ADAR1's enhancement of HIV-1 replication and its relation with PKR activator

Roman Radetskyy

Division of Experimental Medicine

Department of Medicine

McGill University, Montreal, Québec, Canada

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Abstract

Over 36 million people are estimated to be infected with Human Immunodeficiency Virus (HIV) worldwide. Current highly active antiretroviral therapy prevents the progression of the acquired immunodeficiency syndrome. However, a regular drug intake imposes an important economic burden on the health system and comes with a number of side effects, therefore, a cure is needed. Protein Kinase R (PKR), as part of the interferon response, shuts down protein synthesis in the infected cells upon sensing the viral double-stranded RNA, thus, stopping the virus production. Upon stress, cells can also activate PKR through a PKR activator protein (PACT). HIV-1 evolved with several ways to counteract the inhibition of RNA translation by PKR. The viral protein, Trans-Activator of Transcription (Tat) can inhibit PKR. In addition, several cellular proteins such as TAR RNA binding protein and the interferon-inducible RNA-specific adenosine deaminase (ADAR1) also decrease PKR activation level upon HIV-1 infection. More recently, we have observed that in the presence of HIV-1, PACT loses its primary function of activator and becomes an inhibitor of the innate immune response mediated by PKR. Having shown that PACT and ADAR1 interact, we have put forward the hypothesis that the observed change of function may be due to ADAR1 and a viral element.

We approached this project by examining the PKR profile in primary lymphocyte and monocytes cells of individuals with an active HIV-1 replication. The analysis revealed an inverse correlation between ADAR1p150 isoform expression and PKR phosphorylation. This prompted us to genetically modify human T lymphocyte cell lines, Jurkat, to overexpress the ADAR1p150 gene using a lentiviral vector as a transfer tool, as well as CRISPR / Cas9 technology in order to specifically knockout this isoform. This has shown

that ADAR1p150 has a pro-viral effect on HIV-1 kinetics, by boosting viral production in overexpression model, or delaying it by a week in knockout cells. We then identified that PACT and ADAR1 most likely interact through the dsRBDs of the later, and are in a complex with the viral Tat. A mass spectrometry analysis of interacting partners of PACT and ADAR1 from HIV-1 producing revealed PACT's interaction with p17, matrix protein, and identified some unique interaction partners exclusively present in the infection. The presented results contribute to the general understanding of the HIV-mediated control of cellular factors during the infection in host cells.

Résumé

Plus de 36 millions de personnes dans le monde sont infectées par le virus de l'immunodéficience humaine (VIH) selon l'organisation mondiale de la santé. Il existe actuellement des traitements antirétroviraux très actifs qui empêche la progression du syndrome d'immunodéficience acquise (SIDA). Cependant, un apport régulier en médicaments s'accompagne d'un certain nombre d'effets secondaires et impose un fardeau économique important au système de santé, il est donc nécessaire de trouver d'autres moyens pour complètement guérir de la maladie. La protéine kinase R (PKR), fait partie de la réponse à l'interféron, arrête la synthèse des protéines dans les cellules infectées lors de la détection de l'ARN double brin viral, empêchant ainsi la production virale. En cas de stress, les cellules peuvent également activer PKR par le biais de la protéine activatrice de PKR (PACT). Le VIH-1 a évolué de plusieurs façons pour contrecarrer l'inhibition de la traduction de l'ARN par PKR. La protéine virale, trans-activatrice de transcription (Tat) peut inhiber PKR. De plus, plusieurs protéines cellulaires telles que la protéine de liaison à l'ARN TAR et la désaminase de la riboadénosine de type 1 induite par l'interféron (ADAR1) diminuent également le niveau d'activation de PKR lors de l'infection par le VIH-1. Plus récemment, nous avons observé qu'en présence du VIH-1, PACT perd sa fonction d'activateur et devient un inhibiteur de la réponse immunitaire innée induite par PKR. Après avoir montré que PACT et ADAR1 interagissent, nous avons émis l'hypothèse que le changement de fonction observé pourrait être dû à ADAR1 ainsi qu'à un élément viral.

Dans ce projet, nous avons examiné le profil de PKR dans les lymphocytes et les monocytes primaires chez des individus ayant une réplication active du VIH-1. L'analyse a révélé une corrélation inverse entre l'expression de l'isoforme ADAR1p150 et la

phosphorylation de PKR. Ceci nous a incité à modifier génétiquement des lignées de lymphocytes T humaines, Jurkat, pour surexprimer le gène ADAR1p150 en utilisant un vecteur lentiviral comme outil de transfert, ainsi que la technologie CRISPR/Cas9 pour inactiver spécifiquement cet isoforme. Nous avons montré que ADAR1p150 avait un effet pro-viral sur la cinétique du VIH-1, en augmentant la production virale dans le modèle de la surexpression, ou en la retardant d'une semaine dans les cellules inactivées. Nous avons ensuite identifié que PACT et ADAR1 interagissent fort probablement via les domaines dsRBD de ce dernier, et sont dans un complexe avec la protéine virale Tat. L'analyse par spectrométrie de masse des partenaires interagissant avec PACT et ADAR1 à partir des cellules productrices de VIH-1 a révélé l'interaction PACT et p17, la protéine de la matrice, et a aussi permis d'identifier certains partenaires d'interaction uniques, exclusivement présents pendant l'infection. Les résultats présentés contribuent à la compréhension générale du contrôle effectué par le VIH sur certains facteurs cellulaires pendant l'infection des cellules hôtes.

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Finally, I dedicate this thesis to my family for their support and endless love. Without you, I would not have had the same opportunities in life and hopefully one day I will make you all proud.

♪What is life?

Contribution of Authors

I, Roman Radetskyy, wrote the thesis under the supervision of Dr. Anne Gatignol. The introduction sections 1.2 to 1.5 were published in a review:

Roman Radetskyy, Aïcha Daher, Anne Gatignol,
ADAR1 and PKR, interferon stimulated genes with clashing effects on HIV-1 replication,
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Volume 40, 2018, Pages 48-58,
ISSN 1359-6101,
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In which, section 1.4 was written by Aïcha Daher, while Dr. Anne Gatignol edited and organized the manuscript for the publication. Aïcha Daher performed the blot in the Figure 4.2.2B and designed the cloning strategies of lentivirus transfer plasmids inserts and of ADAR1 domains. She also assisted in lentivirus and CRISPR clonal selection, and subsequent screening experiments. Roman Radetskyy performed interaction and HIV-1 infection experiments, and analyzed the mass spectrometry data.

List of abbreviations:

ADAR1: adenosine deaminase acting on RNA 1
AGS: Aicardi-Goutières syndrome
AIDS: acquired immune deficiency syndrome
APOBEC3: apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
CDC: US Centers for Disease Control and Prevention
ds: double-stranded
dsRBD: dsRNA binding domains
eIF2α: eukaryotic translation initiation factor 2
Env: viral envelope
FDA: Food and Drug Administration
FDR: false discovery rate
Gag: group antigen
GFP: green fluorescent protein
HAART: highly active antiretroviral therapy
HBV: hepatitis B virus
HCV: hepatitis C virus
HIV: human immunodeficiency virus
HLA: human leukocyte antigens
HTLV-III: human T-lymphotropic virus-III
IFITM: interferon-induced transmembrane
IFITs: IFN-induced proteins with tetratricopeptide repeats
IFN-I: type I IFNs
IFNAR1: IFN-α receptor 1
IFNs: Interferons
IN: integrase
IP-10: IFNγ-inducible protein 10
IRF: IFN regulatory factor
IRIC: Institute for Research in Immunology and Cancer
ISGs: interferon stimulated genes
IV: Influenza virus
JAK: Janus kinases
LAV: lymphadenopathy associated virus
LCMV: lymphocytic choriomeningitis
LTR: long terminal repeat
MA: matrix
MAVS: mitochondrial antiviral-signaling protein
MDA-5: melanoma-differentiation-associated 5
MHCs: major histocompatibility complexes
MV: measles virus
Mx: myxovirus resistance
Nef: negative regulatory factor
NC: nucleocapsid
NK: natural killer

NF-κB: nuclear factor kappa B
OAS: oligoadenylate synthetase
OFP: orange fluorescent protein
PACT: PKR activator
PAMPs: pathogen-associated molecular patterns
PBMCs: peripheral blood mononuclear cells
PIC: pre-integration complex
PKR: protein kinase RNA activated
Pol: polymerase
PR: protease
pTEFb: positive transcription elongation factor b
Rev: regulator of virus expression
RT: reverse transcriptase
RTC: reverse transcription complex
RLI: RNAse L inhibitor
RVFV: Rift valley fever virus
SAMHD1: sterile alpha motif and HD-domain containing protein 1
SIV: simian immunodeficiency virus
SLFN: Schlafen
ss: single-stranded
STAT: signal transducers and activators of transcription
TAR: trans-activation response
Tat: trans-activator of transcription
TLRs: toll-like receptors
TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
TRIM: tripartite motif
TRBP: TAR RNA binding protein
Tsg101: tumor susceptibility gene 101
Vif: virion infectivity factor
Vpr: viral protein R
Vpu: viral protein U
Vpx: viral protein Xil
VSV: vesicular stomatitis virus
VV: vaccinia virus
WNV: West Nile virus

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Chapter 1: Introduction

1.1 Human Immunodeficiency Virus-1

1.1.1 Brief History of the Pandemic

The current epidemic of human immunodeficiency virus (HIV) infection is believed to have started in mid-to-late 1970s with 100 000 to 300 000 people infected before the first official recording from 1981. The initial report published in Morbidity and Mortality Weekly Report introduced a case of five young, previously healthy, gay men from Los Angeles infected with a rare lung *Pneumocystis carinii* pneumonia yeast-infection [1]. Presence of other infections strongly indicated pathology of the immune system of those men. In the following days, additional reports of clusters of rare and aggressive cancer, called Kaposi's sarcoma, among gay men in New York and California were received by US Centers for Disease Control and Prevention (CDC). By the end of the year, half of the affected people in those reports were no longer alive. In September of 1982, CDC uses the term acquired immune deficiency syndrome (AIDS) for the first time and defines it as: "a disease at least moderately predictive of a defect in cell-mediated immunity, occurring in a person with no known cause for diminished resistance to that disease" [2]. Initially, the large public believed that only certain groups of people, coined 4-H club, were at risk. This included: hemophiliacs, homosexual men, heroin users and Haitians. However, researchers quickly disproved this myth as cases of AIDS were reported in infants and observed in women that were in a sexual relationship with men that already had AIDS. This led to a hypothesis that an infectious agent might be responsible for the pathology and is transmitted via sexual contact and blood exposure [3].

In 1983, Dr. Françoise Barré-Sinoussi identified a retrovirus, Lymphadenopathy Associated Virus (LAV), which could be the cause of AIDS [4]. Dr. Robert Gallo successfully grew the AIDS virus in immune system cells [5]. The year after, he and his colleague identified retrovirus human T-lymphotropic virus-III (HTLV-III) as the causative agent, and showed that LAV is highly identical to HTLV-III. By 1985, AIDS has become a real global epidemic with at least one case being reported in every region of the world, as first antibody tests become available. The scientific community agrees to name the causative retrovirus as "Human Immunodeficiency Virus". Two years later, the first antiretroviral drug, zidovudine, was approved by the Food and Drug Administration (FDA) is USA [6]. However, it took an extra ten years and several millions of new infections before the highly active antiretroviral therapy combining several drugs became the new standard. This has brought a 60-80% decline in AIDS-related deaths and hospitalizations [7]. By the beginning of the 21st century, 33 million people were infected with HIV and 14 million have died since the start of the epidemic. Therefore, the United Nations has included the goal of reversing the spread of HIV into the Millennium Development Goals, which was achieved in 2015. With the same goal in mind, FDA approved pre-exposure prophylaxis for HIV-negative people that believe to be at risk in 2012. In 2017, half of the people living with HIV were on the antiretroviral treatment, which represents a huge achievement for the global community. Since the highly active antiretroviral therapy (HAART) proved to be an effective treatment, but does not eliminate the virus, researchers are currently searching for a functional or sterilizing cure against HIV [8, 9].

1.1.2 Origin and Diversity of HIV

It has become generally accepted and genetically demonstrated that HIV originated in the Democratic Republic of Congo with simian immunodeficiency viruses (SIV) jumping the cross-species barrier from primates to human hunters. Two morphologically similar, but antigenically different viruses are the cause of the current AIDS epidemic. The more predominant and virulent was coined HIV-1, and is the major focus of the scientific research, including this thesis; the less virulent and predominant was sequentially called HIV-2 [10, 11]. Genetic studies have revealed that HIV-1 stemmed from SIV residing in central chimpanzees, and HIV-2 from sooty mangabeys of West Africa [12, 13]. HIV-1 further consists of four lineages that are believed to be a result of four independent cross-species jumps. All four are known to cause the depletion of T-cells resulting in the development of AIDS. Group M, from the word major, was the first one to be characterized, has infected millions of people and represents the pandemic form of the viral infection [14]. It is further divided into genetic subgroups A-D, F-H, J and K, whose prevalence is geo-dependent. For instance, subgroup B is mainly found in North America, and subgroup C is dominant in South Africa. Moreover, genetic recombinant events between the subtypes are possible leading to a greater diversity [15]. Recombinant Group O, the outlier, discovered in 1990, represents less than 1% of the pandemic, is mostly restricted to the Cameroon and neighboring region [14]. Group N, from non-M and non-O, was discovered in 1998, and so far only 13 cases are known and all are from Cameroon [16]. The last group, P, was first identified from the blood of a Cameroonian woman living in France in 2009, and then found again in 2011, in the blood of Cameroonian man [17, 18]. Interestingly, the viral isolate of this group proved to be closer to the gorilla's SIV [18].

1.1.3 Structure and Genome

Together with SIVs, HIV is a lentivirus of *Retroviridae* family. The ability to cause a pandemic that has not been resolved in 40 years, as well as the great variation on genetic level summarized in previous sections are closely related to the life cycle of this virus family.

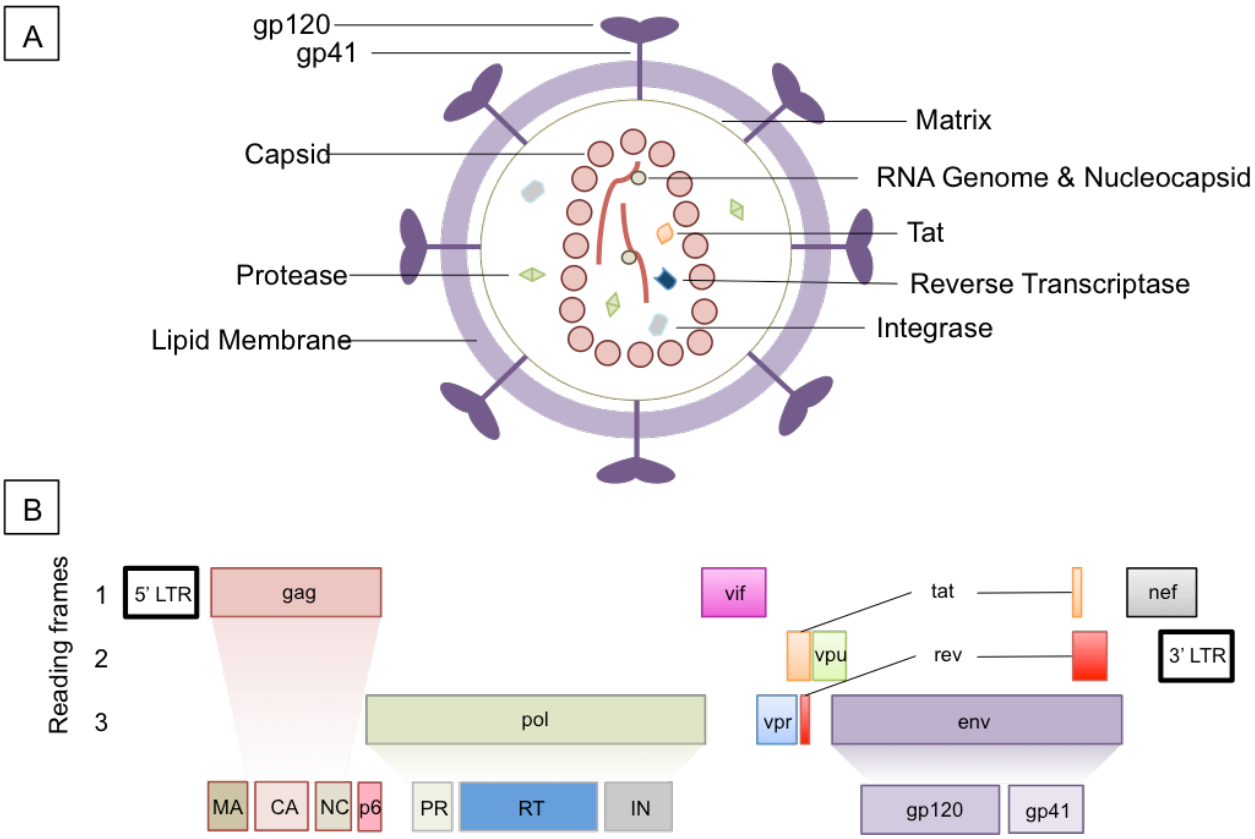

Figure 1.1: Structure of HIV-1 virus particle and genome organization. A. Structure of the viral particle with two genomic plus-strand RNAs and other viral elements. **B.** HIV-1 codes for nine genes existing in three reading frames: gag, pol, vif, vpr, tat, rev, vpu, env and nef. tat and rev are from spliced transcripts. Gag, Pol and Env polyproteins are spliced later in the viral lifecycle into the indicated proteins (adapted from www.scistyle.com)

The viral genome consists of 16 proteins. Three of which are common in all retroviral genomes: group antigens (Gag), polymerase (Pol) and envelope (Env), and are the structural and enzymatic polyproteins. A second set consists of two regulatory proteins:

trans-activator of transcription (Tat) and regulator of virus expression (Rev). The others are called accessory proteins, as they are dispensable for HIV replication in some cell lines and consist of virion infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu) and negative regulatory factor (Nef) (Figure 1.1).

1.1.4 Viral Replication Cycle

1.1.4.1 Entry Phase

The first phase of the viral replication is the entry, which starts with the viral adhesion to the host cell and ends at the delivery of the viral contents into the host cell cytoplasm. Circulating virions can interact with a target cell via unspecific attachment factors that are not essential for the infection, but greatly increase its efficiency [19-21]. The absolute requirement for virus entry is the binding of viral glycoprotein gp120 with the CD4 receptor on the host cells [22]. This acts as a signal for the rearrangement of variable loops of gp120 revealing binding sites for the co-receptors [23, 24]. Co-receptor specificity is another potential classification of HIV-1. R5 HIV-1 uses CCR5, X4 viruses use CXCR4, and R5X4 virus can use both. Interestingly, R5 viruses are more cytopathic, lead to progression to AIDS much faster, and are responsible for the majority of infections [25, 26]. Although controversial and often disputed, the natural restriction against the transmission of X4 viruses is attributed to a combination of physical and biochemical barriers such as the ionic state of the vaginal lumen, co-receptors availability and sensitivity to β-defensins [27-30]. Furthermore, progression to late stages of infection is often preceded by a co-receptor switch, from R5 to X4 and R5X4 [31]. The intermediate step of viral entry is the sliding of the HIV-1 particle to the site of membrane fusion using the host machinery [32, 33]. Co-receptors engagement triggers a series of conformational changes in the Env revealing the

hydrophobic gp41 fusion peptide, which then folds into a six-helix bundle bringing the cell and viral membrane close enough to form a pore [34, 35]. Membranes' fusion releases HIV-1 capsid into the cytoplasm and signifies the beginning of the next viral cycle phase.

1.1.4.2 Uncoating and Reverse Transcription

There are several models as to what happens following the fusion. The general agreement is that the viral capsid disassembles, a reverse transcription complex (RTC) is formed and transcribes viral RNA genome into complementary DNA (cDNA), but the exact timing of these events is unclear [36]. The first model stipulates that the capsid is degraded right after the fusion of the cell membrane with Env [37, 38]. The change of the environment is believed to trigger the assembly of RTC [39]. The argument behind this model is the low amount of capsid protein within intracellular HIV-1 complexes that is observable by transmission electron microscopy [40-42]. According to the second model, the capsid remains intact for some time post-entry, and then undergoes stepwise changes and disassembly in reaction to cell-host elements. Concurrently, transport towards the nucleus and the reverse transcription are occurring. Studies reporting a broad range of different sizes and shapes of HIV-1 capsid in the cytoplasm support this hypothesis [43, 44]. The third model argues that the capsid remains untouched until it reaches the nuclear membrane. The trigger for uncoating is the completion of reverse transcription, which is possible due to selective permeability of the capsid for small macromolecules; thus, regulating the stoichiometry of the transcription step [36, 45].

The uncoating process is coupled with the reverse transcription step. This step of copying RNA into DNA is characteristic of retroviruses and is performed by the reverse transcriptase (RT) discovered in the 1970s [42, 46]. There are two enzymatic activities that

are required for a functional RT: 1) a DNA polymerase capable of copying both RNA and DNA and 2) an RNase activity that degrades RNA exclusively in RNA-DNA duplex [47]. In HIV-1, RT is a heterodimer of a full length and truncated inactive enzyme (p66 and p51 respectively) [48]. Genomic plus-stranded RNA of HIV-1 acts as a template for reverse transcription and a host tRNA lysine 3, incorporated into the virion, acts as a primer and binds at primer binding site around 180 nucleotides from the 5' end of genomic RNA [49]. DNA is synthesized, and the RNA in the dual DNA-RNA complex is degraded. Both ends of the viral RNA are actually repeats regions, called R. Once the DNA synthesis reaches the end of 5', the newly synthesized minus-strand DNA is transferred to the R at the 3' end of genomic RNA [50, 51]. It then becomes a primer for the synthesis of the full-length minus-strand DNA of HIV-1 genome. As the synthesis goes, the RNase H cleavage of RNA does so too, except for a purine-rich sequence in the RNA genome, known as polypurine tract, that is resistant to the RNase activity. HIV-1 has two of those tracts, one in the center and one in the 3'end. The later acts as a primer for the synthesis of 5' end of the plus-strand DNA, that in its own, following a second strand-transfer, will act as a primer for the full-length, plus-strand, genomic HIV-1 DNA. The end result of this replication cycle step is a DNA product that is longer than the original RNA genome with identical ends that are called long terminal repeats (LTR) that will play an important role in the next phase, integration [47].

1.1.4.3 Integration, Transcription and Translation

Following their synthesis, LTR's ends are processed by the viral integrase (IN) to an invariant cytosine-adenine dinucleotide with 3'-hydroxyl group [52]. The formation of this nucleophile group marks the transition of RTC into the pre-integration complex (PIC). PIC is known to consist of several viral and cellular proteins, such as Vpr and p75/LEDGF, which

will interact with nuclear importins for successful translocation into the cell nucleus [53]. Once there, IN cuts the chromosomal DNA in a staggered fashion while the 3'-hydroxyl group of viral DNA attacks the opened 5'-phosphates of the host DNA; thus, completing a strand transfer step [54]. Host cell enzymes will then join the viral DNA with the chromosomal DNA via non-homologous end joining process, resulting in an integrated provirus that can be used for the production of new virus [55]. The site of integration is not random and there is a preference for regions with transcriptionally active genes [56].

The observed preference for integration may be partially explained by the fact that HIV-1 provirus requires cellular factors, such as nuclear factor kappa B (NF-κB), Sp1, the TATA box binding protein and RNA polymerase II, which binds to the 5' end LTR. However, this complex is only capable of producing extremely low levels of viral transcripts that are then spliced, exported and translated. Three HIV-1 proteins are produced in the early stages of transcription from the doubly spliced viral RNAs: Rev, Nef and Tat [57]. The later plays a crucial role in the following stage of viral life cycle by enhancing viral replication. It does so by binding to the positive transcription elongation factor (pTEFb) in the nucleus and brings it to the TAR hairpin, which is present at the 5' end of all new viral RNA transcripts [58, 59]. The kinase unit of pTEFb, cyclin-dependent kinase 9, phosphorylates the C-terminal domain of RNA polymerase II; thus, stimulating transcriptional initiation and elongation [60-62]. In this late stage of transcription, Rev binds to Rev response element present in full-length and singly-spliced transcripts that were too bulky to leave the nucleus on their own and facilitates their export via the CRM1/Exportin 1 pathway [63]. The unspliced 9kB RNA codes for Gag and Gag-Pol, as the singly spliced RNAs code for Env, Vif, Vpr and Vpu [57]. The viral mRNAs are mostly translated by cellular cap-dependent

mechanisms; however, a functional internal ribosome entry site has been also identified in the genomic RNA [64].

