Characterize the Anti-HIV-1 Activity of a Kinase Inhibitor Kenpaullone and The HIV-1 Integrase Association with DIC1 and DYNLT1

By

Bihe Chen

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Department of Medical Microbiology

University of Manitoba

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Abstract

Advances in the antiretroviral therapy (ART) have dramatically reduced the death rate from human immunodeficiency virus type 1 (HIV-1) induced acquired immune deficiency syndrome (AIDS). However, it is still necessary to develop anti-HIV-1 new drugs. In this study, two projects were conducted and may contribute to the new drug development. The first project is focused on characterizing the anti-HIV activity of a kinase inhibitor Kenpaullone (Ken). We found a cyclin dependent kinase (CDK) and glycogen synthase kinase-3β (GSK-3β) inhibitor named Ken can significantly inhibit HIV-1 replication. Mechanistic analysis by RT-PCR revealed that Ken inhibited HIV-1 replication by disrupting transcription possibly through CDK-dependent pathways. The second project is focused on understanding the association between HIV-1 integrase (IN) and dynein components. Our investigation indicated that HIV-1 IN is associated with DIC1 and DYNLT1. Further investigation this IN/dynein component association may help to reveal new anti-HIV targets.

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List of Abbreviations

A3G	Apolipoprotein B mRNA-editing enzyme-catalytic, polypeptide-like 3G
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ATM	Ataxia telangiectasia mutated
BST-2	Bone marrow stromal antigen 2
CA	Capsid
CCD	Catalytic core domain
CDK	Cyclin dependent kinase
CK-2	Casein kinase-2
Co-IP	Co-Immunoprecipitation
cPPT	Central polypurine tract
CRF	Circulating recombinant forms
CTD	C-terminal domain
Cy5	Cyanine 5
CypA	Cyclophilin A
DC-SIGN	Dendritic cell–specific intercellular adhesion molecular 3-grabbing non-integrin
DHC	Dynein heavy chains
DIC	Dynein intermediate chain
DIS	Dimerization initiation site
DLC	Dynein light chain
DSIF	DRB sensitivity-inducing factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESCRT	Endosomalsorting complexes required for transport
FasL	Fas ligand
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
G protein	Guanine nucleotide-binding protein
Gluc	Gaussia Luciferase
GSK-3β	Glycogen synthase kinase-3β
HAART	Highly active antiretroviral therapy
HAT	Histone acetyltransferase
HIV	Human immunodeficiency virus
HSV1	Herpes simplex virus 1
IC_{50}	50% inhibitory concentrations

IIN-1	Integrase interactor 1
IKK	IκB kinase
IN	Integrase
JNK	Jun N-terminal kinase
LCK	LCK proto-oncogene, Src family tyrosine kinase
LEDGF	Lens-epithelium-derived growth factor
LIC	Light intermediate chains
LTR	Long terminal repeat
MA	Matrix
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MLK3	Mixed lineage kinase 3
MT	Microtubule
MTOC	Microtubule-organizing center
myristoyl-CoA	Myristoyl coenzyme A
NC	Nucleocapsid
NES	Nuclear export signal
NLS	Nuclear localization signals
NNRTI	Nonnucleoside reverse transcriptase inhibitors
NPC	Nuclear pore complex
NRTI	Nucleotide reverse transcriptase inhibitor
NTD	N-terminal domain
N-TEF	Negative transcription elongation factor
PAK	P21-activated kinase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Primer binding site
PD-1	Programmed cell death protein 1
PI(3,4)P2	Phosphatidylinositol (3,4)-bisphosphate
PI(4,5)P2	Phosphatidylinositol (4,5) bisphosphate
PI3K	Phosphoinositide 3-kinase
PIC	Pre-integration complex
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PPT	Polypurine tract
PR	Protease
P-TEFb	Positive transcription elongation factor
RNAP II	RNA polymerase II
RRE	Ref response element

RT	Reverse transcriptase
RTC	Reverse transcription complex
SA	Splice acceptor
SD	Splice donor
SIV	Simian immunodeficiency virus
SL	Stem loop
SRp	Serine arginine-rich proteins
SRPK	SR protein kinase
TAR	Tat-acting region
TFIIH	Transcription factor II Human
TGN	Trans–Golgi network
TSCC	Tongue squamous cell carcinoma
URF	Unique recombinant forms
βTrCP	Beta-transducin repeat containing protein

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1 General Review

1.1 HIV Epidemiology

HIV is one of the major worldwide public health problems. Since the first discovery of HIV in 1983, it has become a global epidemic and the sixth leading cause of death [1]. According to the most recent UNAIDS report, in 2013, 35 million people worldwide were living with HIV, and 2.1 million new infections were recorded [2]. The burden of the HIV epidemic is unequally spread between regions, with the highest concentration in Sub-Saharan Africa where the adult HIV-1 prevalence reaches 4.9% and the lowest concentration in East Asia where the adult HIV-1 prevalence is 0.1%. In the past decade, expanding access to anti-HIV treatment and prevention strategies has dramatically altered HIV epidemiology. The worldwide prevalence of HIV has increased by 16.7% due to the prolonged life of HIV-positive people on HIV treatment while the worldwide incidence decreased by 36.3% mainly due to reduced heterosexual transmission. In contrast to downward global trends, new infections have increased in five regions: East Asia, Eastern Europe, Central Asia, the Middle East and North Africa.

1.2 HIV Transmission and Pathology

HIV is predominantly transmitted through direct contact with blood or other body fluids containing the virus. The most common routes include sexual transmission, mother-to-child transmission, unsterilized needles sharing during drug injection, and contaminated blood product exposure.

After HIV infection, the virus replicates at a rapid rate and causes acute infection in which flu-like symptoms including fever, headache, myalgia, lymphadenopathy, and pharyngitis can be observed in 80% of infected individuals [3]. In this stage, viral proliferation is gradually restricted by a provoked immune response to a low level while the number of HIV-targeted CD4+ T cells decreases rapidly. Following acute infection is a chronic asymptomatic phase. In this phase, the plasma viremia becomes relatively stable while the number of CD4+ T cells keeps declining. Over time, the progressive loss of CD4+ cells weakens the immune system and leads to the acquired immunodeficiency syndrome (AIDS) wherein the CD4+ T cell count falls below 200 per microliter of blood [4]. Without anti-HIV treatment, the time for an HIV-infected person to develop AIDS is between eight to ten years and the typical survival time for AIDS patients is three years [5].

1.3 HIV Virology

1.3.1 HIV Classification

HIV is a primate lentivirus originated from another primate lentivirus named simian immunodeficiency virus (SIV) [6]. In contrast to HIV, SIV appears largely nonpathogenic in its natural hosts such as the old world monkeys and large apes. The two types of HIV (HIV-1 and HIV-2) are generated by zoonotic transmission of SIV from its natural hosts to humans through blood and/or body fluids exposure during hunting, butchering, and trading [7]. SIV transmitted from apes develops into HIV-1 while SIV transmitted from sooty mangabey monkeys develops into HIV-2 [6].

According to the genetic diversity between different HIV-1 strains, they are classified into four

distinct lineages known as M (main), N (non-M, non-O), O (outlier), and P (putative) [8,9]. HIV-1 group M is the leading cause of the global HIV pandemic spread throughout the world and represents more than 95% of global HIV-1 infections [10]. Group O is less prevalent than group M. It is restricted to West–Central Africa and is responsible for less than 1% of global HIV-1 infections. Group N and group P are restricted to Cameroon with a very low occurrence. HIV-1 group M can be further divided into nine subtypes A–D, F–H, J, and K while Group O has a higher genetic diversity and can be further divided into subtypes I–V [6]. Recombination between different subtypes within a single HIV-1 group or different HIV-1 groups occurs during multiple infections and generates circulating recombinant forms (CRFs) or unique recombinant forms (URFs). The global distribution patterns are different between subtypes and recombinants [11].

Unlike the global transmission of HIV-1, HIV-2 is largely restricted to Africa. HIV-2 can be further classified into at least eight different lineages known as A-H. Compared to HIV-1, HIV-2 strains are significantly less transmissible, and are only responsible for 0.03% of global infections. Indeed, only HIV-2 group A and B are capable of spreading efficiently among humans while group C–H can only achieve “dead-end” transmissions [6]. The limited transmissibility is due to the low viral loads and high CD4 counts in HIV-2 infection. The two characteristics of HIV-2 infection also result in slower or even failed AIDS development in HIV-2 infected individuals [12].

1.3.2 HIV-1 Structure

The mature HIV-1 particles are spherical in shape with an overall size ranging from 119 to 207 nm [13]. The HIV-1 virion is coated in a bilayer lipid envelope that is derived from host plasma membrane during budding. Two embedded viral proteins, protruding surface protein gp120 and

anchoring transmembrane gp41, bind to each other to form heterodimers. Three of these heterodimers together comprise a mushroom shaped 'spike' on the surface of the virion. Lining the inside surface of the lipid envelope, matrix proteins (MA) interact directly with gp41 to support gp41/gp120 spikes on the envelope. Inside the virion, about 2000 copies of capsid protein (CA) form a cone-shaped core that contains two identical copies of HIV-1 RNA genome and viral proteins (Fig. 1). Besides viral proteins and genome, various host proteins such as signal receptor, kinases, cytoskeleton proteins, and host restriction factors have been detected on the surface or within the HIV-1 virion as bystanders or interaction partners of viral proteins.

1.3.3 HIV-1 Genome

The HIV-1 genome is a 9.2kb, linear, single-stranded, positive sense RNA molecular that encodes for a classical retroviral genome structure: 5'LTR-*gag*-*pol*-*env*-LTR3'.

Gag, *pol*, and *env* encode for different polyprotein precursors that can be processed into individual proteins. Initially, the pol-encoded precursor is part of gag-pol precursor named $Pr160^{GagPol}$. $Pr160^{GagPol}$ is generated by a frameshifting event triggered by the cis-acting element during HIV-1 *gag* translation. When ribosomes encounter the cis-acting element, they shift to the pol-reading frame once in twenty times. The autocatalytic cleavage of $Pr160^{GagPol}$ leads to the formation of HIV-1 protease (PR). The functional homodimeric HIV-1 protease cleaves Pol precursor away from Gag and further cleaves it into three enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN) [14]. The *gag* gene encoded precursor $Pr55^{Gag}$ can also be proteolytically processed by PR into mature Gag proteins: MA, CA, nucleocapsid (NC), and $p6^{Gag}$ [14]. Unlike $Pr160^{GagPol}$ and $Pr55^{Gag}$, the Env precursor gp160 is processed by a cellular protease

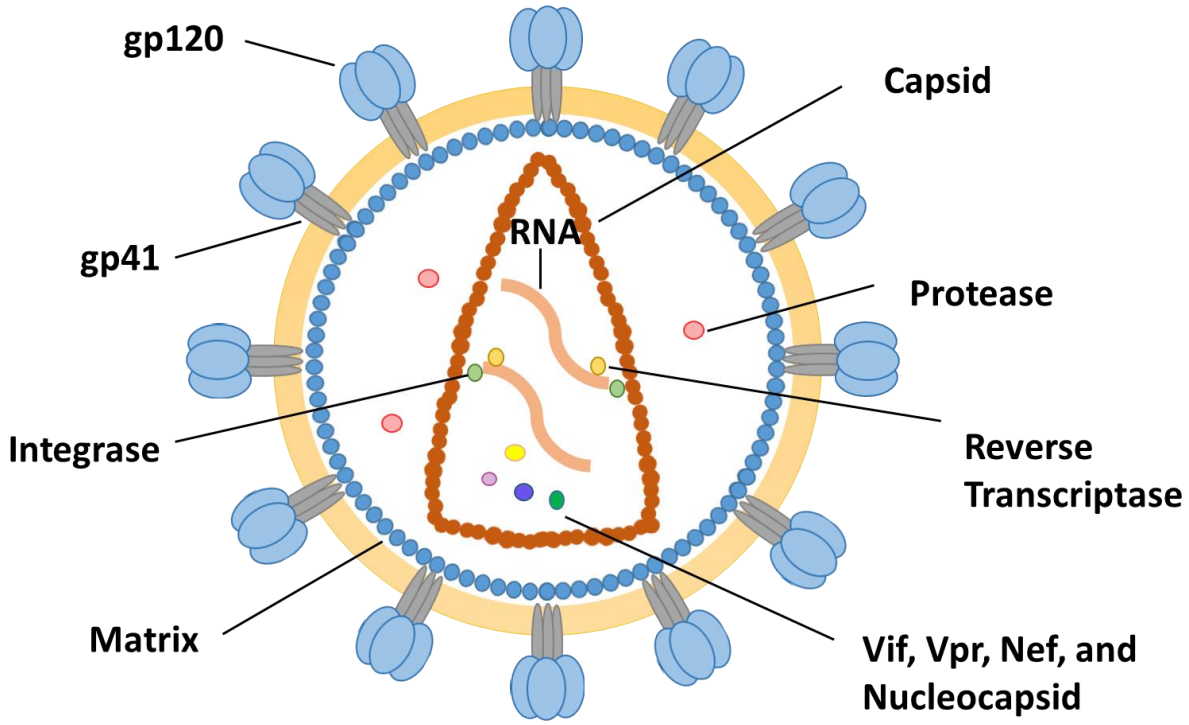

Fig. 1 HIV-1 virion structure. The HIV-1 virion is coated in a host plasma membrane-derived bilayer lipid envelope that is studded with surface protein gp120 and anchoring transmembrane gp41. MA is found between the envelope and the cone-shaped viral core composed of CA. Inside the viral core is HIV-1 RNA genome and viral proteins.

named furin in Golgi apparatus in which gp160 is transported towards the cell surface. This cleavage produces two glycoproteins: gp120 and gp41 [14]. In addition to the proteins mentioned above, the HIV-1 genome also encodes another six proteins: Rev, Tat, Vpr, Vpu, Vif, and Nef (Fig. 2).

1.3.3.1 HIV-1 Genomic Structural Elements

Except for proteins, HIV-1 genome also encodes several elements that contribute to the successful establishment of HIV-1 replication. These elements include LTR (long terminal repeat) Tat-acting region (TAR), primer binding site (PBS), polypurine tract (PPT), central polypurine tract (cPPT), psi RNA packaging signal, dimerization initiation site (DIS), splice donor (SD), splice acceptor (SA), and ref response element (RRE).

LTR is a 640 bp region that functions as a binding site for numerous cellular and viral transcription factors that can regulate the viral gene expression. It can be further divided into three regions: U3, R, and U5. U5 and R regions are located at the 5' terminus of viral RNA while U3 is located at the 3' terminus of viral RNA. During reverse transcription, U5 and R regions are copied and transferred to the 3' terminus of viral RNA as a primer for U3 region copying to make LTR. The newly generated LTR serves as a template for another LTR that is transferred to the 5' terminus of viral DNA [15]. U3 can be further divided into three regions: modulatory region, enhancer region, and core region. The three regions contain binding sites for numerous cellular factors that are involved in HIV-1 gene expression (detailed reviewed in [16]). The NF-kB binding sites in the core region and the SP1 binding sites in the enhancer region are proposed to be the most important activators in HIV-1 gene expression [17,18].

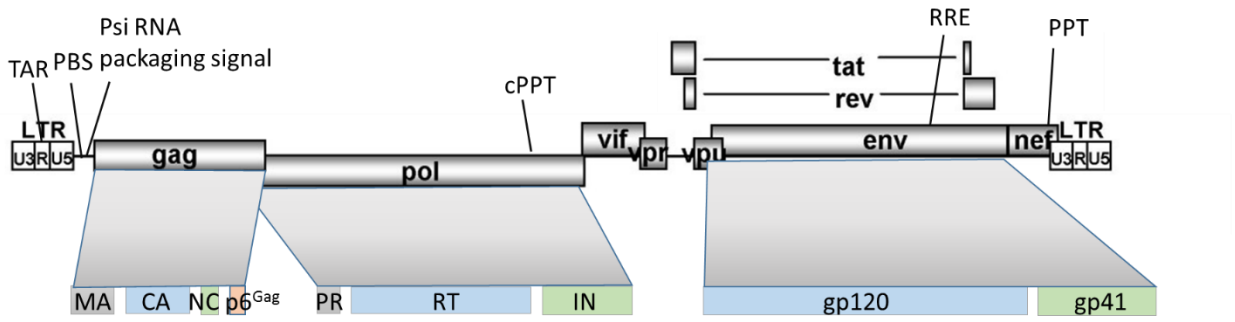

Fig. 2 HIV-1 genomic organization. HIV-1 genome contains three major gene: the gag region which encodes for four of the structural proteins MA, CA, NC, and $p6^{Gag}$; the *pol* region which encodes for the enzymatic proteins PR, RT, and IN; the *env* region which encodes for the other two structural proteins gp120 and gp41. In addition to the structural and enzymatic proteins, the HIV-1 genome also encodes for another six proteins: Rev, Tat, Vpr, Vpu, Vif, and Nef. Several HIV-1 genomic structural elements are shown in the figure includes LTR, TAR, PBS, PPT, cPPT, psi RNA packaging signal, RRE.

TAR is a small stem-bulge-loop structure mapped between nucleotide positions +1 to +59 at the 5' end of all HIV mRNAs [19]. It was originally identified as the binding site for HIV-1 Tat protein. The proper interaction between Tat and TAR is essential for the Tat-mediated transactivation.

PBS, cPPT, and PPT are three elements involved in reverse transcription. PBS is located at the 5′ end of genomic RNA. It comprises 18 nucleotides that are strictly complementary to the nucleotides at the 3′-terminal of $tRNA^{(3,Lys)}$, which is the primer for the negative-strand DNA synthesis [20]. CPPT is located at the center of the genome and probably increases the ability of positive-strand DNA synthesis [21]. PPT is located at the 3′ end of genomic RNA and serves as the primer for the synthesis of positive-strand DNA [22].

Psi RNA packaging signal is composed of four stem loop structures (SL1-SL4). SL1, SL2, SL3 can interact directly with nucleocapsid at high affinity and direct the packaging of viral RNAs into progeny particles [23]. SL4 also binds to NC at low affinity and facilities HIV-1 packaging by stabilizing the psi structure [23,24].

DIS is a GC-rich loop that mediates the dimerization of the RNA genome. DIS contains an unpaired self-complementary sequence that can interact with the unpaired self-complementary sequence in another DIS to generate a 'kissing-loop' complex for RNA genome dimerization [25]. This process is important for packaging of the RNA genome into the virion during assembly [26].

SD and **SA** are splice sites used by HIV-1 to generate different mRNA. There are five SD and nine SA along the HIV-1 genome. HIV-1 uses multiple SD and SA sites to generate more than 30 spliced mRNA species [27].

RRE is a highly branched structure located between nucleotides 7709–8063 within the HIV *env*

region. It functions as an RNA scaffold for HIV-1 Rev assembly. The Rev-RRE oligomeric complex is exported into the cytoplasm where it dissembles. The released viral RNAs are packaged into virions as genomes or translated into viral proteins [28].

1.4 HIV-1 Proteins

The HIV-1 genome encodes for 15 different viral proteins. According to their function, these proteins can be further classified into four categories: the structural proteins that are important for the HIV-1 morphogenesis and morphological structure; the enzymatic proteins that play catalytic roles during HIV-1 replication; the accessory proteins that favor HIV survival in host environment; and the regulatory proteins that are essential for the regulation of HIV gene expression.

1.4.1 HIV-1 Structural Proteins

1.4.1.1 MA

HIV-1 MA is a 17 kDa protein cleaved from the N-terminus of the $Pr55^{Gag}$ precursor. It plays a critical role in different stages of HIV-1 replication cycle. MA is a karyophilic protein carrying two functional nuclear localization signals (NLS) [29]. Some studies suggest that MA is essential in regulating HIV-1 nuclear migration and nuclear import as a part of HIV-1 reverse transcription complex (RTC) and preintegration complex (PIC) [30]. Although MA lacks nuclear export signal (NES), it is important for $Pr55^{Gag}$ nuclear export [31]. During HIV-1 assembly, MA targets $Pr55^{Gag}$ to the cell plasma membrane and facilitates the incorporation of the HIV-1 Env glycoproteins into the virions. The successful establishment of HIV-1 assembly requires the posttranslational

myristoylation of the MA N-terminus and the unsuccessful myristoylation caused by mutation leads to a block in HIV-1 virus budding [32].

1.4.1.2 CA

HIV-1 CA is a 24 kDa protein essential for the structure of the mature HIV-1 viral core. During HIV-1 early stage replication, the viral core needs to be properly disassembled to expose the enclosed RNA genome for reverse transcription. Substantial evidence shows that the capsid core is only partially disassembled until it is transported to the nuclear pore [33,34]. Either stabilized or destabilized capsid core caused by CA mutation or chemical treatment disrupts HIV-1 infectivity [35]. Collectively, these observations suggest that the capsid is one of the viral determinants of HIV-1 uncoating.

1.4.1.3 NC

HIV-1 NC is a 7kDa multifunction nucleic acid binding protein that contributes to HIV reverse transcription, integration, and assembly of progeny virion. In reverse transcription, NC induces the structural changes of $tRNA^{(lys,3)}$ and HIV-1 RNA to favor the initial primer placement, RT processivity and efficient negative-end and positive-end transfer [36]. NC involvement in HIV-1 integration is demonstrated by both in vitro and in vivo studies. Although the mechanism is still largely undetermined, several studies suggest that NC increases strand transfer efficiency by competing with IN for non-specific DNA binding [37]. During HIV-1 assembly, Gag recognition of Psi signal at the 5' end of the genomic RNA via NC region is required for RNA packaging. Moreover, HIV-1 NC also mediates RNA dimerization, tRNA incorporation, and Gag multimerization [38].

1.4.1.4 $P6^{Gag}$

$P6^{Gag}$ is a 6kDa protein cleaved from the C-terminus of the $Pr55^{Gag}$ precursor. It carries two late domains that serve as binding sites for cellular budding machinery components including Tsg101 [39] and AIP-1/ALIX [40]. The interaction between $P6^{Gag}$ and cellular budding machinery components triggers the viral particle detachment from the cell membrane through a cellular machinery involved in small vesicle formation during late endosome budding [41]. Furthermore, it contributes to viral particle assembly by facilitating the Vpr incorporation [42].

