα3 is mediated by two highly conserved motifs in IN-CTD and these motifs are essential for HIV nuclear import. Finally, IN interacts with DYNLL1 and this viral and cellular protein interaction is required for

proper HIV uncoating and/or cDNA synthesis. However, contrary to our earlier assumption, IN and DYNLL1 interaction is unlikely to mediate HIV RTC/PIC recruitment to dynein complex or retrograde transportation. Therefore, how RTC/PIC recruits to dynein complex is still an open question. Overall, the findings presented in this thesis work highlight the significance of IN and cellular factors interactions for completion of steps of early stage HIV replication.

Chapter 6

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Chapter 7.

Publications

The publications arising from this thesis work

1. **Jayappa KD**, Ao Z, Wang Z, Mouland AJ, Yao X. Human Immunodeficiency Virus Type 1 Employs The Cellular Dynein Light Chain 1 Protein For Proper Uncoating and Reverse Transcription at Early Post Infection (Manuscript under revision in Journal of Virology)

2. **Jayappa KD**, Ao Z, Yao X. The HIV-1 passage from cytoplasm to nucleus: the process involving a complex exchange between the components of HIV-1 and cellular machinery to access nucleus and successful integration. Int J Biochem Mol Biol. 2012;3(1):70-85. Epub 2012 Feb 25.

3. **Jayappa KD**, Ao Z, Yang M, Wang J, Yao X. Identification of critical motifs within HIV-1 integrase required for importin α3 interaction and viral cDNA nuclear import. J Mol Biol. 2011 Jul 29;410(5):847-62.

4. Ao Z*, **Danappa Jayappa K*,** Wang B, Zheng Y, Kung S, Rassart E, Depping R, Kohler M, Cohen EA, Yao X. Importin α3 Interacts with HIV-1 Integrase and Contributes to HIV-1 Nuclear Import and Replication. J Virol. 2010 Sep; 84(17): 8650-63. **(* co-first authors**)

Other publications arising during this graduate program

5. Wang X, Ao Z, **Jayappa KD**, Shi B, Kobinger G and Yao X. R88-APOBEC3Gm Inhibits the Replication of Both Drug-resistant Strains of HIV-1 and Viruses Produced From Latently Infected Cells. Molecular Therapy Nucleic Acids (2014) 3.

6. Chen L, Ao Z, **Jayappa KD**, Kobinger G, Liu S, Wu G, Wainberg MA, Yao X. Characterization of Antiviral Activity of Benzamide Derivative AH0109 against HIV-1 Infection. Antimicrob Agents Chemother. 2013 Aug; 57(8):3547-54.

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10. Zheng Y, Ao Z, **Jayappa KD**, Yao X.. Characterization of the HIV-1 integrase chromatin- and LEDGF/p75-binding abilities by mutagenic analysis within the catalytic core domain of integrase. Virol J. 2010 Mar 23; 7:68.**)**

11. Zhujun Ao, **Kallesh Danappa Jayappa**, Meaghan Labine, Yingfeng Zheng, Chris Matthews, Gary Kobinger and Xiaojian Yao. Characterization of Anti-HIV Activity Mediated by HIV-1 Integrase C-terminal Domain Polypeptides Expressed in Susceptible Cells. Journal of Antivirals & Antiretrovirals, 2010, 2(1): 020-028.