1.1.4.4 Viral Assembly and Maturation

HIV-1 virion assembly is mediated by the Gag polyprotein and can assemble into immature viral particles on its own [65]. The polyprotein is composed of three polypeptides matrix (MA), CA and nucleocapsid (NC), as well as three shorter peptides SP1, SP2 and p6 [66]. The aforementioned immature particle is made of roughly 2500 radially oriented Gag and 125 Gag-Pol proteins, with the N-terminal MA facing outward and the C-terminal p6 facing the interior [67]. MA becomes bound to the Env components gp120 and gp41, and the CA domain of one Gag polyprotein binds to the adjacent SP1 of another Gag [68, 69]. NC is responsible for the HIV-1 genome packaging, two positive-strand genomic RNAs interact with it at the Ψ element located near their 5' end [70]. As the multimerization of Gag is happening, p6 recruits the endosomal sorting complexes required for transports, which eventually leads to the budding of the growing spherical lettuce [71]. As it gets released, the protease (PR) domain of Gag-Pol gets activated and cleaves Gag at five positions. Cleavage rate varies a lot from one site to another, leading to a specific sequence of maturation. The proposed model is that the separation of NC-p6 from Gag condenses viral RNA, following is the cut at the MA-CA and SP2-p6 junctions that separates functional domains and forms ribonucleoprotein complex, and finally the cleavage of S1 and CA leads to the formation of a mature viral core [72]. Gag-Pol is cleaved into PR, RT and IN, and this all together frames a mature and infectious viral particle, and concludes HIV-1 replication cycle [73].

1.1.5 Pathology of HIV-1 Infection

From a clinical perspective, HIV-1 infection is divided into three phases depending on plasma viral levels and $CD4^+$ T cell count: 1) an initial/primary acute infection, 2) asymptomatic period or chronic infection and 3) AIDS.

1.1.5.1 Acute Infection

Most common symptoms reported during a primary HIV-1 infection are fever, sore throat, fatigue, rash and myalgia that on average last 14 days [74, 75]. Those nonspecific symptoms make it difficult to correctly diagnose the nature of infection without additional biochemical tests. It is important to understand that the transmission rate of HIV-1 is relatively low with reported ranges of 0.1% to a maximum of 10% for heterosexual exposures [76]. Moreover, gene analyses identified that a productive clinical infection via sexual transmission is a result of a single virus in 77% of cases [77]. During this early stage, HIV-1 undergoes high rate of replication in gut-associated lymphoid tissues due to the absence of an effective immune response and then spreads to other lymph nodes [78]. The plasma viremia can reach as high as 10 million copies per milliliter [79]. Consequentially, $CD4^+$ T cells are depleted by almost a half [80]. Innate immunity and adaptive antibody responses fail to control the virus at this stage, and it is believed that $CD8^+$ T cells take charge of the protection in this time window [81].

1.1.5.2 Chronic Infection

From week 12 post-infection, the first functional neutralizing antibodies appear, marking the beginning of the chronic phase. The viral levels stabilize and reach a set point, which represents the equilibrium between viral replication/mutation and anti-viral immune response[82]. In other words, the error-prone nature of RT, $3x10^{-5}$ per base,

contributes to the generation of viral clones that are resistant to the existent antibodies (same is true for the HAART and vaccine development), forcing the immune system to re-adapt and generate a new one [82, 83]. In the meantime, $CD4^+$ T cells recover although never reaching the same number as during pre-infection. Moreover, the set point correlates with the disease progression and can act as a predictor of future development. A person can be in chronic infection for years. Continuous immune activation by the virus leads to deregulation in other immune cells such as B and natural killer (NK) cells, and finally leading to the breakdown of the immune system, AIDS [84]. There is however a certain percentage of people who are naturally able to suppress HIV-1 replication below the detectable limit and are called "elite controllers", but the exact "controlling" element remains undefined [85, 86].

1.1.5.3 AIDS

A diagnosis of AIDS is given to a HIV positive individual when he/she has a $CD4^+$ cell count less than 200 cells/μL, or $CD4^+$ cells account for less than 14% of lymphocytes, or has been diagnosed with one or more AIDS-defining illnesses. Those illnesses range from opportunistic infections that are normally harmless to rare cancers of natural and/or viral origins that eventually lead to death [87].

1.1.6 HIV-1 Therapy

As of the writing of this thesis, there is only a single person that has been known to achieve a functional cure against HIV-1, Timothy Brown. He developed acute myeloid leukemia, while being HIV-1 positive. Treatment against this form of cancer is to receive allogeneic hematopoietic stem cells transplant. The transplant he received was special in that it contained a homozygous CCR5Δ32/Δ32 deletion, a mutation that makes cells

resistant to infection by HIV-1 R5 viruses [88]. More importantly, he remains without the viral rebound proving the concept for a functional cure against HIV-1 [89]. This had given a proof of concept to the idea of using HIV-1 resistant cell transplant, leading to several studies on gene therapies. Most of them revolve around modifying the CCR5 receptor, targeting viral RNA via RNA interference pathway and introducing decoy substrate. Most have shown very modest results in the past, and some are still in clinical studies [90-96].

40 years of HIV-1 research has not only given us knowledge about the viral biology, but also allowed to develop more than 25 antiretroviral drugs targeting different steps of the virus replication cycle. The different classes of antiretrovirals are entry, RT, IN and PR inhibitors (Figure 1.2). In March of 2018, FDA approved a novel class of drug, called post-attachment inhibitor, for multidrug-resistant HIV-1 cases. The drug is special in a way that it targets CD4 receptor and not a viral component for the first time [97]. In the early 1990s, the drugs were given as mono-therapy, but consistent viral resistance has pushed into using a cocktail of drugs [98]. Mathematical models predict that if at least three mutations are necessary to provide drug-resistance, then the virus fitness will be severely impacted [98, 99]. The combination therapy, also known as HAART, suppresses viral replication, reduces viral load below the detection limit, and as the end result decreases morbidity and mortality associated with AIDS. With proper adherence to the prescription, viral replication remains undetectable lifelong for most patients; therefore, increasing the life expectancy of HIV positive individuals [99-101].

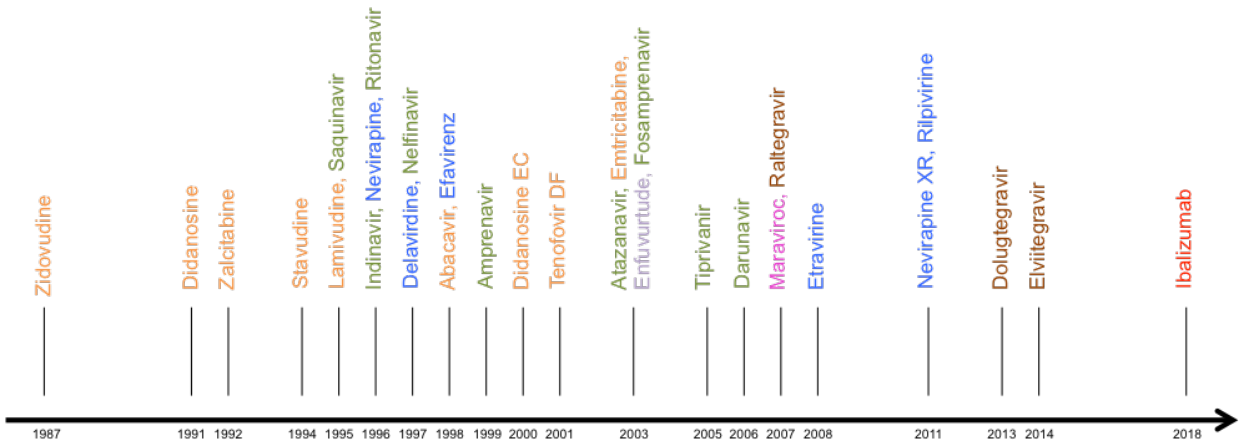

Figure 1.2: Timeline of FDA approval of antiretroviral drugs from 1987 to present. Nucleoside reverse transcriptase inhibitors are in orange, non-nucleoside reverse transcriptase inhibitors are in blue, protease inhibitors are in green, entry inhibitors are purple, red and pink (mechanism of action and targets are different for each color), and intergrase inhibitor are brown.

In 2010, pre-exposure prophylaxis trial of two antiretrovirals has shown a reduction of HIV-1 transmission by 44% in homosexual community [102]. Other studies have also shown effectiveness of prophylactic antiretroviral gels and drugs, however, it was demonstrated that adherence was the influencing and the deciding factor of the outcome, especially in some of the communities [103, 104].

1.2 Innate Immunity against HIV-1

1.2.1 Interferon pathway in the cell

Interferons (IFNs) are cytokines, which belong to the antiviral innate immune system that eukaryotic cells developed through evolution to survive viral infections. IFNs are classified into three groups (types I, II and III) based on their structure, complementary receptors and biological activity [105]. Production of type I IFNs (IFN-I) is a result of interaction of pathogen-associated molecular patterns (PAMPs) with germline-encoded cellular receptors called Pattern Recognition Receptors. Viral PAMPs include single-

stranded (ss) and double-stranded (ds) DNA or RNA molecules, as well as viral glycoproteins [106, 107]. Their detection by Toll-like Receptors (TLRs), nucleotide oligomerization domain proteins, C-type lectin receptors and retinoic acid-inducible gene I (RIG-I)-like receptors leads to the release of soluble IFNs [108, 109]. Once in the extracellular space, secreted IFNs interact with their corresponding cell surface receptors in either an autocrine or paracrine manner. After binding to IFN-α receptor (IFNAR1), IFN-I immediately activates the Janus kinases/signal transducers and activators of transcription (JAK/STAT) signalling pathway, which results in the formation of a trimer composed of IFN regulatory factor (IRF)-9 and the phosphorylated proteins STAT1 and STAT2 [110, 111]. This complex translocates to the nucleus and binds to the IFN Stimulated Response element DNA on responsive promoters to stimulate the induction of hundreds of IFN-stimulated genes (ISGs)(Figure 1.3) [112, 113]. This sets up an environment that prevents viral replication and spread, subsequently leading to adaptive immunity [114, 115].

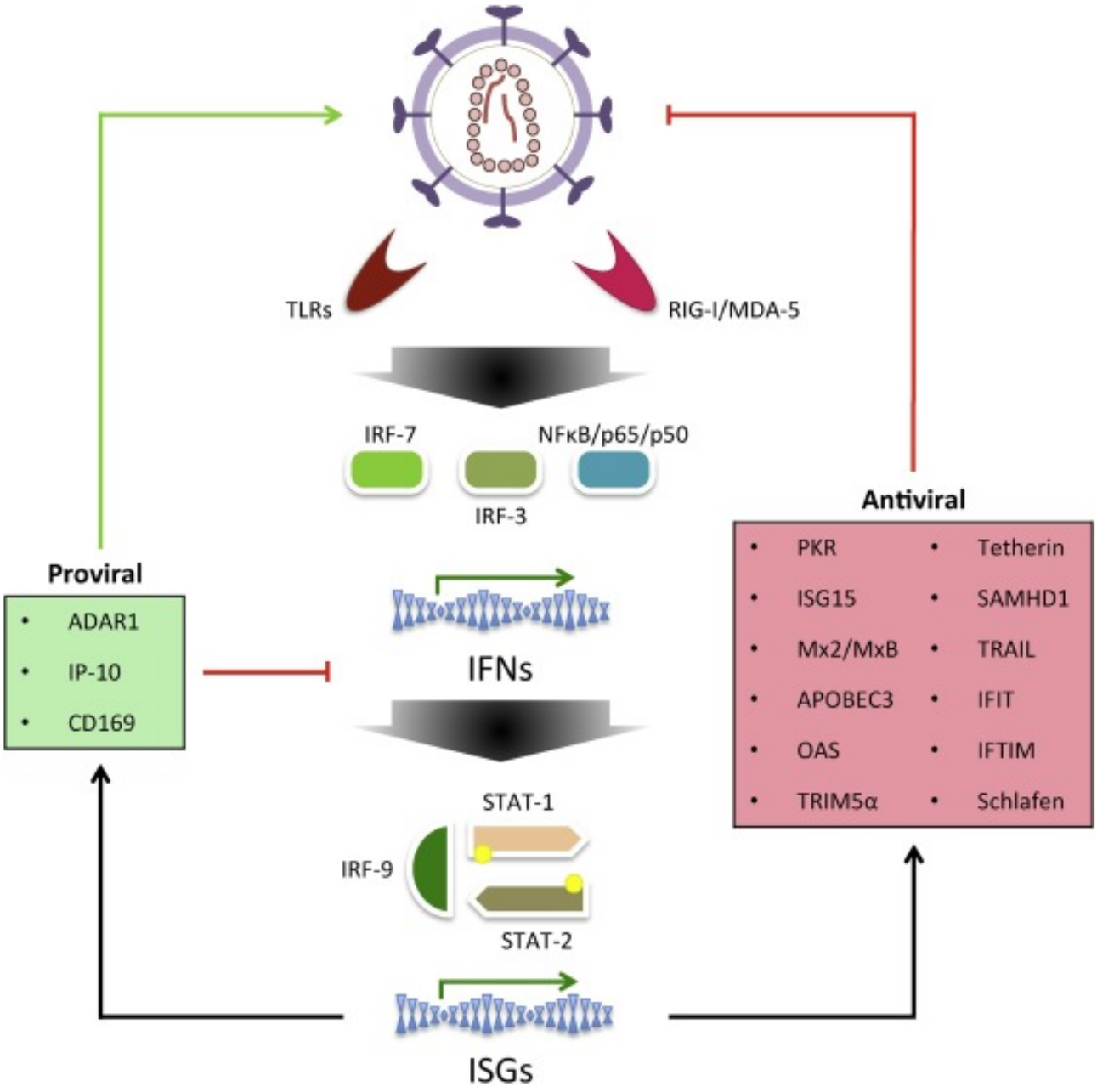

Figure 1.3: From PAMPs to ISGs, the induction of antiviral and proviral molecules by the innate immune response to an HIV-1 infection. Toll-like and RIG-I receptors activate transcription factors, IRF-7,-3 or IκB, upon sensing viral antigens. Newly induced type I IFNs trigger the assembly of STAT-1/2 and IRF-9 complex which then promotes the expression of Interferon Stimulated Genes. Antiviral ISGs inhibit HIV-1 propagation at multiple steps of viral life cycle. Proviral ISGs can either inhibit the production of IFNs in infected cells or directly enhance the virus.

1.2.2 Interferon is needed for viral clearance

IFN treatment of virally infected cells can clear the infection through multiple mechanisms [116]. IFN inhibits HIV-1 replication in primary macrophages and lymphocytic T cells, and induces the production of ISGs, blocking several steps, including entry, reverse transcription, protein synthesis, virion assembly and release [117-119]. Several IFN types are used therapeutically against hepatitis C and B viruses (HCV and HBV), Kaposi's sarcoma-associated herpes virus and papillomaviruses [120, 121]. Approaches using combination therapy with pegylated IFN in HIV-1-infected patients with co-infections and IFN treatment during interruption of antiretroviral therapy have also been assessed and have shown lower viral rebounds in the patients receiving IFN [122, 123]. Additional evidence of IFN's role comes from the increased influenza virus (IV) replication, dissemination and lethality in mice deleted of IFNAR1or STAT1 genes [124, 125]. For individuals with reduced production of IFN-I and –III in myeloid and peripheral dendritic cells, a seasonal flu can be life-threatening [126]. In the same manner, mice lacking the IFNAR1 were acutely susceptible to West Nile virus (WNV), lymphocytic choriomeningitis virus, vesicular stomatitis virus (VSV) and vaccinia virus (VV) [127-130]. So far, due to viral and cellular counteractions of the ISGs, IFN cannot clear HIV-1 infection, but therapeutic administration of IFNα in infected patients has shown a significant reduction in plasma HIV-1 viral load [131, 132].

1.2.3 IFN can be detrimental in the chronic phase

Production of IFNs is generally very prominent at the beginning of the infection and goes down in the following days regardless of viral clearance to prevent excessive immunopathology. Indeed, when they persist during the chronic phase of infection, IFNs

can be detrimental to the cell [115]. During chronic SIV infection, its natural host, the African green monkey, exhibits low IFN-I levels and does not progress to AIDS, while the Rhesus macaque, the non-natural host, develops AIDS and manifests high levels of IFN-I signaling signatures [133, 134]. Equivalently, chronic HIV-1 infection is characterized by elevated levels of pro-inflammatory cytokines, including IFN-I and is correlated to immune activation and a poor prognosis for AIDS progression [135, 136]. Therefore, these deleterious effects, mainly in the chronic phase, preclude long-term therapeutic usage of IFNs [137, 138].

Although IFN response proves to be crucial for the initial and subsequent control of a viral infection, many viruses are able to evade it and successfully propagate in the host. This can be due to viral countermeasures that affect IFN production, signaling or the activity of ISGs [120, 139]. HIV-1 is an example of such viruses and has evolved to develop ways of countering different ISGs aimed at it [140]. In the following sections, we will discuss the most studied ISGs that are known to target HIV-1. Yet, the main focus will be on the emerging literature arguing that some ISGs can actually contribute to the HIV-1 infection, more specifically the Adenosine deaminase acting on RNA (ADAR) 1.

1.3 ISGs and HIV-1

ISGs are produced following IFN signaling as shown in Figure 1. Although originally thought to be only antiviral proteins, some were shown to be restriction factors with or without known evasion mechanisms, while others have proapoptotic or proviral functions.

1.3.1 ISGs as restriction factors against HIV-1 and viral evasion

Restriction factors were originally defined as intrinsic proteins that counteract viral replication and have been positively selected through evolution to counteract viral evasion

mechanisms [141]. Further analysis has shown that restriction factors defined for HIV-1 are ISGs and therefore part of the innate immunity.

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) family of proteins restricts HIV-1 replication and APOBEC3G is packaged into the assembling virion in the absence of the Vif [142-144]. Once the virion reaches its target cell, APOBEC3G deaminates up to 10% of cytidine residues in the nascent ss negative-strand cDNA resulting in guanosine-to-adenosine hypermutation and loss of genetic integrity [145, 146]. Vif binds to APOBEC3G and recruits it to a cellular ubiquitin ligase complex for subsequent proteasomal degradation [147].

Tetherin/Bst2 was identified by looking at the HIV-1 accessory protein, Vpu, requirement for efficient virion release from some cell lines upon IFN treatment [148, 149]. In the absence of Vpu, HIV-1 particles get tethered at the surface of the producing cell, accumulate in endosomes and are blocked from being released into extracellular space [150]. Vpu recruits ubiquitin ligase, thus, mediating surface downregulation and degradation of tetherin but also displaces tetherin from the site of viral assembly [151-153]. The HIV-2 Env and the HIV-1 Group M Nef also antagonize Tetherin/Bst2 [154, 155].

Sterile alpha motif and HD-domain containing protein 1 (SAMHD1) was identified as the restriction factor targeted by HIV-2 Viral Protein X (Vpx) in primary dendritic cells [156, 157]. SAMHD1 acts as a deoxynucleoside triphosphate triphosphohydrolase that converts dNTPs into the deoxynucleoside and inorganic triphosphate upon stimulation by dGTP or GTP [158, 159], which protects the cell from viral replication and cancer development by controlling the DNA replication cycle [160, 161]. Vpx counteracts this by

interacting with SAMHD1 and recruiting E3 ubiquitin ligase leading to proteasomal degradation [157].

1.3.2 ISGs as restriction factors against HIV-1 with undefined evasion mechanisms

Many ISGs restrict the replication of viruses and can have broad or specific antiviral activity. Although their mechanism of action has sometimes been elucidated, the way viruses counteract or bypass their activity remains to be determined [162].

The Myxovirus resistance (Mx)2/MxB is a GTPase located at the nuclear membrane that prevents HIV-1 cDNA from entering the nucleus [163, 164]. The mechanism of action of Mx2/MxB and its modulation involves interacting with HIV-1 capsid and inhibiting nuclear import of viral complexes [165-167].

Major histocompatibility complexes (MHCs; also called human leukocyte antigens, HLA in humans) are cell surface glycoproteins that exhibit antiviral activity by presenting virus-derived peptides at the cell surface for recognition by cytotoxic T lymphocytes. The HIV-1 Nef protein is able to decrease their presence at the cell surface by internalization through endocytosis [168]. This process is inefficient and Nef downregulates approximately 20% of HLA-A, whereas only a specific Nef variant downregulates HLA-B [169]. In contrast, HLA-C is suppressed by Vpu [170].

ISG15, a ubiquitin-like protein, was first described in a study characterizing proteins induced by IFN-I [171]. Further research into IFN response against HIV-1 revealed that ISG15 prevents release of new virions without affecting HIV-1 protein synthesis. The first suggested mechanism was that ISG15 inhibited ubiquination of Gag and tumor susceptibility gene 101 (Tsg101), a regulator of vesicular trafficking process, thus, preventing their interaction and subsequent release of HIV-1 viral particles into the

extracellular space [172]. ISG15 also conjugates to another protein of the endosomal sorting complex, CHMP5, and inhibits HIV-1 at the budding step [173]. Later, ISG15 was shown to be ISGylated to Gag by cellular protein, HERC5, leading to a block of HIV-1 particle assembly at the plasma membrane [174]. There is no unique viral protein that directly targets ISG15, but rather, Vpu inhibits NF-κB, an inducible transcription factor that is involved in the regulation of numerous ISGs, including ISG15 [175, 176].

IFN-induced proteins with tetratricopeptide repeats (IFITs) are known to suppress viral replication of VSV, IV, WNV and Rift valley fever virus (RVFV) and were found to be overexpressed in HIV-infected macrophages [177, 178]. A knockdown of IFIT1 to 3 in the monocyte-derived macrophages increases HIV-1 production by 50%. The exact mechanism and step at which IFITs restricts HIV-1 is currently unknown [179].

The interferon-induced transmembrane (IFITM) proteins are similarly known to restrict multiple viruses, including HIV-1. They inhibit HIV-1 replication by interfering with virus entry [180]. IFITM proteins impair viral fusion by antagonizing the envelope glycoprotein and thus reducing virion infectivity [181-183].

Treatment of human foreskin fibroblasts with IFN-I, exogenous dsRNA and DNA induces the expression of Schlafen (SLFN) genes, with SLFN11 being one of the most prominent. Further testing has shown that SLFN11 is able to inhibit HIV-1 production from HEK 293 T cells via selective suppression of viral protein expression. This selectivity stems from SLFN11's ability to exploit viral codon bias towards A/T nucleotides compared to the cellular preferences and counteracting HIV-1 influence over the tRNAs [184]. Moreover, HIV-1 elite controllers revealed elevated expression of SLFN11 in their $CD4^+$ cells [185].

1.3.3 ISGs as restriction factors against HIV-1 with cellular evasion mechanisms

The dsRNA-dependent protein kinase R (PKR) is one of the most studied ISGs for its role in the resistance to viral infections. PKR is an IFN-I inducible serine/threonine protein kinase that is activated by dsRNA. It was first discovered for its translation blockage in cells infected by VV and treated with IFN [186]. Once activated by phosphorylation, PKR phosphorylates the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α), which induces a block in protein translation of cellular and viral RNA. PKR is activated at the beginning of HIV-1 infection and is able to stop virus protein production and replication [187-189]. HIV-1 has evolved with countermeasures that inhibit PKR. They include the viral Tat protein, but mainly, cellular mechanisms involving the Trans-activation response (TAR) RNA Binding Protein (TRBP), ADAR1 and the PKR Activator (PACT) [190-192]. Detailed mechanisms are described in section 1.4.

The enzyme oligoadenylate synthetase (OAS) binds to dsRNA, which activates its function. Once activated, OAS produces 2'5' oligo adenylates (2-5A) from cellular ATP. 2-5A then activates the latent RNAse L, which subsequently degrades viral and cellular RNA, thereby preventing RNA translation [193, 194]. The products of RNAse L in turn activate RIG-I, which further amplifies IFN production [195]. HIV-1 has evolved to induce the production of the RNAse L inhibitor (RLI also called HP68 or ABCE1), which regulates the viral capsid assembly [196, 197].

1.3.4 ISGs as restriction factors with cross-species specificities

A screen of rhesus macaque genes for proteins restricting HIV-1 infection in human cells identified the tripartite motif (TRIM) 5α and TRIMCyp proteins that have similar activities in other mammalian species [198-200]. Indeed, TRIM5α proteins proved to be poor

inhibitors of retroviruses in their natural hosts; for instance, the human homolog is ineffective against HIV-1, but is quite effective against viruses in other species representing a barrier for cross-species transmission [201]. TRIM family proteins bind to HIV-1 capsid, which must occur within 15-30 minutes of viral entry for inhibition to be effective [202]. This interaction results in a pre-mature capsid fragmentation that disrupts the reverse transcriptase complex and blocks that step of the viral cycle [203]. The mechanism is dependent on microtubules and dynein motor complexes [204].

1.3.5 ISGs with proapoptotic functions

Although most ISGs are beneficial for the host with antiviral activities, some have detrimental roles through a proapoptotic function, which likely contributes to the chronic inflammation during HIV-1 infection. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce cell-death in virally infected or malignant cells via binding with its receptors R1 and R2 [205]. In the case of HIV-1 infection, exposure to TRAIL leads to the depletion of B cells and uninfected $CD4^+$ T-cells, while infected cells are resistant [206-208]. This was shown despite the initial finding that *ex vivo* treatment of HIV-infected cells with high concentration of TRAIL results in a decreased reservoir of HIV-positive cells [209]. The conferred resistance was found to be mediated by an IFN-inducible isoform of TRAIL, called TRAILshort, that can differentially bind to the TRAIL receptors, preventing TRAIL-induced apoptosis; thus, conferring protection to virus-infected and bystander cells [210].