1.4.1.5 Gp120/gp41

GP120 and gP41 are produced by gp160 precursor proteolytic processing mediated by cellular protease furin in the Golgi apparatus. Gp120 is composed of five constant regions (C1-C5) and five variable regions (V1-V5) alternating with each other [43]. These regions form a cage-like structure composed of an inner and an outer domain connected by a bridging sheet containing two strands from both the inner and outer domain. The constant domains are mainly located in the inner domain and are important for gp120 folding, while the variable regions are mainly located in the outer region and are important for the immune evasion. Gp41 consists of four regions: N-terminal fusion peptide, two heptad repeat regions (NHR and CHR), and the C-terminal transmembrane region. Three copies of each of gp120 and gp41 bind to each other in a non-covalent manner to form a spike on the virion surface, which is essential in host receptor binding and membrane fusion.

1.4.2 HIV-1 Enzymatic Proteins

1.4.2.1 PR

PR is an 11 kDa aspartyl-type protease that is responsible for generating individual proteins by recognizing and cleaving HIV $Pr55^{Gag}$ and $Pr160^{GagPol}$. PR is initially synthesized as part of $Pr160^{GagPol}$. During viral assembly, the autoprocessing of $Pr160^{GagPol}$ leads to the release of free PR. How autoprocessing is regulated is largely unknown, although precursor dimerization has been proposed as an essential step in this process. The mature PR also functions as a homodimer and the two Asp residues in the conserved Asp-Thr-Gly sequence of each of the monomer act as the center catalytic site [44].

1.4.2.2 RT

RT is an asymmetric heterodimer that is responsible for converting the single-strand HIV-1 RNA genome into a double-strand DNA. Mature RT is comprised of two polypeptide subunits, p51, and p66. The p66 is generated by PR mediated cleavage of $Pr160^{GagPol}$. It processes two domains critical for the enzymatic function of RT, the RNase H and DNA polymerase domains. The association of two p66 subunits forms a p66/p66 homodimer in which the RNase H domain of one of the p66 is cleaved by PR to produce p66/p51 heterodimer [45]. The deletion of the RNase H domain and the conformational change in the DNA polymerase domain makes p51 structurally essential, but catalytically inactive [46].

1.4.2.3 IN

IN is a 32 kDa protein that mainly functions as a catalyst mediating the integration of HIV-1 DNA

into the host chromosome. To accomplish integration, IN catalyzes two sequential reactions: the 3' end processing in which IN cleaves two dinucleotides from the 3' terminus of each viral DNA strand; and the strand transfer in which the processed 3' ends are inserted in to the targeted cellular chromosome [47]. IN also facilities HIV replication by regulating the other early stage replication events including uncoating [48], reverse transcription [48], and nuclear import [49,50].

1.4.3 HIV Regulatory Proteins

1.4.3.1 Tat

Tat has a variable molecular mass in different viral strains ranging from 9 to 11 kDa. The major function of Tat is to enhance HIV-1 transcription by recruiting positive transcription elongation factor (p-TEFb) to TAR at HIV-1 promoter. In the absence of Tat, RNA polymerase II (RNAP II) mediated HIV-1 transcription is restricted by TAR associated negative transcription elongation factors (N-TEFs) and DRB sensitivity-inducing factor (DSIF) and thus mainly generates short transcripts. In the presence of Tat, P-TEFb is recruited to TAR region and stimulates mRNA elongation by inducing activation of Pol II and disassociation of N-TEF and DSIF. This process is regulated by histone acetyltransferases (HATs) induced Tat modification, and Tat-mediated chromatin remodeling. Tat acetylation at Lys28, Lys50, and Lys51 decreases the TAR binding affinity and thus reduces HIV-1 transcription efficiency [51]. Tat-mediated chromatin remodeling destabilizes the interaction between histone and DNA to help HIV-1 transcription. Tat is also involved in HIV-1 reverse transcription, but its function is controversial. Some studies suggested Tat is a suppressor, while others suggest it as an activator [52]. In addition to its role in the HIV-1 replication cycle, it can also be released from the infected cell, binding to specific receptors and

inducing various signal pathways [53].

1.4.3.2 Rev

Rev is a 19 kDa phosphoprotein that plays a role in the exportation of viral RNA and mRNA from the nucleus to the cytoplasm via Rev shuttling cycle. The accomplishment of the Rev shuttling cycle depends on NLS and NES. After expression, Rev is imported into the nucleus in a classical nuclear import pathway mediated by NLS/importin-β interaction [54]. In the nucleus, up to 13 copies of Rev protein binds to RRE to form a Rev-RRE oligomeric complex. The ribonucleoprotein complex is exported into the cytoplasm in the classical nuclear export pathway mediated by NES/Crm1 interaction [55].

1.4.4 HIV-1 Accessory Proteins

1.4.4.1 Vif

Vif is a 23 kDa protein that is capable of neutralizing a cellular antiviral factor named apolipoprotein B mRNA-editing enzyme-catalytic, polypeptide-like 3G (also known as APOBEC3G or A3G). A3G is a defensive protein of innate immunity that can inhibit HIV-1 reverse transcription by causing lethal guanine to adenine hypermutations. Vif is able to recruit E3 ubiquitin ligase to A3G and induce the polyubiquitynation and degradation of A3G [56]. In addition, Vif interferes A3G translation by interacting with A3G mRNA [57,58]. Lastly, Vif impairs the A3G incorporation into the newly synthesized virion by competing with A3G for the binding site in NC during HIV-1 budding [59]. In addition to the A3G counteracting activity, Vif is believed to have a role in virus assembly and to enhance viral core stability [60].

1.4.4.2 Vpr

Vpr is a 14 kDa protein that mainly localize to the nucleus. In accordance with its karyophilic activity, several studies suggested that Vpr is involved in the HIV-1 nuclear import in a non-conventional way, by interaction with importin-α. In the nucleus, Vpr enhances viral replication by transactivating LTR [61]. It has been proposed that Vpr binds to p300 and the transcription initiation complex, recruiting additional factors to the promoter to aid HIV-1 transcription and pathogenesis [62]. Besides these well-studied functions, Vpr has been shown to have several potential roles in HIV reverse transcription. An early study reported that Vpr interacts with Lys-tRNA synthetase and inhibits its enzymatic activity, thus impairing reverse transcription initiation [63]. Vpr also interacts with human uracil DNA glycosylase 2, which belongs to the base excision repair system that might be involve in reverse transcription fidelity.

1.4.4.3 Vpu

Vpu is a 16 kDa transmembrane protein that localizes mainly in the endomembrane system, including the endoplasmic reticulum (ER), the trans–Golgi network (TGN), and endosomes. It recruits SKP1-cullin1-F-Box (SCF) multi-subunit E3 ubiquitin ligase to various substrates by interacting with β-TrCP1 and β-TrCP2 adaptors and thus leads to lysosome or proteasome-mediated substrate degradation. Vpu has two major targets: CD4 receptor [64], and bone marrow stromal antigen 2 (BST-2) restriction factor which is able to tether HIV-1 virion to the cell membrane [65]. The down-regulation of CD4 favors HIV-1 replication by preventing superinfection to avoid apoptosis, and releasing gp160 precursor from CD4 to allow its processing [66]. The down-regulation of BST-2 contributes to the virus release [65].

1.4.4.4 Nef

Nef is a 27 kDa protein that is mainly involved in cell surface receptor modulation [67]. It can mediate the down-regulation of CD4, CXCR4, and CCR5 which impair the re-infection of preinfected cells. It can also eliminate newly synthesized major histocompatibility complex class I and class II (MHCI and MHCII) to restrict immune response. In both cases, Nef recruits clathrin molecules to numerous targeted receptors by binding to clathrin adaptor (AP-1 or AP-2), and therefore induces endocytosis or directs the targeted protein to lysosomes for degradation. Co-stimulatory proteins CD80 and CD86 are also targeted by Nef, but through Rac-mediated endocytosis. The down-regulation of the co-stimulatory inhibits T cell activation to escape immune surveillance.

1.5 HIV-1 Replication Cycle

HIV-1 replication is a complicated multiple step process whose accomplishment requires the cooperation of both viral and host proteins. As represented in Fig 3, HIV-1 replication cycle can be divided into early stage and late stage. The early stage contains 6 steps, from HIV-1 entry to DNA integration, while the late stage contains 5 steps, from gene expression to the virus maturation.

1.5.1 HIV-1 Entry

HIV-1 entry to CD4+ T cells initiates from the virion attachment to the cell surface. The attachment is mainly mediated by the interaction between Env and cell attachment factors such as α4β7 integrin and dendritic cell–specific intercellular adhesion molecular 3-grabbing non-integrin (DC-

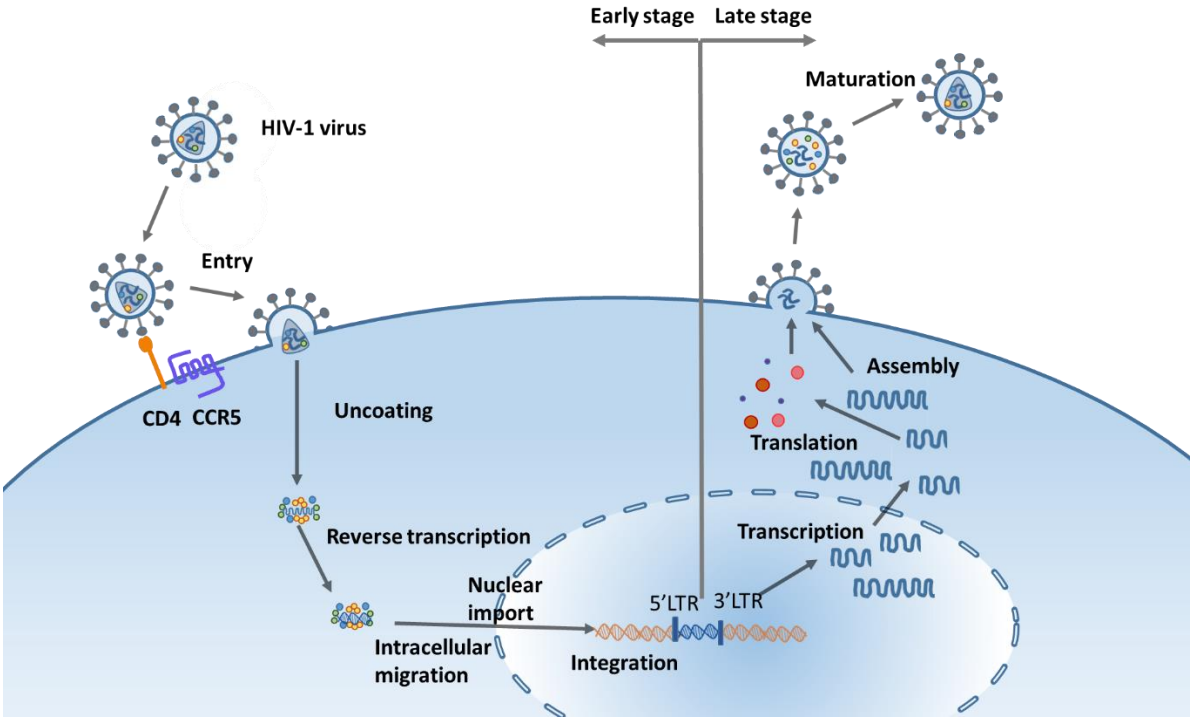

Fig. 3 HIV-1 life cycle. HIV-1 replication cycle can be divided into early and late stage. The early stage includes the HIV-1 entry, viral core uncoating, HIV-1 genomic RNA reverse transcription into double strand DNA, PIC/RTC migration toward nucleus, PIC nuclear import and viral DNA integration. The late stage includes HIV-1 transcription, mRMA translation, viral assembly, budding and maturation.

SIGN) [68]. Although these factors are not required for HIV-1 Infection, they facilitate the Env attachment to viral receptor and co-receptor by reducing the spatial distance. Subsequently, Env binds to CD4 through the CD4 binding site that is located at a depression formed in between the outer and inner domains of gp120. This interaction induces a series of conformational changes at the V1/V2 loop, V3, and the bridging sheet that expose and generate the binding site for CCR5 co-receptor and/or CXCR4 co-receptor [69].

CCR5 is a β-chemokine receptor expressed on macrophages, naïve, and memory T cells while CXCR4 is a α-chemokine receptor expressed on activated T cells. HIV strains can be classified according to the co-receptor affinity. HIV-1 strains which use CCR5 are known as R5 HIV; HIV strains which use CXCR4 are known as X4 HIV, and HIV strains which use both co-receptors are known as R5X4 HIV. This co-receptor tropism is dependent on the V3 charge of gp120. Interestingly, R5 HIV is predominant in up to 90% of HIV-1 early stage patients, while X4 HIV emerges in 50% of HIV late stage patients [70].

Upon co-receptor binding, gp41 is activated and leads to a sequence of conformational change. The N-terminal fusion peptide is exposed and inserts into the targeted cell. Subsequently, the CHR folds back to NHR in each gp41 subunit to form a stable six-helix bundle that draws the viral and cell membranes together. The energy released in the conformational change also enables membrane fusion [71].

1.5.2 HIV-1 Uncoating

Following HIV entry, the HIV capsid core, which contains the HIV genome and viral proteins, are released into the host cytoplasm. The capsid core is inherently unstable and disassembles before

nuclear import in a process known as uncoating. Initially, uncoating was thought to take place immediately after viral entry because the incorporated capsid protein is largely absent from RTC isolated early after infection. However, later investigation of HIV mutants indicated that capsid protein is important for protecting the viral genome from degradation, and mediating nuclear import [72,73]. Based on these results, it has been proposed that HIV uncoating is a gradual process under precise regulation.

Although the detailed regulatory mechanism is unclear, viral proteins, cellular proteins, and intracellular transportation system are likely required for proper uncoating kinetics. Cyclophilin A (CypA) is the best-characterized cellular determinant of HIV uncoating. CypA is a peptidyl prolyl isomerase that interacts directly with capsid protein. Upon binding, CypA induces a conformational change of capsid protein and prevents the interaction of the capsid core with restriction factor, to destabilize the capsid core. Mutagenesis study has revealed that intact HIV IN is essential for the CypA mediated capsid protein disassociation. Lack of functional IN reduces CypA incorporation and alters the HIV uncoating kinetics [74]. Several recent studies suggest that dynein and kinesin motor complexes are also involved in HIV-1 uncoating. Blocking dynein or kinesin mobility by chemical inhibitors, or siRNA knockdown, delays uncoating [75]. Previous work in our laboratory also have demonstrated that the association between HIV-1 IN and dynein light chain 1 (DYNLL1) play an important role in HIV-1 proper uncoating [48]. Besides viral and host proteins, reverse transcript product has been proposed as a potential uncoating regulator. During HIV reverse transcription, viral RNA is converted to DNA with a central DNA Flap. Inhibition of this HIV reverse transcription by a reverse transcriptase inhibitor, or introduction of a mutation to the

DNA Flap encoding area, resulted in capsid core accumulation in the cytoplasm [76].

1.5.3 HIV-1 Reverse Transcription

HIV uncoating changes the structure of the viral core and forms RTC in which efficient HIV reverse transcription takes place. Completion of HIV reverse transcription transits RTC into the PIC for nuclear entry. Multiple viral and cellular proteins are associated with the RTC and PIC, but their structures are unknown.

HIV reverse transcription is catalyzed by RT. RT functions as a DNA polymerase that can generate DNA by copying from a DNA or RNA template. It also functions as an RNase H that can degrade RNA in an RNA-DNA hybrid. Similar with many other DNA polymerases, RT-mediated HIV reverse transcription requires the cellular $tRNA^{Lys,3}$ binding to the PBS as the primer. The newly synthesized DNA anneals to the R region at 3' end of the viral RNA to enable synthesis of the rest of the minus-strand DNA. During the minus-strand DNA synthesis, the whole viral RNA is progressively degraded, except the PPT sequence that is resistant to RNase H and serves as the primer for the plus strand DNA synthesis. In the plus strand DNA synthesis, the first 18 nucleotides from the $tRNA^{Lys,3}$ are also copied then the $tRNA^{Lys,3}$ is cleaved by RT leaving a single A ribonucleotide. The newly synthesized DNA anneals to the minus-strand DNA at the region copied from PBS to enable the extent of DNA synthesis in both directions [15].

HIV reverse transcription is regulated by viral proteins and cellular proteins. Given that HIV reverse transcription is closely related to HIV uncoating, it is not surprising that the interactions between capsid protein and CypA as well as IN and DYNLL1 are involved in reverse transcription regulation. In fact, inhibiting the interaction between CA/CypA and IN/DYNLL1 results in

decreased HIV reverse transcription [48,77].

1.5.4 HIV-1 Intracellular Migration

After HIV-1 entry, the incoming virus needs to move from the cell periphery area to the perinuclear area. Restricted by the crowded and complex organization of the cytoplasm environment, the intracellular migration is proposed to be mediated by the host intracellular transportation system, such as actin and microtubule filaments, rather than free diffusion.

In CD4+ T cells, actin is mainly arranged in bundles underneath the cellular membrane. The interaction of HIV Env with receptors and co-receptors induces a series of signaling pathways resulting in the activation of Arp2/3 actin nucleator and cofilin. The activated Arp2/3 actin nucleator and cofilin promote actin polymerization, and the activated cofilin also loosens the actin network, together allowing HIV migration through the cell cortex [78]. Although it is clear that actin is essential for the HIV intracellular transportation, no functional interaction between HIV and actin motor complex has been demonstrated. This might be due to the technical difficulties with current approaches or due to novel motor involvement.

Microtubule is exploited by HIV for long distance travel towards the cell nucleus. An early study demonstrated that depolymerizing of microtubule by nocodazole significantly reduces HIV infectivity [79]. Later, visualization of single virion linked this phenomenon to impaired motility. Microinjection of antibody to inhibit the movement of the cytoplasmic dynein, which is the motor protein responsible for directing cargo proteins towards the nucleus, also reduced transport efficiency [80]. Collectively, these evidence suggest that intact microtubule and dynein are essential in HIV transportation.

1.5.5 HIV-1 Nuclear Import

To deliver the HIV cDNA into the nucleus, PIC need to cross the nuclear membrane through the nuclear pore complex (NPC). The NPC is a basket shaped supramolecular complex that spans the nuclear membrane bilayer and allows the import and export of molecules. The molecular size limit for passive diffusion through the NPC is 9 nm, which is much smaller than the size of PIC (50 nm) [81]. For this reason, the PIC nuclear entry is an active nuclear import process. Classical active nuclear import is mediated by karyophilic proteins carrying NLS, which can be recognized by nuclear import factors such as importin-α. The interaction between NLS and Importin-α recruits importin-β to target the NPC. Subsequently, the cargo-receptor complex is translocated into the nucleus by an unclear mechanism. The incoming cargo-receptor complex is subsequently disassociated by the GTP-bound form of the small Ran GTPase (RanGTP) to release the cargo.

As a karyophilic RTC/PIC component, IN has been proposed as the determinant for nuclear import. Previous work in our lab have demonstrated that the interaction of HIV-1 IN with importin α3 and importin 7 play important roles in HIV-1 cDNA nuclear import [50,82]. Several studies suggested that another karyophilic protein Vpr is involved in the HIV-1 nuclear import in a non-conventional way, by interaction with importin-α. Besides IN and Vpr, MA also have intrinsic karyophilic properties and can interact with importin-α. Although some functional studies have reported MA as potential determinants, whether and how it contributes to HIV-1 nuclear entry remains controversial. MA contains an NLS at both N-terminus and C-terminus, however, MA itself is absent from the nuclear, and MA defective HIV can still replicate efficiently so that MA is unlikely to be responsible for this process [83,84].

1.5.6 HIV-1 Integration

Once the PIC has translocated into the nucleus, the viral DNA can be either integrated or unintegrated. Unintegrated viral DNA can form both 2-LTR circles by ligating the ends of viral cDNA and 1-LTR by LTR recombination. Although the circular unintegrated viral DNA is stable, it is unable to produce virus. For this reason, proper integration is important for productive replication.

HIV integration is initiated in the cytoplasm where IN cleaves a pGT dinucleotide from each of the 3' end of viral DNA. After nuclear entry, the two 3-hydroxyl residues attack the DNA phosphodiester bonds on different strands of the host DNA with a five base pair interval. The ligation of viral DNA to the host DNA results in a five base gap at each of the junctions, and two unpaired bases at 5' end of the viral DNA. Subsequently, the unpaired bases from the viral DNA are removed, and the gap is filled by an unknown cellular repair pathway [47].

Analysis of HIV integration sites in different cell types suggests that active transcription units are strongly preferred by HIV. Several host proteins, including integrase interactor 1 (INI1) and lens-epithelium-derived growth factor (LEDGF), have been hypothesized to guide the integration site selection [85]. After integration site selection, HIV cDNA is tethered to the targeted host chromosome by LEDGF and nucleoporin 62. Inhibition of the IN association with LEDGF or nucleoporin 62 significantly impair viral DNA integration.

1.5.7 HIV-1 Transcription and Translation

The integrated viral genome functions as the template for transcription to produce mRNAs. The successful establishment of HIV translation requires functional LTR and coordination of numerous host and viral factors.

The first stage of HIV transcription is called the early Tat-independent stage, in which the HIV translation simply relies on cellular transcriptional factors. Due to the restrictive chromatin environment, as well as the presence of N-TEF and DSIF, the translation efficiency is limited. HIV mRNA produced in this stage undergoes multiple splicing into 2 kb mRNAs that are translated to yield Rev, Tat, and Nef.

The second stage of HIV transcription is called late Tat-dependent stage, in which Tat goes to the nucleus element, binding to TAR to increase transcription efficiency. The Tat-TAR interaction recruits cyclin dependent kinase 9 (CDK9)/cyclin T1, which is the component of p-TEFb. CDK9 catalyzes the phosphorylation of the C-terminal domain (CTD) in RNAPII to enable the elongation of the viral mRNA. CDK9 also catalyzes the phosphorylation of SPT5 in N-TEF and DSIF to disassociate N-TEF from TAR. Once transcription is activated, Tat recruits HATs for chromatin remodeling to release transcription start sites from nucleosomes.

To produce mRNA encoding for different HIV proteins, 50% of the HIV mRNAs produced in the late Tat-dependent stage undergo alternative splicing. According to the alternative splicing method, spliced mRNAs can be classified into two groups: 4 KB incompletely spliced mRNAs which encode the Env, Vpu, Vpr, and Vif proteins, and 2KB completely spliced mRNAs which encode the Tat, Rev, Vpr and Nef proteins. The other 50% of the HIV mRNAs produced in the late Tat-dependent stage are 9 KB unspliced mRNA which encodes the $Pr55^{Gag}$ and $Pr160^{GagPol}$ polyprotein and also can be packaged as genomic RNA [86].