The overexpression and activation of PKR induce apoptosis mediated by the phosphorylation of eIF2α [211, 212]. PKR also induces neuroinflammation through the induction of proinflammatory cytokines such as NFκB, tumor necrosis factor α and interleukin-1β [213, 214]. PKR contributes to the activation of the inflammasome complex

by releasing the high mobility group box 1 complex and by interaction with its activator PACT [215, 216]. In the context of HIV-1 infection, PKR expression is increased in the acute phase of viral infection [217] and its activation can lead to neuronal apoptosis [218]. In contrast, lentivirus-mediated overexpression of PKR in hematopoietic stem cells does not induce apoptosis and protects the transduced cells from HIV-1 infection suggesting that PKR-induced apoptosis is cell-specific [219].

2'5' OAS induces apoptosis mediated by several viruses [220-222]. During HIV infection, OAS increase in the brain correlates with HIV-related neurological disorders suggesting that it contributes to chronic inflammation and neurocognitive impairment [223]. The relationship between induction of apoptosis and pathogenicity in HIV-infected patients remains to be determined.

1.3.6 ISGs that have proviral roles

Although ISGs have mainly antiviral activities, some of them were found to promote viral replication opening the idea of some antagonistic functions within the ISG family (Figure 1.3).

IFNγ-inducible protein 10 (IP-10), also known as CXCL10, was first isolated from a lymphoma cell line treated with recombinant IFN-γ [224]. It binds to CXCR3, promoting the chemotactic activity of cells expressing the receptor such as lymphocytes, NK, dendritic and macrophage cells. This ISG induces apoptosis, regulates cell growth and proliferation in infectious and inflammatory diseases, and in cancer [225]. Furthermore, IP-10 levels in plasma have been correlated with HIV-1 rapid disease progression [226]. There was an observable drop of IFN-γ production and a decrease in proliferative capacity in cells treated with physiological levels of IP-10, representing a negative feedback loop. Blocking IP-10

with a neutralizing antibody led to a significant increase of proliferation and IFN-γ production from peripheral blood mononuclear cells (PBMCs) and NK cells [227]. This inhibition also increased degranulation and production of perforin from $CD8^+$ cells important for its antiviral function [228].

CD169/Siglec-1 is an ISG induced by IFN-I in myeloid cells. It acts as an attachment factor of HIV-1 virions to dendritic cells via cell-derived glycosphingolipid (GM3) on viral surface [229]. HIV-1 is then incorporated into non-lysosomal compartments which act as barriers against circulating neutralizing antibodies and other phagocytic cells, subsequently leading to the DC-mediated trans-infection pathway [230]. Moreover, CD169 enhanced fusion and entry of virus particles into the $CD4^+$ T-cells even in the presence of IFN-I, highlighting its proviral role [231].

ADAR1 is an ISG that mediates adenosine deamination to change it into inosine. Inosine is then recognized as a guanosine by the translational machinery, which changes the amino acid sequence and increases protein diversity [232]. ADAR1 was originally thought to be antiviral but several studies showed that it increases the replication of several viruses including VSV, measles virus (MV) and HIV-1 [233, 234]. The mechanism of this increase was attributed to RNA editing and PKR inhibition, but additional mechanisms were recently identified and explained in section 1.5.

1.4 PKR: an ISG blocking HIV-1 replication with viral and cellular evasion mechanisms

PKR is a 68kDa protein containing two dsRNA binding domains (dsRBD) at its N-terminal region and a C-terminal catalytic kinase domain [235, 236]. PKR is constitutively expressed in the cells in its monomeric inactive form. Binding to dsRNA longer than thirty nucleotides at low concentration promotes formation of homodimers that promote

autophosphorylation at residue Thr446 and Thr451 therefore augmenting its catalytic activity [237, 238]. Activated PKR then phosphorylates several substrates, among them eIF2α at its residue Ser51, which stops the formation of the translation initiation complex eIF2-GTP-Met-tRNAi necessary for the initiation of protein synthesis. This leads to both viral and host inhibition of global protein synthesis [239, 240].

1.4.1 PKR activation during HIV-1 replication

PKR is also a dsRNA sensor in response to viral infection [241]. During HIV-1 infection, the TAR RNA, a structured stem-bulge-loop, is the main activator of PKR in the early stage [242]. PKR is activated transiently at the beginning of HIV-1 infection of lymphocytic Jurkat T cells as well as in PBMCs and is inhibited during active HIV replication [187, 190]. This activation coincides with the presence of low levels of HIV-1 mRNAs, which all have the TAR RNA at their 5' ends. TAR RNA binds PKR and promotes its dimerization and activation [243]. HIV-1 has evolved with different strategies that counteract PKR activation. Among them is the viral protein Tat along with several positively acting cellular factors like TRBP, ADAR1 that counteract PKR activation and PACT that reverses its function in HIV-1 expressing cells [190, 192, 244].

1.4.2 Viral evasion: PKR inhibition by Tat

The HIV-1 Tat protein interacts directly with PKR both *in vitro* and in the cells and modulates its inhibition [245-247]. Tat inhibits the activation of the kinase both by sequestering dsRNA and independently of RNA by direct binding. The amino acid sequence of Tat required for PKR interaction consists of residues 40 to 58 including the arginine-rich region essential for Tat function and for TAR RNA binding. The interaction between Tat and TAR during HIV-1 replication contributes to increasing the formation of a complex that

inhibits PKR activation containing ADAR1 and PACT [248]. Tat also acts as a pseudosubstrate for PKR as it shares a sequence homology with eIF2α [190, 242]. However, the sequence homology is stronger with K3L from VV and positive selection of PKR was demonstrated with K3L [249, 250]. These analyses suggest that PKR is a restriction factor that has been selected by Poxviruses through evolution and has maintained its activity with HIV-1 when it emerged [192].

1.4.3 Cellular evasion: PKR inhibition by TRBP

TRBP is a cellular dsRBP protein that was first discovered based on its capacity to bind HIV-1 TAR RNA [251-253]. TRBP consists of two dsRBDs with a KR-helix motif within dsRBD2, which is essential for TAR RNA binding [254-256]. These dsRBDs also contribute to protein-protein interactions with PKR and PACT. TRBP has a third domain called Medipal, which mediates protein-protein interactions for Merlin, Dicer and PACT [257]. In the context of HIV-1, TRBP was the first cellular protein to be characterized for its anti-PKR effect. The inhibition of PKR by TRBP is due to direct binding, sequestration of dsRNA as well as by interaction and inhibition of PACT [258, 259]. TRBP rescues HIV expression and replication in the cells where the virus was suppressed by overexpressed PKR [189, 258]. Cells like human astrocytes that express low levels of TRBP due to a weak promoter show an increased PKR activation either when transfected by PACT or with a molecular clone of HIV-1 [259-262]. In astrocytes, activated PKR is correlated with low translation of HIV-1 mRNA and low viral replication [260, 263, 264].

1.4.4 Cellular evasion: PKR inhibition by ADAR1

The activity of ADAR1 to increase viral replication was first identified in the context of VSV where the protein augments host susceptibility to the infection by counteracting

PKR activation [265]. The expression of ADAR1 also enhances the replication of MV by triggering PKR activation and preventing MV-induced apoptosis [266]. An additional proof of ADAR1 inhibition of PKR came from ADAR1 knocked-down cells that increased PKR phosphorylation, decreased VSV and MV replication and increased apoptosis [266, 267]. During MV infection, ADAR1 suppresses PKR activation and subsequent stress granule formation [266, 268]. Furthermore, ADAR1 increases the replication of human T cell lymphotropic viruses I and II by inhibiting PKR activation [269].

The expression of ADAR1 is induced in Jurkat cells and in PBMCs after HIV-1 infection and this expression is concomitant with the augmentation of HIV-1 protein expression and the decrease of PKR and eIF2α phosphorylation. PKR and ADAR1 interact directly and this binding is enhanced during HIV-1 infection [187, 188, 270]. ADAR1's inhibition by short hairpin RNAs decreases HIV-1 protein expression, whereas ADAR1's overexpression alleviates PKR-mediated inhibition of HIV-1 protein expression and virus production [187, 188]. ADAR1 contributes to the inhibition of PKR by direct binding through its first dsRBD domain and is present in a multiprotein complex formed around PKR during the infection of lymphocytes with HIV-1 [190].

1.4.5 A cellular activator of PKR, PACT, becomes a cellular inhibitor

PACT is a stress-modulated activator of PKR with proapoptotic functions in the absence of dsRNA or viral infection [271]. This 34 kDa dsRNA-binding protein shares a strong homology with TRBP but has an opposite effect on PKR by inducing PKR phosphorylation under oxidative stress conditions [272]. PACT heterodimerizes with PKR and activates it by direct protein-protein interaction. It has two dsRBDs as well as a third Medipal domain required for PKR activation [257, 273, 274].

TRBP binds to PACT through each dsRBD and each Medipal domains and inhibits PACT's activity on PKR [257]. PACT-induced PKR activation is restored in Tarbp2$^{-/-}$ murine tail fibroblasts and in HEK 293 T or HeLa cells when TRBP expression is reduced by small interfering (si)RNAs. In HEK 293 T and HeLa cells, arsenite, peroxide, and serum starvation-mediated stresses dissociate the TRBP-PACT interaction and increase PACT-induced PKR activation. In an astrocytic cell line where TRBP is weakly expressed, PACT induces PKR and eIF2α phosphorylation in the absence of stress [259].

In the context of HIV-1 replication, PACT expression is increased when the virus is expressed at high levels and the protein interacts with the multiprotein complex formed with PKR, ADAR1 and TRBP. PACT overexpression induced an increased viral expression and production with concomitant PKR and eIF2α inactivation in HIV-transfected HEK 293 T cells as well as U251MG astrocytes [188]. The results in astrocytes show that PACT's inhibition of PKR in HIV-producing cells could not be attributed to TRBP inhibition but indicated that PACT becomes a PKR inhibitor in the cells expressing HIV-1 [188, 191, 192]. Based on these results, an HIV component or an HIV-induced component changes the function of PACT to become a PKR inhibitor in HIV-infected cells. The explanation of this change could be that the proteins that inhibit PKR act in concert. In addition to TRBP-PACT, TRBP-PKR, ADAR1-PKR and PACT-PKR, PACT and ADAR1 interact directly [188]. Furthermore, an *in vitro* kinase assay showed that ADAR1, PACT, TAR RNA and Tat synergistically inhibit PKR phosphorylation, suggesting that PACT reversion may be the result of this combination (Figure 1.4) [248, 275].

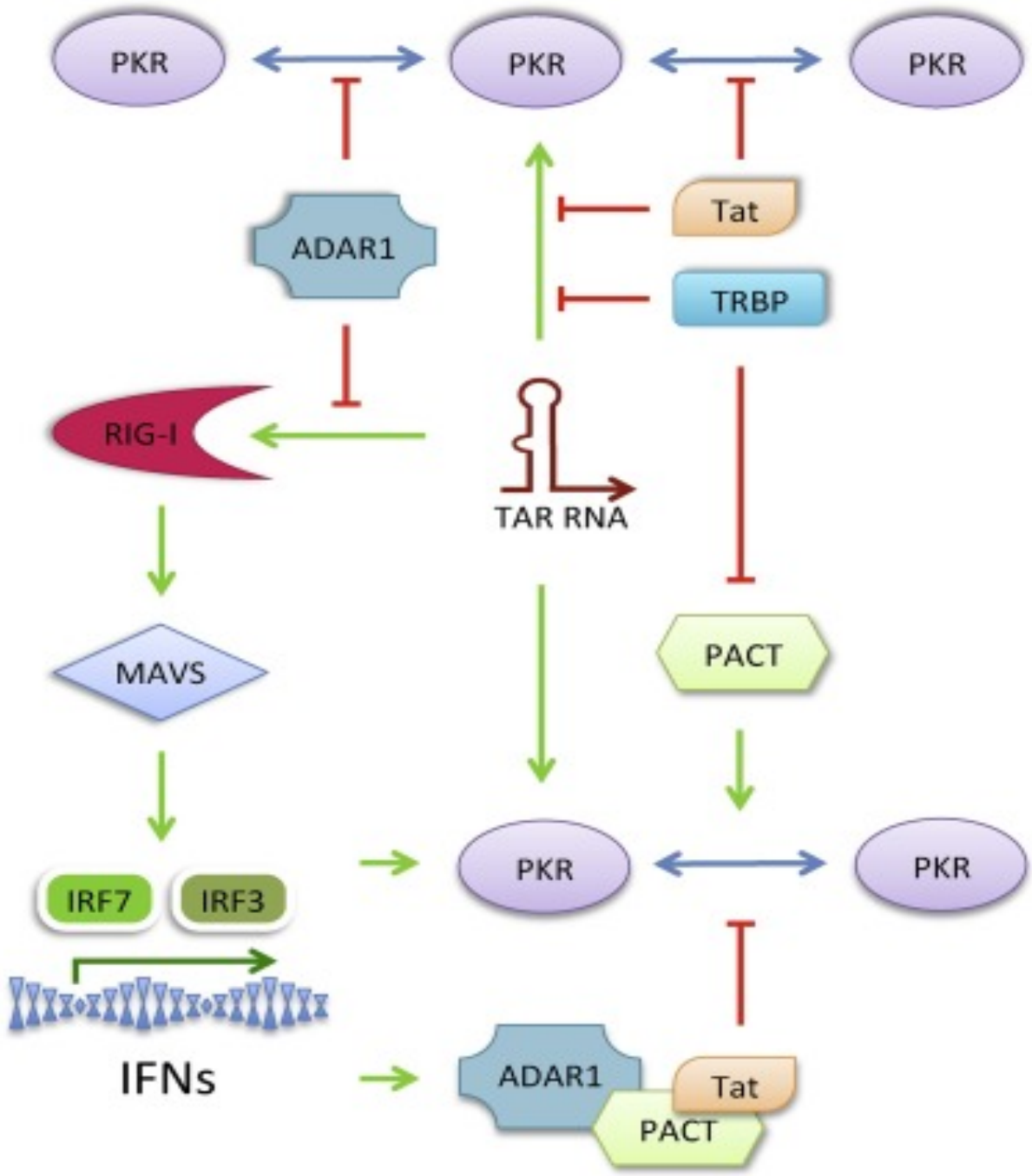

Figure 1.4: Protein Kinase R (PKR) is regulated by cellular and viral components. Sensing of TAR RNA by PKR is prevented by Tat and TRBP via competitive binding and sequestration mechanisms. TRBP has been also shown to regulate the activity of PACT, stress-inducer of PKR. Both Tat and ADAR1 can prevent the dimerization of PKR separately or in complex with PACT. Furthermore, ADAR1 edits viral RNA to prevent its detection by RIG-I/MAVS pathway, thus, downregulating the antiviral IFN response.

Overall, PKR is an ISG acting as a restriction factor whose activation reduces HIV-1 expression and replication. Its positive selection by VV has further evolved with HIV. In addition to the viral Tat, HIV-1 has evolved to replicate in cells expressing a large amount of TRBP. It induces the production of ADAR1 and reverses the function of its activator PACT, that all together contribute to counteract PKR activation [190, 192, 244]. It has been surprising to observe that ADAR1, an ISG, counteracts PKR, another ISG, but ADAR1 has additional functions that contribute to HIV-1 enhancement of replication [233, 234].

1.5 ADAR1: an ISG promoting HIV-1 replication

1.5.1 ADAR1 function in the cell

ADAR1 catalyzes one of the most common RNA base modifications in mammals: adenosine to inosine. The protein exists in two isoforms as a result of different promoters and alternative splicing events [276-278]. Full-length isoform p150 is IFN-inducible and is localized in both the nucleus and the cytoplasm [279]. The p110 isoform of ADAR1 is constitutively expressed and is localized exclusively in the nucleus [277]. The p150 form has two Z-DNA binding domains at the N-terminus and the p110 form has only one. Both forms have three centrally located dsRBDs and an active C-terminal catalytic deaminase domain (Figure 1.5) [233, 280]. The known targets of the catalytic activity of the enzyme are cellular pre-mRNAs, noncoding RNAs including micro (mi)RNAs, and viral dsRNAs [280, 281]. The substrates of ADAR1 can be of variable length, starting at about 20 base pairs long, that are likely edited selectively, and reaching an upper range hundreds of base pairs, which are characterized by more promiscuous editing leading to about 50% of all adenosine residues of the RNA being deaminated to an inosine [282, 283].

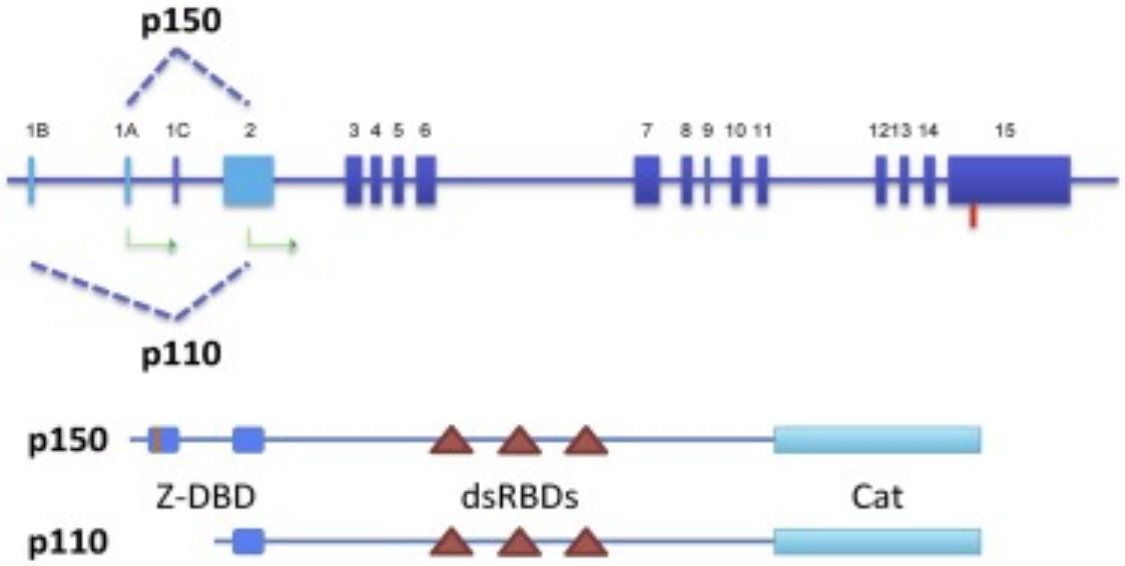

Figure 1.5: ADAR1 gene generates two isoforms p150 and p110 via alternative splicing event. Interferon-induced p150 isoform is translated from the splicing of exon 1A with its own start site to exon 2 of ADAR gene. This result in expression of an extra Z-DNA Binding Domain contains a Nuclear Export Signal (orange). p110 is expressed from exon 1b spliced to exon 2 and uses its start site, therefore, resulting in a shorter isoform of the protein.

In the attempt to understand the specific role of the deaminase, ADAR1$^{-/-}$ and ADAR1 p150$^{-/-}$ mice were genetically engineered; however, these deletions turned out to be embryonically lethal due to failed erythropoiesis and fetal liver disintegration [284]. An inducible mouse model of ADAR1 gene disruption has revealed that it is essential for maintenance of adult hematopoietic cells, as its loss leads to a global increase of IFN-I and II and ISGs, leading to high rates of cellular apoptosis [285]. In the human context, an autoimmune disease, Aicardi-Goutières syndrome (AGS), associated with an aberrant production of IFN-α, has been linked to mutations in the ADAR1 gene [286, 287]. In the AGS cohort of the group that made the initial observation, ADAR1 displayed eight amino-acid substitutions. Five of these lie along the site of the protein-dsRNA interaction surface; two lie internal to the domain and are thought to destabilize protein structure, and the last substitution is in the Z-DNA binding domain [287]. These observations underline the

regulatory role of ADAR1 in innate immunity and hint that RNA binding and editing properties are central to it. Moreover, rescued mouse embryos resulting from the cross of IFNAR1 and ADAR1 null mice have a similar phenotype to the ADAR1$^{-/-}$ embryos suggesting that up-regulation of IFN signaling is not the main reason for embryonic lethality [288]. Another argument for the ADAR1's anti-apoptotic role is its localization to the stress granules induced with polyI:C or sodium arsenite [289].

1.5.2 ADAR1 as an RNA sensing inhibitor limiting IFN production

Exogenous RNA activates the IFN-I signaling cascade through specific receptors, such as TLR-3, RIG-I and melanoma-differentiation-associated gene (MDA)-5 [290-292]. ADAR1 limits RIG-I, but not TLR-3 detection of RNA, thus interfering with RNA sensing and subsequent immune activation of the cell via competitive binding [293]. This regulation is inherent to the IFN-stimulated p150 isoform of the gene and is mediated via the MDA-5 and mitochondrial antiviral-signaling protein (MAVS) pathway because the deletion of either MDA-5 or MAVS rescued the embryonically lethal deficiency of ADAR1 in mice [294]. Furthermore, in cells exposed to poly I:C, short dsRNA of 20 base-pairs containing inosine-uridine pairs bound to RIG-I and MDA-5 inhibit the downstream activation of IRF-3, and consequently the induction of ISGs and apoptosis, suggesting that IU-dsRNAs generated by ADAR1 may have the same function [295]. The role of RNA editing by ADAR1 in this mechanism was demonstrated with an engineered mouse model in which ADAR1 had a single mutation in the catalytic domain, therefore unable to edit the RNA, but with functional RNA and Z-DNA binding domains. These mice exhibited a similar phenotype of embryonic lethality and increased the induction of ISGs as the ADAR1 null mice. However, when crossed with mice lacking MDA-5, the ISG expression was comparable to the wild

type levels and mice reached adult stage, although slightly smaller than the control animals [296]. Together these results led to a formulation of a model that ADAR1 marks RNA as self via editing events, thus preventing activation of the immune system via the MDA-5 pathway [297].

The role of ADAR1 as a marker of the RNA as self in HIV-1 infection was analyzed in primary macrophages using its down-regulation by siRNA. This decrease in ADAR1 led to an increased expression of MDA-5, IFN-β and ISGs, including the proviral CXCL10/IP-10 [227, 298]. HIV-1 replication was inhibited by as much as 85% in the ADAR1-deficient macrophages, supporting the proviral role of ADAR1. The knockdown of the MDA-5 in the macrophages did not have any effect on the HIV-1 production, but the addition of a chemical inhibitor of the next step of the downstream RIG-I/MDA-5/MAVS signaling pathway released the block of HIV-1 replication in ADAR1 knockdown macrophages. These results suggest that the RNA sensing inhibition by ADAR1 is at least partially responsible for its proviral role in HIV-1 infected macrophages (Figure 1.4) [298].

1.5.3 ADAR1 as an RNA editing protein

In addition to the modulation of the innate immune response, RNA editing A to G patterns typical of ADAR enzymes have been observed in several RNA viruses and the direct activity of ADAR1 has been shown for hepatitis delta virus, MV, RVFV, VSV and HIV-1 [233]. The initial study of the relationship between HIV-1 and ADAR1 demonstrated that increasing amounts of catalytically active ADAR1, but not ADAR1 lacking editing activity, in virus producing HEK 293 T and COS7 cells resulted in upregulation of viral protein production in a dose dependent manner [299]. RNA analysis of these cells showed a decrease in unspliced viral RNA and an increase in Tat-encoded multiply spliced RNA.

ADAR1-mediated editing was found in the Env gene in the vicinity of the Rev Response Element sequence. A mutant similar to the edited sequence showed enhanced replication rates, suggesting a proviral role for ADAR1 via viral RNA editing [299]. Another study showed that ADAR1 overexpressing cells were more efficient at viral release of the virions, and that those HIV-1 virions were also more infectious. In this study, the 5' UTR sequence of viral transcripts had A-to-I mutations in the overexpressing cells. Rev and Tat had mutations in their coding sequences resulting in five amino acid changes and one silent mutation respectively, showing that ADAR1 can edit and modify HIV-1 RNA [270]. Therefore, the RNA-editing activity of ADAR1 on the viral RNA also contributes to enhancing HIV-1 replication [233].

1.5.4 ADAR1 as an anti-PKR protein

The activity of ADAR1 as an anti-PKR was shown in the context of several viruses as described in section 1.4.4 and shows that an ISG can counteract the activity of another ISG [233, 234]. This activity was identified in HIV-1 expressing cells [187, 270]. These studies also showed that the expression of ADAR1 is enhanced during viral replication in Jurkat cells and in PBMCs and that ADAR1's interaction with PKR is enhanced at the peak of infection [187, 188]. To determine the impact of an impaired ADAR1 function on HIV-1 replication, PBMCs from AGS donors carrying a mutation in the ADAR1 gene were infected with HIV-1. The analyses revealed that HIV-1 replication was impaired at the post-transcriptional step. They suggested that a decrease of eIF2α phosphorylation could be the cause, but the direct link between these mutations and PKR inhibition remains to be determined [300]. From these studies, the interaction between ADAR1 and PKR largely contributes to PKR inhibition and increases HIV-1 replication.

1.5.5 Other possible functions of ADAR1 during HIV-1 replication cycle.

A co-immunoprecipitation assay of ADAR1 from HIV-1 producing cells revealed that it interacts with p55 Gag protein, and subsequently is incorporated into virus-like particles [301]. Whether this characteristic contributes RNA sensing inhibition, RNA editing, PKR inhibition or all these functions when the incoming virus particle enters the cell should be determined in the future (Figure 1.6). ADAR1 has also been shown to interact with Dicer, edit miRNAs and interfere with the RNA interference pathway [302]. This is another important function that has not yet been studied in the context of HIV-1. Because HIV-1 induces an upregulation of ADAR1 p150 in lymphocytes [187, 188] it is likely that it may change Dicer function and edit more miRNAs in HIV-infected cells and could contribute to viral pathogenesis as seen with other viruses [303].

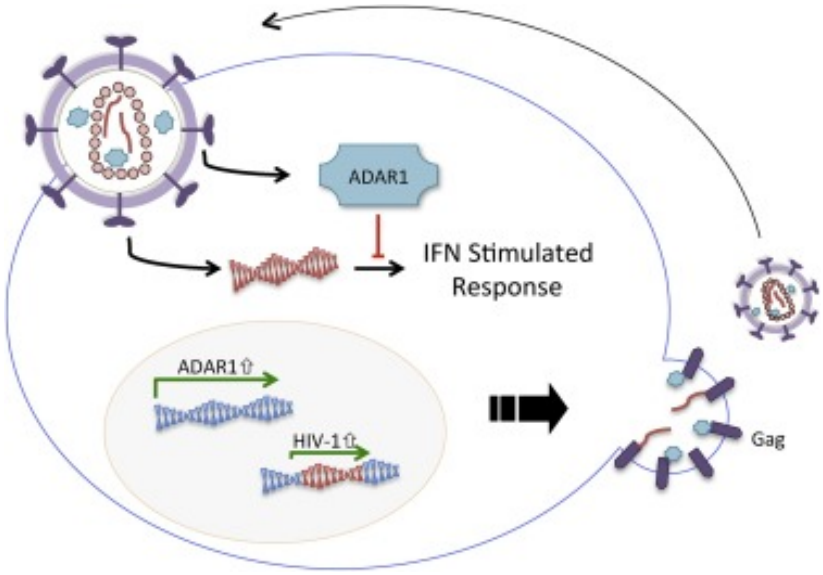

Figure 1.6: ADAR1 is incorporated into virions and inhibits IFN stimulated response in HIV-1 infected cells. HIV-1 producing cells overexpress ADAR1, which gets incorporated into the newly synthesized virions via its interaction with Gag protein. Upon viral infection, readily available ADAR1 inhibits interferon response in the cell leading to an enhanced viral production.

Chapter 2: Objectives and Hypothesis

As mentioned in the literature review, the IFN response holds multiple tools to its disposition to suppress virus infections. HIV-1 has evolved many ways to pass undetected or neutralize the defense mechanisms. Previous findings in our lab have indicated that the IFN response, more precisely, PKR activation and its blockage of protein synthesis is suppressed in HIV-1 producing cells. The general objective of this project is to identify the elements causing the aforementioned inhibition. TRBP was the first cellular factor identified to inhibit the PKR reaction against the virus. The same was later demonstrated for ADAR1, and eventually PACT proved to become an inhibitor of PKR, and not its activator, as canonically believed, in HIV-1 infected cells. The working hypothesis of this project is that ADAR1, PACT and a viral element are in a multiprotein complex that affects innate immune pathways, specifically PKR, which is crucial for initial establishment and replication of HIV-1 in a cell. To test our hypothesis three objectives have been defined for this project:

Objective 1: Determine the expression of ADAR1, PKR and PACT in PBMCs of HIV-1 positive individuals

To answer this objective, we have analyzed the protein profile of PBMCs donated by individuals that were HIV-1 negative, HIV-1 positive treated with HAART, or newly diagnosed that have not received antiretroviral treatment yet.

Objective 2: Determine the effect of ADAR1p150 overexpression in Jurkat cell line infected with HIV-1

Based on the results obtained in objective 1, we have genetically engineered Jurkat cell line to overexpress ADAR1p150 via lentivirus transduction. The newly generated

clones were infected with HIV-1 and viral kinetics were monitored over first three weeks of infection.

Objective 3: Determine the effect of ADAR1p150 knockout in Jurkat cell line infected with HIV-1

We have genetically engineered Jurkat cell line using CRISPR/Cas9 to knockout ADAR1p150 isoform. The newly generated clones were infected with HIV-1, PKR profile and viral kinetics were analyzed over first three weeks of infection.

Objective 4: Map the interaction between ADAR1 and PACT, and determine their interacting partners in HIV-1 producing cells

As previously noted in the lab [188], ADAR1 and PACT interaction was stronger in HIV-1 infected than in non-infected cells. Therefore, we have cloned different mutants of ADAR1 gene, and performed immunoprecipitations to identify domains necessary for the interaction. We also identified a viral element, Tat, to be in the aforementioned complex. In order to identify other interacting partners, we performed a mass spectrometry on immunoprecipitated ADAR1 and PACT from HIV-1 producing cells.

Chapter 3: Materials and Methods

3.1 Cell culture

HEK 293 T cells (ATCC CRL-11268) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 2mM L-glutamine, 10% Fetal Bovine Serum (FBS) (Hyclone) and 1% penicillin-streptomycin (Life technologies) at 37°C in 5% CO2.

Jurkat cells were maintained in Roswell Park Memorial Institute Medium (RPMI) 1640 supplemented 10% FBS (Hyclone) and 1% penicillin-streptomycin (Life technologies) at 37°C in 5% CO2.

PBMCs were obtained from healthy donors that tested negative for HIV, HTLV-I/II, and HCV, and from HAART treated and untreated HIV-1 infected individuals. Cells were maintained in RPMI medium supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1% penicillin-streptomycin. Ethics review board of McGill University approved the blood sample collection.

3.2 Plasmids and cloning

pCMV2-Flag-PACT, pcDNA3.1-ADAR1-p110-V5, pcDNA3.1-ADAR1-p80-V5, pcDNA3.1-ADAR1-p70-V5 and pcDNA3.1-ADAR1-delta cat-V5 were previously described [188]. HIV-1 molecular clone, pNL4-3 was obtained from Dr. M A. Martin. pCMV-Myc-Tat was obtained from Dr. R. Patel (University of South Carolina, USA).

3.2.1 Lentiviral plasmids

pSUPER lentiviral transfer vector, VSV-G and pCMVΔR-8.74 were obtained from Dr. Charneau (Institut Pasteur, France). We amplified the promoter region and the whole ADAR-1 gene plus the V5 tag using pcDNA3.1-ADAR1-p150-V5 as a template.

Forward primer: 5'AATTAATCGATCGATGTACGGGCCAG3'

Reverse primer: 5'AAGGAGCTAGCAACTCAATGGTGATG3'

To construct the control lentiviral transfer vector, the same primers were used to amplify the promoter region and V5 tag, the template being pcDNA3.1-V5. All the PCR products were cut by ClaI and NheI were sub-cloned into the lentiviral transfer vector cut with the same enzymes.

pHIV-EGFP, second lentiviral transfer vector, was obtained from Dr. Bryan Welm and Dr. Zena Werb through Addgene. New packaging, psPAX2, and envelope plasmids, pMD2.G, were ordered from Dr. Didier Trono. pHIV-EGFP vector already had EF-1α promoter just before the multiple cloning site, as well as the green fluorescent protein (GFP). We then amplified the whole ADAR-1 gene and the V5 tag using pcDNA3.1-ADAR1-p150-V5 as a template.

Forward primer: 5'ATTGTTAACGCCACCATGAATCCGCGGCAGGGGTAT3'

Reverse primer: 5'CCCGGCTCTAGAGGTTTAAACTCAATGGTGATGGTG3'

To amplify the V5 tag as a control lentiviral transfer vector, we used the same reverse but designed a new forward primer.

Forward primer for V5: 5'ATTGTTAACGCCACCATGGGAATTCTGCAGATATCC3'

All the PCR products were cut by HpaI and XbaI and sub-cloned into the lentiviral transfer vector with the same enzymes.

pHIV-7 was obtained from Dr. Rossi laboratory (City of Hope, California). Since it didn't have a promoter before the multiple cloning sites, we added the promoter region, and then the ADAR-1 gene plus the V5 tag using pcDNA3.1-ADAR1-p150-V5 as a template.

Forward primer: 5'ATTGCGGCCGCCGATGTACGGGCCAGATATAC3'

Reverse primer: 5'TCCGCGCGTCTAGAAACTCAATGGTGATGGTG3'

The PCR product was cut by NotI and XbaI and sub-cloned into the lentiviral transfer vector using the same enzymes.

For the control transfer vector, an intermediate cloning was generated to introduce HpaI restriction site to the empty plasmid pHIV-7 using oligos:

Sense: 5'GGCCGCTATTGTTAACTTAATTAAGCCCGGGCAAAT3'

Anti-sense: 5'CTAGATTTGCCCGGGCTTAATTAAGTTAACAATAGC3'

The promoter region and the V5 tag were then amplified using pcDNA3.1-V5 as a template.

Forward primer: 5'GCTCGTTAACCGATGTACGGGCCAGATATACGCG3'

Reverse primer: 5'TCCGCGCGTCTAGAAACTCAATGGTGATGGTG3'

The PCR product was cut by HpaI and XbaI and sub-cloned into the intermediate empty vector cleaved with the same enzymes.

3.2.2 CRISPR/Cas9

CRISPR/Cas9 NickaseNinja plasmid was ordered from DNA2.0 (now ATUM). The gRNAs against ADAR1p150 were designed and cloned by the company platform and services. GeneArt CRISPR Nuclease Vector with OFP Reporter Kit was ordered from Thermo Fisher. Five gRNAs were designed using the publically available web tool for non-commercial purposes, called CHOPCHOP [304, 305] (Table 3.1). We cloned the gRNAs into the GeneArt plasmid using the company's protocol available on their web domain.

Table 3.1: Sequence of gRNAs used in GeneArt CRISPR/Cas9 system to generate ADAR1p150 knockout

gRNA1	*5'AATGAATCGCGGGCGCTCCGCGG3'*
gRNA2	*5'CCATCTTAACCGGAGGGGCCCGG3'*
gRNA3	*5'TGAATCCGGGCGCAAGCGGCAGG3'*
gRNA4	*5'TTTCCGGGCCAAACAGGGGAAGG3'*
gRNA5	*5'AGCAAACGAAGAAATTGAACCGG3'*

3.2.3 ADAR1 dsRBDs

To construct all the combination of the ADAR1 dsRBDs domains, pcDNA3.1-ADAR1-p150-V5 was used as a template. The domains were amplified by PCR using the primers as described in Table 3.2.

Table 3.2: List of primers used in the cloning of dsRBDs of ADAR1

dsRBDs 1-2-3	Forward	*5'TCGGATCCAATGTCACCCTACAAGAAA3'*
	Reverse	*5'GACCTCGAGCTTCTGCCTTCTCGTT3'*
dsRBDs 1-2	Forward	*5'TCGGATCCAATGTCACCCTACAAGAAA3'*
	Reverse	*5'GACCTCGAGCGTTATCAGAAGCCAT3'*
dsRBDs 2-3	Forward	*5'TCGGATCCAATGACCCCCACCCCTTCA3'*
	Reverse	*5'GACCTCGAGCTTCTGCCTTCTCGTT3'*
dsRBD 1	Forward	*5'TCGGATCCAGCCACCATGGAGTGCCAGCTGAAGAAC3'*
	Reverse	*5'GACCTCGAGCTGATTTTCCACTGTCCTTGGC3'*
dsRBD 2	Forward	*5'TCGGATCCAGCCACCATGGAGTCCCAGACCCCCACCCCT3'*
	Reverse	*5'GGCCACCTCGAGCGTTATCAGAAGCCATGGAGTTGGTCGC3'*
dsRBD 3	Forward	*5'TCGGATCCAGCCACCATGGTCAGGAAGATTGGCGAG3'*
	Reverse	*5'CCGGACCTCGAGCTTCTGCCTTCTCGTT3'*

The PCR products cut by BamHI and XhoI were subcloned into pcDNA3.1 V5 cut by the same enzymes.

3.3 Sequencing

All the sequencing following plasmid cloning and CRISPR/Cas9 knockout was performed by Genome Quebec at McGill University, Montreal.

3.4 Transfection

Cells were plated 24 hours prior to transfections to be at 60-70% confluency. Plasmids were transfected into cells using Polyethylenimine (PEI) at a 1:3 DNA:PEI ratio and the media was changed 4 hours after. Jurkat transfection with CRISPR/Cas9 NickaseNinja and GeneArt CRISPR/Cas9 was done using the TransIT-Jurkat (Mirus) at 1:3 DNA:TransIT ratio. 48 hours post-transfection, cells were lysed in ice-cold lysis buffer phenoxypolyethoxylethanol (NP)-40 (Sigma) and 10% Glycerol supplemented with

protease and phosphatase inhibitors (Roche). Cell lysates were then subjected to Western blot and immunoprecipitation assay.

For lentivirus production, HEK 293 T cells were transfected using TransIT (Mirus) at a 1:3 DNA:TransIT ratio and Sodium Butyrate was added at 1mM to the media. The lentivirus plasmids ratios varied between 3:5:1 and 3:7:1 ratio for transfer plasmid to packaging to envelope respectively. 48 hours post-transfection, supernatant was harvested and Lenti-X concentrator (Takara) was added at ¼ the volume. The solution was put to rotation for an hour at 4°C and then span-down at 1500g for 45 minutes. The pellet concentrated with lentivirus was resuspended in PBS and stored at -80°C for later uses.

3.5 Transduction

Jurkat and HEK 293 T cells were plated at 60% density and transduced with normalized amounts of lentivirus at 4000-5000 CPM per T-25 flask. Polybrene was added to the media at 8 μg/mL. Transduction efficiency was assessed by GFP signal via fluorescent microscopy 72 hours post-transduction.

3.6 Infection

Jurkat cells were plated at 60% density and transduced with normalized amounts of HIV-1 virus at 7800-10000 CPM per T-75 flask. Polybrene was added to the media at 4 μg/mL.

3.7 Cell sorting

72 hours post-transfection or transduction with CRISPR/Cas9 or lentivirus respectively, cells were collected and span-down at 1200 rpm for 5 minutes. They were passed through cell strainer and resuspended in 1%FBS PBS to arrive at concentration of 1000000 cells/mL. A sample of 0.5 mL was then used for analysis. Internal expression of

GFP and orange fluorescent protein (OFP) were measured at 510nm and 583nm respectively. Cells were sorted one cell per well into 96 well plates using High Speed Cell Sorter FACSAria Fusion.

3.8 Western Blot

Cellular and viral lysates (between 50-120 µg) were boiled for 10 min at 95°C in SDS loading buffer (0.5 M Tris HCl, 25% SDS, 20% Glycerol and 0.01 % Bromophenol blue) prior to loading into a 10% or 12% SDS PAGE gel. The proteins were then transferred onto a nitrocellulose membrane by semi-dry transfer (Bio-Rad Laboratories) for 1 hour at 10V, using a transfer buffer (48mM Tris-HCl, 39mM glycine, 0.375% SDS and 20% ethanol). Consequently, the membranes were then blocked in 5% milk diluted in Tris saline buffer supplemented with 0.1% Tween20 (TBS-T) for 1 hour and washed 2 times with TBS-T prior to adding primary antibodies diluted in 3% bovine serum albumine overnight at 4°C (Table 3.3). The following day, membranes were washed 4 times in TBS-T for 5 minutes, and then incubated with TBS-T and secondary antibodies for 1 hour. Membranes were washed 3 times for 10 minutes in TBS-T and Western lightning ECL solution (GE healthcare life sciences) was added prior to visualization.

Table 3.3: List of antibodies and their origin used in western blots

Antibodies	**Animal**	**Company**
ADAR1	*Rabbit*	*Dr. B.L. Bass*
Actin	*Mouse*	*Clone C4 EMD Millipore*
p-eIF2α	*Rabbit*	*Invitrogen*
eIF2α	*Rabbit*	*Cell Signaling*
Flag	*Mouse*	*Sigma*
GAPDH	*Mouse*	*Santa Cruz*
GFP	*Mouse*	*Santa Cruz*
Myc	*Mouse*	*Santa Cruz*
PACT	*Rabbit*	*Gatignol Lab*
p-PKR (pT446)	*Rabbit*	*Clone E120 Abcam*

PKR 70-10	*Mouse*	*Dr. A Hovanessian*
p-STAT1	*Rabbit*	*Cell Signaling*
STAT1	*Mouse*	*Santa Cruz*
VDAC	*Mouse*	*Santa Cruz*
V5	*Mouse*	*Invitrogen*

3.9 Reverse Transcriptase Assay

5µl supernatants from HIV-1 transfected HEK 293 T cells were added to 25µL of a non-radioactive cocktail (60mM Tris-HCl pH 7.8, 75mM KCl, 5mM MgCl2, 0.1% NP-40, 1.04 mM EDTA). Subsequently, 25 µL of a radioactive RT cocktail (60mM Tris-HCl pH 7.8, 75mM KCl, 5mM MgCl2, 0.1% Nonidet P-40, 1.04 mM EDTA, 10 µg/mL polyA , 0.33 µg/mL oligo dT) supplemented with 5 µL αP32 radiolabeled TTP (Perkin Elmer) and 4 µL Dithiothreitol per 500 µL of RT cocktail and was mixed with the non-radioactive cocktail containing the virions. The mixture (55 µL) was incubated for 2 hours at 37°C and 5 µL of the reaction were spotted onto a DEAE paper (Perkin Elmer). The DEAE paper was left to dry for 10 min prior to 4 times, 5 minutes washes in 2X SSC buffer (3 M NaCl and 300 mM Citrate) and 2 washes of 1 minute in 95% ethanol and left to dry again. Papers were sealed in a plastic bag (Perkin Elmer) and radioactivity amount, expressed in cpm, were counted on a MicroBeta Jet scintillation counter (Perkin Elmer).

3.10 Immunoprecipitation

Protein G agarose fast flow compact beads (Sigma) were blocked for non specific interaction with 3% BSA TNET buffer (50 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP40 and 3% BSA) prior to adding 2 µg of anti-Flag mouse antibody (Sigma). Next, the beads with antibodies were incubated with rotation overnight at 4°C for 24 hours. After washing the beads 3 times with ice-cold lysis buffer, 1.5 mg of lysates were added. They

were then incubated overnight with rotation at 4°C. The next day, the beads were washed 3 times with ice-cold lysis buffer and 2 times with ice-cold phosphate buffered saline (PBS). Elution of proteins was done with in SDS loading buffer (0.5 M Tris HCl, 25% SDS, 20% Glycerol and 0.01 % Bromophenol blue).

3.11 Mass spectrometry

Immunoprecipitations were performed as described in section 3.10. The mass spectrometry analyses were performed by Quebec Genome Center at Laval University and proteomic group of Institute for Research in Immunology and Cancer (IRIC) at Montreal. Quebec Genome Center required 3 washes with lysate buffer and 2 washes with ammonium bicarbonate at 20mM. IRIC proteomic group required 10 washes with ice-cold PBS. Results were visualized via Scaffold 4 with peptide/protein threshold set at 1% FDR and minimal peptide/protein count set at two or one for IRIC analysis.

Chapter 4: Results

4.1 Interferon-stimulated isoform of ADAR1 is overexpressed and sustained in PBMCs of HIV-1 infected individuals

To better understand the relevance and mechanics of PKR pathway inactivation in HIV-1 infection, PBMCs provided by Dr. J.P. Routy, of viremic individuals with either active viral infection or viral infection controlled with HAART were lysed and compared to lysates from healthy individuals (Figures 4.1.1 and 4.1.2). Patients #1, 2, 3, 10, 11 and 12 are HIV-1 negative, clinically having the highest average count of CD4 cells. In parallel, CD4 cells are significantly lower in the viremic, non-treated group, represented by patients #4, 5, 6, 13, 14 and 15, in some cases twenty times less. The viral load of this study group varied between 3.04 and 5.21 log copies of viral RNA per mL of blood. Patients #7, 8, 9, 16, 17 and 18 have their viral load suppressed by antiretroviral drugs, and some recovered their CD4 cell counts to comparatively healthy levels (Figures 4.1.1A and 4.1.2A).

The first set of the western blot on samples from patients was performed by Samantha Burugu, the previous master student in the laboratory, and revealed an increase of ADAR1 expression exclusively in the viremic non-treated group (data not shown). Extra samples were asked to repeat the experiment and confirm the finding. In our first western blot, ADAR1 p110 isoform was overexpressed in two out three viremic non-treated samples, and in a single sample from healthy and treated subjects each. High expression of p150 isoform of ADAR1 was detected in the two viremic non-treated patients same as for p110, but not in other study groups (Figure 4.1.1B). Phosphorylation of PKR and eIF2α followed the same pattern and were phosphorylated in PBMCs of patients #5, 6, 8 and 9. PACT was also more expressed in those samples, in addition to #3 (Figure 4.1.1B).

A

Patient #:	1	2	3	4	5	6	7	8	9
Age	60	48	42	37	43	38	29	45	29
Sex	F	F	M	M	M	M	F	M	F
CD4 count	-	862	810	370	204	212	554	318	866
CD8 count	-	609	1405	851	361	518	288	431	466
VL log	0	0	0	4.43	4.82	4.82	<1.6	<1.6	<1.6
Additional information	-	-	-	CMV Varicella Rubella	-	-	CMV	CMV	CMV

B

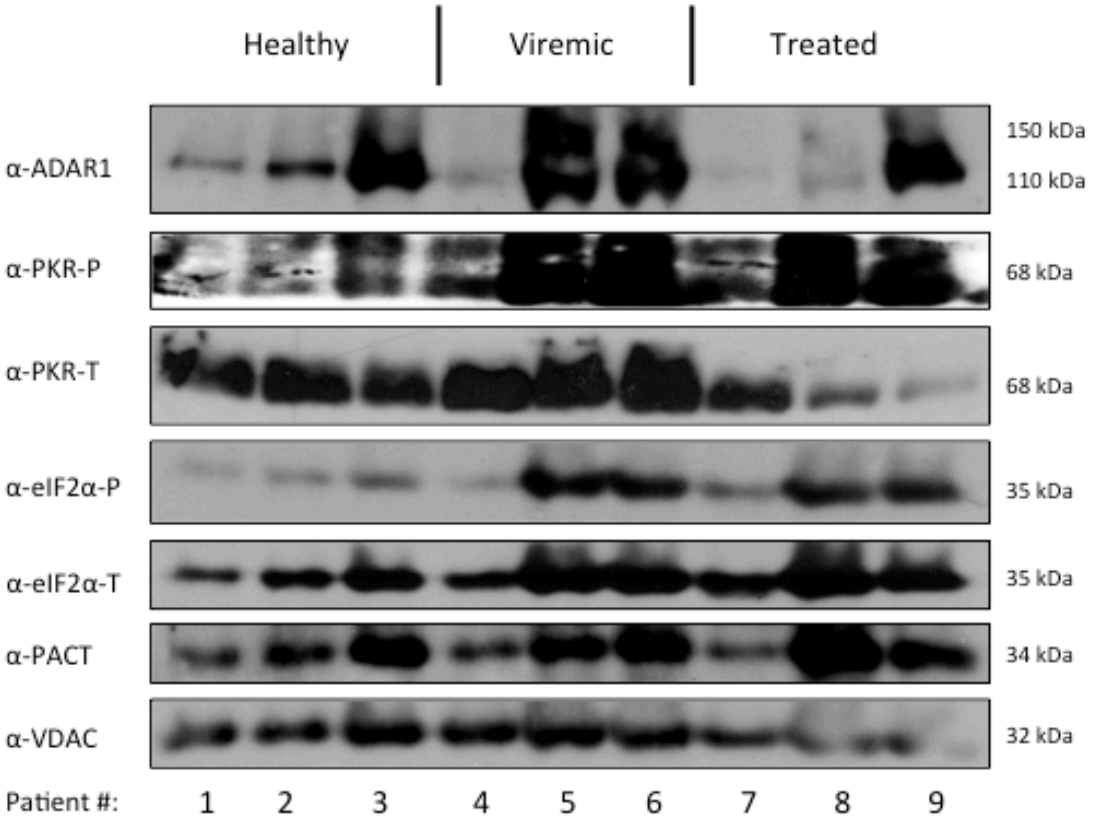

Figure 4.1.1: Expression of ADAR1 p150 isoform correlates with the viral replication in HIV-1 positive individuals. **A**. Patient's information, including CD4 and CD8 cells count per mL of blood, viral load expressed in logarithmic scale per mL of blood, age and sex. Patients #1, 2 and 3are healthy HIV-negative; #4, 5 and 6 are diagnosed with HIV-1 but have not received HAART treatment; patients# 7, 8 and 9 are also HIV-1 positive, but have been on HAART and viral load is below the detection limit. Dr. J.P. Routy provided patients' data. B.120 µg of protein lysates from PBMCs of patients were subjected to a 10% SDS-PAGE. The samples were then blotted against ADAR1, PKR-P, PKR-T, eIF2α-P, eIF2α-T, PACT, and VDAC.