The newly synthesized viral mRNAs need to be translocated from nucleus to cytoplasm for translation or assembly. The completely spliced HIV mRNA are exported in the same pathway

used by cellular mRNA, while the incomplete spliced and unspliced HIV mRNA are exported with the help of Rev. Binding of Rev to the RRE region recruits CRM1 and RanGTP, to allow incompletely spliced and unspliced HIV mRNA to be exported in the classical pathway.

1.5.8 HIV-1 Assembly

HIV assembly packages Env protein, $Pr55^{Gag}$, $Pr160^{GagPol}$, cellular $tRNA^{Lys,3}$, and two copies of genomic RNA into an immature particle on the plasma membrane. The $Pr55^{Gag}$ drives the major steps in this process, including membrane association, Gag multimerization, and viral protein and genomic RNA incorporation. The accomplishment of $Pr55^{Gag}$ function in assembly mainly depends on three domains: MA is responsible for membrane association and Env incorporation; CA contributes to Gag multimerization and $Pr160^{GagPol}$ incorporation; NC facilities RNA incorporation. The role of these subdomains are not completely independent of each other. In fact, $Pr55^{Gag}$ mediated HIV assembly events occur simultaneously, and have functional overlappings [87].

$Pr55^{Gag}$ is synthesized by free ribosomes and cotranslationally myristoylated by myristoyl coenzyme A (myristoyl-CoA) in the cytoplasm. Soon after synthesis, $Pr55^{Gag}$ is transported to the cell periphery. Although the detailed mechanism of $Pr55^{Gag}$ trafficking is unclear, there is evidence suggesting that the endosomal pathway and microtubule network are involved in this process. At the plasma membrane, $Pr55^{Gag}$ interacts with the plasma membrane-specific lipid phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) through the MA domain. The interaction with PI(4,5)P2 induces the conformational change of the $Pr55^{Gag}$, exposing the myristoyl group in the MA domain to increase the affinity of $Pr55^{Gag}$ for membrane binding. Besides the myristoyl switch, the ionic interactions between the positively charged basic residues and the negatively charged

phospholipids also contribute to the $Pr55^{Gag}$ anchoring.

Generally, $Pr55^{Gag}$ molecules are inserted into the plasma membrane as monomers or dimers. To generate a particle, $Pr55^{Gag}$ proteins need interact with each other. The oligomerization of $Pr55^{Gag}$ is mainly mediated by homotypic interactions, especially between the CA CTD domains. Besides homotypic interactions, the interaction between the viral RNA and NC domains has also been reported to have a structural ‘‘scaffolding’’ role that triggers $Pr55^{Gag}$ oligomerization. The highly ordered Gag oligomerization further triggers the myristoyl switch to enhance $Pr55^{Gag}$/membrane binding. More importantly, it induces the formation of a curved lattice, bulging out the plasma membrane that provides the force for HIV assembly [88].

Genomic RNA needs to be encapsulated into the virion. After synthesis, two HIV-1 genomic RNAs are dimerized by generating a “kissing-loop” structure through the complementary of DIS in different RNA strands. Subsequently, the dimeric genomic RNA is recruited to the assembly site by binding to the NC domain of $Pr55^{Gag}$ through the ψ element. As the ψ element locates after the splicing doner and is removed in the spliced RNAs, this dedicated binding site selection guarantees the full-length genomes are preferably incorporated into the virion over the spliced RNAs.

The host cell contains more than 30 different tRNAs, but only $tRNA^{Lys,3}$ is selectively incorporated into the virion. This selection is mediated by human lysyl-tRNA synthetase (LysRS), which binds to the $tRNA^{Lys,3}$ and the $Pr55^{Gag}$ at the same time. Interestingly, the association between $Pr55^{Gag}$ and $tRNA^{Lys,3}$ is insufficient for the virion incorperation of $tRNA^{Lys,3}$. Several studies have demonstrated that, for successful incorporation, $tRNA^{Lys,3}$ needs to interact with the RT domain of $Pr160^{GagPol}$

simultaneously.

Although $Pr160^{GagPol}$ has the same MA and CA domains as $Pr55^{Gag}$, it cannot incorporate independently. It is generally accepted that $Pr160^{GagPol}$ is concentrated at the assembly site through the interaction between the CA domains of $Pr55^{Gag}$ and $Pr160^{GagPol}$, but the motif in the CA domain responsible for this interaction is still controversial.

The assembling HIV particles are covered by Env spikes whose incorporation is indispensable of $Pr55^{Gag}$. Env precursor gp160 is synthesized and cotranslationally inserted into the rough endoplasmic reticulum. Subsequently, gp160 is transported through the cellular secretory pathway to the Golgi, where it is cleaved to yield gp120 and gp41. Multimerization of gp120 and gp41 generates Env, which is delivered to the plasma membrane for virion incorporation [89]. Originally, the Env was thought to incorporate into the virion in a passive or random way, as completely removing the cytoplasmic tail of Env only slightly attenuated Env incorporation in some cell lines. Later, an increasing amount of evidence suggested that an interaction with the MA domain is required for Env incorporation. Several studies demonstrated that Env interacts directly with the MA domain and disrupting this interaction by MA domain mutation or deletion can induce Env incorporation deficiency. Furthermore, Env incorporation deficiency of the MA mutant virus can be rescued by simultaneous deletion of Env cytoplasmic tail. Together, these observations indicated that full-length Env incorporation requires the MA domain.

1.5.9 HIV-1 Budding

To generate individual virions, the fully assembled virion needs to be released from the plasma membrane. The detachment of the nascent HIV-1 virus is mediated by $p6^{Gag}$ through a well-

established pathway named endosomal sorting complexes required for transport (ESCRT) pathway.

$P6^{Gag}$ contains two motifs, the PTAP domain and the YPXL domain, that are essential for recruiting the early-acting factors of the ESCRT pathway. The PTAP domain recruits the ESCRT-I complex by binding to its subunit TSG101, and the YPXL domain recruits the ESCRT-I factor ALIX. These interactions lead to the recruitment of late-acting ESCRT factors CHMP2, CHMP4, and VPS4. Although the mechanism of ESCRT-I induced late-acting ESCRT factor accumulation is not yet clear, the ALIX pathway is well understood [90]. The interaction with YPXL domain triggers the ALIX dimerization and activation. The activated ALIX recruits CHMP4 and induces the latter to polymerize into spiraling filaments at the budding site. CHMP4 recruits CHMP2 which functions as a bridge for VPS4 binding.

Since the CHMP4 formed filaments spiral toward the cell, they are likely to function as the main constrictor pulling the opposing membranes together. The successful establishment of membrane fission also requires VPS4, which may have a role in promoting the spiraling filaments formation, or driving fission to completion. In the final stage of membrane fusion, VPS4 provides energy for the disassembly of the other engaged factors.

1.5.10 HIV-1 Maturation

The HIV-1 viral particles newly separated from the plasma membrane are in an immature, noninfectious form that needs to undergo a series of protein cleavage, conformational changes, and subunit rearrangements to be converted into mature infectious form.

HV-1 maturation is mainly driven by PR. Initially, PR exists in an inactive form as part of the

$Pr160^{GagPol}$ precursor. During HIV-1 assembly, budding, and maturation, PR is gradually activated and released from the $Pr160^{GagPol}$ precursor, probably through an autoprocessing way. Subsequently, PR cleaves $Pr160^{GagPol}$ at five different sites generating MA, CA, PR, RT, and IN. PR also cleaves $Pr55^{Gag}$ at five different sites to produce four viral proteins and two space peptides (SP): MA, CA, NC, $p6^{Gag}$, SP1, and SP2.

The $Pr55^{Gag}$ cleavage takes place in a certain sequence that is at least partially regulated by the efficiency of proteolytic processing. Accordingly, these cleavage sites can be further classified into three different categories: rapid site (SP1/NC), intermediate site (SP2/p6, MA/CA), and slow site (NC/SP2, CA/SP1). The cleavage at SP1/NC favors the condensation of the ribonucleoprotein (RNP) within the viral center [91]. The cleavage at MA/CA disassociates CA from the membrane-incorporated MA. The cleavage at CA/SP1 disassembles the immature lattice and forms the new viral capsid core [91]. The cleavage at NC/SP2 frees NC and further promotes RNP condensation [92]. Together, these sequential events result in the formation of a mature HIV-1 particle with cone-shaped capsid core that contains condensed RNP.

1.6 Anti-HIV Drugs

The antiretroviral drugs currently used for HIV treatment belong to four distinct classes: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), entry inhibitors, integrase inhibitors, and protease inhibitors.

NRTIs are prodrugs that need to be phosphorylated by host kinase to form the functional ddNTPs [93]. DdNTPs are competitors of normal dNTP substrates. As dNTPs, ddNTPs can incorporate into the viral DNA chains during HIV-1 reverse transcription. Compared with normal dNTP substrates,

ddNTPs lack a 3’-hydroxyl group essential for the formation of 3’-5’-phosphodiester bond with the later incorporated substrates. Given that, ddNTPs work as a DNA chain elongation terminator during both viral RNA-dependent DNA and DNA-dependent DNA synthesis. Currently, there are nine Food and Drug Administration (FDA)-approved NRTIs including abacavir, didanosine, emtricitabine, lamivudine, stavudine, zidovudine, tenofovir, disoprovil, and fumarate.

NNRTIs directly interact with HIV-1 RT and induce the formation of a binding pocket close to the dNTP binding site of HIV-1 RT. Although the conformational change has no effect on the DNA and dNTP binding, it impairs RT-mediated DNA polymerization. Several hypotheses have been proposed to explain the mechanism underlying the anti-HIV activity of NNRTI including the following: restriction of mobility within protein structure; distortion of the polymerase motif; alternation of the dissociation/association frequency between RT and nucleic acid substrate [94]. Currently, there are five FDA-approved NNRTIs including etravirine, delavirdine, efavirenz, nevirapine, and rilpivirine.

Entry inhibitors can be divided into two distinct classes, the co-receptor antagonist, and the fusion inhibitor. Maraviroc is the only co-receptor antagonist approved by FDA thus far. It is a nonpeptidic inhibitor that is capable of binding in the hydrophobic transmembrane pocket of CCR5. Upon interaction, Maraviroc induces the conformational change of the CCR5 and prevents gp120 binding [95]. To date, Enfuvirtide is the only fusion inhibitor approved by FDA. It is a 36 amino-acid synthetic peptide designed to mimic the CHR fragment of gp41. As the interaction between CHR and NHR of gp41 induced stable six-helix bundle formation is the critical step in HIV-1 fusion, the binding of Enfuvirtide to NHR significantly disrupts the fusion process [96].

Protease Inhibitors Several protease inhibitors have been approved by FDA: atazanavir, darunavir, fosamprenavir, indinavir, nelfinavir, ritonavir, saquinavir, and tipranavir. Except for tipranavir, the other six protease inhibitors are designed to mimic the natural PR cleavage site. Unlike the PR substrates, these protease inhibitors replace PR targeted P1–P1′ amide bond with non-hydrolysable isosteres [44]. Upon binding, they block the proteolytic activities of PR, resulting in the inability of $Pr55^{Gag}$ and $Pr160^{GagPol}$ cleavage and the formation of immature non-infectious viruses.

IN inhibitors are the newest class of antiretroviral drug in HIV-1 treatment. All three currently FDA-approved integrase inhibitors (dolutegravir, elvitegravir and raltegravir) target the strand transfer during HIV integration. The IN inhibitors specifically bind to the IN–DNA complexes and interact with the two Mg^{2+} cofactors in the IN catalytic site [97].

1.7 HARRT and Current Challenges

In the history of anti-HIV-1 therapy development, the highly active antiretroviral therapy (HAART) is considered as a milestone achievement. Proper adherence to HAART can durably suppress HIV-1 replication to minimal levels, delay the progression to AIDS, and prolong the life of HIV-1 infected individuals. HAART treatment usually begins with the combination of two NRTIs and an NNRTI. If the HIV becomes resistant to the treatment, or the treatment induces serious side effects, the treatment plan might be changed into the combination of two NRTIs and a protease inhibitor.

Despite the success of HAART, significant problems still exist in current HIV-1 treatment including the failure to eradicate HIV, severe side effects, and the development and spread of drug resistant

strains. Current HAART allows the functional cure of HIV-1 positive patients. However, it is unable to eliminate HIV-1 thus the patients must continue the treatment lifelong. The HAART treatment is accompanied by a large diversity of side effects. The beginning of HAART treatment is frequently accompanied by rashes, hypersensitivity reactions, and immune reconstitution syndrome. Over time, dyslipidemia, diabetes, avascular necrosis, peripheral lipoatrophy, and central fat accumulation can be observed. Suboptimum treatment or incomplete adherence to therapy may lead to the selection of drug resistance mutations. As the ART targets are relatively conserved, strains resistant to one medicine may also prove resistant to other medicines in the same class, thus further limiting the choice of medicine. Furthermore, drug resistant strains may also be transferred to treatment naive individuals and cause HIV-1 drug resistant strain spread [97].

1.8 The Critical Role of Protein Phosphorylation in HIV-1 Life Cycle

1.8.1 Cellular Kinase Pathways Involved in the Establishment of HIV-1 Infection

Kinase-mediated phosphorylation plays important roles throughout HIV replication and pathogenesis. In fact, significant changes have been detected at more than 200 phosphorylation sites in CD4+ T cells after HIV-1 infection.

1.8.1.1 LIMK and Abl Pathways in HIV-1 Cell Entry

The first challenge the HIV-1 meet after entry is the restriction of the cortical actin on the inner surface of the cellular membrane. To overcome the physical barrier, HIV-1 uses co-receptors to induce a kinase-signaling pathway to manipulate the actin rearrangement for viral migration.

HIV-1 co-receptor CCR5 and CXCR4 are transmembrane receptors that bind to heterotrimeric guanine nucleotide-binding protein (G protein) complex with the inner cytosolic domain. In the inactive state, heterotrimeric G protein complex contains three different subunits Gα, Gβ, and Gγ. Upon activation, Gα is disassociated from the complex and the freed Gα and Gβ/γ are able to transmit signals separately. Gα can be further divided into four different classes: Gαs, Gαi, Gαq, and Ga12/13. In the case of HIV-1, Gαi [98], and Gαq [99] are the major G proteins activated by HIV-1 Env for signal transduction.

One of the downstream effectors of G protein during HIV-1 infection is Rho family GTPases including Rac, CDC42, and RhoA [99]. The activation of Rac or CDC42 stimulates p21-activated kinase (PAK) which phosphorylate LIM kinase (LIMK) at Thr508 causing LIMK activation [100]. LIMK phosphorylates cofilin, which is a ubiquitous actin-binding protein that is able to cause actin

depolymerization by enhancing actin disassembly. Phosphorylation at Ser3 inhibits cofilin activity and facilitates actin polymerization [101]. Another downstream effector of Rac is Wave2 complex that can interact with Arp2/3 actin nucleator to induce actin remodeling. The activation of this Rac/Wave2 signaling pathway requires another kinase named Abl both upstream and downstream. In upstream, Abl phosphorylates Ras GEF complex to promote Rac GEF Tiam-1 induced Rac activation. In downstream, Abl phosphorylates Wave2 to favor interaction with Arp2/3 [102]. Activated Arp2/3 binds to a pre-existing actin filament and induces polymerization and side branching.

1.8.1.2 PI3K, LCK/PKC θ and CDK Pathways in HIV-1 Transcription

HIV transcription is under the control of LTR, the viral promoter containing binding sites for numerous cellular and viral factors. Effective HIV transcription relies on two critical cellular factors: NF-κB, which is a transcriptional activator, and RNAP II, which is an mRNA elongator. Both are regulated by kinase pathways.

NF-κB is one of the downstream effectors of PI3K. During HIV infection, PI3K can be activated in several different ways including Env interaction with the CD4 receptor and/or co-receptor [103], Tat or cytokines interaction with corresponding receptors [104], and Nef/PI3K direct interaction. NF-κB is also the downstream effector of protein kinase C (PKC) θ. Env engagement with CD4 activates LCK proto-oncogene, Src family tyrosine kinase (LCK) [105]. Subsequently, the activated LCK recruits and activates PKC θ [106]. Although the detailed pathway is not fully revealed, the activation of PI3K, LCK or PKC are likely to trigger the activation of IκB kinase (IKK) for IκB degradation to release NF-κB from NF-κB /IκB complex. The released NF-κB is subsequently

translocated from the cytoplasm into the nucleus and binds to specific sites in LTR to promote HIV transcription [107].

Regulation of RNAP II is primarily mediated by CDK pathways. Mammalian RNAP II contains a conserved CTD, which consists of 52 tandem copies of the repeat heptad sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. During HIV transcription, RNAP II can be phosphorylated and dephosphorylated at Ser2 and Ser5 of each repeat in an inequivalent manner.

The initiation of HIV transcription relies on the assembly of preinitiation complex at the promoter. The preinitiation complex contains RNAP II, six general transcription factors (transcription factor II A, B, D, E, F, and H), and mediator complex. In this stage, Ser5 of RNAP II CTD is phosphorylated by CDK7/cyclin H in transcription factor II Human (TFIIH) and CDK8 in the mediator complex. Subsequently, the Ser5 phosphorylated RNAP II leaves the promoter and catalyzes transcription elongation which is favored by Tat and pTEF-b. In the absence of tat, RNAP II mediated transcription is paused downstream of the transcription start site. In the presence of Tat, pTEF-b is recruited to TAR region where CDK9/cyclin T1 in pTEF-b mediates the phosphorylation of N-TEF and DSIF as well as Ser2 in RNAP II CTD. The phosphorylated N-TEF is disassociated from its binding site in TAR region, and this changes DSIF into a positive elongation factor that enhances the processivity of RNAP II. The function of CDK9/cyclin T1 is also modulated by CDK9 phosphorylation. A recent study suggests CDK2/cyclin E as a potential regulatory factor for CDK9/cyclin T1 as inhibition of CDK2 expression by iron chelators or knockdown approach that reduces CDK9 phosphorylation at Ser90 and impairs HIV replication.

1.8.1.3 SPRK and PI3K/Akt Pathways in HIV-1 mRNA Splicing

To generate genetic templates coding for viral proteins required for HIV-1 replication with one single template, about 50% of produced HIV mRNA take advantage of the host alternative splicing system to form mature mRNA of different sizes.

Major steps of alternative splicing including splicing site selection, cleavage, and ligation are catalyzed by the spliceosome. The assembly and function of spliceosome are regulated by serine arginine-rich proteins (SRps), and the function of SRp is affected by SR protein kinase (SRPK) mediated phosphorylation [108]. Thus, it is not surprising that SRp phosphorylation affects HIV-1 replication. It has been reported that HIV-1 expression can be up-regulated 20-fold by increasing the activity SRPK2 and can be down-regulated to variable levels in the presence of different SRPK inhibitors [109].

Interestingly, HIV-1 alternative splicing is also affected by PI3K/Akt pathway [110]. Activated by HIV infection, PI3K is relocated to the plasma membrane and phosphorylates phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 serves as a docking site for Akt kinase binding. Inhibition of PI3K by chemical compounds impair Akt kinase and SRp phosphorylation, which result in altered splicing pattern and reduced HIV infectivity. However, how SRp is phosphorylated in this pathway is controversial, and some evidence suggests that Akt directly induces SRp is phosphorylation while another suggests that Akt use SRPK as a potential downstream effector for SRp phosphorylation.

1.8.1.4 P38 and ERK Pathways in HIV-1 Pathogenesis

HIV progression is accompanied by increased viral load and continuous CD4+ T cell depletion.

Although CD4+ T cell loss can be caused by different mechanisms, apoptosis is the major one. It has been reported that HIV manipulates P38 and extracellular signal-regulated kinases (ERK) pathways to induce cell apoptosis.

P38 and ERK belong to the mitogen-activated protein kinase (MAPK) family that can be activated by a diverse array of stimulation including cell activation as well as cytokine and chemokine stimulation. The P38 and ERK function in the opposite way. P38 is often implicated in cell death and cell cycle arrest while ERK frequently contributes to cell survival and proliferation. Interestingly, both pathways are required for successful HIV replication and are activated during HIV-1 infection.

P38 can be activated through Env binding to CD4 and CXCR4 as well as extracellular Tat and Nef stimulation [111,112]. The activation of p38 phosphorylate various downstream transcriptional factors such as P53 and NF-κB and is accompanied by upregulated expression of proteins associated with apoptosis such as Fas ligand (FasL), programmed cell death protein 1 (PD-1), TNF-α, and IL-10. ERK can be activated through Env binding to CD4 and as well as extracellular Vpr and Nef stimulation [103,113]. Activation of ERK results in increased IL-2 expression and suppressed apoptosis.

Given that P38 and ERKs play antagonistic roles in apoptosis, cell destiny depends on the strength and duration of the two pathways. Several studies suggest that the activation pattern of the two kinases are different from one another during HIV-1 infection. P38 activation is sustained while ERK activation is strong but transient, with the peak at around five minutes [113]. How HIV infection regulates the strength and duration of the two pathways is currently unknown. Although

a recent study hypothesized that an alternative pathway for P38 activation might be involved in this process, no firm evidence has been provided.

1.8.2 HIV-1 Protein Phosphorylation during HIV-1 Replication

1.8.2.1 CA

As the major core component, CA significantly affects core stability and uncoating rate. Several studies demonstrate that phosphorylation plays an important regulatory role in CA disassembly. The first study identified three phosphorylation targets in CA: Ser109, Ser149, and Ser178 [114]. Introducing serine to alanine mutation at these sites significantly reduced uncoating and reverse transcription efficiency [114,115]. Since these sites are localized in the C-terminus domain of CA, which is involved in CA dimerization [116], this result suggests that phosphorylation at these sites might contribute to proper HIV uncoating by altering the HIV core structure. A recent study reported that virion-incorporated ERK2 is able to mediate CA phosphorylation at Ser16 during HIV maturation [117]. The phosphorylated Ser16 along with the adjacent Pro17 is the targeting site of peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1) [118]. Upon the interaction between PIN1 and CA through pSer16-Pro17 motif, the capsid structure is altered to facilitate CA disassociation. Abolishing the interaction between PIN1 and CA by PIN1 knock down or pSer16-Pro17 motif mutagenesis severely interfered with HIV uncoating [118].