A second set of PBMCs from different patients was obtained from Dr. J. P. Routy. One viremic non-treated patient had highly phosphorylated PKR with extremely low ADAR1 p150 expression (patient #15, Figure 4.1.2B). Additionally, in that sample, PACT levels were low. ADAR1 p110 isoform was detectable in patient's samples #10, 13, 14, 15 and 16. The interferon stimulated ADAR1 was present in the rest of viremic group, patient samples #13 and 14 (Figure 4.1.2B). PKR was also phosphorylated in samples #13 and 18, but much less than in #15. This, however, did not affect eIF2α phosphorylation, as it was similar in all the samples except #10. PACT was lower in two viremic non-treated patients #13 and 15 when compared to the rest of the subjects (Figure 4.1.2B).

A

Patient #:	10	11	12	13	14	15	16	17	18
Age	55	50	52	42	26	51	40	60	78
Sex	M	M	M	M	F	M	M	M	M
CD4 count	1366	813	675	42	627	43	519	972	412
CD8 count	504	391	205	215	732	1363	1025	553	389
VL log	0	0	0	5.21	3.04	4.89	<1.7	<1.6	<1.7
Additional information	CMV Toxoplasmosis Syphilis	CMV	-	CMV Varicella HCV	CMV	CMV Varicella Rubella	CMV Hepatitis	CMV	CMV HCV

B

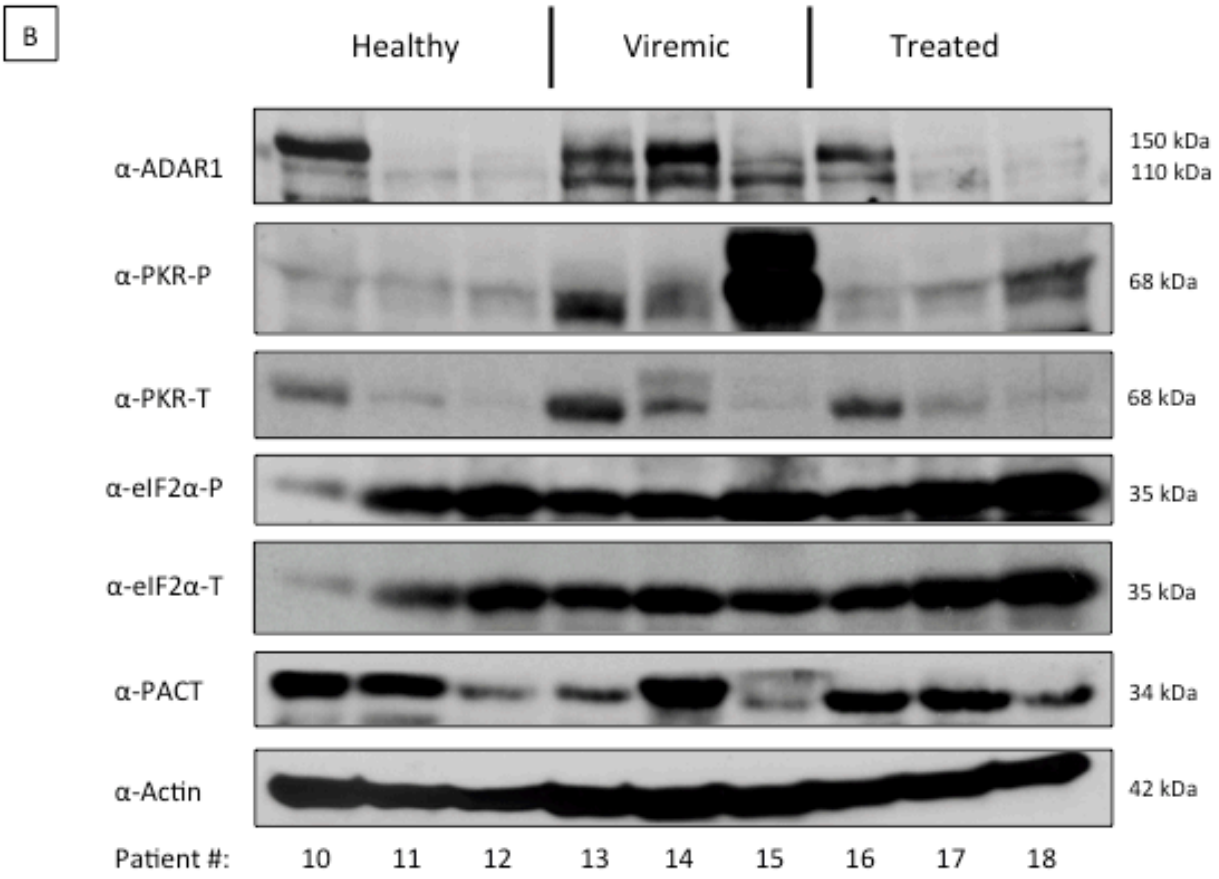

Figure 4.1.2: Expression of ADAR1 p150 isoform is inversely correlated with PKR phosphorylation in HIV-1 viremic individual. A. Patient's information, including CD4 and CD8 cells count per mL of blood, viral load expressed in logarithmic scale per mL of blood, age and sex. Patients #10, 11 and 12 are healthy HIV-negative; #13, 14 and 15 have been diagnosed with HIV-1 infection but have not received HAART treatment at the moment of blood collection; patients# 16, 17 and 18 are also HIV-1 positive, but have been on HAART long enough for it to suppress viral load below the detection limit. Dr. J.P. Routy provided patients' data. B. 120 µg of protein lysates from PBMCs of patients were subjected to a 10% SDS-PAGE. The samples were then blotted against ADAR1, PKR-P, PKR-T, eIF2α-P, eIF2α-T, PACT and Actin. (Performed by Aïcha Daher)

4.2 Effect of ADAR1p150 overexpression on HIV-1 replication in Jurkat cells

To further study the reason behind the increased expression of ADAR1p150 isoform in the PBMCs of several individuals with active HIV-1 replication, we genetically engineered Jurkat cells overexpressing the said gene. For this purpose, we chose a lentivirus approach, since it can stably integrate into the host genome insuring a long and stable expression of a protein (Figure 4.2.1).

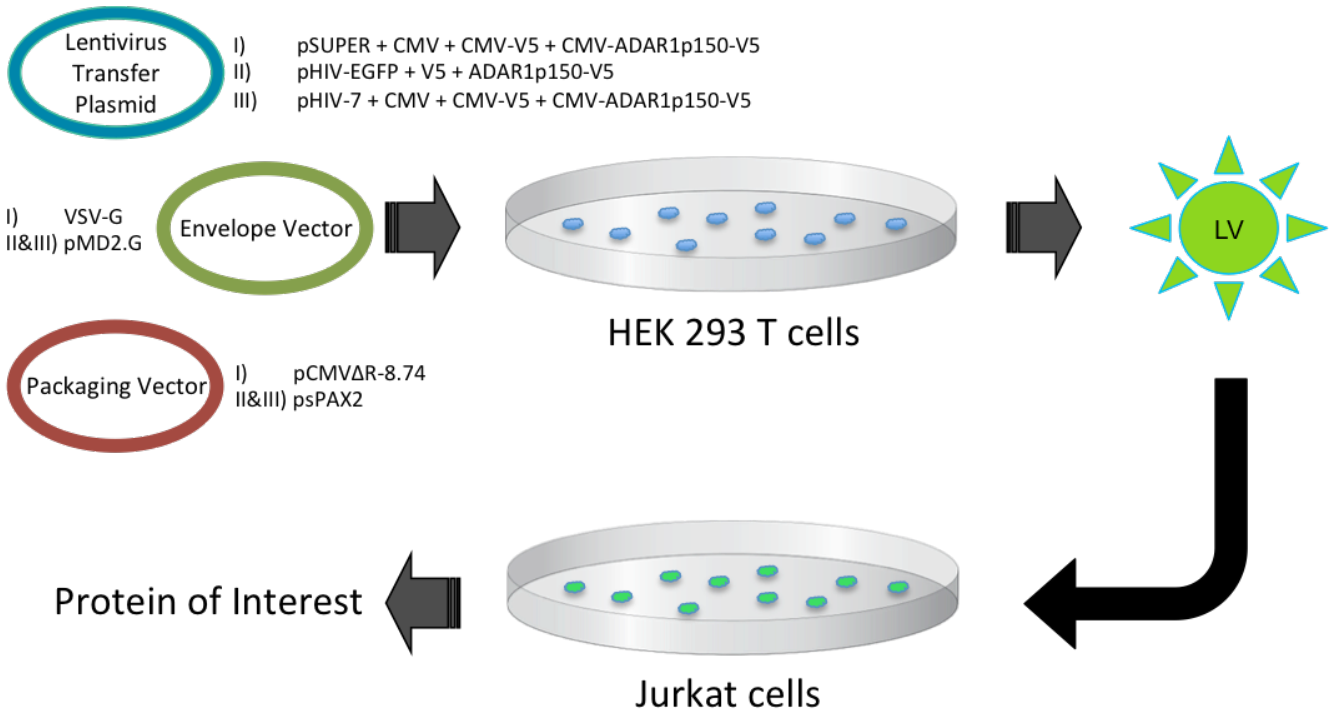

Figure 4.2.1: Flowchart of lentivirus production and transduction for a transient gene expression. Lentiviral particles are produced from transfection of HEK 293 T cells with a lentivirus transfer plasmid coding for the gene of interest, an envelope vector and a packaging vector. Cell line of interest is transduced with the infectious lentiviral particle. Successfully transduced cells express integrated marker of selection and the protein of interest. Combinations of plasmids were used as indicated by roman numbers.

4.2.1 Optimization of Jurkat cell line transduction with pHIV-EGFP based lentivirus

We initially cloned ADAR1p150-V5 from pcDNA3.1-ADAR1p150-V5 and V5-tag from pcDNA3.1-V5 into pSUPER transfer plasmid and used with VSV-G and pCMVΔR-8.74 plasmids gifted by Dr. Pierre Charneau (combination I, Figure 4.2.2). However, the expression of GFP marker of selection and expression of lentiviral transfer plasmid was extremely low, close to 1% (data not shown). Therefore, we re-cloned ADAR1p150-V5 and V5 into pHIV-EGFP transfer plasmid obtained from Dr. Bryan Welm and Dr. Zena Werb through addgene platform. New packaging and envelope plasmids, psPAX2 and pMD2.G respectively, were ordered from Dr. Didier Trono through addgene also. Transfection of HEK 293 T cells in 3:3:1 ratio of transfer: packaging: envelope plasmids (combination II, Figure 4.2.1) yielded GFP signal, and western blot showed that ADAR1p150-V5 was expressed following the cloning (Figure 4.2.2A and B). Transduction of Jurkat with the produced lentiviral particles proved to be challenging despite the similar levels of GFP expression from HEK 293 T lentivirus production. We therefore tested several modifications to our transfection in HEK 293 T and Jurkat transduction (Figure 4.2.2C). Whereas increasing the proportion of packaging plasmid did not modify viral particle expression significantly, the addition of TransIT, Sodium Butyrate, an inhibitor of histone deacetylation [306], to HEK 293 transfection led to higher transduction. In the transduction step, the addition of polybrene to the media improved the efficiency. Spinoculation or higher FBS percentage in culture media (data not shown) had no significant effect. Overall, the addition of TransIT. Sodium Butyrate and polybrene improved Jurkat transduction with HIV-EGFP based lentivirus, but was not sufficient for EGFP-V5-ADAR1 expression.

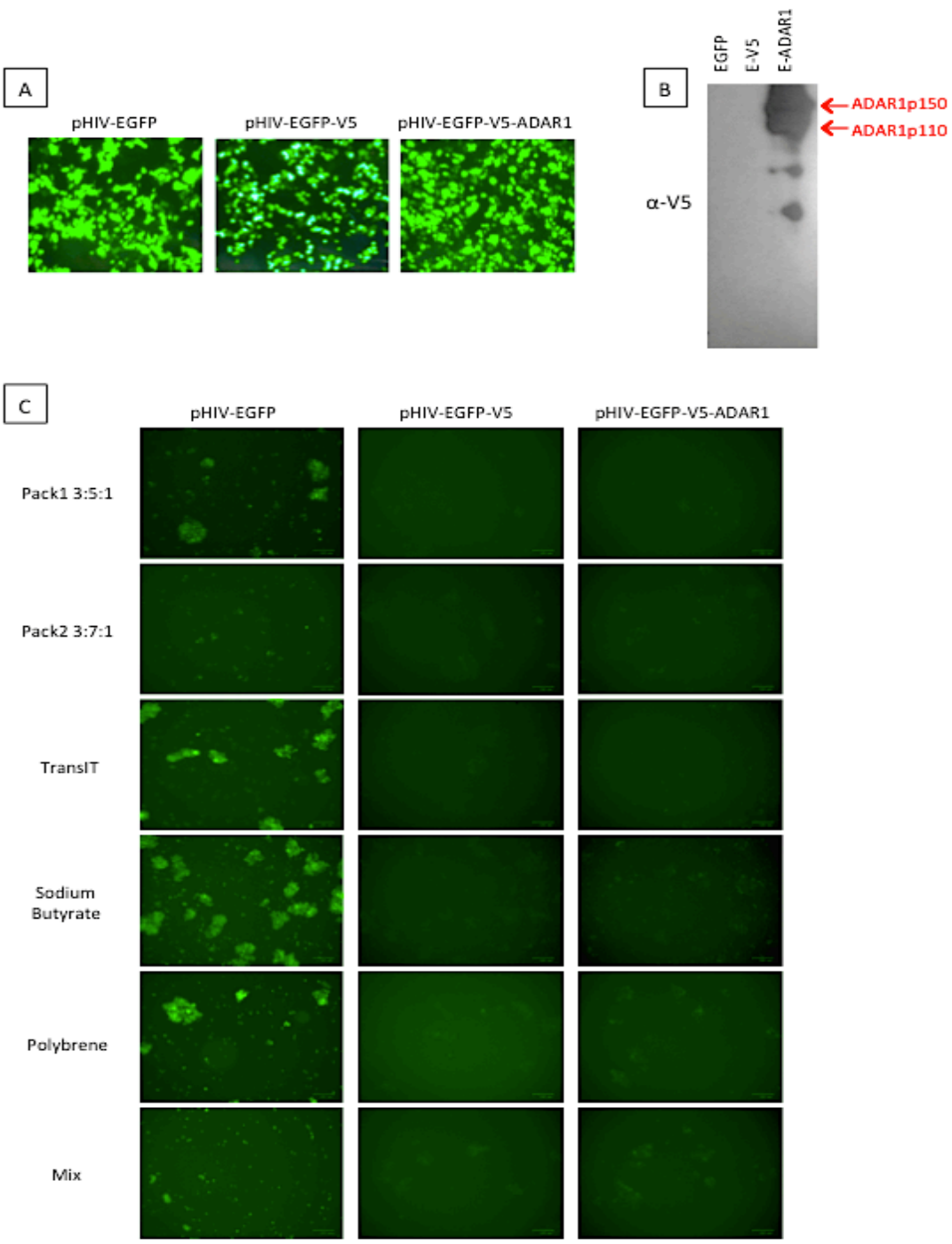

Figure 4.2.2: Production and transduction optimization of lentivirus particles expressing ADAR1p150-V5 from pHIV-EGFP transfer vector. A. HEK 293 T cells were transfected with three plasmids: 2µg of pMD2.G (envelope), 6µg of psPAX2 (packaging) and 6µg of either pHIV-EGFP, pHIV-EGFP-V5 or pHIV-V5-ADAR1 depending on the condition. The expression of the plasmids forming the lentivirus particle was indirectly assessed via GFP expression. **B.** ADAR1-V5 expression from the lentivirus plasmid as shown on a Western Blot blotted against V5 tag. **C.** Pack1 condition was considered to be control for the ratio of transfer: packaging: envelope plasmids, all other conditions had one element changed. Changes include the amount of packaging plasmid input, additions of TransIT from Mirus Bio, Sodium Butyrate at 1mM and polybrene at 8 µg/mL. Mix condition represents all modifications together.

4.2.2 pHIV-7-CMV based lentivirus transduce Jurkat and HEK 293 T cell lines with higher efficiency than pHIV-EGFP based

Despite the optimization efforts, transduction of Jurkat with pHIV-EGFP transfer plasmids expressing V5 and ADAR1p150-V5 was relatively low compared to the original lentivirus. Therefore, we re-cloned our gene of interest into a pHIV-7 plasmid, to which we also added a CMV promoter upstream of V5 and ADAR1-V5 inserts. Despite the expression of ADAR1p150-V5 in HEK 293 T cells from both lentiviral plasmids, western blot analyses of lysates from transduced Jurkats did not detect its expression (combination III, Figure 4.2.1) (Figure 4.2.3A). On the other hand, transduction in terms of GFP signal as well as its expression as measured by western blot proved to be successful (Figure 4.2.3B and A). Variants of pHIV-7 lentivirus were more fluorescent than those of pHIV-EGFP on days 5 and 8. Interestingly, on day 5, cells were fluorescent, however, blotting did not support such observations.

Jurkat cell line is a poor expresser of proteins when compared to HEK 293 T. To rule out the possibility that our gene loses the ability to be expressed once the lentiviral transfer plasmid is integrated into the host genome of the lymphocytic cells, we transduced HEK 293 T cells. Following the same procedures as with Jurkats, we detected low levels of tagged ADAR1 in cellular lysates by blotting against V5 (Figure 4.2.4A). In comparison, the blot against GFP was several times stronger, also confirmed by fluorescence microscopy (Figure 4.2.4A and B). Altogether, we have identified a combination of lentivirus transfer, packaging and envelope plasmids that under optimized conditions can transduce in high ratio lymphocytic cell line to overexpress our gene of interest.

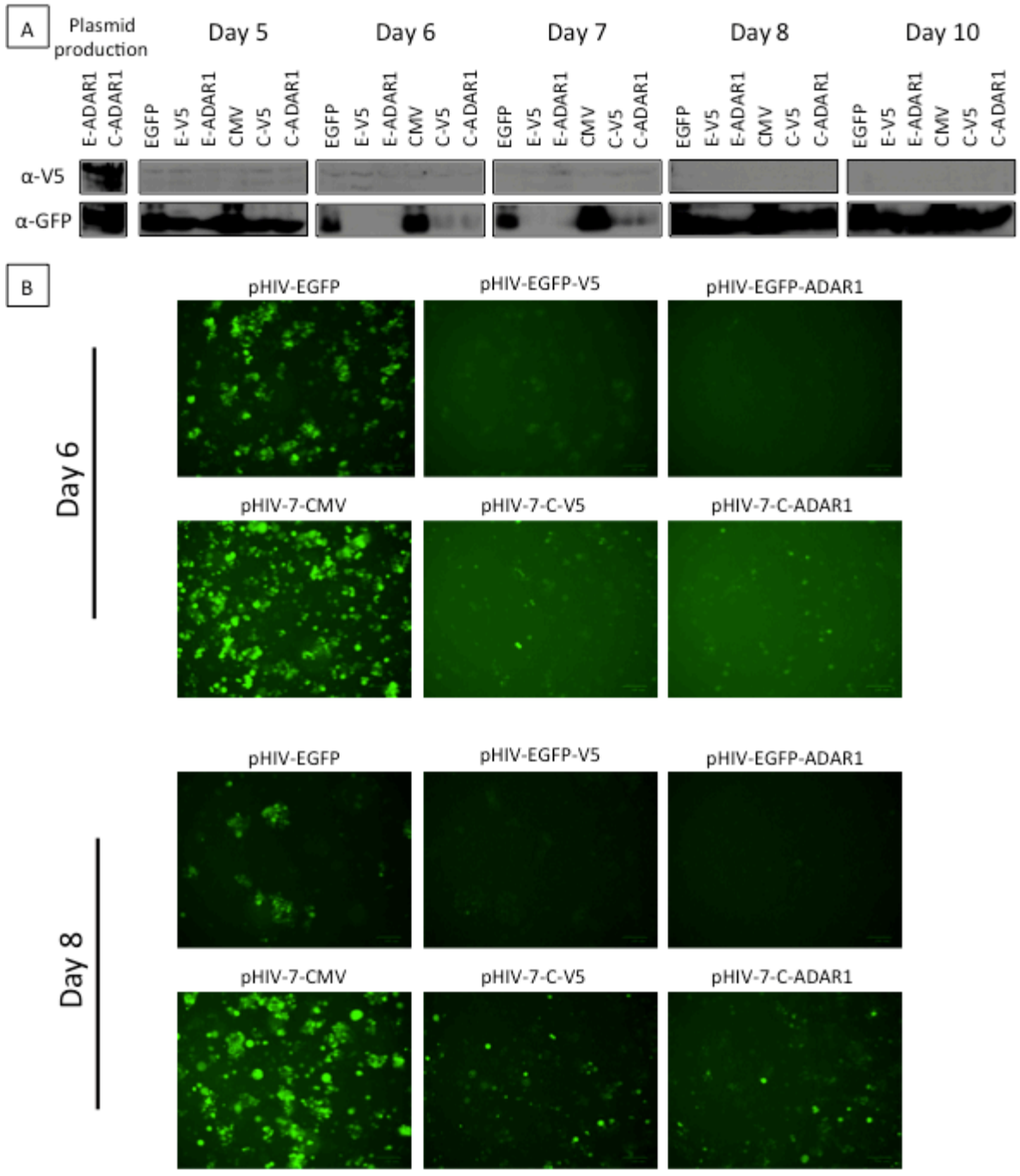

Figure 4.2.3: Transduction of Jurkat cells with lentivirus particles from pHIV-EGFP and pHIV-7-CMV transfer plasmids over 10 days. A. ADAR1p150-V5 and GFP expression from HEK 293 T cells transfection and Jurkat cells transduction over five days as determined with 70 µg of Jurkat cellular lysates via Western Blot, blotted against V5 and GFP. **B.** Transduction of Jurkat cells on days 6 and 8 as seen through GFP imaging. E: pHIV-EGFP and C: pHIV-7 with CMV promoter based viruses.

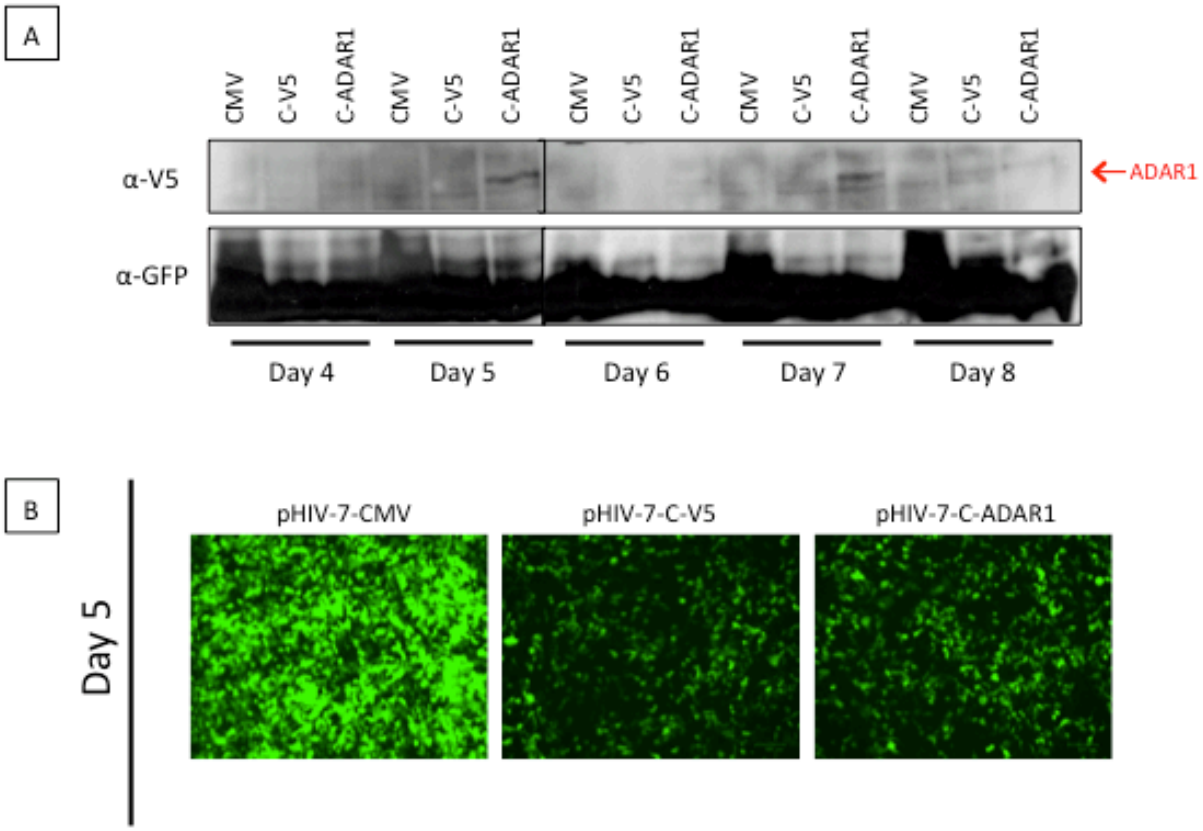

Figure 4.2.4: Transduction of HEK 293 T cells with lentivirus particles from pHIV-7-CMV transfer plasmid over eight days. A. ADAR1p150-V5 and GFP expression from HEK 293 T cells transduction over five days as determined with 70µg of cellular lysates via Western Blot, blotted against V5 and GFP. **B.** Transduction of HEK 293 T cells on day 5 as seen through GFP imaging. C: pHIV-7 with CMV promoter based virus.

4.2.3 Clonal expansion following GFP-sorting generates transduced Jurkat cell line overexpressing ADAR1p150

Despite the optimization of the transduction protocol with pHIV-7-CMV transfer plasmid and its incorporation in HEK 293 T cells and Jurkat (verified by GFP expression), ADAR1p150-V5 was not expressed in Jurkat and low in HEK 293 T. Because our aim is to obtain a lymphocytic cell line expressing ADAR1p150, we transduced Jurkat cell line again, but this time, we sorted and expanded GFP positive clones via flow cytometer, and then evaluated their expression of ADAR1p150-V5. Cells were transduced and sorted via GFP

marker into 96 wells plates. One half of the plate held cells with normal GFP expression, while in the half we sorted cells with the high-intensity signal (Figure 4.2.5). Cell sorting allowed us to also quantify the general transduction ratio of our lentivirus particles. The empty backbone lentivirus transduction was at 71.6%, the V5-cloned lentivirus at 43.7% and the ADAR1p150-V5 lentivirus at 21.6%. These rates conform to the usual proportions we would previously see in our experiments.

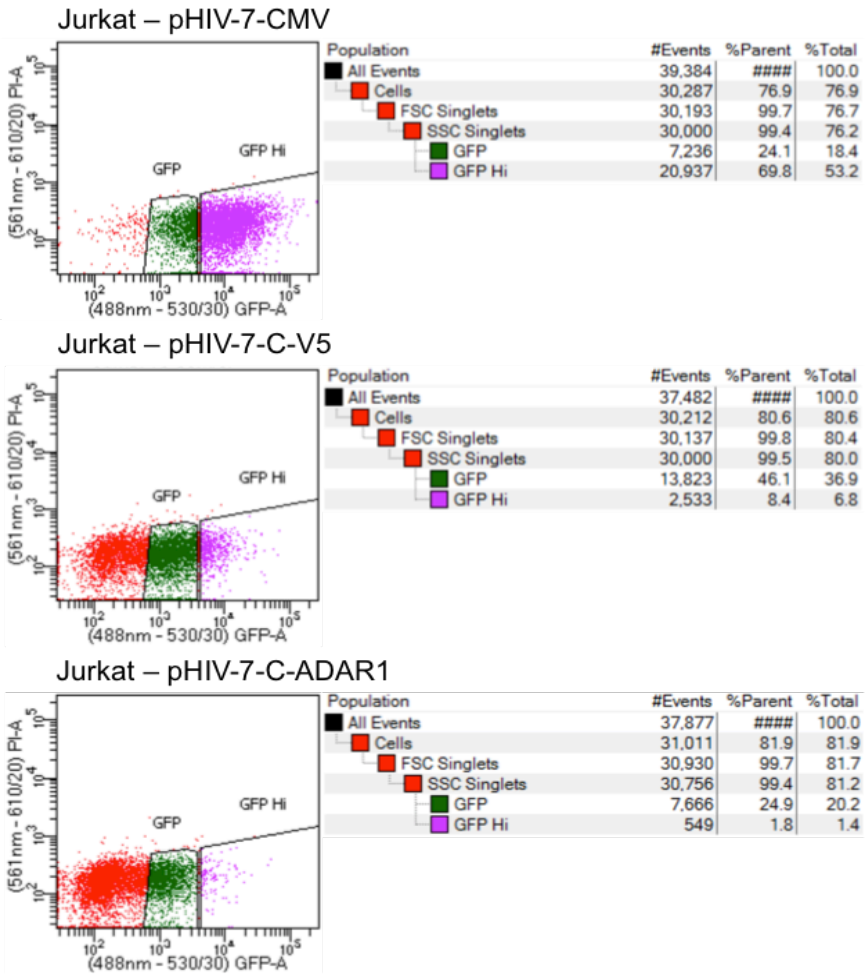

Figure 4.2.5: Flow cytometry dot plot of GFP-positive lentivirus transduced Jurkat cells. Jurkat cells transduced with pHIV-7-CMV, pHIV-7-C-V5 and pHIV-7-C-ADAR1 lentivirus were gated for average and high GFP signal at 510nm and sequentially sorted into 96 well plates.

Clones were visually checked for growth and GFP signal. The controls, original lentivirus pHIV-7-CMV and pHIV-7-C-V5, were also sorted and put into culture. 25 ADAR1 clones were selected, called from A1 to A25, and the ones with most wild type like growing kinetics were screened for ADAR1p150-V5 expression using a V5 antibody (Figure 4.2.6A top). A4, A7, A8, A9, A16 and A25 appeared to express either the p110 or the p150 ADAR1 protein and were further screened with a greater amount of protein lysate loaded into the 10% SDS-PAGE gel (Figure 4.2.6A bottom). A8 was the only one to express high levels of both isoforms of ADAR1-V5. A7 also expressed low amount of p150 that was more visible in a longer exposure (data not shown). A16 clone showed the greatest levels of expression of p110, but the interferon-stimulated isoform was not present. We have also performed PCR on clones' DNA to verify the insertion of ADAR1-V5 or V5-tag with the CMV promoter (Figure 4.2.5B). PCR confirmed gene integration for all ADAR1 clones, despite the fact that A25 did not express ADAR1-V5 in western blot screen (Figure 4.2.6B and A). Only one of the two clones of pHIV-7-C-V5 transduction had positive PCR product, despite both being GFP positive. For the future experiments, we retained clones C1, V2, A7 and A8. Their GFP expression is represented by their respective fluorescence (Figure 4.2.6C).

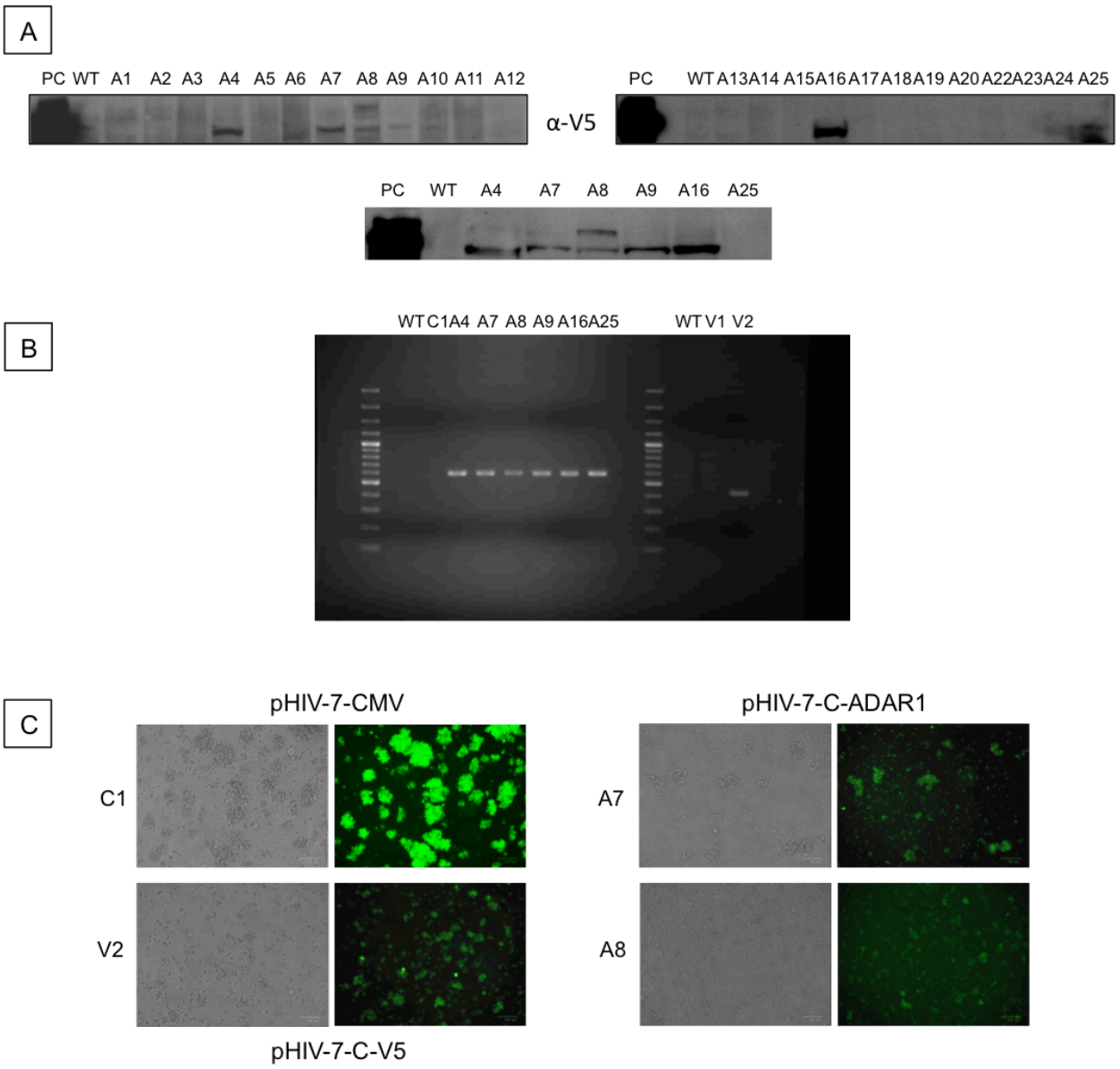

Figure 4.2.6: ADAR1 integration screening of Jurkat cell line transduced with pHIV-7-CMV-ADAR1 lentivirus following clonal expansion. A. Western blot of Jurkat clones that were GFP positive following clonal expansion. 70µg of cellular lysates were run on 10% SDS-PAGE gel and blotted against V5. **B.** PCR of Jurkat clones genome of integrated lentivirus including ADAR1 sequence and V5 tag, for the V5 clones: V1 and V2, as well the pHIV-7-CMV: C1. PCR includes the last region of ADAR1 and V5 tag or CMV region and V5-tag. **C.** GFP expression of Jurkat clones with integrated pHIV-7-CMV, pHIV-7-C-V5 and pHIV-7-C-ADAR1.
WT: Jurkat wild type cells **PC**: positive control of HEK 293 T transfected with pcDNA3.1-ADAR1p150-V5
C1: pHIV-7-CMV transduced Jurkat cellular clone **V#:** pHIV-7-C-V5 transduced Jurkat cellular clones
A#: pHIV-7-C-ADAR1p150-V5 transduced cellular clones

4.2.4 Constitutive overexpression of ADAR1p150 in Jurkat increases HIV-1 replication

Jurkat WT, V2, A7 and A8 clones, constitutively overexpressing V5 and ADAR1p150-V5 respectively from the CMV promoter, were infected with HIV-1 and followed over 22 days. At day 6, the viral production from A8 peaked, followed with a steep decline that then regained its momentum at day 12 (violet). The RT activity from other three samples followed a common trend in which RT activity starts to increase by day 12, while still remaining lower than A8 clone (Figure 4.2.7). Although this experiment is preliminary and should be reproduced, LV A8 expressing ADAR1p150 increases HIV-1 viral replication.

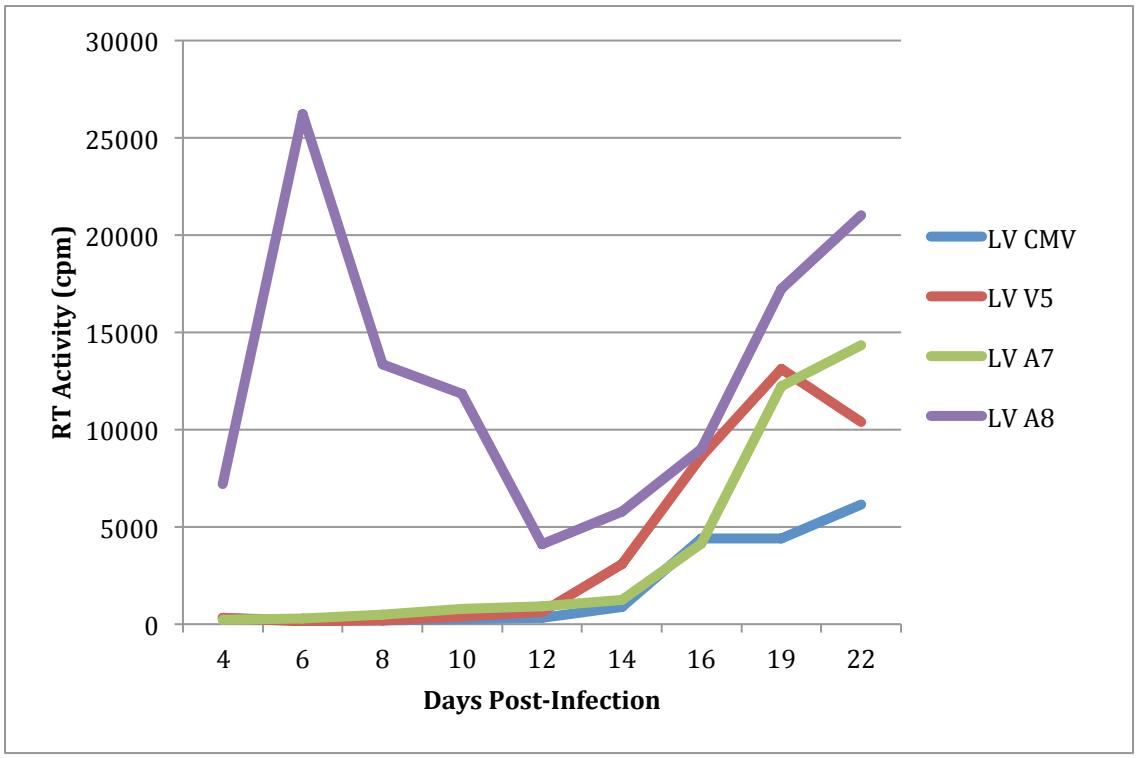

Figure 4.2.7: Constitutive overexpression of ADAR1p150 in Jurkat increases HIV-1 replication. Jurkat WT, V2 (LV V5), A7 (LV A7) and A8 (LV A8), ADAR1 overexpressing clone, were infected with HIV-1 and the viral RT activity was measured over 23 days post-infection.

4.3 Effect of ADAR1p150 knockout on HIV-1 replication in Jurkat cells

To better understand the role of ADAR1p150 and to elucidate the interferon response to HIV-1 infection, we also created a knockout model of the gene. We chose CRISPR/Cas9 approach and have designed gRNAs specifically targeting the interferon-stimulated isoform of ADAR1 in Jurkat cell line (Figure 4.3.1).

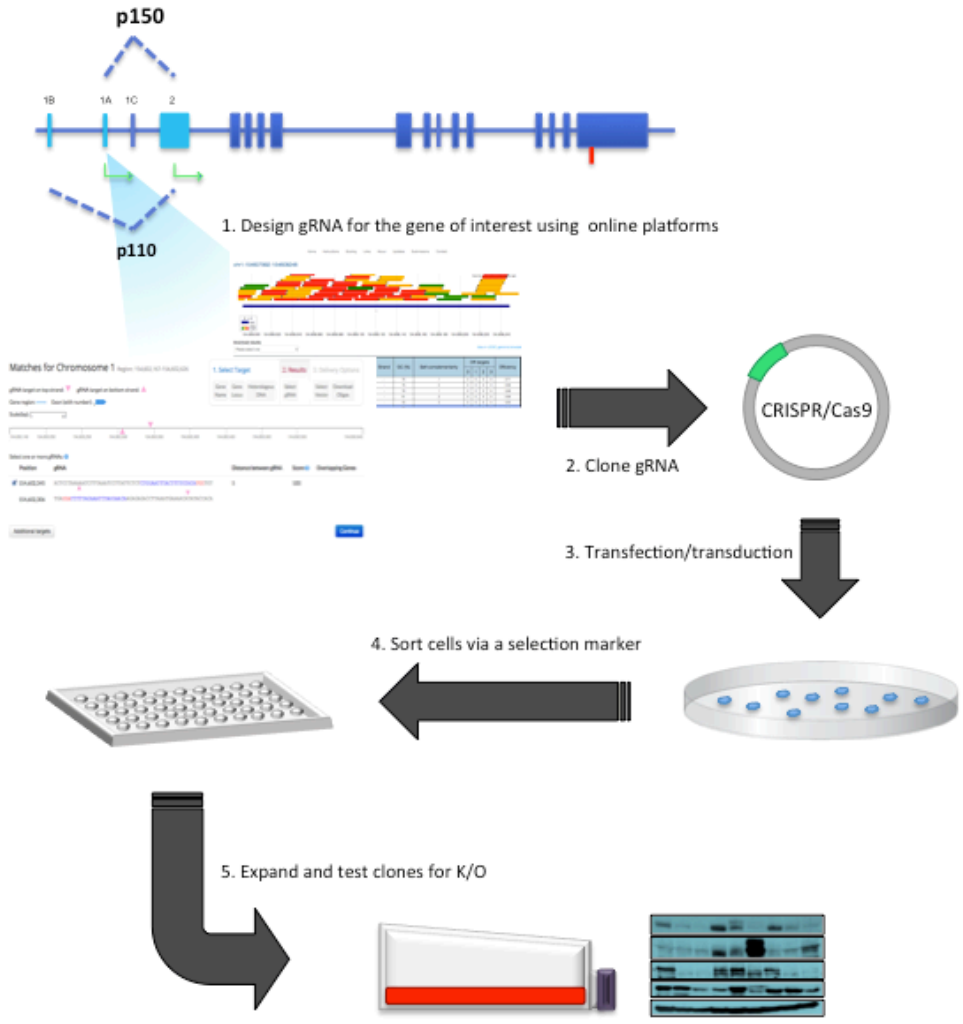

Figure 4.3.1: Flowchart of CRISPR/Cas9 design for a gene knockout. 1. Online platforms offer great tools to design a gRNA targeting a gene of interest or a chromosomal segment with theoretical predictions of efficiency and off-targets. 2. Once the gRNA(s) is selected, it can be synthesized and cloned into a vector coding for CRISPR/Cas9. 3. The vector is then delivered into the cells of interest via transfection, transduction or other available methods. 3.48 hours post-delivery of CRISPR/Cas with gRNA into the cells, a selection marker can be applied depending on the vector characteristics. 5. Selected clones are expanded and then tested for successful K/O (knockout) by a variety of genetic and/or proteomic tests.

4.3.1 Design of CRISPR/Cas9 against ADAR1p150 and generation of knockout clones

In our first attempt, we ordered CRISPR/Cas9 NickaseNinja plasmid with a gRNA targeting the exon 1A of ADAR1 gene designed by homemade platform, pre-cloned and ordered from the company DNA2.0 (today ATUM). Following the transfection and selection of over 500 GFP positive cellular clones on two separate attempts, none were replicating. Therefore, we decided to switch to GeneART CRISPR Nuclease with OFP reporter kit from Thermo Fisher. To design our gRNAs, we have used a publically available web tool, called CHOPCHOP. Five top gRNAs were chosen based on a predicted efficiency of the knockout and absence of theoretical off-targets. Due to the previous experience with Jurkats and considering the fact that ADAR1$^{-/-}$ mouse is embryonically lethal [284], we have decided to transfect three cell lines: HEK 293 T, HeLa and Jurkat, with all five gRNA-specific variants of GeneArt CRISPR plasmids. As an example of transfection efficiency of cells with CRISPR, 31.3% of HEK 293 T, 35.2% of HeLa and 2.7% of Jurkat cells expressed the reporter gene OFP from the GeneArt plasmid containing gRNA1 (Figure 4.3.2). Each CRISPR gRNA variant was sorted into a single 96 well plate based on the intensity of OFP signal, half of the plate had average intensity clones; and the other, high-intensity clones.

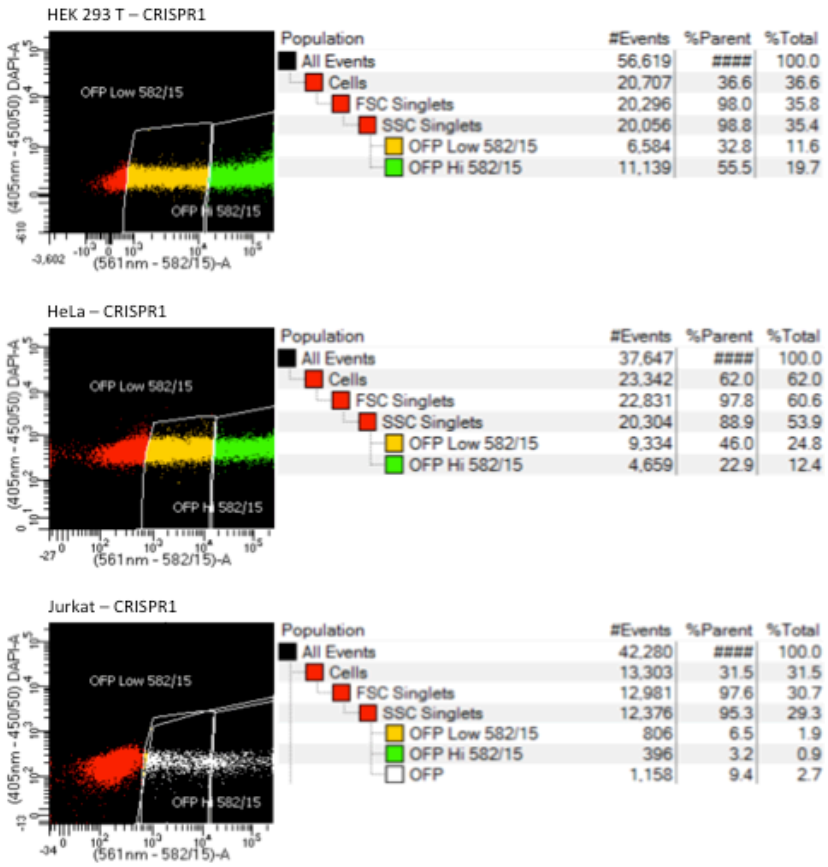

Figure 4.3.2: Flow cytometry dot plot of OFP-positive HEK 293 T, HeLa and Jurkat cells transfected with CRISPR/Cas9 expressing gRNA1. Cells were transfected with 1.5µg of GeneArt CRISPR/Cas9 with gRNA1, and then sorted via flow cytometer excited at 583 nm according to average and high OFP signal into 96 well plates.

4.3.2 Selection and characterization of ADAR1 p150 knockout Jurkat clone

Since the GeneArt CRISPR vector was transfected into cells and transfections are transient, we could not rely on fluorescence for subsequent selection. Therefore, we attempted to screen our clones via genomic cleavage kit that detects in/del mutations via PCR of genomic region suspected to have undergone a non-homologous end joining. However, it did not yield any positive results. As previously mentioned, ADAR1p150 isoform is an interferon-stimulated gene, we have decided to use this to our advantage: stimulate our clones with 10 000 U/mL of IFN-α/β and screen via a western blot. PKR phosphorylation was used as a positive control for IFN stimulation in the cells. Several

clones for each of the three cell lines were screened, but halted upon identification of a Jurkat knockout clone (Figure 4.3.3). We have noticed that one of the HEK 293 T cells knockout reverted back to the wild type after two weeks in culture (data not shown). Therefore, we have additionally screened the Jurkat Cr1.1 clone a month and a half (Figure 4.3.3) and six months into culture (data not shown) for a reversion, which did not occur.

To characterize the knockout generated by CRISPR/Cas9 in the Jurkat clone, we have amplified and sequenced the genomic region of ADAR1's 1A exon. We identified a point mutation, a thymine insertion, generating a frame shift preventing mRNA translation, and therefore, expression of ADAR1p150 isoform (data now shown).