1.8.2.2 IN

The progressive CA disassociation during uncoating disturbs the structural integrity of viral core, exposing the internal viral proteins to the cellular environment that increases the risk of protein

degradation. IN is an unstable protein that can be degraded in ubiquitination pathway by the cellular protease. To favor their own replication, HIV have developed a strategy using phosphorylation to counter degradation. In activated CD4+ T cells, IN is phosphorylated by the c-Jun N-terminal kinase (JNK) at Ser57 [119]. IN modification allows the binding of PIN1, which in turn induces IN conformational change of the binding site from cis isomers to trans configuration. The interaction between PIN1 and IN is accompanied by a significantly prolonged IN lifespan [119]. Moreover, disrupting the interaction of IN with PIN by mutagenesis, knockdown, or using a chemical inhibitor results in an increased ubiquitination level [119]. Collectively, this evidence suggests that the phosphorylation might be involved in IN stabilization against ubiquitination-induced degradation.

1.8.2.3 Nef

Nef is a multifunctional protein of great significance in HIV pathogenesis. It contains multiple phosphorylation sites and the modification at these sites affects many aspects in HIV biology. Although many Nef-interacting cellular kinases have been identified, only a small proportion induce Nef phosphorylation.

An early study shows that a multiprotein complex is able to phosphorylate Nef at the N-Terminus upon binding. While tyrosine kinase Lck is identified as one of the complex components, it does not directly interact with Nef [120]. Besides, Nef N-Terminus is still phosphorylated in the absence of Lck [120]. Based on these results, it has been hypothesized that the multiprotein complex contains another kinase that is able to induce Nef phosphorylation. This hypothesis is confirmed by a follow-up investigation in which Nef was found to be phosphorylated at Ser6 by PKCδ and θ

in the complex [121]. Nef S6A mutant with PKCδ and θ binding deficiency is more likely to distribute to the plasma membrane rather than the normal distribution site around the nuclear. In addition, the Nef S6A mutant does not affect Nef induced CD4 expression downregulation on the cell surface. PKCδ and θ knock down significantly affected viral infectivity in both stimulated and resting PBMCs [121].

PKC is another member of the PKC superfamily that has been reported to induce Nef phosphorylation [122,123]. Different from PKCδ and θ, PKC induces Nef phosphorylation at Thr15 and is not the determinant of Nef distribution while it enhances Nef induced CD4 expression downregulation [123]. Nef can also be phosphorylated by cAMP-dependent kinase (PKA) at Ser6. Inhibiting PKA induced Nef phosphorylation by introducing serine to alanine mutation at Ser6 resulted in reduced HIV infectivity in resting human PBMCs [124].

1.8.2.4 Vif

It has been known for a long time that Vif can be phosphorylated at different sites including Thr96, Ser144, Thr155, Ser165, and Thr188 [125,126]. Although several mutations preventing Vif phosphorylation at a specific site have been demonstrated to be replication defective at various levels, how phosphorylation contributes to Vif activity and HIV infectivity is largely unknown [125]. One of the major functions of Vif is to neutralize the antiviral activity of A3G, which can induce viral DNA guanine to adenine hypermutations. To achieve this goal, HIV Vif protein forms an E3 ubiquitin ligase containing Cullin 5, Rbx1 proteins and Elongin B and C, to target A3G for proteasomal degradation [127]. It not surprising that the early studies proposed that phosphorylated Vif might play a crucial regulatory role in this process. However, this hypothesis

is not supported by later investigations as mutation of the conserved phosphorylation residues impairs HIV replication but has little to no effect on Vif binding to A3G or A3G degradation [56]. The assembly of the Vif-E3 ubiquitin ligase requires direct interaction between Vif and Elongin C through the conserved Vif BC-box motif (S144LQ(Y/F)LAL150) [127]. A recent study shows that this interaction is negatively regulated by Ser144 phosphorylation, as stimulating Ser144 phosphorylation is able to weaken the interaction [126]. Given that Vif phosphorylation is unlikely to be involved in countering A3G, and phosphorylated BC-box can be recognized by numerous cellular proteins including p120 RasGAP [128], Crk-L, and Nck [129], it has been proposed that Vif phosphorylation might trigger intracellular signal transduction to favor HIV replication. However, no firm evidence has been provided to support this hypothesis.

1.8.2.5 Vpu

The interaction between cell surface receptor CD4 and viral Env is the prerequisite for HIV infection. Reinfection of previously infected cells is termed as superinfection that is able to induce apoptosis. In HIV-infected cells, newly synthesized CD4 and gp160 bind and form a complex retaining ER. To release gp160 for viral assembly and reduce CD4 expression on the cell surface for the prevention of superinfection, Vpu mediates CD4 degradation in the ER. This process requires Vpu to be phosphorylated at specific sites.

Several initial observations have shown that Vpu binding to the cytoplasmic domain of CD4 is essential for CD4 degradation. Later, ubiquitous casein kinase-2 (CK-2) induced Vpu phosphorylation at Ser52 and Ser56 was found to be indispensable in this process [130]. However, Vpu phosphorylation is not required for CD4 binding as the phosphorylation-defective mutants

have intact CD4 binding capability [64]. The role of Vpu phosphorylation was revealed when a beta-transducin repeat containing protein (βTrCP) was identified as a Vpu binding partner [131]. βTrCP is involved in multiple proteasomal degradation pathways. It recognizes its substrates through a conserved motif (DSpGψXSp) containing two phosphoserine. In the case of Vpu, it requires the phosphorylation at Ser52 and Ser56. βTrCP recruitment to CD4 through Vpu binding allows the ubiquitination and the subsequent degradation of CD4 [131].

1.8.2.6 Tat

Tat is expressed early in the HIV replication. After expression, Tat is translocated to the nucleus, binding to the TAR region to enhance HIV gene expression. Evidence suggests that Tat phosphorylation at different sites affects Tat nuclear entry and/or TAR binding, but the functional consequence is controversial.

Endo-Munoz et al. first reported that PKR is able to phosphorylate Tat at Ser62, Thr64, and Ser68 [132]. By using phosphorylation-defective mutants, they found that the TAR binding affinity of Tat is positively related to the Tat phosphorylation level and efficient phosphorylation of Tat significantly increased the LTR-directed HIV transcription. A recent study also reports that PKR is able to phosphorylate Tat at Thr23, Thr40, Ser46, Ser62, and Ser68 [133]. In contrast to the early study, they found that Tat phosphorylated at these sites disrupts Tat function. Mimicking phosphorylation by Asp mutation at these sites results in blocked Tat nuclear localization as well as reduced Tat/TAR and Tat-cyclin T1 binding.

1.8.2.7 Vpr

Many previous investigations report G2/M cell cycle arrest following HIV infection. Since HIV LTR

is more transcriptional active in the G2 phase, a prolonged G2 phase by cell cycle arrest favors HIV transcription. Moreover, G2/M cell cycle suppresses immune clearance of HIV-1 by preventing T cell proliferation and inducing cell apoptosis. Several studies suggest that phosphorylated Vpr is an important factor involved in G2/M cell cycle arrest.

In infected cells and virions, Vpr phosphorylation at three different serine residues (Ser79, Ser94, and Ser96) have been reported [134]. The mutant analysis shows that replacing Ser79 with Ala abolished the G2/M cell cycle arrest while the same mutation at the other two sites had no effect. Interestingly, although the adjacent Arg80 is not a phosphorylation site, substitution Arg for Ala significantly reduced Vpr phosphorylation, which suggested an important role of Arg80 in kinase recognition. In a recent study, the kinase responsible for Ser79 was identified as protein kinase A (PKA) [135]. Consistent with the earlier investigation, PKA is likely to use Ser79 and Arg80 as its recognition motif. Inhibiting PKA/Vpr interaction by chemical compound and using Vpr S79A or R80A mutant significantly reduced G2/M cell cycle arrest.

1.8.2.8 $P6^{Gag}$

Proper virion assembly and effective cell membrane disassociation are necessary for HIV production, and both steps are indispensable for $p6^{Gag}$. Although $p6^{Gag}$ is widely accepted as a phosphoprotein, the functional consequence of $p6^{Gag}$ phosphorylation is controversial.

The original analysis of $p6^{Gag}$ phosphorylation state at different replication steps shows that $p6^{Gag}$ modification is mediated by both cellular and virion-associated kinases during budding [136]. The virion-associated kinase was identified as ERK2, which selectively phosphorylate $p6^{Gag}$ at Thr23 in a conserved MAPK recognizing site [137]. The mutant analysis shows that loss of phosphorylation

at the Thr23 leads to budding deficiency, morphology and size alternation, as well as reduced infectivity. The cellular kinase was identified as atypical protein kinase C (aPKC) which mediates the $p6^{Gag}$ phosphorylation at Ser40. Inhibiting aPKC activity by pseudosubstrate peptide or aPKC knockdown significantly reduced Vpr incorporation into virions [138]. However, both studies are challenged by the finding that introduced mutations into individual phosphorylation sites in $p6^{Gag}$ had no effect on virus release, morphology, and infectivity [139]. The conflicting results might be due to cell line difference or the specific mutation introduced [140]. Extensive study is still needed to address the functional significance of $p6^{Gag}$ phosphorylation.

1.8.2.9 MA

MA is a major structural protein that plays an important functional role in both early and late replication stages. MA can also be phosphorylated at multiple sites by unknown kinases. Several studies indicate that MA phosphorylation has a role in driving HIV cDNA to the nucleus. However, the mechanism is controversial.

One group reported that phosphorylation of MA is essential for the association with the viral core, and the karyophilic nature of MA drives the viral core towards the nucleus [141,142]. However, the single mutation analysis failed to identify the specific site essential for this process. Another group reported that the simultaneous mutation at different serine residues significantly decreased the phosphorylation level of MA and reduced HIV-1 infectivity without impairing viral assembly [143]. Based on this, they proposed that phosphorylation at multiple sites changes the surface charge of MA and thus enables MA to guide HIV-1 viral core to the nucleus. However, other groups suggested that MA phosphorylation does not enhance its karyophilic potential.

1.9 Anti-HIV Activity of Kinase Inhibitors

HIV Env interaction with receptor and co-receptor induced downstream tyrosine kinase activation such as Abl, LCK, and ERK is necessary for HIV infection and replication. The anti-HIV activity of several tyrosine kinase inhibitors has been demonstrated in vitro. Nilotinib is a specific Abl inhibitor that can impair HIV entry by inhibiting Abl activation induced actin rearrangement. It has been shown to be able to reduce HIV replication by >90% without significant cytotoxicity. Dasatinib is a relatively broad-spectrum tyrosine kinase inhibitor. By blocking receptor and co-receptor signaling pathways, it can reduce HIV replication by 3.4 logs in primary CD4+ T cells isolated from HIV-1 infected patients [144].

As previously mentioned, CDK-mediated phosphorylation of RNAPII, N-TEF and DSIF is essential in effective HIV RNA elongation. Thus, inhibiting CDK enzymatic activity is considered a possible method for HIV replication interference. The anti-HIV activity of several CDK9 inhibitors including 5,6-dichloro-1-β-D-robofuranosyl benzimidazole (DRB), Flavopiridol, Roscovitine, and their derivatives have been proved [145,146]. However, the target specificity of DRB and Flavopiridol are not high enough. In inhibitor selectivity tests, both drugs were found to bind and inhibit a large diversity of human kinases unrelated to their primary targets with high affinity. Currently, Flavopiridol is in Phase II clinical trial for relapsed chronic lymphocytic leukemia treatment, but the strong side effects such as vascular thrombosis make it unlikely to be developed into a clinical agent. Roscovitine is a highly selective CDK inhibitor that specifically targets CDKs 1, 2, 5, and 7. It can significantly inhibit both wild type and drug-resistant HIV replication in different cell lines by neutralizing Tat-induced transcriptional transactivation and inducing apoptosis of infected cells

[147]. A roscovitine derivative, r-roscovitine (also known as CYC202) is under Phase II clinical trial for breast and lung cancer treatment that will be helpful to evaluate its clinical potential [148].

HIV basal transcription depends on NF-κB activity. For this reason, the upstream regulator of NF-κB such as PKC and p38 have been proposed as possible targets for anti-HIV drug development. Early studies demonstrate that PKC inhibitors H7, 2-amihopurine, staurosporine, and GO 6976 can reduce HIV replication at different levels in cell lines [149]. Among these PKC inhibitors, GO 6976 exhibited the highest anti-HIV activity and the lowest cytotoxicity [150]. P38 inhibitors SB203580 and RWJ67657 have also been shown to markedly suppress HIV replication by disturbing the late stage of HIV replication in cell lines [151]. Since PKC and p38 are involved in numerous intracellular signaling pathways, the detailed anti-HIV mechanism of these drugs is currently unclear. Furthermore, further studies need to be conducted to evaluate the benefit and risk of these drugs in vivo.

Another target of interest is ataxia telangiectasia mutated (ATM) which is a PI(3)K)-like serine/threonine-protein kinase activated by DNA double-strand breaks regulating the cellular response to DNA damage. Therefore, ATM plays an important protective role at multiple steps during HIV replication such as reverse transcription and integration. Several studies have shown that ATM inhibitor KU55933 can significantly impair HIV replication at nontoxic concentrations. Quantification of reverse transcription product, 2-LTR (the marker for nuclear import) and integrated DNA by RT-PCR revealed that KU55933 treatment specifically interrupts DNA repairment in HIV post-integration stage [152]. Although KU55933 has not been licensed, these observations provide evidence that support ATM as a promising pathway for anti-HIV drug design.

1.10 General Properties of Kenpaullone

9-Bromo-7,12-dihydro-indolo[3,2-d][1]benzazepin-6(5H)-one (kenpaullone, Ken) is an ATP-competitive kinase inhibitor that functions by occupying the ATP binding site in the targeted kinase. It is a potent inhibitor of GSK-3β, and CDKs. The following are the 50% inhibitory concentrations (IC_{50}) against each kinase: GSK-3β (IC_{50} 230 nM), CDK1/cyclin B (IC_{50}, 400 nM), CDK2/cyclin A (IC_{50}, 680 nM), CDK2/cyclin E (IC_{50}, 750 nM), and CDK5/p25 (IC_{50}, 850 nM) [153]. Since CDK and GSK-3β play important roles in tumor and neuronal cell death separately, Ken has been considered a potential treatment for tumors and neurodegenerative diseases.

1.10.1 Anti-tumor Activity of Ken

Uncontrolled cell proliferation resulting from dysfunctional cycle control is one of the major characteristics of the tumor. The cell cycle can be divided into four phase: G1, S, G2, and Mitotic phase. The transition from one phase to the next is under the regulation of CDKs. The CDK family consists of 21 members, and four of them (CDK1, 2, 4, and 6) have been shown to play crucial regulatory roles in cell cycle procession. The activity of CDKs is modulated by different subunits known as cyclins. CDK2 interacts with Cyclin E and A successively to promote G1/S transition and S phase progression. CDK1 interacts with Cyclin B to drive cells to mitosis. Increased CDK activity has been detected in various human cancers such as breast cancer, leukemia, and non-small cell lung cancer [154].

As a potent CDK inhibitor, the antitumor activity of Ken has been evaluated in different cell lines. It has been shown that ken is able to induce 50% growth inhibition in colon cancer, melanoma, and non-small cell lung cancer cells with concentrations of less than 100 μM [155]. Moreover, Ken

treatment has been reported to induce S/G2-M cell cycle arrest in canine mammary tumor and Madin–Darby canine kidney cells [156]. However, the anti-tumor activity of Ken seems to be different between cell types. In tongue squamous cell carcinoma (TSCC), Ken treatment antagonizes the IL-18 protein induced cell apoptosis and favored TSCC proliferation [157]. In LNCaP human prostate cancer cells, Ken treatment enhances androgen receptor dependent prostate cancer growth [158]. For this reason, although Ken is a promising anti-tumor drug in vitro, more studies need to be conducted in vivo to find an appropriate therapeutic dose range.

1.10.2 Neuroprotective Ability of Ken

Neuronal cell death is the core reason for neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer disease, and Parkinson's disease. Numerous studies have suggested GSK-3β as a key factor contributing to neuronal cell death. In fact, GSK-3β upregulation has been reported in many different neurodegenerative diseases and the inhibition of GSK-3β are proved to efficiently reduce the apoptosis of neuronal cell and suppress the progression of neurodegenerative diseases [159]. Although the detailed mechanism is unclear, there is evidence suggesting that GSK-3β antagonize the neuroprotective Wnt/β-catenin signaling pathway and active mixed lineage kinase 3 (MLK3) to induce cell apoptosis [160].

The neuroprotective effects of Ken was first discussed by Jordà et al [161]. In their study, they show that Ken can significantly inhibit colchicine-induced cerebellar granule neurons apoptosis. Later Skardelly et al reported that Ken is able to suppress oxygen–glucose deprivation induced apoptosis of ReNcell CX human neural progenitor cells [162]. A recent study also demonstrated that Ken can strongly promote the survival of the motor neuron differentiated from induced

pluripotent stem cells by two fold [163].

1.11 HIV-1 IN

1.11.1 HIV-1 IN Structure

IN is a 288 amino acid protein consisting of three functional and structurally distinct domains: the N-terminal domain (NTD; amino acids 1–49), the catalytic core domain (CCD; amino acids 50–212), and the C-terminal domain (CTD; amino acids 213–288). Two flexible loops (amino acids 44-55 and amino acids 187–194) serve as linkages between the domains. Early structural biology analysis revealed that each domain is dimeric. More recent two-domain crystal structures showed that CCD and CTD or NTD and CCD are connected to each other in dimeric organizations with dimer interfaces different from those observed in individual NTDs and CTDs. Currently, it is still unclear how these two domains are dimerized in full-length IN.

The N-terminal domain contains an HHCC motif that possess two conserved His and Cys residues analogous to the zinc-binding residues of zinc fingers. It has been shown that the effective Zn-coordinating enhances HIV-1 IN catalytic function by promoting protein multimerization. The catalytic domain carries a D, D-35-E motif that is comprised of three residues: Asp64, Asp116, and Glu152. It has been proposed that these residues coordinate either magnesium or manganese divalent metal ion required for catalytic activity. A mutation at any of these residues severely diminishes the IN catalytic activities and reduces viral infectivity. This domain is also involved in viral DNA binding mainly through Q148, K156, and K159 residues. The C-terminal domain is less conserved than the other two domains. It has been known for a while that the C-terminal domain functions as a stabilizing platform for DNA binding; however, the mechanism underlying CTD/DNA binding is controversial. One theory proposed that the cleft formed at the interface between the

two CTD subunits is essential for DNA binding while another theory proposed that a positively charged region on the CTD surface enables the non-specific binding of viral DNA.

Multimerization is known to be essential for IN function. The earliest evidence was provided by complementation studies in which the mixture of individually nonfunctional N-terminal deleted IN and C-terminal restored catalytic function to near wild-type levels. Current experimental evidence suggests that dimerized IN binding to both ends of viral DNA is essential for 3' processing, whereas tetramer of IN stabilized by viral DNA is necessary for viral DNA strand transfer.

1.11.2 HIV-1 IN and HIV-1 Early Stage Replication

HIV-1 IN effects throughout the early stage of HIV-1 replication including uncoating, reverse transcription, nuclear import, and integration [164].

1.11.2.1 Uncoating

The involvement of IN in HIV-1 uncoating has been suggested by several recent studies. The first evidence comes from the analysis of two HIV-1 strains containing IN mutation, one carrying IN C130S point mutation and the other IN deletion mutation. Both strains are defective in production of reverse transcription products. Given that both strains have normal cell attachment, entry, and intact RT, the result indicates that the infection impairment might occur in uncoating [165]. In the subsequent study, the stability of viral cores isolation from wt and IN mutant viruses were compared and in both IN mutants, the stability of the viral core was decreased. The study also linked the accelerated uncoating of the IN mutant virus to the decreased CypA–CA interaction that is important for viral core stability [74].

Previous work in our laboratory also revealed the functional role of IN in HIV-1 uncoating. The

HIV-1 IN (Q53A/Q252A) mutant virus defective in dynein light chain 1 (DYNLL1) association exhibits premature uncoating. However, in contrast to the earlier investigation, no differences in CypA incorporation were detected between the HIV-1 wt and HIV-1 IN (Q53A/Q252A) mutant virus [48].

1.11.2.2 Reverse Transcription

HIV-1 IN has been demonstrated to be an HIV-1 RT-interacting protein [166]. A cell-free reverse transcription assay demonstrated that the addition of IN increased RT-mediated reverse transcription by fourfold and increased elongation by threefold [167]. Collectively, these observations suggest that a functional association between HIV-1 IN and RT contributes to reverse transcription efficiency.

The interaction between IN and cellular proteins also has been proved to play an important role in HIV-1 reverse transcription. Several investigations suggest that the interaction of IN with SIP1/Gemin2 is essential for the assembly of IN and RT on HIV-1 genomic RNA for efficient reverse transcription [168]. Previous work in our laboratory also suggested the functional role of IN/ DYNLL1 in HIV-1 reverse transcription. Compared with the wild-type strains, the HIV-1 point mutant (Q53A/Q252A) defective in dynein light chain 1 association exhibits significantly lower levels of HIV-1 reverse transcription [48].

1.11.2.3 Nuclear Import

Initially, HIV-1 IN was proved a strong nucleophilic protein that localized predominantly in the nucleus when transiently expressed as a fusion protein. The strong nucleophilicity indicated a potential role of IN in HIV-1 cDNA nuclear import. Later, a comparison among wild-type HIV-1,

MA NLS-Vpr dual HIV-1 mutant, and MA NLS-Vpr-IN triple HIV-1 mutant shows that normal nuclear translocation of NC and RT can be observed in MA NLS-Vpr dual mutant infected cells but not in MA NLS-Vpr-IN triple HIV-1 mutant infected cells [169].

IN is reported to interact directly with several nuclear import receptors including Impα1, Impα3, Imp7, and tranportin 3. The critical motifs for HIV-1 IN/Impα1 interaction are two NLSs (^{186}KRK and ^{211}KELQKQITK) located in the C-terminal domain. Introducing mutation to the two NLS restricted IN mutant to the cytoplasm [169]. The interaction between HIV-1 IN and Impα3 depends on two NLSs (^{211}KELQKQITK and ^{262}RRKAK) located in the C-terminal domain. HIV-1 that has specific mutations in the two NLSs, showed a deficiency in nuclear import [82]. Similarly, IN interacts with Imp7 through regions (^{235}WKGPAKLLWKG and ^{262}RRKAK) within the C-terminal domain. The IN/Imp7 interaction-deficient mutant was unable to replicate and was impaired at reverse transcription and nuclear import stages [50]. The interaction between tranportin 3 and IN can be dramatically reduced by the IN R262A/K264A mutant. The virus with IN R262A/K264A mutation retains intact reverse transcription activity but is two-fold defective in nuclear import [170].

In addition to the importin family, several studies provided evidence for the direct interaction between IN and Nup153. Disrupting the IN/Nup153 interaction by overexpressing the c-terminal of Nup153 or gene knockdown affected HIV-1 cDNA nuclear import [171].

1.11.2.4 Integration

HIV-1 IN catalyzes two well-characterized reactions during HIV-1 integration: 3’ end processing and DNA strand transfer. 3’ end processing occurs in the cytoplasm within PIC. In this reaction,

two nucleotides are removed from the each 3’ end of the viral DNA and exposes the terminal 3’-hydroxyl group. The following DNA strand transfer happens in the nucleus. In the reaction, HIV-1 IN mediates the 3’ ends attack on the target DNA and leads to separation of a pair of phosphodiester bonds to allow viral DNA insertion. In vitro study demonstrated that purified IN is sufficient for catalytic activity in both reactions, and substantial evidence suggests the importance of host cell factors in the accomplishment of HIV-1 DNA integration in infected cells [172].

P300 is a cellular acetyltransferase that is able to bind and acetylate three lysine residues in the IN C-terminus (Lys264, Lys266, and Lys273). The modification of IN by p300 enhances the interaction affinity between IN and DNA, promoting the DNA strand transfer activity of the protein. Carrying IN mutations at the acetylation sites abolish virus replication [173].

LEDGF bind to IN both in vitro and in vivo [174]. Inhibiting LEDGF/p75 expression by knock down approach significantly reduced HIV replication and this reduction can be rescued by using an LEDGF/p75 siRNA-resistant expression. LEDGF favors HIV replication by protecting IN from proteasomal degradation and mediates chromosome tethering [175,176]. An LEDGF binding defective IN mutant Q168A was identified to display wild type IN activity in vitro. Whereas HIV-1 viruses containing IN Q168A were able to generate 2-LTR circles at the same level as the wild-type virus but were defective for replication as the result of impairment in chromosome tethering [176].

1.12 Intracellular Transportation System

The cell cytoplasm is rich in organelles and macromolecules. The intracellular aqueous

environment restricts the free movement of macromolecules or macromolecular complexes in the cytoplasm. In fact, only macromolecules under 500 kDa, with a hydrodynamic diameter less than 40 nm, are able to diffuse within the cytoplasm at a rate dependent on their radius [177]. To achieve the active transport across this dense environment, eukaryotic cells take advantage of motor proteins that move along actins or microtubules. Actins function as tracks for short-range transport while microtubules function as tracks for long-range transport. Microtubules are polarized with a highly dynamic “plus” end that is able to grow and shrink rapidly, extending towards the cell periphery, and a relatively stable “minus” end that is tethered to the microtubule-organizing center (MTOC). Dynein and kinesin complexes are microtubule motors that mediate minus-end and plus-end transport, respectively [178] (Fig. 4).

Following cell entry, several viruses need to travel an extensive distance through the cytoplasm and reach the nucleus or the perinuclear area where replication takes place. Similar to other macromolecular complexes, passive diffusion is not a practical method for viral particles to reach their destination. It has been proposed that many viruses are transported along microtubules by hijacking the dynein motor complex.

1.13 Dynein Complex

The dynein motor complex is a 1.6 MDa complex built around two copies of ATPase force-generating subunits called dynein heavy chains (DHCs). Two dynein intermediate chains (DICs) and two light intermediate chains (LICs) bind directly to the DHCs. Three dynein light chain (DLC) pairs, DYNLL, DYNLT, and DYNLRB, bind to DICs at separate sites (Fig. 5). Cargoes are adapted to dynein either by direct interaction with dynein components or through other adapter proteins

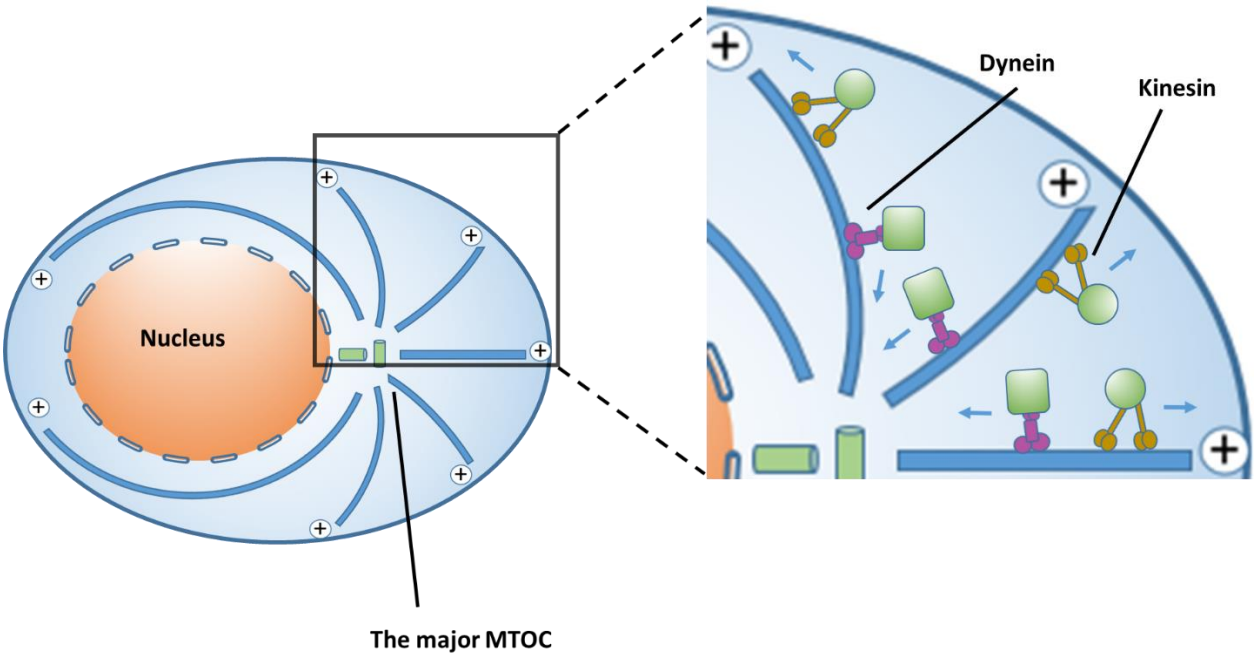

Fig. 4 Microtubule dependent intracellular transportation system. Microtubules are polarized with a highly dynamic “plus” end that is able to grow and shrink rapidly, extending towards the cell periphery, and a relatively stable “minus” end that is tethered to the MTOC. Dynein and kinesin complexes are microtubule motors that mediate minus-end and plus-end transport, respectively

including Bicaudal-D, lissencephaly 1, and nuclear distribution protein E (NudE) or nuclear distribution protein E-like (NudEL), Rod-Zw10-Zwilch, Spindly, and dynactin [179].

1.13.1 DIC

DIC functions as a scaffold in the dynein complex by binding directly to the other four components including DHC, DYNLL, DYNLT, and DYNLRB. DIC binds to DHC through a β propeller structure containing seven tryptophan-aspartic acid (WD) repeats at the C-terminus of DIC. Just upstream of the seven WD repeats is the binding region for the roadblock light chain family [180]. Next is a 61-amino-acid region necessary for the dimerization of intermediate chains. Upstream are the binding sites for the other two DLCs [181,182]. The N-terminus domain contains a coiled-coil region essential for interacting different regulatory proteins.

Humans have two dynein intermediate chain genes that generate at least six different isoforms of dynein intermediate chains by alternative splicing. Those isoforms exhibit different expression levels in different tissues and cells. It has been proposed that the isoforms of intermediate chains contribute to distinct cargo-binding specificity and movement velocity. Several studies support the following theory: dynein complex containing DIC-2C isoform carries membrane-bounded organelle cargos for rapid axonal transport whereas dynein complex with the other isoforms carries cytosolic proteins for slow axonal transport [183].

1.13.2 DYNLL

The DYNLL family has two mammalian isoforms, DYNLL1 and DYNLL2, which are capable of forming homodimers and heterodimers. Dimerized DYNLL1 and DYNLL2 can form two identical parallel grooves where DYNLL interacts with different binding patterns at the dimerization

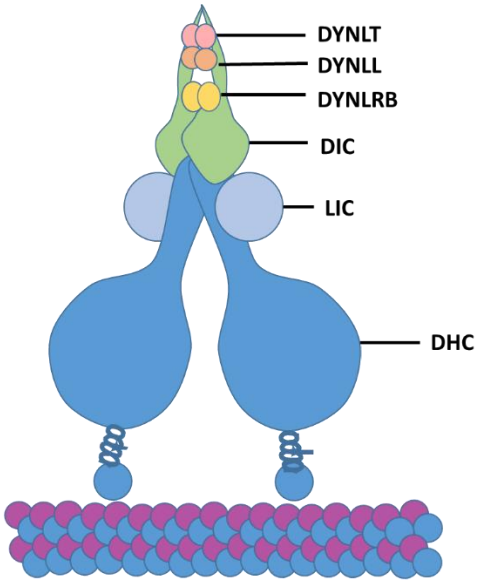

Fig. 5 Dynein complex. The dynein motor complex is built around two copies of ATPase force-generating subunits called dynein heavy chains (DHC). Two dynein intermediate chains (DIC) and two light intermediate chains (LIC) bind directly to the DHCs. Three dynein light chain pairs, DYNLL, DYNLT, and DYNLRB, bind to DICs at separate sites.

interface [184]. The proteins reported to interact with DYNLL and be transported on microtubules include Bassoon, Egalitarian, hDlg1/SAP97, KIBRA etc. [185,186]. Notably, many DYNLL-binding motifs are located in intrinsically disordered protein segments and often located close to coiled-coil or other dimerization domains of the interacting partners [187]. Based on this, DYNLL has been suggested to function as an essential hub protein that promotes dimerization and stabilization of its interaction partners.

The function of DYNLL1 might be modulated by viral-induced modification. Under cellular conditions, DYNLL is mostly dimeric. When DYNLL1 dimer is phosphorylated at Ser88, it will dissociate to stable monomers and lose its target-binding function [188]. DYNLL1 phosphorylation at Ser88 has been attributed to Pak1, which can be activated by infection of many important pathogenic human viruses including HIV and respiratory syncytial virus [189]. The transition between dimer and monomer could have important roles in the dynein-virus association.

1.13.3 DYNLT

The human DYNLT family consists of six members: DYNLT1, DYNLT3, TCTEX1D1, TCTEX1D2, TCTEX1D3, and TCTEX1D4 [190]. Among these members, DYNLT1 and DYNLT3 are better studied. DYNLT1 and DYNLT3 are capable of forming homodimers and heterodimers. DYNLT1 and DYNLT3 homodimers compete for binding to DICs while DYNLT heterodimers do not bind to DICs [191]. Both DYNLT1 and DYNLT3 are thought to mediate the attachment of cargoes to the dynein motor and thus allow intracellular transportation.

DYNLT1 is 14 kDa protein encoded on q25.2-q25.3 of chromosome 6. As one of the dynein cargo adaptors, it directly interacts with a large diversity of proteins. Based on the comparison of

DYNLT1 binding sites in different reported DYNLT1 ligands, two consensus sequences, (R/K)(R/K)XX(R/K) and (VS(K/H))T/S)X(V/T)(T/S)(N/Q)V, have been proposed as the critical motif for DYNLT1 recognition [182,192]. However, both consensus sequences are shared by a portion of DYNLT1 interacting proteins.

Similar to DYNLL1, the function of DYNLT1 is likely to be regulated by phosphorylation. Site-directed DYNLT1 mutants mimicking DYNLT1 phosphorylation at Ser82 or Thr94 are able to abolish the interaction between DYNLT1 and DIC [188,193]. This phosphorylation drives DYNLT1 disassembly from the dynein complex and might have a role in cargo transportation.

1.13.4 DYNLRB

The DYNLRB family has two mammalian isoforms: DYNLRB1 and DYNRB2. DYNLRB1 is ubiquitously expressed in all tested human tissues while DYNRB2 is expressed in a few tissues such as kidney and testis [194]. Both DYNLRB isoforms are capable of forming homodimers and heterodimers. Unlike the other two DLC families, the function of the DYNLRB family is largely unclear. Several proteins have been reported as the interacting partners of DYNLRB, including GlcNAc kinase and small GTPase, but none of the interaction is related to the dynein-mediated intracellular transportation [195,196]. A recent study identified DYNLRB as a member of a protein superfamily responsible for the regulation of NTPase [197]. As DHC is a kind of ATPase, DYNLRB might have a role in the regulation of dynein complex.

1.14 Dynein-virus Association

1.14.1 HIV-1

After HIV-1 Env-mediated cell entry, the HIV-1 core containing HIV-1 RNA genome is released into the cytoplasm. The disassembly of CA forms RTC where HIV-1 RNA reverse transcription takes place. The completion of HIV-1 reverse transcription transfers RTC into PIC, which is a complex capable of nucleus entry and integration. At the same time, the RTC/PIC is translocated from the cell periphery area to the cell nucleus. It has been proposed that the migration of the incoming virus is mediated by the dynein and microtubule-dependent intracellular transportation system. This hypothesis is supported by several observations. Later, by visualizing individual particles in living cells, HIV-1 PICs movements were shown to be predominantly towards the nucleus. Furthermore, the PICs are associated with microtubules and concentrated at the MTOC. Disrupting dynein/microtubule-mediated intracellular transportation system by nocodazole-induced microtubule depolymerization or anti-dynein antibody injection induced dynein dysfunction inhibited HIV-1 PIC transportation towards MTOC and resulted in the peripheral accumulation of PIC [80].

1.14.2 HSV-1

Herpes simplex virus 1 (HSV-1) is a sensory neuron invasive virus. During infection, HSV-1 binds to cell surface receptors and enters the cell by membrane fusion. Upon membrane fusion, the envelope and outer tegument proteins dissociate from the capsid and inner tegument proteins. The released nucleocapsid-tegument complex is retrograde transported to nuclear pore complex,

where viral DNA is injected into the nucleus. The incoming cytosolic HSV-1 capsids co-localize with microtubules and concentrate around the MTOC. Using MT-depolymerizing agents such as colchicine and nocodazole will lead to capsids scattered throughout the cytoplasm and HSV-1 infection blockage in cultures and animal models [198]. Moreover, the dynein components including DIC1, DHC are known to co-localize with inbound cytosolic HSV-1 capsids and HSV-1 motility can be blocked by ATP depletion [198]. In experiments using yeast two-hybrid and pepscan, several viral proteins including UL34 and UL35 are reported as potential dynein complex interaction candidates [199].

HSV-1 UL34 is an integral membrane protein anchored to the inner nuclear membrane and required for efficient egress of progeny virions from the infected cell nucleus. While it is a structural component of the perinuclear virion, it dissociates from the egressing virion at the outer nuclear membrane during de-envelopement. Surprisingly, UL34 is reported to be associated with the neuronal isoform of DIC1 in GST pull down [200]. However, UL34 is such an essential protein in the HSV-1 life cycle that null mutant and partial deletion mutants can only poorly replicate in specific cell lines that make the importance of this interaction hard to evaluate in vivo. HSV-1 UL35 is an HSV-1 capsid protein reported to be associated with DYNLT1 and DYNLT3 [201]. However, HSV-1 UL35 null mutants show no significant effect on dynein-dependent retrograde viral transport in cell cultures and in animal experiments that rely on axonal transport [202,203].

1.14.3 Adenovirus

The adenovirus is a DNA virus that typically causes mild infections involving the upper or lower respiratory tract, gastrointestinal tract, or conjunctiva. After receptor-mediated endocytosis,

pentons and capsids are digested by the host cell lysozyme, releasing a partially uncoated particle into the cytoplasm. The core migrates to the nucleus where the replication and transcription take place. Early studies revealed that microinjection of anti-dynein antibody abolished the viral nuclear localization. Cell treatment with nocodazole or transfection with p50 dynamitin abrogates retrograde transport by at least 50%. Recently, Bremner et al. reported that dynein and dynein regulatory protein NudE/NudEL co-localized with post-endosomal adenovirus particles in immunoprecipitation experiments. The study also shows that the viral capsid hexon subunit interacts directly with the dynein intermediate chain. Using immunoprecipitation and antibody microinjection experiments, the adenovirus hexon-binding site was selectively localized to a single site within the intermediate chain and no significant interactions were observed with any of the three dynein light chains DYNLL1, DYNLT1, or DYNLRB1 [204].

Type 2 adenovirus E3 protein is a polypeptide involved in the downregulation of host immune response. It is associated with DYNLT1 through binding to a small GTPase [196]. Since this Type 2 adenovirus E3 protein is not a structural component of the virion, the biological significance of the interaction remains unclear.

2 Methods and Materials

2.1 General Regents

2.1.1 Chemicals

The Cell Proliferation Reagent WST-1 and high pure RNA isolation kit were obtained from Roche. Gluc kit was obtained from Targeting system. Moloney murine leukemia virus reverse transcriptase (M-MLV RT) was obtained from Promega. NP-40 and protease inhibitor cocktail was obtained from Calbiochem. The Western blot (WB) detection enhanced chemiluminescence (ECL) kit was purchased from PerkinElmer Life Science. 4',6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen.

2.1.2 Antibodies

Antibodies for cellular and viral proteins: Mouse anti-DYNLT1 antibody was obtained from Santa Cruz Biotech. Rabbit anti-DIC1 antibody was obtained from Abcam.

Epitope tag antibody: Horseradish peroxidase (HRP)-conjugated anti-GFP antibody was purchased from Miltenyi Biotec.

Secondary antibodies: HRP-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were obtained from Amersham Biosciences. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody and Cyanine 5 (Cy5)-conjugated anti-mouse antibody were purchased from Kirkegaard & Perry Laboratories.

Enzyme-linked immunosorbent assay (ELISA) kit for $p24^{Gag}$ detection was obtained from AIDS Vaccine Program of the Frederick Cancer Research and Development Center

2.1.3 Plasmids

2.1.3.1 HIV IN Expression Plasmids

To express full-length IN and region deleted IN mutant in cells, corresponding plasmid were constructed using CMV-AcGFP-C1 plasmid (Clontec) as the vector. For CMV-AcGFP-IN construction, the IN cDNA fragment was obtained by double digestion of YFP-IN expresser and inserted between BglII and BamHI restriction enzyme sites on the vector. For the construction of region deleted IN mutant CMV-AcGFP-IN 1-212 and CMV-AcGFP-IN 50-288, the cDNA fragments were obtained by PCR amplification from CMV-AcGFP-IN plasmid and were inserted between HindIII and BamHI restriction enzyme sites on the vector.

2.1.3.2 Proviral Plasmids

HxBru and pNL4.3-Nef+/GFP proviral plasmids were previously described [49,205]. HxBru/Nef-/Gluc+ and HxBru GLuc+/RT-/IN-/Nef-/Vif-/Env- proviral plasmids were constructed in the similar way as HxBru/Nef-/luc+ and HxBru Luc+/RT-/IN-/Nef-/Vif-/Env- whose nef gene had been replaced by Gluc gene [206].

2.1.4 Cells

2.1.4.1 Cell Lines

Human embryonic kidney cells 293T were cultured in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), penicillin at a final concentration of 100 I.U./mL, and streptomycin at a final concentration of 100 μg/mL. ACH2 cells, C8166 T cells, Jurket T cells, U937 monocytic cells were maintained in Roswell Park Memorial Institute (RPMI)-1640

medium containing 10% FCS, penicillin at a final concentration of 100 I.U./mL, and streptomycin at a final concentration of 100 μg/mL.

2.1.4.2 Human Primary Peripheral Blood Mononuclear Cells (PBMCs)

Human primary PBMCs were isolated form whole blood donated by healthy adult volunteers by Ficoll-Paque method. Isolated PBMCs were stimulated by 3ug/mL phytohemagglutinin (PHA) in RPMI-1640 supplemented with 10 U IL-2, 10% FCS and penicillin at a final concentration of 100 I.U./mL, and streptomycin at a final concentration of 100 μg/mL. At 3 days of stimulation, PHA was washed away and stimulated PBMCs were maintained in RPMI-1640 supplemented with 10 U IL-2, 10% FCS, penicillin at a final concentration of 100 I.U./mL, and streptomycin at a final concentration of 100 μg/mL.

2.2 General Methods

2.2.1 Virus Production

2.2.1.1 HxBru/Nef-/Gluc+, pNL4.3-Nef+/GFP+, and HxBru viruses

To produce HxBru/Nef-/Gluc+, pNL4.3-Nef+/GFP+, and HxBru viruses, corresponding proviral plasmids were transfected into 293T cells respectively. At 48h post-transfection, supernatant was collected and viruses were concentrated by ultracentrifugation (35,000 rpm for two hours at 4°C). The virus titers were measured by $p24^{Gag}$ ELISA

2.2.1.2 Single cycle Gluc virus

To produce single cycle Gluc virus, HxBru Gluc+/RT-/IN-/Nef-/Vif-/Env- was cotransfected with

delta 8.2, VSVG into the 293T cells. At 48h post-transfection, supernatant was collected and viruses were concentrated by ultracentrifugation (35,000 rpm for two hours at 4°C). The virus titers were measured by $p24^{Gag}$ ELISA

2.2.1.3 Production of nevirapine-resistant HIV-1 virus

Nevirapine-resistant HIV-1 virus (Nevi virus) was obtained from the NIH AIDS Research and Reference Reagent Program (catalog number: 1392) [207]. To produce Nevi virus, the C8166 T cells were infected with the Nevi virus. At 48 hours post-infection, supernatant was collected and viruses were concentrated by ultracentrifugation (35,000 rpm for two hours at 4°C). The virus titers were measured by $p24^{Gag}$ ELISA

2.2.2 Infectivity Measurement

2.2.2.1 Gaussia Luciferase (GLuc) Measurement

GLuc gene is originally isolated from the marine copepod Gaussia princeps. It encodes for a 19 kD secretary monomeric enzyme that catalyzes the oxidative decarboxylation reaction that turns coelenterazine into coelenteramide. This process is accompanied by the emission of blue light (wavelength 475 nm). It is wildly used as a reporter to detect gene expression. In our study, GLuc is used for measuring the HIV-1 replication levels. Briefly, the infected cells were cultured in 1 mL media at a density of 0.5×10^6 cells/well in a 24 well plate. At 48 h post-infection, the supernatants were collected, and the Gluc activity was measured with the Gluc kit according to the manufacturer's instructions

2.2.2.2 ELISA

For each sample, 200 uL supernatant was collected and 20uL 10% Triton X-100 was added to the supernatant. The mixtures were incubated for 0.5 hours at 37°C for inactivation. Maxisorp plates were coated with coating anti-p24 antibody (1:1000 diluted in coating buffer) at 4°C overnight. On the next day, the wells were washed with PBST for 3 times and blocked with blocking buffer for two hours at 37°C. Then the wells were washed with PBST for three times, p24 standards and samples were added to the wells and incubated at 4°C overnight. After washing with PBST for five times, the plate was blocked with blocking buffer for two hours at 37°C. Then the wells were washed with PBST for five times, detection anti-p24 primary antibody was added to the wells and incubated for two hours at 37°C. After incubation the wells were washed with PBST for five times, detection HRP conjugated secondary antibody was added to the wells and incubated at 37°C for one hour. After incubation, the wells were washed with PBST for five times and TMB substrate was used for development. The absorbance was measured using microplate reader using 450 nm as the primary wave length.