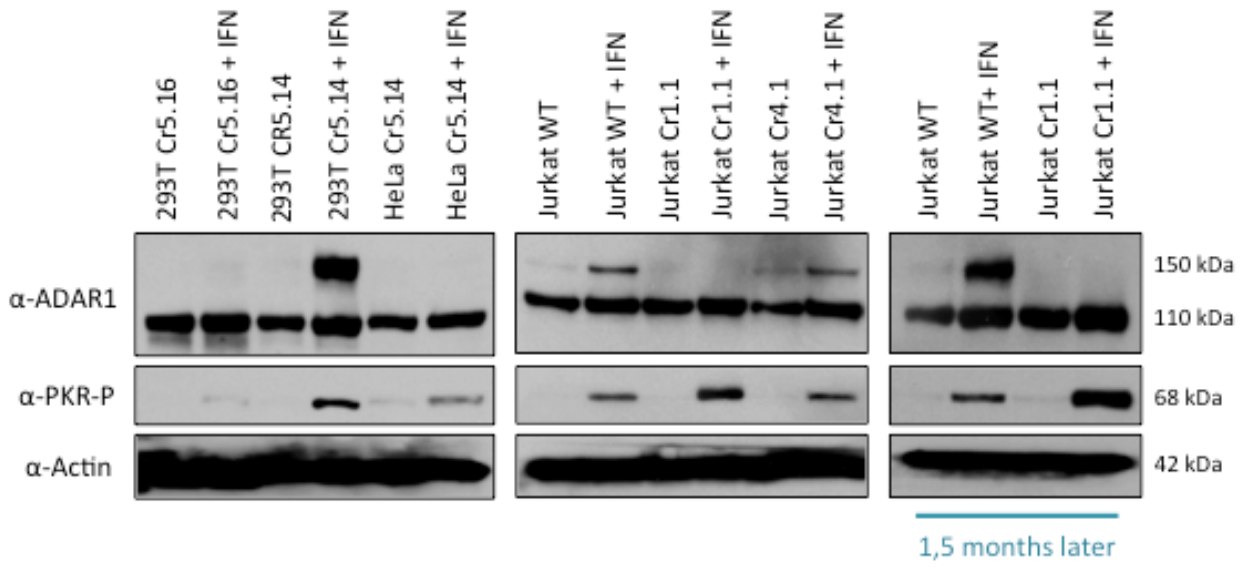

Figure 4.3.3: Screening of ADAR1p150 knockout from HEK 293 T, HeLa and Jurkat cells treated with CRISPR-Cas9. Cellular clones of each cell line were treated with 10 000 U/mL of Interferon, and then lysed for a Western Blot analyses, blotted against ADAR1, PKR-P and Actin. Jurkat Cr1.1 clone was in culture for additional 1.5 months to check for reverse mutations.

4.3.3 Knockout of ADAR1 p150 isoform in Jurkat cell line delays HIV-1 infection

To partially answer our question on the activity of ADAR1p150 in HIV-1 replication, we infected wild type and Cr1.1 Jurkat cells with the virus, and treated the cells with IFN-α/β to stimulate an immune response. Cellular lysates and supernatant were collected over 21 days post-infection for protein profiling and RT activity measurements (Figure 4.3.4). Western blot against PKR profile of wild type infection in comparison to Cr1.1 only revealed two differences. First one being that ADAR1p150 in wild type started to be detectable on day 17, while in Cr1.1 it was naturally absent. The second difference was the expression of viral Gag proteins: p24, p55 and their intermediates revealed by an anti-capsid (p24) antibody. In the wild type, p55 was detectable since day 4 and was continuously increasing with each collection sample reaching a peak around day 19. The intermediary was detectable since day 13 and was also continuously stronger. The earliest detection of p24 was on day 4, and its production substantially started growing at day 11 (Figure 4.3.4A). On the other hand, p55 production in Cr1.1 started to rise only by day 15, although being detectable at day 4 too. A significant signal from the intermediaries appeared but at day 19. p24 expression started to go up around days 13-15. Furthermore, RT activity of the viral particles produced from wild type (blue) and Cr1.1 (green) behaved in a similar manner: for the first it started around day 11, and for the later, at day 17 (Figure 4.3.4B).

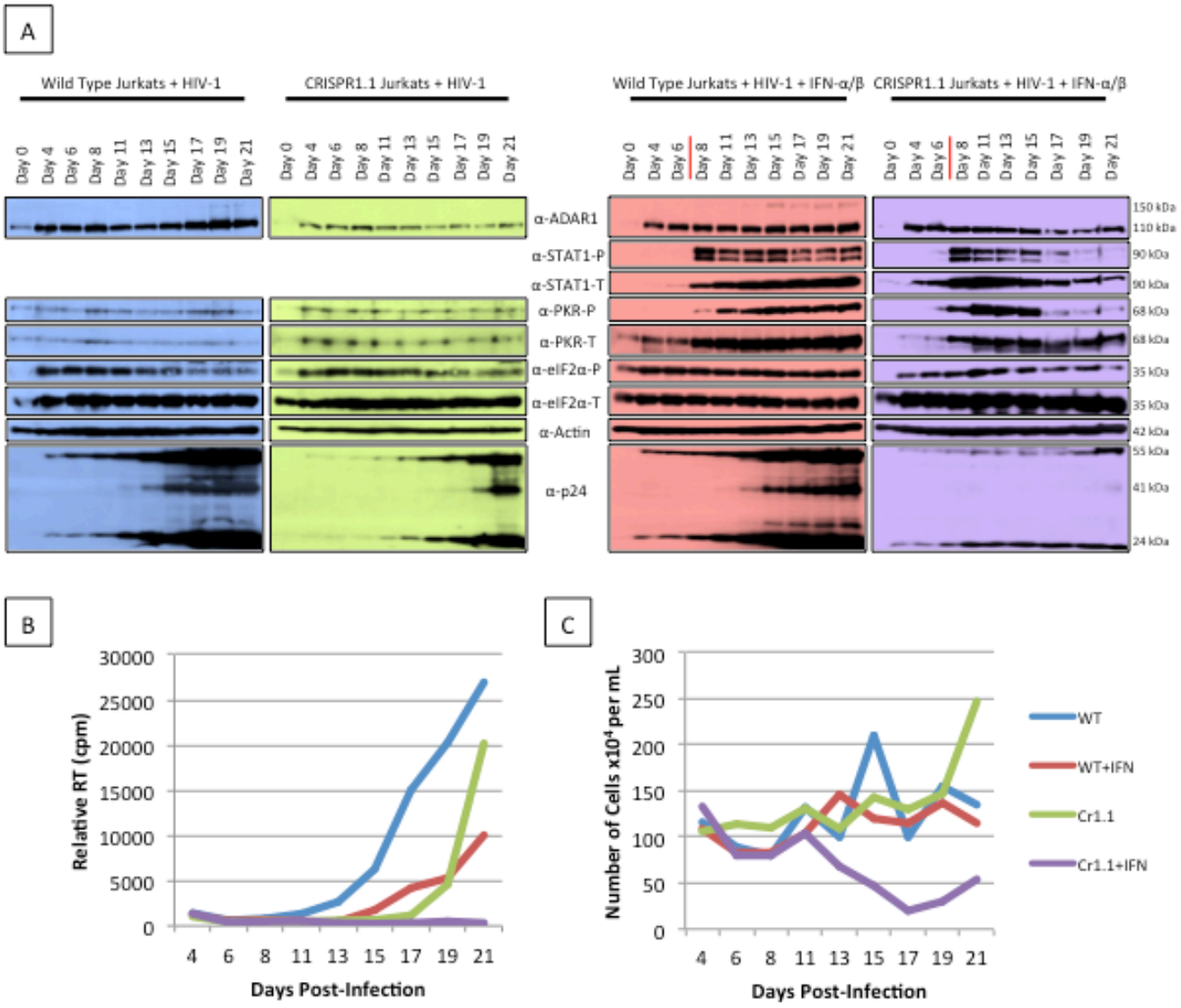

Figure 4.3.4: Knockout of ADAR1 p150 isoform in Jurkat cell line delays HIV-1 production. A. Western Blot of 100µg of cellular lysate of Jurkat wild type and CRISPR1.1 clones treated and non-treated with IFN-α/β at day 7 (red line), blotted against ADAR1, PKR-P, PKR-T, STAT1-P, STAT1-T, eIF2α-P, eIF2α-T, actin and p24 over 21 days. **B.** Reverse Transcriptase activity of HIV-1 virus produced from wild type and CRISPR1.1 Jurkats over 21 days post-infection. **C.** Cell count of the Jurkat WT and CRISPR1.1 infected with HIV-1 and trated with IFN-α/β over 21 days post-infection. Gels in **A** have been colorized to correspond to the legend in **B** and **C**.

We repeated the experiment and introduced IFN-α/β treatment to infected cells on day 7 post-infection. Immediately after, the expression of ADAR1p150 in the wild type Jurkats, although in low levels became noticeable, and STAT1 and PKR became

phosphorylated in both cell lines. The behavior of phosphorylation levels was different between the wild type cells and Cr1.1 clone. In the wild type, the amount of phosphorylated STAT1 remained constant over 13 days, despite a small increase in the total STAT1 during that time. Phospho-PKR followed a similar trend. In contrast, at the addition of IFN-α/β to Cr1.1, phosphorylation of both STAT1 and PKR went up and dropped by day 17 (Figure 4.3.4A). The peak of PKR phosphorylation in Cr1.1 treated with IFN-α/β was at day 11, the same time point from which the number of cells (purple) started to sharply decrease, only to start recovering at day 19 (Figure 4.3.4C). Phosphorylation levels of eIF2α remained constant for both cell lines with a slight decrease towards the end of the observation period in Cr1.1. Production of the viral p24 in wild type treated with IFN-α/β was lower to the non-treated wild type. Moreover, treated Cr1.1 clone had the lowest production of the viral proteins between the four groups. The intermediates were practically non-present, with a small signal appearing at day 21. p55 and p24 levels were detectable since day 4, yet, they stayed at similar levels for the whole observation period (Figure 4.3.4A). Correspondingly, the RT activity of virus from Cr1.1 clone (purple) remained extremely low, constant and never took off (Figure 4.3.4B). RT activity from treated wild type (red) behaved very similarly to the non-treated Cr1.1 condition (green), the only noticeable difference being the sudden rise at day 21 for the Cr1.1 (Figure 4.3.4B). The increase of RT activity correlated with the increase in a number of cells per mL for the non-treated Cr1.1 on the last day of collection (Figure 4.3.4B and C). To summarize, we see a delay of HIV-1 production from ADAR1p150 knockout cells, while IFN-α/β treatment keeps viral replication at a minimum for the first three weeks.

4.4 ADAR1 and PACT interactomes

4.4.1 ADAR1 and PACT interaction requires dsRBDs of ADAR1

The initial hypothesis of this project was that interaction between ADAR1, PACT and a viral element are in a multi-protein complex that inhibits PKR activation during HIV-1 infection and replication. We have chosen to map the interaction between ADAR1 and PACT to use this knowledge in the potential development of a peptide mimetic to prevent the formation of aforementioned complex.

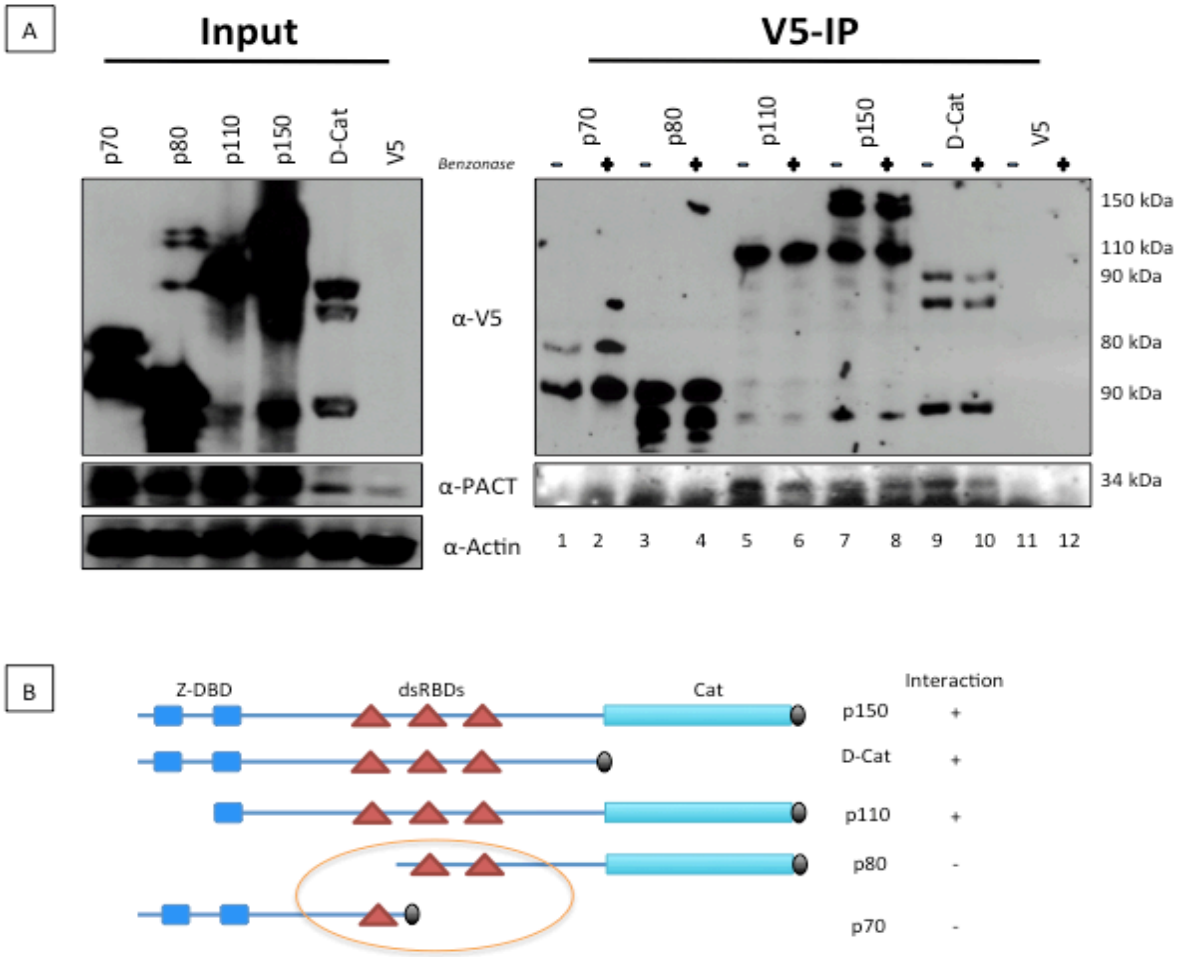

Figure 4.4.1: dsRBDs of ADAR1 are necessary for its interaction with PACT. A. Immunoprecipitaion of PACT by V5-tagged isoforms of ADAR1 from 1.5 mg of cellular lysates from HEK 293 T cells. Beads were treated with Benzonase, a nuclease against single-stranded, double-stranded DNA and RNA. **B.** Schematic map of V5-tagged isoforms of ADAR1 and their PACT interaction profile.

To reach the first goal, we transfected HEK 293 T cells with ADAR1 isoforms and mutants tagged with V5: p150, D-Cat, p110, p80 and p70 previously made in the lab (schematic, Figure 4.3.1B), to perform a V5 immunoprecipitation. Isoforms p110, p150 and mutant D-Cat pulled down the endogenous PACT, while mutants, p70 and p80, did not. Presence of the RNase, Benzonase, did not affect the results of the pull-down (Figure 4.4.1A). The common feature among ADAR1 variants that interacted with PACT in this assay is the intact dsRBDs region of the protein (Figure 4.4.1B).

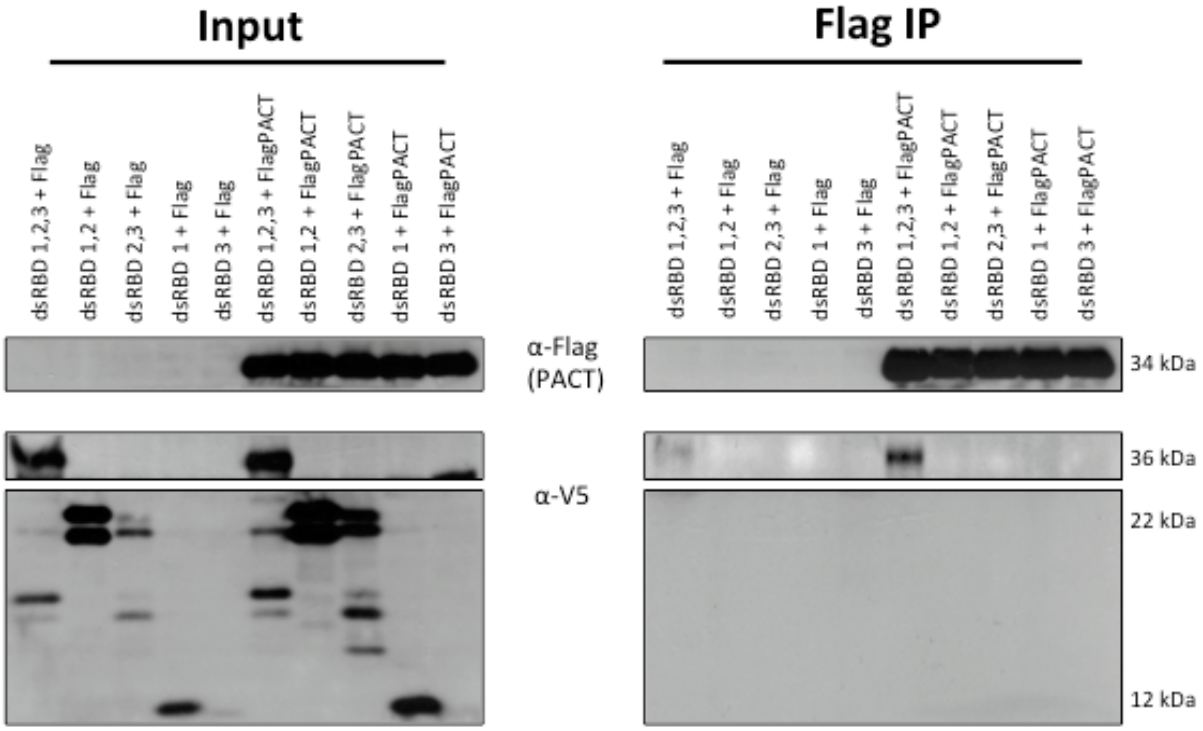

Figure 4.4.2: All three dsRBDs are necessary for the PACT-ADAR1 interaction. Immunoprecipitation of V5-tagged ADAR1's dsRBDs with Flag-PACT from 1.5 mg of HEK 293 T cells transfected with Flag-PACT and corresponding V5-tagged domains. Immunoprecipitate were run on a 15% SDS-PAGE gel and blotted against Flag and V5.

To identify the ADAR1-PACT binding domain in ADAR1 more precisely, we next cloned different combinations of dsRBDs (1-2-3, 1-2, 2-3), as well as single domains into the

pcDNA3.1-V5 plasmid. Domains were co-transfected with pCMV2-Flag-PACT into HEK 293 T cells and a Flag immunoprecipitation was performed. dsRBD3 construct did not express well. Western blot of this assay revealed that only dsRBDs 1-2-3 construct was pulled-down (Figure 4.4.2). Although not entirely complete due to the very low expression of dsRBD3 and the lack of dsRBDs 1-3 combination, this assay suggests that all three dsRBDs might be necessary for ADAR1-PACT interaction.

4.4.2 Tat is in a multi-protein complex with ADAR1 and PACT

PACT was identified as the PKR activator assuming that it is its canonic role [271]. Therefore, the change into a PKR inhibitor characterized in HIV-1 infection is very likely to be triggered by a viral element [188]. One of the *in vitro* studies showed that PACT and ADAR1 inhibit PKR more efficiently in presence of Tat on PKR activation, suggesting a multi-protein complex between the three [248]. We decided to test this direction by co-transfecting pcDNA3.1-ADAR1p150-V5, pCMV2-Flag-PACT and pCMV-Myc-Tat into HEK 293 T cells. Immunoprecipitation of Flag-PACT revealed its interaction with both ADAR1 and Tat as visualized in different gels. 10% and 12% polyacrylamide gels were used to visualize ADAR1-V5 and Myc-Tat fusion proteins respectively (Figure 4.4.3). We have also noted that co-transfection with Myc-Tat increased the expression of Flag-PACT and ADAR1-V5 (lanes 5 and 7), as well as the amount of precipitated proteins (lanes 12 and 14, Figure 4.4.3). The exact role of Tat in the complex with PACT and ADAR1 on PKR inhibition is yet to be determined, but it may be one of the viral factors causing the PACT's switch from activator to inhibitor of the aforementioned kinase.

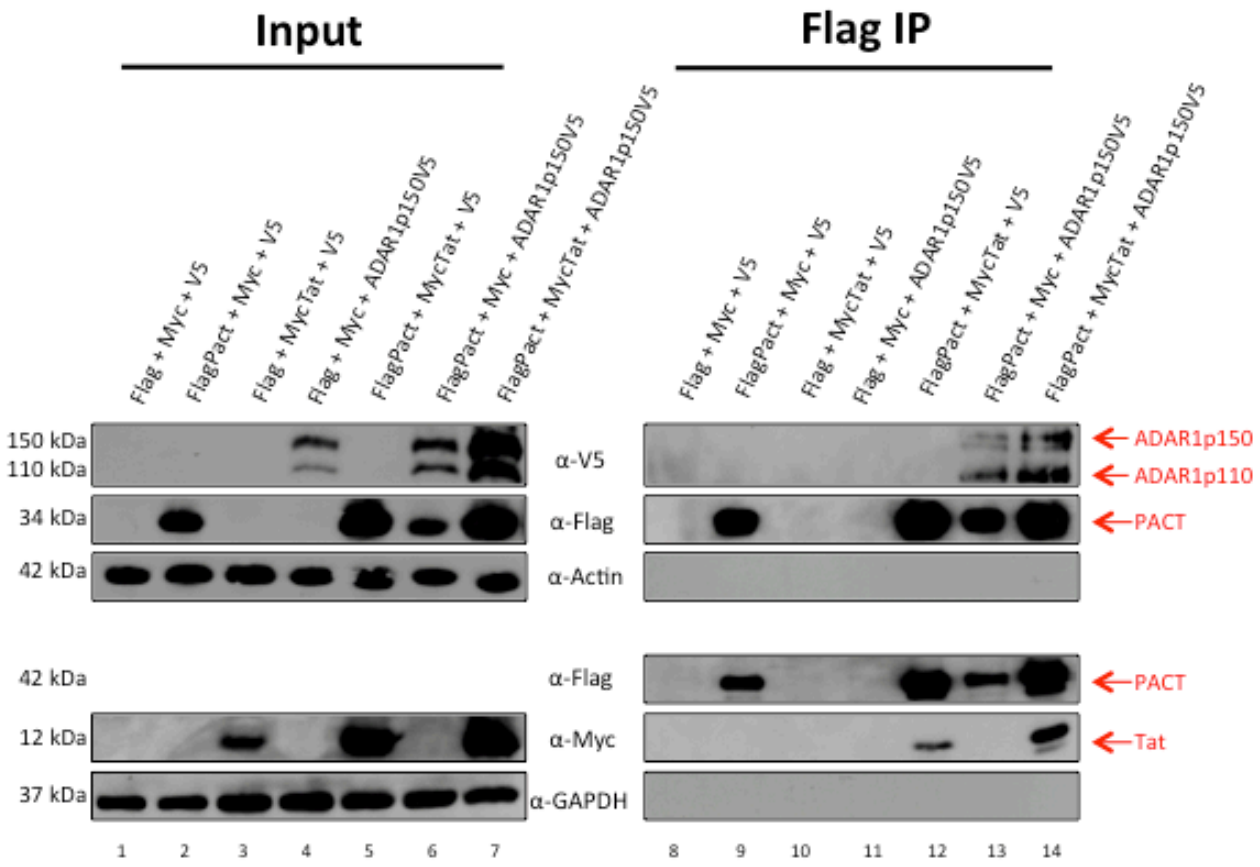

Figure 4.4.3: PACT forms a multi-protein complex with ADAR1 and Tat in HEK 293 T cells. HEK 293 T cells were transfected with 0.75 µg of pCMV2-Flag (lanes 1, 3 and 4) or pCMV2-Flag-PACT (lanes 2, 5, 6 and 7), and pcDNA3.1-V5 (lanes 1, 2, 3 and 5) or pcDNA3.1-ADAR1p150-V5 (lanes 4, 6 and 7)), and 1.0 µg of pCMV-Myc (lanes 1, 2, 4 and 6) or pCMV-Myc-Tat (lanes 3, 5 and 7). The cellular lysates were collected 48 hours post-transfection. 100 µg of cell extract from each condition was used for the input (lanes 1-7) and 1.5 mg were used in the immunoprecipitation assay (lanes 8-14) and loaded on a 10% and a 12% polyacrylamide gels. The 0.45 µm membranes (top) were then blotted with anti-V5, anti-Flag and anti-Actin. The 0.20 µm membranes (bottom) were blotted with anti-Flag, anti-Myc and anti-GAPDH.

4.4.3 PACT interactome in HIV-1 producing cells

Tat presence in the multi-protein complex does not exclude other viral elements or cellular from contributing to the HIV-1 mediated control of PKR activity. To attempt to isolate these proteins, we co-transfected HEK 293 T cells with pCMV2-Flag-PACT and HIV-1 molecular clone, pNL4-3, immunoprecipitated Flag-PACT and sent the samples for mass spectrometry analysis. RT activity measurement acted as a control of successful co-transfection, and it revealed that addition of Flag-PACT doubles the RT activity when

compared from expression of HIV-1 molecular clone alone (Figure 4.4.4A). The mass spectrometry analysis was performed by Quebec Genome Center at Laval University and visualized via Scaffold 4 with peptide/protein threshold set at 1% FDR and minimal peptide/protein count set at two. There were over 800 hundred peptides and proteins discovered, with 20 being unique to PACT and 58 to HIV-1+PACT sample, and 42 were in common between the two conditions (Figure 4.4.4B). For this analysis, we were mainly interested in the HIV-1+PACT unique pull-downs. Gene ontology of those peptides revealed that most of them contribute to cell processing (32) and metabolic process (Figure 4.4.4C). Few also are known to be involved in the viral processing (5) and immune system process (5) (Figure 4.4.4C). However, mass spectrometry did not detect the proteins, we found via immunoprecipitations analyzed by western blot in previous experiments.