2.2.2.3 WST-1 Cell Proliferation Assay

The WST-1 cell proliferation assay is a method for cell viability quantification. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene Disulfonate) is a cell-impermeable tetrazolium salt that can be cleaved to formazan at the cell surface by plasma membrane electron transport, predominantly by NAD(P)H dehydrogenase in viable cells. For this reason, the amount of formazan dye formed in the cell culture after incubation is positively correlates to the metabolically active cell content. As the cleavage is accompanied by absorbance change, the

formazan dye amount can be measured by microplate reader.

In our study, the WST-1 cell proliferation assay was used to estimate the viability of C8166T cells treated with Ken at different concentrations. Briefly, C8166 T cells were cultured at a density of 1×10^5 cells per well in a 96-well plate and incubated with different concentrations of Ken ranging from (0 nM to 9600 nM) for three days. Then, 10 µL WST-1 reagent was added to each well and the cells were incubated at 37°C for four hours. After incubation, the absorbance was measured at 490 nm using microplate reader.

2.2.3 Quantitative Detection of HIV-1 mRNA Production

To detect the effect of Ken on HIV-1 mRNA production levels, real time PCR (RT-PCR) was performed. Briefly, $1X10^6$ ACH2 cells were culture in the presence or absence of Ken. 24 hours after treatment, cells were collected and total RNA was extracted using high pure RNA isolation kit following the manufacturer's instructions. The isolated RNA was subjected to reverse transcription using M-MLV RT.

The cDNA was amplified by SYBR Green PCR system using Taq.

The primers used for HIV-1 cDNA detection are as following:

TD-Gag-Fr: 5'-ATCAAGCAGCCATGCAAATG-3'

TD-Gag-Rv: 5'-CTGAAGGGTACTAGTAGTTCC-3'

HIV-1 cDNA content was normalize by amplifying the human β-globin gene.

The primers used for human β-globin cDNA amplification are as following:

β-globin-Fr: 5'-CAACTTCATCACGTTCACC-3'

β-globin-Rv: 5'-GAAGAGCCAAGGACAGGTAC-3'

2.2.4 Transfection

For 293T cell transfection, cells were cultured in 10 cm dishes at 40-50% confluence. After 15 h's growth, transfections were performed following the standard calcium-phosphate-DNA precipitation method.

For HeLa cell transfection, cells were cultured on glass coverslips in 24 well plate at 65-75% confluence. After 15 h's growth, transfections were performed following the standard calcium-phosphate-DNA precipitation method.

2.2.5 Co-Immunoprecipitation

The interaction between HIV-1 IN and DIC1 or DYNTL1 was analyzed by Co-Immunoprecipitation (Co-IP). The CMV-AcGFP-control or CMV-AcGFP-IN wt/mt plasmid was transfected into 293T cells. At 48 h post-transfection, the cells were harvested and washed with PBS by resuspension and centrifugation for twice. Then, 90% of the cells were lysed in lysis buffer (lysis buffer: RPMI medium containing 0.12% NP-40 and protease inhibitor cocktail) on ice for 30 min. To further disrupt the cells, 100uL glass beads were added to each sample, and the mixtures were vortexed three times for ten secs each. The cell lysates were clarified by centrifugation and incubation with preclear beads. The supernatants were incubated with anti-GFP antibody overnight under rotation at 4℃ and incubated with 60 μL protein G-Sepharose beads under rotation at 4℃ for two hours. The beads were collected by low-speed centrifugation and washed by cold lysis buffer for 3 times. The immunoprecipitaties were eluted into 45 μL SDS loading buffer and subjected to western blot (WB) analysis. The presence of GFP and GFP-IN wt/mt was detected by HRP-conjugated anti-GFP antibody. The IN-bound proteins were detected by anti-DYNLT1 or anti-DIC1

antibodies. To detect the expression of GFP and GFP-IN wt/mt as well as endogenous DYNLT1 and DIC1, the other 10% of the collected cells were lysed in RIPA buffer (PBS containing 5% sodium deoxycholate and protease inhibitor cocktail) and the lysates were subjected to WB analysis with corresponding antibodies.

2.2.6 Western blot

For protein detection, cell lysates were resolved in a 12% SDS-Page gel and transferred onto nitrocellulous transfer membrane by electrophoresis (80 mA, overnight at 4 °C). The next day, the membrane was washed three times with PBS and blocked in 5% milk bath for one hour at room temperature. After washing for three times with PBS, the membrane was incubated with corresponding primary antibodies (anti-DIC1 and anti-DYNLT1 antibodies were diluted to 1:1000 with PBS; HRP-conjugated anti-GFP antibody was diluted to 1:6000 with PBS) overnight at 4 °C. Then, the membrane was washed three times with PBS and incubated with corresponding secondary antibodies (anti-rabbit was diluted to 1:5000 with PBS; anti-mouse was diluted to 1:7500 with PBS) for one hour at room temperature. Finally, the membrane was washed for five times with PBS and incubated with ECL WB Substrate for one minute for film image.

2.2.7 Immunofluorescence

To determine whether HIV-1 IN is associated with dynein, we performed the colocalization analysis by immunoflourescence. Briefly, HeLa cells were seeded on 12 mm^2 glass coverslips in 24-well plates at 65-75% confluence. After 15 hours of growth, Hela cells were transfected with AcGFP-IN or AcGFP-control expressors. After 12 hours of transfection, cells were rinsed with PBS for 3 times followed by fixation and permeabilization with methanol acetone (1:1 ratio) for 30

min at room temperature. After washing with PBS, the fixed cells were blocked by 5% FBS for 30 mins at room temperature then incubated with rabbit anti-GFP (1:500) and anti-DIC1 (1:250) primary antibodies overnight at 4 °C. The next day, the samples was washed with PBS three times and incubated with (FITC)-conjugated anti-rabbit (1:1000) and (Cy5)-conjugated anti-mouse (1:1000) fluorescent conjugated antibodies for one hour at room temperature. Subsequently, the cells were washed with PBS three times and cell nuclei were stained with DAPI for 5 minutes at room temperature. After washing with PBS, the coverslips were sealed with nail polish. The cells were visualized under the fluorescent microscope (Axiovert 200 Carl Zeiss microscope).

3 Characterize the Anti-HIV-1 Activity of a Kinase inhibitor Ken

3.1 Introduction

In the early 1980s, the first cases of AIDS were reported, bringing the unprecedented threat into awareness. In the following decade, the small-scale epidemic of AIDS among specific populations, such as males who have sex with males and drug users, rapidly spread to the general population throughout the world. The unleashed spread of AIDS led to extensive scientific and medical study of this disease [5].

Initially, the treatment of AIDS patients was hindered by a limited understanding of the pathogenesis of AIDS. Later, HIV-1 was identified as the cause of this devastating disease and its life cycle was gradually revealed, which allowed the initiation and development of antiviral therapies. In 1987, the first ART named AZT was approved by the FDA. In the subsequent three decades, the anti-HIV drug development flourished with more than 30 different drugs approved [5]. The progress of anti-HIV drug development and optimization of clinical therapy have achieved great success in control of the disease progression of HIV-1 infected individuals. At present, the standard treatment is HAART, which is able to transfer HIV-1 into a manageable chronic disease.

Despite its many successes, HAART has its own disadvantages that need to be taken into consideration. The primary concern is the associated emergence of drug-resistant strains [208]. The rise of drug-resistant strains occurs as the result of low fidelity of HIV-1 replication and ongoing anti-HIV treatment. The lack of a proofreading mechanism enables HIV-1 mutants to develop naturally at high frequency. In the presence of anti-HIV drugs, the drug-resistant

mutations are selected due to loss of restriction. As HAART is unable to eradicate HIV-1, the undesired selection can hardly be avoided. Moreover, the problem of drug-resistant strain emergency can be aggravated by the suboptimum treatment or incomplete adherence to therapy that are commonly observed in clinical treatment. As the ART targets are relatively conserved, strains resistant to one medicine may also resistant to the other medicines in the same class, thus further limiting the choice of medicine. In addition, the drug-resistant strains may be transferred to treatment-naive individuals and cause HIV-1 drug-resistant strain spread. In Canada, the overall prevalence of acquired drug resistance is approximately 9%, and the overall prevalence of acquired multidrug resistance to more than one class of antiretroviral drugs is approximately 1% [209].

The shortcomings of HAART makes the development of novel and potent anti-HIV drugs an urgent matter. Given that host kinases play essential roles in both HIV life cycle and pathogenesis, they are considered as one of the potential targets. HIV-1 Env interaction with receptor and co-receptor activates the Abl/Rac/Wave2 kinase pathway to induce actin remodeling, thus facilitating the incoming HIV-1 to overcome the actin barrier. Specific inhibition of Abl by Nilotinib has been proved to impair HIV-1 entry and reduce HIV-1 replication by >90% without significant cytotoxicity [144]. The efficient synthesis of HIV RNA relies on CDK9-mediated phosphorylation of RNAPII, N-TEF, and DSIF. Inhibition of CDK9 by DRB, Flavopiridol, Roscovitine, and their derivatives have been proved to impair HIV entry and reduce HIV replication [145,146].

In the present research, we screened 196 kinase inhibitors and identified a CDK and GSK-3β inhibitor kenpaullone (Ken) as a potential new anti-HIV-1 drug. Based on this result, we

established five objectives: characterize the anti-HIV-1 activity of Ken; test the anti-HIV-1 ability of Ken in different cell lines; identify steps in the HIV-1 replication cycle that could be blocked by Ken; determine the mechanism of the anti-HIV-1 activity of Ken; test the anti-HIV-1 ability of Ken in human primary PBMCs.

3.2 Results

3.2.1 Drug Screening

To discover kinase inhibitors with potent anti-HIV activity, we conducted a primary drug screening of 196 different synthesized molecules (library obtained from Enzo Life Sciences), targeting more than 80 different kinases that play essential roles in viral replication and/or cell function. To this end, we treated HIV-1 infected C8166 T cells with the same concentration of different kinase inhibitors and compared the HIV-1 replication and cell proliferation levels at 72 hours post-infection. Specifically, 2×10^6 C8166 T cells were infected with pNL4.3-Nef+/GFP+ virus (25 ng p24 equivalent) for 1.5 hours and were then washed twice in sterilized PBS to remove the remaining virus and resuspended in 10 mL complete media. 100 µL of cells was aliquoted into a 96-well plate and treated with different inhibitors at a final concentration of 5 µM. At 72 hours post-infection, levels of HIV replication were determined by measuring p24 concentrations in the supernatants. In this screening, Ken stood out in its ability to suppress HIV replication with little toxicity.

3.2.2 Characterize the Anti-HIV-1 Activity of Ken

Since the primary drug screening indicated Ken as a potential new anti-HIV drug, in this section, we want to further confirm the anti-HIV-1 activity of Ken. Two factors are of interest: the median effective concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50}) for cell proliferation. In new drug development, the two factors are used to assess the suitability and performance of the drug candidate. In our case, EC_{50} indicates the Ken concentration required for 50% inhibition of HIV-1 replication in comparison with the control experiment, while the IC_{50} indicates the Ken concentration that results in 50% cell growth inhibition in uninfected cells.

To determine the EC_{50} of Ken, we infected 0.5×10^6 C8166 T cells with HxBru/Nef-/Gluc+ virus (5 ng p24 equivalent) in 1 mL complete media in the presence of various concentrations of Ken ranging from 0 nM to 900 nM. At two hours post-infection, cells were washed twice in sterilized PBS to remove the remaining virus and then cultured in 1 mL complete media containing the same concentrations of Ken as before. At 72 hours post-infection, the viral replication was assessed by measuring the Gluc activity in the supernatants. The result shows that Ken inhibits HIV replication in a dose dependent manner and the EC_{50} is about 600 nM (Fig. 6 B). Furthermore, HIV-1-induced multinucleated giant cells (syncytium) were visualized by microscopy. Compared with the untreated cells (Fig. 6 C, left panel), the syncytium formation is significantly inhibited in Ken treated cells (Fig. 6 C, right panel).

To determine the IC_{50} of Ken, we cultured C8166 T cells at 0.1×10^6 cells per well in 100 µL complete media with different concentrations of Ken ranging from 0 nM to 9600 nM as indicated in Fig. 6D. At 72 hours posttreatment, the effect of Ken treatment on cell proliferation was

determined by WST-1 assay as mentioned in materials and methods and the result indicated the IC_{50} is approximately 9500 nM (Fig. 6D). Together, these results indicate that Ken is able to significantly inhibit HIV-1 replication at a non-toxic concentration.

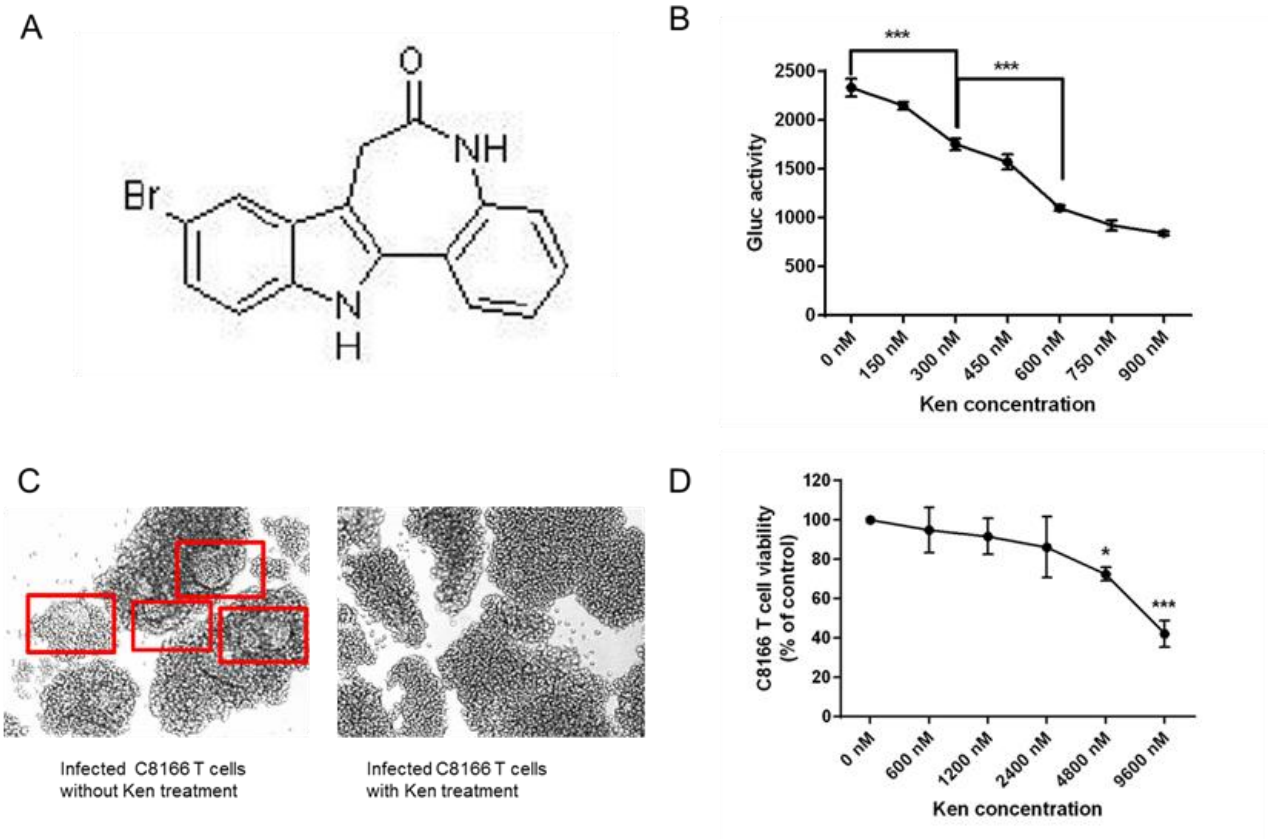

Fig 6. Characterize the anti-HIV-1 activity of Ken. (A) Chemical structure of Ken. (B) Determination of the EC_{50} of Ken against HIV-1. C8166 T cells were infected with HxBru/Nef-/Gluc+ virus in the presence of various concentrations of Ken. At two hours post-infection, cells were washed twice in sterilized PBS to remove the remaining virus and then cultured in 1 mL complete media containing the same concentrations of Ken as before. At 72 hours post-infection, the Gluc activity in the supernatants was measured. Data shown are means ± standard deviation of results from three independent experiments. Statistical significance was determined by Tukey-kramer test ***p<0.001 (N=3). (C) C8166 T cells were infected with HxBru/Nef-/Gluc+ virus. At 2 hours post-infection, cells were washed twice in sterilized PBS to remove the remaining virus and then cultured in 1 mL complete media containing the same concentrations of Ken as before. At 72 hours post-infection, HIV-1-induced syncytium formation in the absence (left) or presence (right) of 600 nM Ken were viewed by microscopy. (D) Determination of the IC_{50} for cell proliferation of Ken. C8166 T cells were maintained at 37°C in the presence of various concentrations of Ken. At 72 hours posttreatment, cell proliferation was detected by WST-1 assay. Data shown are means ± standard deviation of results from three independent experiments. Statistical significance was determined by Dunnett's test ***p<0.001 (N=3)

3.2.3 Ken Inhibits HIV-1 Replication in Different CD4+ Lymphocytic Cell Lines

HIV-1 is a highly variable virus containing four distinct lineages (M, N, O, and P) and the M lineage responsible for the HIV-1 global pandemic can be further classified into nine major groups. When considering the recombination between different strains, the number of circulating HIV-1 strains is virtually numerous. In addition, HIV-1 targeted cells are CD4+ lymphocytic cells, which contain many different cell types including monocytes, T cells, etc. Based on these facts, an ideal new drug candidate should exhibit comparable abilities against different HIV-1 strains in different cell types.

For this reason, we tested the anti-HIV-1 ability of Ken against a clinically isolated HIV-1 reverse transcriptase inhibitor-resistant strain (Nevi virus) in two cell lines derived from different cell type: human monocytic U937 cell line and Jurkat T cells. In particular, we infected 1×10^6 U937 cell and Jurkat T cells with Nevi virus (15 ng and 9 ng p24 equivalent respectively). Four hours after infection cells were washed twice with sterilized PBS to remove the remaining virus. Subsequently, cells were cultured in two wells with or without 1200 nM Ken. At 72 hours post-infection, the supernatants were collected and the HIV-1 replication levels were monitored by $p24^{Gag}$ ELISA. The results show the anti-HIV activity of Ken is not restricted to laboratory strains and C8166 T cells as it is able to significantly inhibit the replication of HIV-1 reverse transcriptase inhibitor-resistant strains in both monocytic cell line U937 and Jurkat T cell line (Fig. 7A and 7B). The broad-spectrum anti-HIV-1 activity of Ken suggests it as a promising anti-HIV new drug.

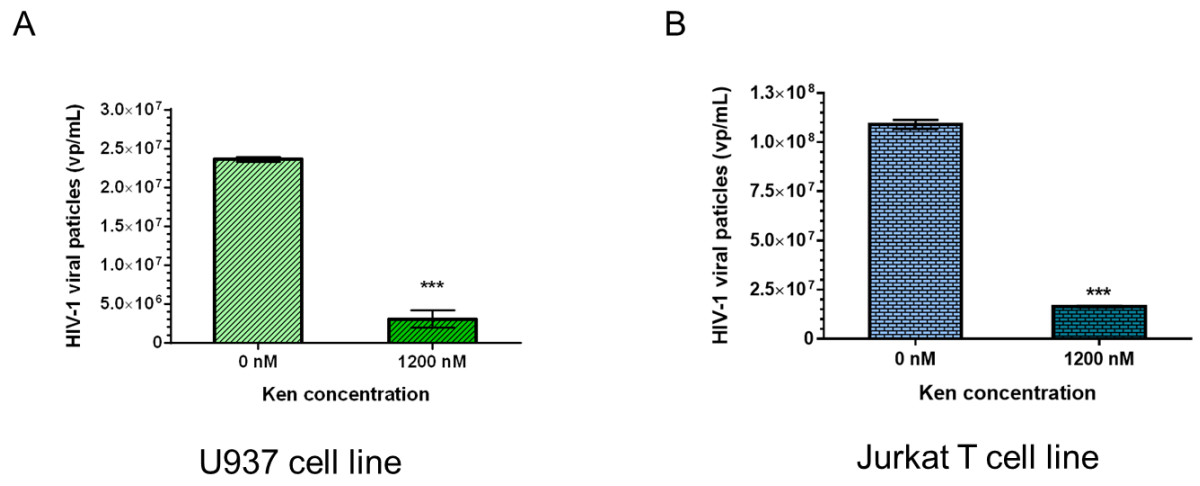

Fig 7. Ken inhibits HIV-1 replication in different CD4+ lymphocytic cell lines. (A)(B) Monocytic U937and Jurkat T cells were infected with an HIV-1 reverse transcriptase inhibitor-resistant strain. At four hours post-infection cells were washed twice with sterilized PBS to remove the remaining virus. Subsequently, cells were cultured in two wells with or without 1200 nM Ken. At 72 hours post-infection, HIV-1 replication was monitored by measuring HIV-1 p24 antigen in the supernatants. Viral particle concentration was calculated according to the p24 concentration. Data shown are means ± standard deviation of results from four independent experiments. Statistical significance was determined by Student's t-test, ***P<0.001 (N=4)

3.2.4 Ken Inhibits HIV-1 Transcription

To gain insight into the mechanism underlying the HIV-1 inhibition activity of Ken, we first asked which HIV-1 replication stage is affected by Ken treatment. According to previous studies, the average duration of HIV-1 early stage replication is approximately 24 hours and the average time taken to complete a single HIV-1 replication cycle is approximately 36 hours. The addition of Ken before the completion of the targeted stage will result in normal inhibition of HIV-1 replication while addition of Ken after the completion of the targeted stage will result in decreased or abolished inhibition of HIV-1 replication.

For this reason, we divided C8166 T cells into two groups: the first group was treated with Ken for two hours before HIV-1 infection and the second group was not treated with Ken until 20 hours after infection. Specifically, for the first group, 0.5×10^6 C8166 T cells were treated with different concentrations of Ken ranging from 0 nM to 900 nM. At two hours posttreatment, cells were infected with single cycle Gluc virus (5 ng p24 equivalent) in 1 mL complete media. At two hours post-infection, cells were washed twice with sterilized PBS to remove the remaining virus and then cultured in 1 mL complete media containing the same concentrations of Ken as before. For the second group 0.5×10^6 C8166 T cells were infected with single cycle Gluc virus (5 ng p24 equivalent) in 1 mL complete media. At two hours post-infection, cells were washed twice with sterilized PBS to remove the remaining virus cultured in 1 mL complete media. At 20 hours post-infection, cells were treated with different concentrations of Ken ranging from 0 nM to 900 nM. At 48 hours post-infection, the viral replication levels in both groups were assessed by measuring the Gluc activity in the supernatants. The result clearly shows that Ken treatment 20 hours after

HIV-1 infection can achieve similar inhibition activity as Ken treatment two hours prior to HIV-1 infection. This result indicates that Ken inhibits HIV-1 late stage replication.

To pinpoint the specific step of HIV-1 replication targeted by Ken, we analyzed the effect of Ken on ACH-2 cell line which is a latent T cell clone containing one integrated proviral copy in the genome. Specifically, 1×10^6 ACH-2 cells were cultured in 1 mL complete media and treated with different concentrations of Ken as indicated in Fig. 8 B and C. After 24 hours of treatment, HIV-1 replication was monitored by measuring HIV-1 p24 antigen in the supernatants. At the same time, the ACH-2 cells were collected and the HIV-1 gag mRNA production was measured by RT-PCR. The results show that the Ken treatment can inhibit HIV-1 replication and HIV-1 gag mRNA production in ACH-2 cell line by 50% which indicates that Ken inhibits HIV-1 replication at the transcription step.

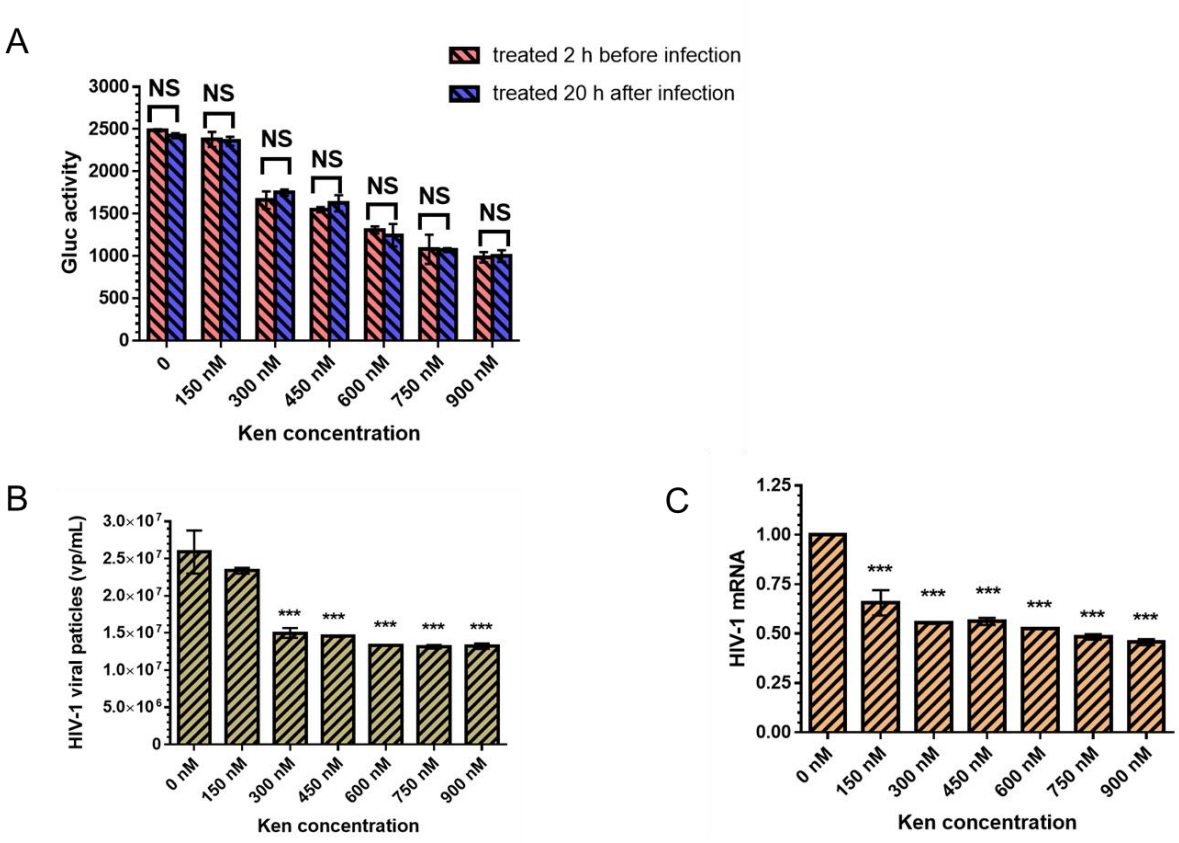

Fig. 8 Ken inhibits HIV-1 transcription. (A) C8166 T cells were infected with HIV-1 single cycle Gluc virus. Ken treatment was performed at two hours before infection or 20 hour post-infection, respectively. At 48 hours post-infection, Gluc activity in supernatants was measured. Data shown are means ± standard deviation of results from three independent experiments. Statistical significance was determined by Student's t-test, ***P<0.001 (N=3). (B)(C) ACH-2 cells were treated with different concentrations of Ken. At 24 hours post-treatment, HIV-1 replication was monitored by measuring HIV-1 p24 antigen in the supernatants and HIV-1 gag mRNA production was measured by RT-PCR. Viral particle concentration was calculated according to the p24 concentration. Data shown are means ± standard deviation of results from three independent experiments. Statistical significance was determined by Dunnett's test ***p<0.001 (N=3)

3.2.5 Ken Inhibits HIV-1 Replication through CDK Pathways

Ken is a potent inhibitor of CDK and GSK-3β. Previous studies indicate that CDK pathways play important roles during HIV-1 transcription. For this reason, we hypothesized that Ken inhibits HIV-1 replication through CDK pathways. To test this hypotheses, we compared the anti-HIV-1 activity and cytotoxicity of CDK inhibitors (olomoucine, N9-isoproplyl-olomoucine, roscovitine) with GSK-3β inhibitors (indirubin, indirubin-3'-monoxime). All the three CDK inhibitors are purine analogues that compete with ATP at the ATP binding site in the targeted kinases [210]. Olomoucine, and N9-isoproplyl-olomoucine selectively target CDK1, CDK2 and CDK5. Besides CDKs, olomoucine also inhibit ERK1 to a limited extent (IC_{50}=25 μM) [211]. Comparing with olomoucine and N9-isoproplyl-olomoucine, roscovitine has a relatively broader selectivity, it can inhibit CDK1, CDK2, CDK4, CDK5, CDK6, CDK7 and CDK9. However, only the following four kinases can be substantially inhibited: CDK1/cyclin B (IC_{50}, 650 nM), CDK2/cyclin A (IC_{50}, 700 nM), CDK2/cyclin E (IC_{50}, 700 nM), and CDK5/p35 (IC_{50}, 200 nM) [211]. Similar to the CDK inhibitors, indirubin and indirubin-3'-monoxime have high affinity for ATP-binding site in the targeted kinases. Both of them can substantially inhibit GSK-3β and (IC_{50} values of 1 μM and 22 nM respectively) [212].

Specifically, we infected 6.5×10^6 C8166 T cells with HxBru virus (22ng p24 equivalent) in 6.5 mL complete media. After 2 hours of infection, cells were washed twice in sterilized PBS to remove the remaining virus and then resuspended in 13 mL complete media. 1 mL of cells was aliquoted into a 24-well plate and treated with different inhibitors at different concentrations as indicated in Fig 9. At 72 hours post-infection, levels of HIV replication were determined by measuring p24

concentrations in the supernatants and the cytotoxicity of different inhibitors were evaluated by trypan blue dye exclusion.

To normalize the decreased HIV-1 production induced by cytotoxicity caused cell death or cell proliferation impairment, we divided the p24 concentrations in the supernatants by the ratio of cell amount in drug treated group to the cell amount in untreated control. The results show that neither of the two GSK-3β inhibitors is able to inhibit HIV-1 replication while all the CDK inhibitors are able to inhibit HIV-1 replication by 25% to 60%. Collectively, these results suggest that Ken may target CDK pathways for HIV-1 suppression.

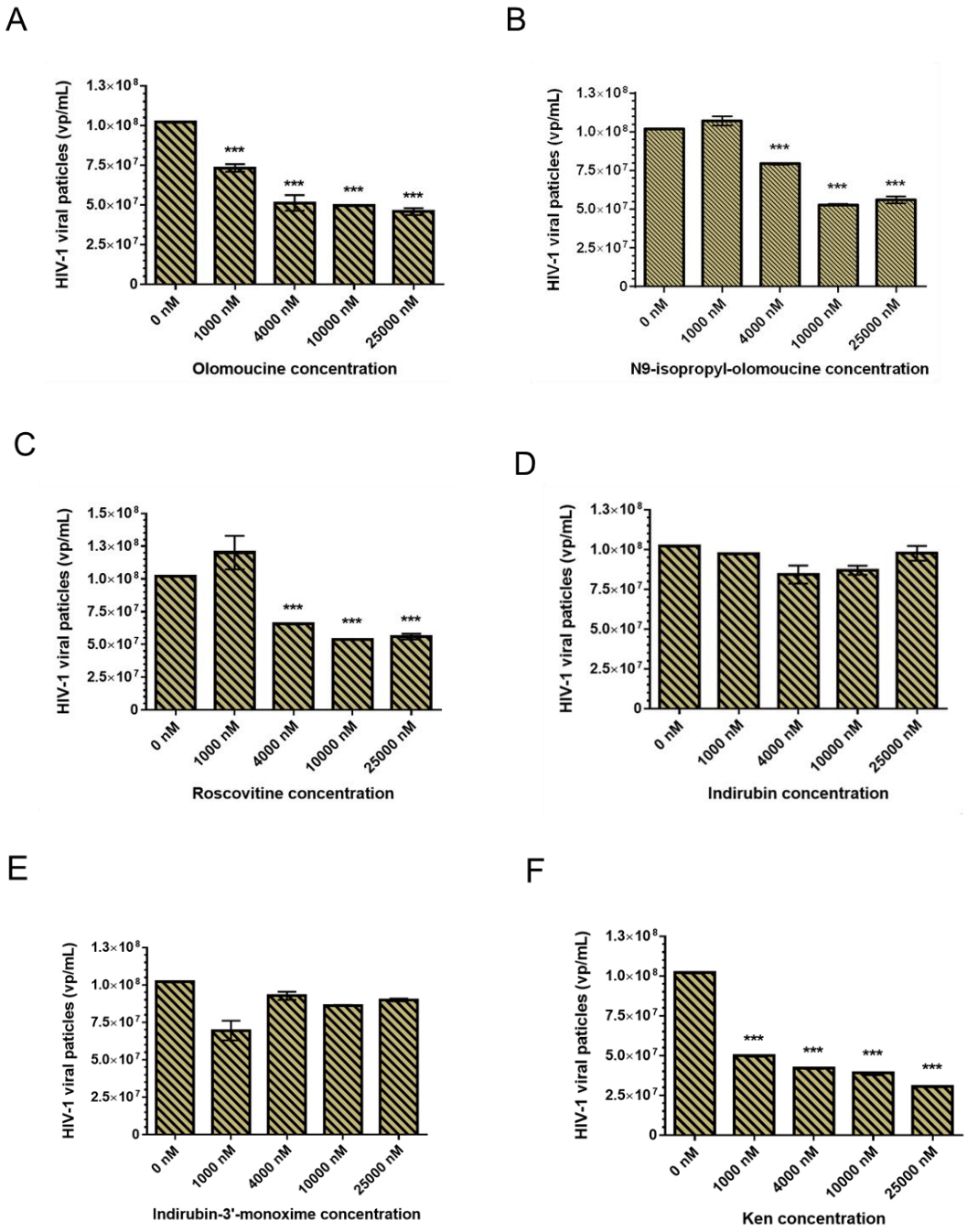

Fig. 9 Ken inhibits HIV-1 replication through CDK pathways. (A-F) C8166 T cells were infected with HxBru virus in the presence of various concentrations of CDK inhibitors (olomoucine, N9-isoproplyl-olomoucine and roscovitine) and GSK-3β inhibitors (indirubin, indirubin-3'-monoxime and kenpaullone). 6.5×10^6 C8166 T cells were infected with HxBru virus. After 2 hours of infection, cells were washed twice in sterilized PBS and then aliquoted into a 24-well plate. At 72 hours post-infection, levels of HIV replication were determined by measuring p24 concentrations in the

supernatants and the cytotoxicity of different inhibitors was evaluated by trypan blue dye exclusion. P24 concentrations are normalized by the cell viability. Viral particle concentration was calculated according to the p24 concentration. Data shown are means ± standard deviation of results from four independent experiments. Statistical significance was determined by Dunnett's test ***$p<0.001$ (N=4)

3.2.6 Ken Inhibits HIV-1 Replication in Human Primary PBMCs

The potent anti-HIV-1 activity of Ken in cell lines makes it of interest to know the anti-HIV-1 activity of Ken in human primary PBMCs. To this end we stimulated human primary PBMCs with 3ug/mL PHA in complete RPMI-1640 supplemented with 10 U IL-2, At 3 days of stimulation, PHA was washed away and activated PBMCs were maintained in complete RPMI-1640 supplemented with 10 U. 2×10^6 activated human primary PBMCs were infected with Nevi virus (12 ng p24 equivalent). At four hours after infection, cells were washed with sterilized PBS twice to remove the remaining virus and then resuspended in 4 mL complete RPMI-1640 supplemented with 10 U IL-2. Subsequently, cells were treated with different concentrations of Ken as indicated in Fig.10. 48 hours and 72h hour after infection, supernatants were collected for $P24^{Gag}$ ELISA. The results clearly show that Ken is able to inhibit HIV-1 replication in human primary PBMCs.

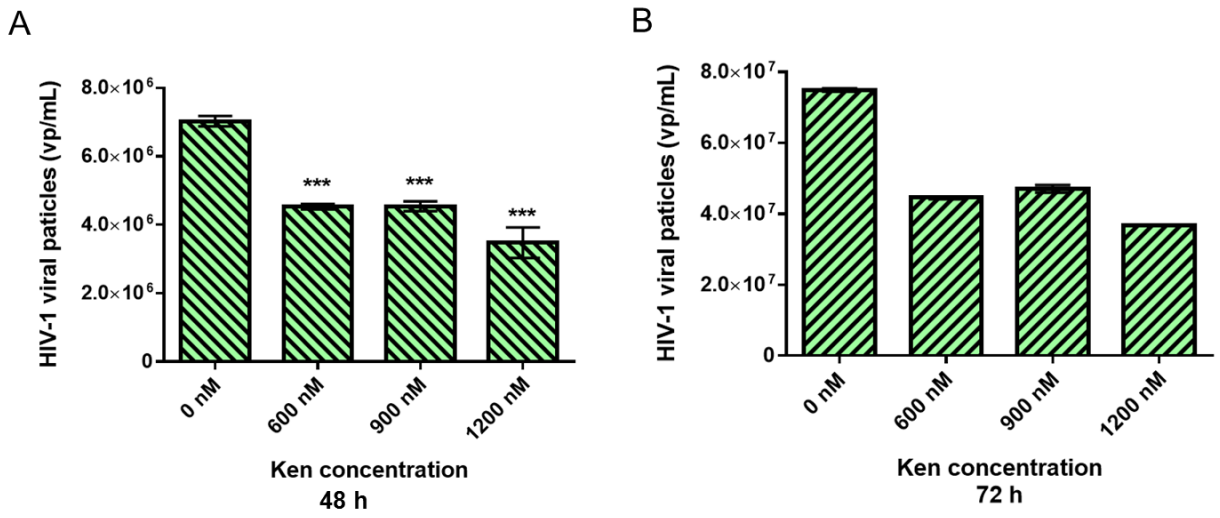

Fig. 10 Ken inhibits HIV-1 replication in human primary PBMCs. (A)(B) 2×10^6 activated human primary PBMCs were infected with Nevi virus (12 ng p24 equivalent). At four hours post-infection cells were washed with sterilized PBS for twice and separated into 4 wells. Subsequently, Cells were treated with different concentrations of Ken. Supernatants were collected at 48h p.i. and 72h p.i. for p24 assay. Viral particle concentration was calculated according to the p24 concentration. Data shown are means ± standard deviation of results from four independent experiments. Statistical significance was determined by Dunnett's test ***$p<0.001$ (N=4)

3.3 Discussion

HAART is considered the most effective weapon again HIV-1; however, its clinical utility is limited by drug-resistant HIV-1 infections, nonadherence to therapy, as well as short- and long-term side effects. For this reason, there is a continued interest in the development of novel and effective therapeutics. Current ARTs mainly target viral protein. Once mutations occur at these targets, the ART treatment will become less efficient. Given that HIV-1 utilizes the host mechanism for successful replication and pathogenesis, targeting host proteins is an attractive strategy in HIV-1 treatment. Extensive study in the past decades have provided substantial evidence showing cellular kinase signaling pathways are tightly regulated during HIV-1 infection and disease progression. Therefore, cellular kinase signaling pathways might be a promising target for the development of novel anti-HIV therapeutics. Although no kinase-targeting drugs have been tested in clinical trials, the anti-HIV-1 activity of a number of kinase inhibitors have been demonstrated in culture-based assays. In order to find potential novel drugs, we scanned 196 different kinase inhibitors targeting more than 80 different kinase pathways and identified Ken as an effective new anti-HIV-1 drug. In further characterization of this drug, we found the EC_{50} of Ken against HIV-1 replication in C8166 T cells is around 600 nM lower than its IC_{50} for cell proliferation, which is 9500 nM. To rule out the possibility that the anti-HIV-1 activity of Ken is restricted to a single cell line, we analyzed the anti-HIV-1 activity of Ken in monocytic cell line U937 and Jurkat T cell line. The results clearly indicate that Ken exhibited potent anti-HIV-1 activity at 1200 nM in both cell lines. In addition to cell lines, we tested the anti-HIV-1 activity of Ken in human primary PBMCs and the anti-HIV-1 activity was also detected.

The wide use of HAART is accompanied by the emergence and spread of drug-resistant strains. In treatment-experienced patients, up to 50-70% experience viral rebound and drug-resistant strains can be detected in most of them. Data from prevalence analysis suggests that transmission of drug-resistant strains to treatment-native individuals is between 10% and 17% in high-income countries. According to the WHO drug-resistance report, in Europe and North America, drug resistance most frequently involved the two reverse transcription inhibitors: NRTI and NNRTI, with a prevalence of 7.4% and 3.4% respectively [213]. Therefore, the effectiveness of a new anti-HIV-1 product against reverse transcription inhibitor resistant strains need to be evaluated before it can be subjected to next stage trial. In our study, we demonstrated that Ken is able to inhibit the replication of a nevirapine-resistant HIV-1 strain by over 80% in U937 and Jurkat T cells at 1200 nM.

The broad-spectrum anti-HIV-1 activity of Ken in different cell lines suggests it as a potential new anti-HIV-1 drug. To understand the mechanism underlying the HIV-1 inhibition activity of Ken, we first analyzed which stage in HIV-1 replication cycle is targeted by Ken. At cellular level, HIV-1 replication cycle can be divided into two stages: early stage refers to the steps from viral attachment to the cell surface to viral DNA integration whereas the late stage begins at transcription and continues through to viral maturation. An early study tracked the timing of these events by Southern (DNA) and Northern (RNA) blot analysis and suggested that HIV integration occurs at 15 hours post-infection and genomic RNA and mRNA begin to appear at 24 hours post-infection. This kinetics of HIV-1 replication is confirmed by several recent studies through imaging methods and quantitative measurement of viral DNA and RNA. For this reason,

we compared HIV-1 replication in cells treated with Ken at two hours prior to HIV-1 infection or 20 hours after infection. The result clearly shows that Ken treatment at different time points did not affect the inhibition activity of Ken, indicating that Ken specifically affects the late stages of HIV-1 replication. To define the specific step of HIV-1 replication targeted by Ken, we utilized ACH-2 cell line for further analysis. ACH-2 cell line is a CD4- T cell clone with one copy of HIV-1 provirus integrated into the cellular genome, thus making it an ideal model to study HIV-1 late stage replication. We treated ACH-2 cells with different concentrations of Ken and collected the cells for HIV-1 mRNA quantification at 24 hours after treatment. The result clearly indicated that Ken treatment is able to impair HIV-1 mRNA production by 50%. In accordance with the reduced HIV-1 mRNA production in Ken treated cells, the P24 contents were also reduced by 50% in supernatants collected from Ken-treated cells. Collectively, these results suggest that Ken mainly inhibits HIV-1 replication by impairing HIV-1 late stage replication specifically at transcription.