The only viral protein that was identified in this assay was Gag p17, matrix protein. We have also compiled some of the 58 proteins unique to HIV-1+PACT condition together with their gene ontology terms (Table 4.4.1). Their roles included but were not limited to regulation of viral genome replication, regulation of translation and transcription, as well the cell cycle such as regulation of proliferation and programmed cell death (Table 4.4.1).

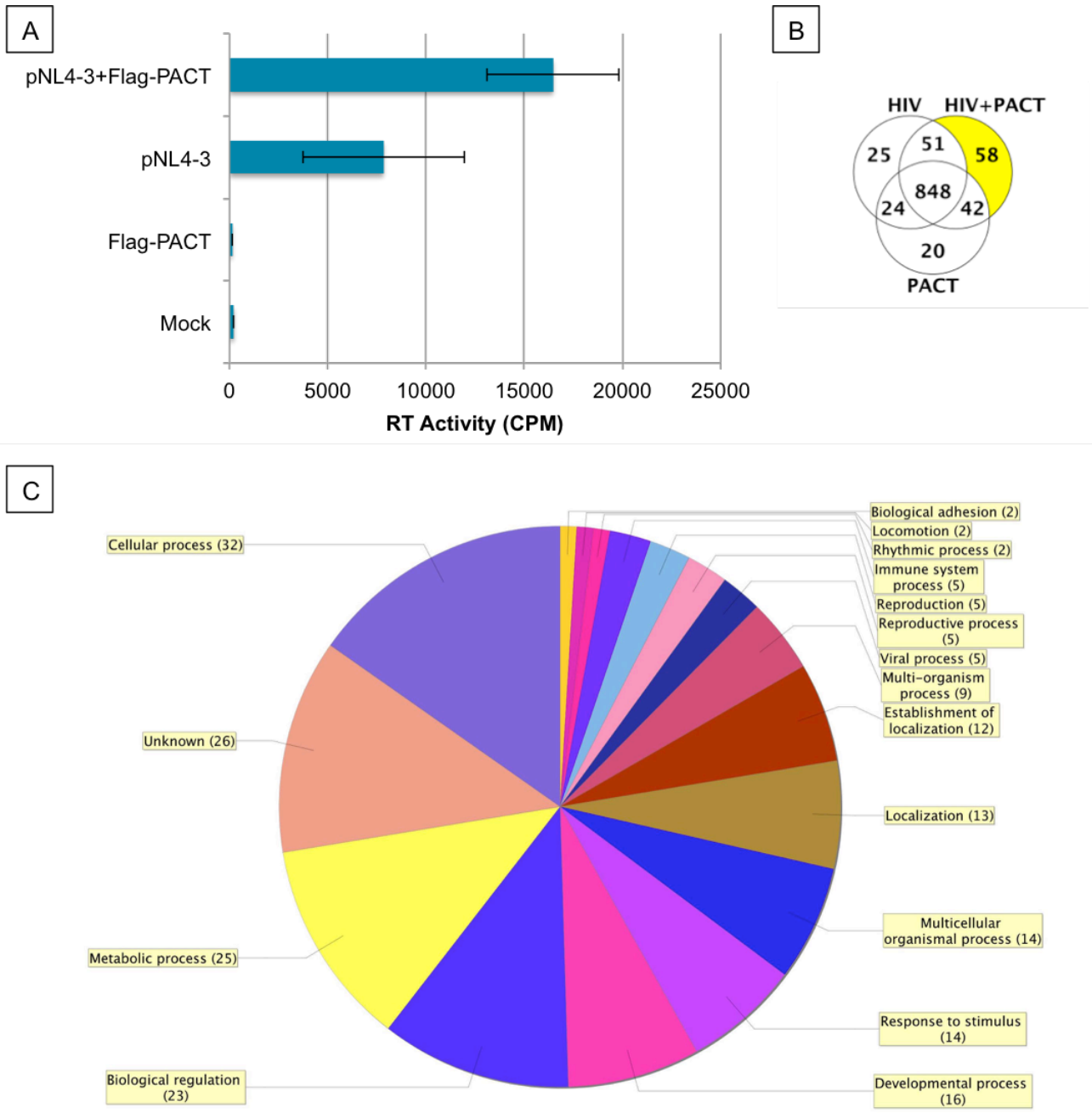

Figure 4.4.4: Mass spectrometry analysis of PACT interactome during HIV-1 production. A. Reverse transcriptase activity of HIV-1 produced from transfection of 3µg of pCMV2-Flag-PACT, shown as PACT, or co-transfected with 1.5µg of HIV-1 molecular clone, pNL4-3 and 1.5µg of pCMV2-Flag-PACT. Mock represents non-transfected cells. **B.** Venn's diagram indicating the numbers of proteins identified by MS that were pulled down in a Flag-PACT co-immunoprecipation. Proteins found in the control, mock-transfected, were excluded from the list in the Venn's diagram. **C.** The biological functions of proteins exclusively pull-down from the pCMV2-Flag-PACT and pNL4-3 co-transfection. Due to the inability of the software to retrieve gene ontology of all the proteins identified by MS, some were marked as unknown.

Table 4.4.1: List of proteins and their gene ontology that were uniquely pull-down from HIV-1 producing cells co-transfected with pCMV2-Flag-PACT.

Protein	Gene ontology or known function
Gag p17	Multiple functions, such as inducing T-cell proliferation
SFRS protein kinase 1	Negative regulation of viral genome replication Positive regulation of viral genome replication Regulation of mRNA processing Regulation of mRNA splicing, via spliceosome Innate immune response
DNA topoisomerase 1	DNA replication Programmed cell death Viral process
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	Golgi organization Cell activation involved in immune response Cellular response to virus Neutrophil chemotaxis Reactive oxygen species biosynthetic process Viral process
Eukaryotic translation initiation factor 2D	IRES-dependent viral translational Initiation formation of translation preinitiation complex
Vesicle trafficking protein SEC22b	Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent Negative regulation of autophagosome assembly Positive regulation of protein catabolic process
Alpha-taxilin	B-cell activation Exocytosis
60S ribosomal proteins L31 and L36	Nuclear-transcribed RNA catabolic process Translation initiation
Calcyclin-binding protein	Negative regulation of cell death
Parafibromin	Negative regulation of apoptotic process Negative regulation of cell proliferation Negative regulation of transcription from RNA polymerase II promoter Protein ubiquitination
Guanine nucleotide-binding protein-like 3	Positive regulation of pri-miRNA transcription from RNA polymerase II promoter Positive regulation of telomere maintenance Regulation of cell proliferation
Staufen	Positive regulation by virus of viral proteins in host cell Positive regulation of viral genome replication vRNA transport Cellular response to oxidative stress
Insulin-like growth factor 2 mRNA-binding protein 3	Negative regulation of translation Regulation of mRNA stability mRNA transport
Myv-binding protein 1A	Circadian regulation of gene expression Negative regulation of transcription

4.4.4 PACT and ADAR1 interactome in HIV-1 producing cells

Before making any conclusion and passing to the confirmation step of mass spectrometry, we performed another set of mass spectrometry from V5 and Flag immunoprecipitation of HEK 293 T cells co-transfected with pNL4-3 and either pCMV-

ADAR1p150-V5 or pCMV2-Flag-PACT respectively. This time, the proteomic group of IRIC at Montreal performed the mass spectrometry. The immunoprecipitation conditions were much more stringent than the Quebec Genome Center protocol, resulting in a much lower quantity of identified proteins even despite changing the requirement of minimum peptide/protein detected from two to one (Figure 4.4.5A). There were only 23 unique proteins from Flag-PACT precipitation from HIV-1 producing cells, and but 5 proteins for ADAR1+HIV-1 condition (Figure 4.4.5). Most of the proteins in both cases were histones and ribosomal units (data not shown). RT activity measurement revealed that addition of ADAR1 to pNL4-3 transfection doubles the RT activity. These levels are comparable to a transfection with the double amount of pNL4-3 plasmid alone. Flag-PACT also increased the enzyme's activity although only by 10 000 CPM (Figure 4.4.5B).

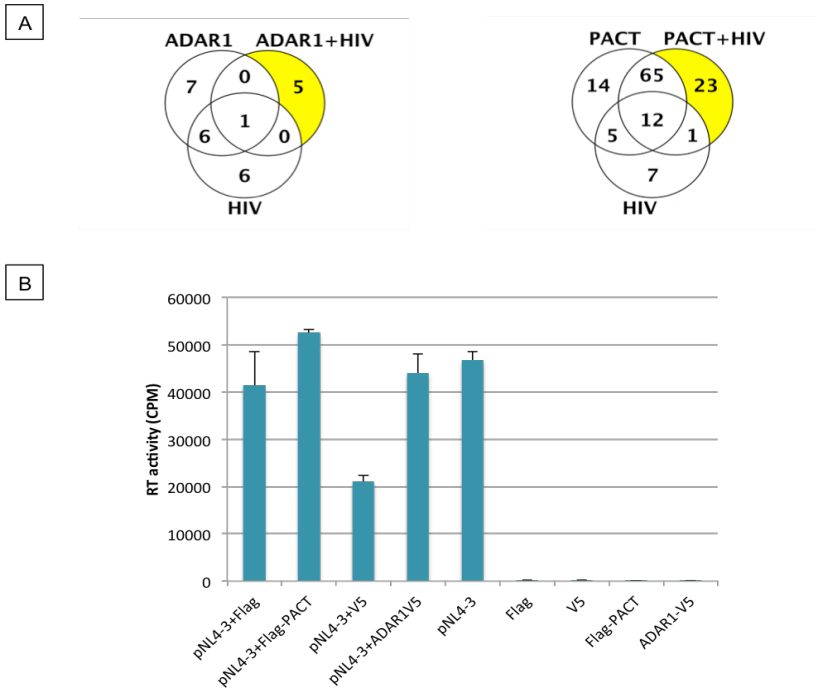

Figure 4.4.5: Mass spectrometry analysis of PACT and ADAR1 interactome during HIV-1 production. A. Venn's diagram indicating the numbers of proteins identified by MS that were pulled down in a Flag-PACT or ADAR1-V5 co-immunoprecipation from 1.5mg of cellular lysate from HEK 293 T cells co-transfected with 1.5µg of either pCMV2-Flag-PACT or pCMV-ADAR1p150-V5 and 1.5µg of pNL4-3. In the pNL4-3 (HIV), Flag, V5, PACT and ADAR1 conditions, cells were transfected with 3µg of plasmid. **B.** Reverse transcriptase activity of HIV-1 produced from (co)-transfections.

Chapter 5: Discussion

Multiple studies have shown that many viral infections can successfully be controlled by ISGs, while others, such as HIV-1, escape the grasp of the immune system. Our laboratory has demonstrated that one of the most prominent ISGs, PKR, is inhibited during HIV-1 production. Moreover, this negative modulation of anti-viral response is not exclusively inherent to the virus, but includes participation of cellular factors such as TRBP, PACT and ADAR1.

5.1 ADAR1p150 isoform is over-expressed in patients with actively replicating HIV-1 and down-regulated with hyper-phosphorylation of PKR

In this part of the project, we attempted to better understand the dynamic of PKR pathway in primary cells of HIV-positive individuals. Cellular lysates of PBMCs from healthy, HIV-positive treated and non-treated donors were analyzed and compared to identify discrepancies in the IFN-PKR pathway. Indeed, in the viremic group with actively replicating and circulating virus, p150 isoform of ADAR1 was generally overexpressed (Figure 4.1.1B). ADAR1p150 is the IFN-stimulated isoform of the protein and its expression directly suggests that the IFN pathway, including PKR phosphorylation, is activated. This is indeed the case for two of the patients' samples (5 and 6, Figure 4.1.1B), yet, in two other samples PKR is also phosphorylated but no ADAR1p150 is observed in the western blot (8 and 9, Figure 4.1.1B). We therefore concluded that the expression of IFN stimulated isoform of ADAR1 might be specifically correlated with actively circulating HIV-1. Considering that previous studies identified ADAR1 as a negative regulator of IFN production, as well as the fact that HIV-1 is capable of suppressing IFN and ISGs, we propose a hypothesis that p150 isoform plays an essential role as a negative modulator of IFN pathway in HIV-1 infection.

Interestingly, the analysis of sample #15 fits the working model of ADAR1 and PACT as cellular pro-viral factors acting against PKR, but in a reverse fashion. The hyper-phosphorylation of PKR co-occurred with extremely low, almost inexistent, levels of ADAR1 and PACT (Figure 4.1.2B). The exact reason of hyper-phosphorylation of PKR is unknown to us, but we did notice the low count of CD4 cells, 42/mL, and high count of CD8 cells, 1363/mL, in the blood of the donor. Clinically these parameters fit with two periods of HIV-1 cycle in the host: early and late infection. During both of those periods HIV is rapidly replicating - leading to CD4 cells exhaustion, while CD8 cells, as well as the rest of immune system, mount an inefficient response [307]. Higher ratio of CD8 over CD4 populations is also a sign of CD8 immunosenescence associated with age and CMV infection [308]. The patient #15, who is 51 years old and CMV positive, meets both of the later criteria (Figure 4.1.2A). All things considered, we believe that PKR hyper activation might be a sign of immune exhaustion, high apoptosis rate and potentially onset of AIDS. An exhausted and defective immune system would not require modulators of the anti-viral response, which would explain the absence of PACT and ADAR1. Furthermore, PKR's substrate, eIF2α phosphorylation levels, were unaffected by the variation of PKR activation, arguing for an inadequate response (Figure 4.1.2). Another viremic non-treated patient sample that had low CD4 count had higher than average levels of phosphorylated PKR combined with low expression of PACT and absence of ADAR1p150 isoform (#13, Figure 4.1.2). Despite revealing some interesting patterns inspiring some of the following parts of this thesis, any definitive conclusion from patients' samples need to be confirmed with the analysis of additional samples.

5.2 Technical challenges towards overexpression and knockout of ADAR1p150 in Jurkat cells

It would be unfair to move onto the next section without briefly mentioning the challenges of generating ADAR1p150 overexpressing and knockout models in Jurkat cells. The main challenges of the overexpression model were to achieve high transduction efficiency of the lentivirus and to have significant expression of ADAR1p150 from transduced cells. In order to address the first, we started with optimization of lentiviral production and transduction. After one entirely unproductive and one intermediate tries, we identified the transfer plasmid, pHIV-7, that following transduction expressed the most of selection marker, GFP (Figure 4.2.2). Other optimization included increasing the ratio of packaging plasmid and addition of Sodium Butyrate to the lentivirus producing cells. Polybrene was also effective in increasing the transduction of Jurkat cells (Figure 4.2.2C). In order to address the poor expression of ADAR1p150 in the Jurkat, we had to sort and amplify single clones despite the relatively high levels of GFP signal (Figures 4.2.4 and 4.2.5).

The knockout model proved to be as demanding to generate. In our first two attempts we sorted and observed over 900 unique clones that were transfected with CRISPR/Cas9-Nickase with gRNAs targeting ADAR1p150 isoform, not a single viable clone was identified. This was not entirely surprising, since $ADAR1^{-/-}$ mice are embryonically lethal. Focusing on the fact that some $ADAR1^{-/-}$ cell lines, such as mouse embryonic fibroblasts, do exist, we designed five new gRNAs against ADAR1p150 isoform. Screening of HEK 293 T cells revealed a clone with a successful knockout, which reverted back to the wild type in a month. This further demonstrated that ADAR1p150 plays an important role

in the cells. A Jurkat ADAR1p150$^{-/-}$ clone with a frame shift mutation in the gene was identified and was shown not to revert back 1.5 and 6 months into the cell culture (Figure 4.3.3A).

5.3 ADAR1p150 isoform positively modulates HIV-1 replication in Jurkat cells

Overall, HIV-1 infection of both models showed that ADAR1p150 isoform has a positive effect on viral production. A preliminary RT activity analysis of HIV-1 infection of a Jurkat clone constitutively overexpressing both isoforms of ADAR1 revealed an elevated and much earlier HIV-1 production. Its proviral effect has shown to act in a double-edged manner, as we see a sudden decline in RT activity following a peak, although later recovered (Figure 4.2.7). It is possible that around days 6-8 post-infection, the rate of cell death due to viral budding exceeded that of cell division in the culture. We believe that such a prominent response in the first week of infection further argues the role of IFN-stimulated isoform of ADAR1 in the establishment of HIV-1 infection in the host.

Complementary assays in ADAR1p150 knockout Jurkat cells infected with HIV-1 showed that RT activity and viral capsid production are delayed by six days. Those viral kinetics were comparable to the infection of wild type Jurkats that were then treated with IFN-α/β at day 7. This finding fits into the model of ADAR1 acting as the negative regulator of IFN and ISGs anti-viral response. Moreover, our data suggests that this role can be attributed to the p150 isoform, hinting that it acts in a negative feedback loop manner. Addition of type I IFN revealed few other aspects of ADAR1p150 role in cells during HIV-1 infection. First, we can see a peak in activation of STAT1 and PKR at days 8 and 11 in knockout clone, Cr1.1, treated with IFN. This is different to the constant or progressive activation in the wild type IFN-treated cells. Peak of PKR-phosphorylation is followed with a

sudden drop in Cr1.1 cell number, suggesting that the absence of ADAR1p150 is partially responsible of mediating pro-apoptotic function of PKR just like in VSV and MV infections [266, 267]. The progressive decrease of STAT1 and phosphorylated PKR in Cr1.1 by day 17 appears to be proportional to the drop in cell numbers and therefore, might be regulated by an apoptotic gene. Interestingly, there is less of Gag intermediates and p24 produced from ADAR1p150$^{-/-}$ cells (Figure 4.3.4). This is in correlation with the low amount of virions released as measured by RT activity. Therefore, the absence of ADAR1p150 decreases the expression of Gag and consequently the formation of viral particles. However, we cannot exclude that the RNA editing role of ADAR1 may potentially be responsible of editing viral RNA affecting cleavage sensitive regions. Thus, absence of its isoform may affect the viral production.

To summarize, ADAR1p150 seems to be necessary for a normal progression of HIV-1 production, at least in the first three weeks of infection. The only proviral role that we can conclude with our data is modulation of PKR and its pro-apoptotic activity leading to the cell death of infected cells. More data and assays such as RNA sequencing could to be done to determine if the RNA editing role of ADAR1 is also involved in the regulation of HIV-1 replication.

5.4 ADAR1 and PACT interactomes

Following the observations of proviral roles of ADAR1 and PACT in HIV-1 infection, as well as identification of their interaction, we hypothesized that the two proteins might act together on PKR inhibition. ADAR1 might also be one of the factors causing PACT's functional switch. In our attempts to characterize the complex, we identified that all three dsRBDs of ADAR1 are necessary for the RNA-independent interaction with PACT (Figures

4.4.1 and 4.4.2). We have also shown that Tat is part of the complex. Moreover, Myc-Tat increased co-expression of Flag-PACT and ADAR1-V5 from their respective plasmids. Tat presence also increased the amount of protein pull-down, suggesting that it may stabilize the aforementioned complex (Figure 4.4.3).

To further answer the question of what makes the change of function of PACT in HIV-1 infection, we have performed two independent mass spectrometry analyses on PACT interactome from HIV-1 producing cells. The first one was performed at Quebec Genome Center and they have identified 58 proteins, and the second one, from IRIC, identified 23 proteins that uniquely interacted with PACT in virus producing cells (Figures 4.4.4 and 4.4.5). Quantitative difference between the two results stems from more stringent conditions requested by proteomic center at IRIC. In parallel to PACT analysis, we also did the ADAR1 interactome from HIV-1 producing cells, which revealed extremely low number of proteins interacting with ADAR1, as well as no commonalities with PACT pull-downs.

Gene ontology terms distribution revealed that majority of proteins unique to PACT interactome in HIV-1 producing cells are mostly involved in cellular process (32), metabolic process (25) and biological regulation (23). Of the more rare terms associated with the proteins were viral (5) and immune system (5) processes. Some of these proteins were listed with more specific annotations for potential analyses (Table 4.4.1).

The only viral protein pulled-down by PACT and identified via mass spectrometry was Gag p17. Preliminary results in our lab described that overexpressed PACT potentially gets integrated into the viral particle and may interact with Gag (S. Burugu, unpublished). Another protein that we knew that interacts with PACT and was identified in the list of mass spectrometry identifications from IRIC analysis is PKR. It was present in similar

amount in both conditions with and without HIV-1. Another kinase, SFRS protein kinase 1, was found to interact with PACT exclusively in HIV-1 producing cells. Its best-known function is on the regulation of pre-mRNA splicing, and some groups have found that it has a proviral effect in HCV infection, but is antiviral in HBV and Herpes Simplex viral infections [309]. Another protein implicated in viral processing is Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1. It plays an important role in maintaining Golgi structure and enabling anterograde and retrograde traffic through the Golgi [310]. Some groups described that it plays an important role in early infection of Dengue virus and indirectly affects HCV replication and other positive single stranded RNA viruses [311]. The third and last protein known to be involved in viral processes is Staufen. Staufen is a dsRNA binding protein, involved in the transport of mRNAs to different subcellular compartments. One study characterized Staufen's PKR inhibitory effect in HCV, while another identified ADAR1 as the inhibitor of mRNA decay mediated by Staufen [312, 313]. Other proteins pulled-down by PACT from HIV-1 producing cells are mostly involved in transcription, cell proliferation and apoptosis (Table 4.4.1). These general profiles fit with our model of PACT inhibiting the pro-apoptotic PKR response against HIV-1 and hints at the involvement in some other steps of viral and cellular cycles through protein-protein interactions.

ADAR1 pull-down from HIV-1 producing cells only had five unique proteins as identified by mass spectrometry (Figure 4.4.5). These proteins were histones and unknown peptides. Thus, no conclusion could be made from the experiment and needs to be redone with either higher amount of ADAR1 or less stringent conditions during the immunopreciptations.

To sum up, ADAR1 and Tat interact with PACT, and contribute to the inhibition of PKR response to HIV-1 elements. We also showed that PACT interactome in HIV-1 producing cells consists of proteins mainly involved in mRNA processing, transcription, proliferation and apoptosis. Further studies on these findings may lead to identification of new pro-HIV roles of PACT.

Chapter 6: Conclusion and Future Directions

To conclude, the first part of this project started with exploratory IFN and PKR pathways analysis of PBMCs from HIV-1 infected viremic patients. It revealed higher levels of IFN-stimulated isoform of ADAR1 in cell extract of some non-treated individuals, and that, in cases with low CD4 cell count, the p150 isoform is in reverse correlation with PKR activation. This prompted us to genetically engineer Jurkat cell models with the overexpression and knockout of ADAR1p150 by using lentivirus and CRISPR/Cas9 techniques respectively. Both models confirmed ADAR1's proviral effect on HIV-1 production. Moreover, IFN treatment of the ADAR1p150 knockout cell line stimulates PKR activation and its pro-apoptotic role in HIV-infected cells leading to cell death.

In near future, we would like to further study the PKR pathway, as well as confirm some of our findings, in PBMCs from HIV-1 infected individuals and establish a statistically significant conclusion about ADAR1p150 overexpression. A look into RIG-I/MDA-5 pathway in PBMCs and ADAR1p150$^{-/-}$ cells may also yield interesting results based on current literature. Additional assays with our models are also needed to have a full picture. To start, we should analyze the PKR activation on our overexpression model. Next, since we found that ADAR1 positively stimulates HIV-1 replication, it would be interesting to look at the viral kinetics such as infectivity of newly produced viral particles. As an IFN regulator, ADAR1p150 overexpression and knockdown is likely to have an effect on levels of other ISGs and cytokines produced throughout infection. Finally, ADAR1 primary role is RNA editing. Recently published data on ADAR1p150 and p110 knockout in HEK 293 T cells and neuronal progenitor cells showed that it primarily edits Alu elements in RNA polymerase II

transcribed mRNA [314]. In the similar manner, we are thinking of looking at editing of HIV-1 RNA and whether it contributes to ADAR1's positive modulation of infection.

The last part of this project turned around PACT interactome with ADAR1 and a viral element. We mapped the interaction from ADAR1's perspective; a next step would be to map it in PACT. Also to further explore our hypothesis of a multi-protein complex inhibiting PKR, we are interested in seeing the effect of varying PACT quantity in our ADAR1p150 knockout cell line on PKR phosphorylation. In the absence of p150 isoform, PACT should conserve its canonical role and further increase the levels of activated PKR. We also plan to further explore PACT and ADAR1 interactomes and empirically verify mass spectrometry data.

The general objective of this project is to contribute to the general understanding of the interaction between HIV-1 and host cells. Since, HIV-1 hijacks and evades the detection by innate immune system, a better understanding of these mechanisms will lead to a development of approaches that will neutralize and prevent the establishment of new infections in the cells.

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