Ken is known as a potent inhibitor of GSK-3β, CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, and CDK5/p25. There is substantial evidence suggesting that the Tat mediated effective HIV-1 mRNA production is indispensable for CDK activity. By interacting with pTEF-b and TAR simultaneously, Tat recruits pTEF-b to the HIV-1 promoter where the CDK9/cyclin T1 subunit of pTEF-b mediates the phosphorylation of N-TEF and DSIF as well as Ser2 in RNAP II CTD. The phosphorylated N-TEF is displaced from the TAR region whereas the phosphorylated DSIF is changed into a positive elongation factor that enhances the processivity of RNAP II. A recent study suggests CDK2/cyclin E as a potential regulatory factor for CDK9/cyclin T1 as inhibition of CDK2 expression by iron chelators or knockdown approach reduces CDK9 phosphorylation at Ser90 and impairs HIV

replication. For this reason, we asked whether Ken inhibits HIV-1 replication through CDK pathways. To answer this question, we compared the anti-HIV-1 activity and cytotoxicity of GSK-3β inhibitors (indirubin, indirubin-3'-monoxime) with CDK inhibitors (olomoucine, N9-isoproplyl-olomoucine, roscovitine). The results show that neither of the two GSK-3β inhibitors is able to inhibit HIV-1 replication whereas all three CDK inhibitors can inhibit HIV-1 replication by 25% to 60%. Collectively, these results suggest that Ken may target CDK pathways for HIV-1 suppression.

Our results clearly indicate that Ken, at non-toxic concentrations, is able to inhibit HIV-1, including the wild type and reverse transcription resistant viruses by blocking viral gene expression. As Ken disrupts a step that has not previously been targeted in current antiretroviral chemotherapy, it can be used in combination with other drugs for more effective HIV-1 suppression

3.4 Future directions

Our result has indicated the Ken possibly inhibits HIV-1 replication through the CDK signaling pathways. As previously reviewed, Ken is a potent inhibitor of several members of the CDK family including CDK1, CDK2, and CDK5. To understand the mechanism underlying the anti-HIV-1 activity of Ken, future study should examine the inhibition for which CDK is responsible for the decreased mRNA production in cells treated with Ken.

In our study, we demonstrated Ken as an effective inhibitor of HIV-1 replication at the cellular level. This raises a question whether Ken treatment will affect the bigger picture of HIV transmission among people. To answer this question, a reinfection assay will be conducted to mimic HIV-1 transmission among individuals. The comparison between the Ken-treated group and the non-treated group will help us evaluate Ken as a prevention method.

Although Ken is a promising new anti-HIV drug, there is still a long way to go before it could be used in clinical settings. At present, more studies need to be conducted to adjust the usage of Ken to reach its full potential against HIV-1 infection. For this reason, our future study will also focus on developing a more effective Ken-based therapy through the following two ways: drug combination and drug modification.

1 Drug combination

Ken targets the late stage of HIV-1 replication, specifically at the mRNA production stage. Combining Ken with other drugs targeting different steps in HIV-1 replication cycle such as viral

entry and maturation may help to increase the effectiveness as well as decrease the dose of a single drug.

2 Drug modification

Drug modification is a commonly used method to enhance the effectiveness of a single drug. Several Ken modifications have proved to have better anti-cancer performance. It is of interest to know whether drug modification may increase the anti-HIV-1 ability of Ken.

4 The IN Association with DIC1 and DYNLT1

4.1 Introduction

HIV-1 replication initiates from the proper engagement of Env with CD4+ T cell surface receptors and coreceptors, followed by the viral and cellular membrane fusion that results in the release of viral contents into the cytoplasm. Uncoating of the incoming virus forms RTC where the HIV-1 RNA genome is reverse transcripted into cDNA. The newly synthesized HIV-1 cDNA remain associated with viral proteins as a complex named PIC, which is delivered to the nucleus for nuclear import and DNA integration. Although this model of HIV-1 early stage replication is generally accepted, there are still unsolved questions to complete the whole story. One of the intriguing questions is how HIV-1 RTC/PIC is transported to the nucleus. The intracellular environment is populated with macromolecules and organelles, which only allows the free migration of molecules with a hydrodynamic diameter less than 20 nm. As the average diameter of HIV PIC is 56 nm, passive diffusion is unlikely to be the method of HIV intracellular traveling [214].

Given that the transportation of cellular macromolecules and organelles towards the nucleus relies on microtubule network and dynein motor complex, it has been hypothesized that HIV-1 utilizes the intracellular transportation system for its own purposes. An early study reported that nocodazole-induced microtubule depolymerization impairs HIV-1 early stage replication. Later, by visualizing individual particles, McDonald D et al. demonstrated that HIV-1 PICs are associated with MT and concentrated in the area of the MTOC [80]. Moreover, disrupting dynein/microtubule-mediated intracellular transportation by microtubule depolymerization or

microinjection of anti-dynein antibody inhibited HIV-1 PIC transportation towards MTOC and resulted in the peripheral accumulation of PIC. Collectively, these results suggest that the microtubule network and dynein motor complex may play an important role in HIV-1 intracellular transportation.

As the major component of RTC/PIC, CA has been proposed to mediate the HIV-1 association with dynein; however, no firm evidence has been provided to support this hypothesis. Given that HIV-1 uncoating at least partially exposes the capsid packaged viral proteins to the cellular environment, it is possible that the interaction between RTC/PIC and dynein is mediated by proteins inside the capsid [215]. Unlike the other viral proteins, IN accompanies the HIV-1 genetic material throughout HIV-1 early stage replication, thus making it a candidate of interest. For this reason, in our current study we have focused on the association between IN and dynein complex. Dynein complex contains three types of light chains: DYNLL1, DYNLT1, and DYNLRB. Among these three different types of light chains, only DYNLL1 and DYNLT1 has been proposed as the potential cargo adaptors [179]. The previous investigation in our laboratory has demonstrated the interaction of HIV-1 IN with DYNLL1, one of the dynein cargo adaptors. However, the functional analysis shows the interaction between HIV-1 IN and DYNLL1 is not involved in the retrograde transportation of HIV-1 because the DYNLL1 knockdown had no effect on HIV-1 nuclear import. Since DYNLT1 is also one of the dynein cargo adaptors and DIC1 is the key structural component in dynein complex, to gain more insight into the mechanism underlying dynein recruitment of HIV-1, my task was to explore the potential association of HIV-1 IN with DYNLT1 and DIC1. By Co-IP, we have demonstrated that IN is associated with two dynein components: DIC1 and DYNLT1.

We also identified that the C-terminus of IN is crucial for the association between IN and DIC1. By immunofluorescence, we have demonstrated that IN co-localizes with DIC1.

4.2 Results

4.2.1 HIV-1 IN is Associated with DIC1 and DYNLT1

For this study, we wanted to investigate whether IN is associated with intracellular DIC1 and DYNLT1. To allow the detection potential protein interactions, we used the overexpression system. In order to express full-length HIV-1 IN in 293T cells, we constructed CMV-pAcGFP-IN wt plasmid by fusing the cDNA encoding the full-length HIV-1 IN (generated from CMV-YFP-IN double digestion) to the 3' end of the GFP tag in CMV-AcGFP-C1 plasmid.

The CMV-AcGFP-control and CMV-AcGFP-IN wt plasmid were transfected into 293T cells separately. At 48 hours post-transfection, the cells were collected and lysed in 0.12% NP40 lysis buffer that contains protease cocktail. The lysates were clarified then subjected to Co-IP using the anti-GFP antibody. The immunoprecipitations were resolved in 12% SDS-PAGE. The co-immunoprecipitated DIC1 and DYNLT1 were analyzed by using the corresponding antibodies. The result revealed that the IN is associated with both DIC1 and DYNLT1.

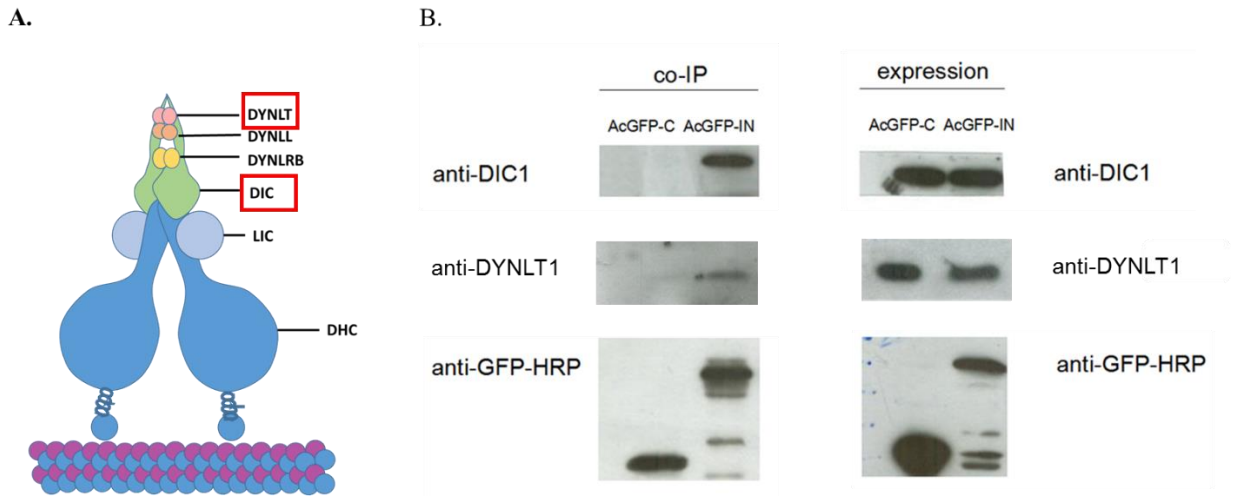

Fig. 11 HIV-1 IN is associated with DIC1 and DYNLT1 (A) Dynein complex structure and dynein components targeted for co-IP analysis (highlighted by box). (B) HIV-1 IN is associated with DIC1 and DYNLT1. AcGFP-Control and AcGFP-IN wt expressers were transfected into 293T cells. After 48h of transfection, cells were lysed and cell lysates were subjected to Co-IP using anti-GFP antibody. The co-pulled down DIC1 and DYNLT1 were detected by WB using anti-DIC1 and anti-DYNLT1 antibodies, respectively. 1/10th of cells were lysed in RIPA buffer and the expression of DIC1 and DYNLT1 were detected by WB using respective antibodies. The expression of AcGFP-C and AcGFP-IN were similarly detected in WB using anti-GFP-HRP antibody

4.2.2 HIV-1 IN C-terminus is Essential for IN/DIC1 Association

As previously reviewed, IN contains three functionally distinct domains. For this reason, we wanted to investigate which region of IN is essential for the association of IN with intracellular DIC1. In order to express region deleted HIV-1 IN in 293T cells, we constructed plasmids CMV-AcGFP-IN 1-212 and CMV-AcGFP-IN 50-288 by fusing cDNA encoding the corresponding HIV-1 IN mutant (generated from PCR using CMV-AcGFP-IN wt as template) to the 3' end of the GFP tag in CMV-AcGFP-C1 plasmid.

The CMV-AcGFP-control, CMV-AcGFP-IN wt, and CMV-AcGFP-IN mt plasmids were transfected into 293T cells separately. At 48 hours post-transfection, the cells were collected and lysed in 0.12% NP40 lysis buffer that contains protease cocktail. The lysates were clarified then subjected to Co-IP using the anti-GFP antibody. The immunoprecipitations were resolved in 12% SDS-PAGE. The co-immunoprecipitated DIC1 was analyzed by using the anti-DIC1 antibody. The result revealed that both IN wt and IN 50-288 mt were associated with DIC1 while the IN 1-212 mt was not associated with DIC1. Together these results indicated that the binding site of DIC1 in HIV-1 IN is located at the IN 212-288, which is the C-terminal domain of IN.

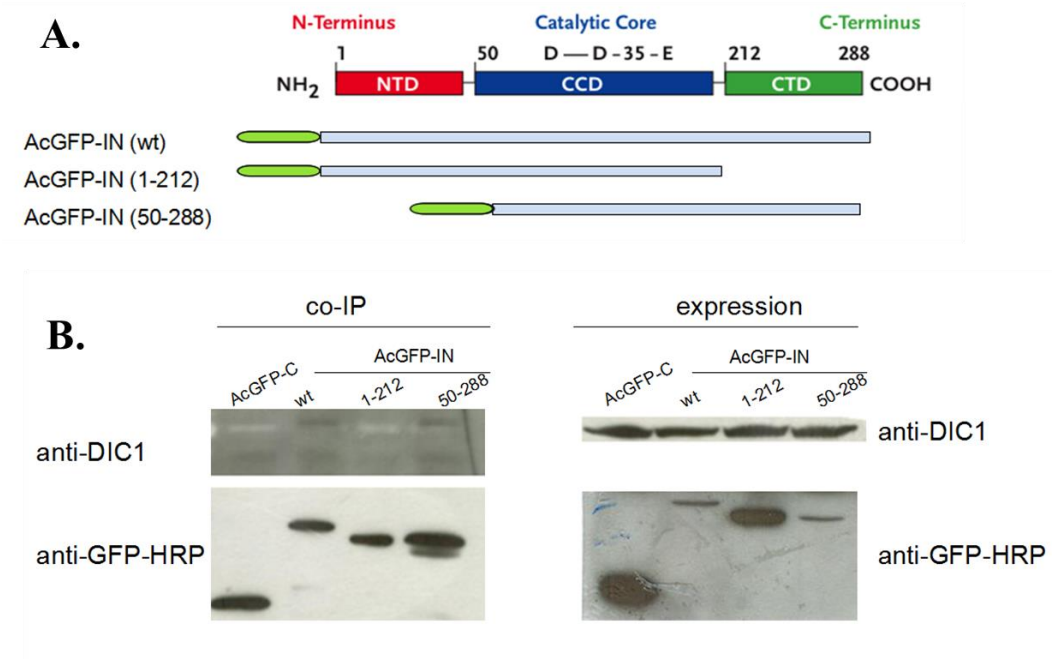

Fig. 12 HIV-1 IN C-terminus is essential for IN/DIC1 association (A) HIV-1 IN wt and HIV-1 IN mt used for Co-IP analysis. (B) HIV-1 IN C-terminal domain is required for IN/DIC1 association. AcGFP-C, AcGFP-IN wt and AcGFP-IN mt expressers were transfected into 293T cells. After 48h of transfection, cells were lysed and cell lysates were subjected to Co-IP using anti-GFP antibody. The co-pulled down DIC1 were detected by WB using anti-DIC1 antibodies. 1/10th of cells were lysed in RIPA lysis buffer and the expression of DIC1 were detected by WB. The expression AcGFP-C, AcGFP-IN wt and AcGFP-IN mt were similarly detected in WB using anti-GFP antibody.

4.2.3 HIV-1 IN Colocalizes with DIC1

To confirm the association of HIV-1 IN with DIC1, we performed the colocalization assay. In this study, Hela cells cultured on glass coverslips were transfected with CMV-AcGFP-IN or CMV-AcGFP-control expressors. After 12 hours of transfection, cells were fixed and permeated. AcGFP-IN and AcGFP-control were stained with FITC conjugated antibody while DIC1 was stained with Cy5 conjugaed antibody as described in the method and materials.

The AcGFP-IN shows two different distribution patterns: concentration around the nucleus (upper panel), and inside the nucleus (middle panel). When AcGFP-IN concentrated around the nucleus, it is colocalized with DIC1; while after nuclear entry, AcGFP-IN lost colocalization with DIC1. On the contrary, AcGFP-control and DIC1 are distributed throughout the cell but they do not colocalize with each other. Together, these results suggest that AcGFP-IN is associated with DIC1 in the cytoplasm before AcGFP-IN nuclear import.

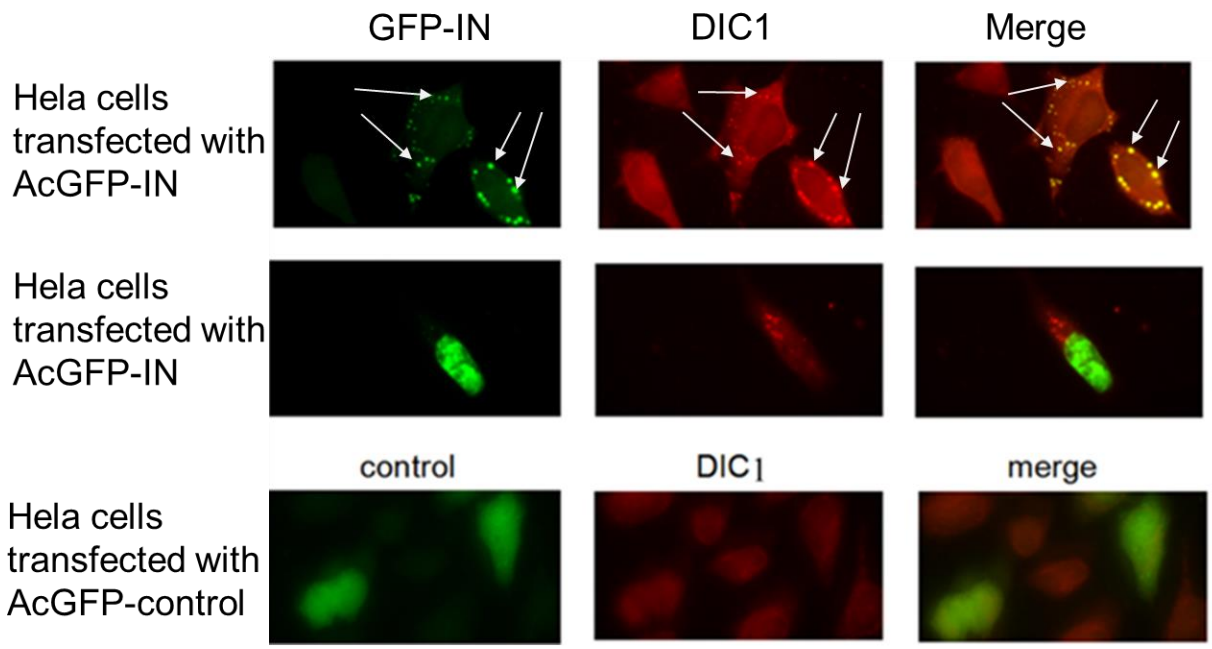

Fig 13. AcGFP-C and AcGFP-IN expressers were transfected into HeLa cells cultured on chamber slides. After nine hours of transfection, cells were fixed and permeabilized with methanol acetone (1:1 ratio) for 30 minutes at room temperature. The fixed cells were blocked by 5% FBS overnight at 4°C. The blocked samples were incubated with rabbit anti-GFP and mouse anti-DIC1 primary antibodies overnight at 4°C. Then, the samples were incubated with corresponding incubated with (FITC)-conjugated anti-rabbit and (Cy5)-conjugated anti-mouse antibody.

4.3 Discussion

Crowded with numerous obstacles, the intracellular environment is more than the aqueous solution in which the free migration of macromolecules is highly restricted. To enable the long distance intercellular transportation of functional cargoes, the cells have evolved an organized microtubule transportation system. Along the microtubules, dynein and kinesin complexes move towards opposite directions that allow retrograde and anterograde transportation respectively. Several previous studies suggest that HIV-1 RTC/PIC utilizes the dynein-dependent microtubule transportation system for migration from cell periphery to the nucleus [80]. However, to date, the mechanism underlying the HIV-1 recruitment of dynein is not known. The previous investigation in our laboratory has demonstrated the interaction of HIV-1 IN with DYNLL1, one of the dynein cargo adaptors. However, the functional analysis shows the interaction between HIV-1 IN and DYNLL1 is not involved in the retrograde transportation of HIV-1 because the DYNLL1 knockdown had no effect on HIV-1 nuclear import [48]. Since DYNLT1 is also one of the dynein cargo adaptors and DIC1 is the key structural component in dynein complex, to gain more insight into the mechanism underlying dynein recruitment of HIV-1, my task was to explore the potential association of HIV-1 IN with DYNLT1 and DIC1.

Through a binding analysis performed by overexpression of AcGFP-IN in 293T cells, we detected the association of AcGFP-IN with endogenous DYNLT1 and DIC1. An immunofluorescence assay performed by overexpression of AcGFP-IN in HeLa cells demonstrated that AcGFP-IN co-localizes with DIC1. Together, these results suggest that AcGFP-IN is likely associated with dynein complex. Subsequent delete mutation analysis indicated that HIV-1 IN212-288 is responsible for the

association between HIV-1 IN and DIC1. Previous work in our laboratory has demonstrated that the HIV-1 IN 212-288 is essential for multistep in HIV-1 early stage replication including HIV-1 reverse transcription and nuclear import [49]. This leads us to ask what the functional role is of the association between HIV-1 IN and dynein complex. The further functional analysis performed in our laboratory demonstrated that HIV-1 DYNLT1 knockdown has no effect on HIV-1 reverse transcription but significantly inhibits the HIV-1 cDNA nuclear import, which suggests that DYNLT1 contributes to HIV-1 RTC/PIC nuclear migration (data not shown). Collectively, these results indicate that the association between HIV-1 IN and dynein complex is responsible for HIV-1 retrograde transportation.

In conclusion, our study provides the first evidence for the HIV-1 IN association with DIC1 and DYNLT1. Further investigation on these virus/host interactions may contribute to our better understanding of HIV biology.

4.4 Future Directions

In our study, we observed that overexpressed HIV-1 IN is associated with endogenous DIC1 and DYNLT1. However, the expression of IN in transient transfection assay is higher than IN amount in HIV-1 infected cells. Furthermore, HIV-1 IN was expressed in the absence of other viral components required for HIV-1 early stage replication. For these reasons, it is necessary to confirm the HIV-1 IN association with DIC1 and DYNLT1 in HIV-1 infected cells. As previously reviewed, rather than functioning as a cargo adaptor, DIC1 functions as the scaffold providing binding sites for the attachment of DLCs. Although overexpressed HIV-1 IN is able to pull down endogenous DIC1, the association between the two proteins might be indirect. For this reason, further study needs to demonstrate the interaction of IN with DIC1 and DYNLT1 recombinant proteins in vitro.

The previous investigation in our laboratory has revealed that the interaction between HIV-1 IN with DYNLL1 contributes to proper uncoating and reverse transcription, but is not required for PIC recruitment to dynein complex. The current study in our laboratory suggests that the association of HIV-1 IN and DYNLT1 contributes to HIV recruitment to dynein complex and HIV-1 RTC/PIC nuclear migration. Since both DYNLL1 and DYNLT1 are important dynein components, it is worth exploring the spatial and temporal sequence of IN association with DYNLL1 and DYNLT1 and how the two associations are regulated.

Addressing these questions will allow us to extend our knowledge from basic study to practical utilization. Since HIV-1 IN CTD is essential for its multimerization, DNA binding and host protein

binding, it is an attractive target for the new drug development. Small molecule inhibitors of the IN CTD function may be developed into a novel ART.

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