Cloning and expression of DDX52 to study its effect on HIV-1 replication and infectivity

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Abstract

Human Immunodeficiency Virus (HIV) is known to mainly affect CD4+ T helper cells and it has 3 stages of infection: acute, chronic and lastly symptomatic stage which is called as Acquired Immunodeficiency Syndrome (AIDS). On the other hand, microRNAs are a small class of RNA consisting of about ~22 nucleotides and are known to play important regulatory roles in animals and plants and has been studied for its effect in gastric cancers, neurodegenerative and psychiatric disorders and also in many viral infections and diseases such as of Hepatitis C and Yellow fever. There are also many studies done regarding the inter-relation between HIV-1 infection and miRNAs; such studies include miRNA-223, miRNA-29a/b, miRNA-155 and microRNA-21 that show either increased or decreased expression during the infection, furthermore, many other studies have been done which are available in the public domain NCBI-GEO database. After retrieving micro-array data from the same and analyzing it, this laboratory has found 16 unique microRNAs to be significantly modulated upon HIV-1 infection. Out of them, miR-197-3p showed consistent pattern of expression, when validated by Real-Time PCR. So, our main concern was to study miR-197-3p and its inter-connection with HIV-1 infection. miR-197-3p has already been studied in the lab and it was seen that during the HIV-1 infection it showed an increased expression. It was also seen that over-expression of this miRNA causes an increase in the viral infectivity. This makes the study of miR-197-3p important for the understanding of miRNA - HIV-1 interactions for the host pathogen relationship. Later, by target prediction we determined that DDX52 (DExD-box

helicase 52) was one of the target genes of miR-197-3p, which is known to code for the protein probable ATP-dependent RNA helicase, known to be expressed highly in kidney. Thus, 3' untranslated region of DDX52 was cloned in a mammalian expression vector and by reporter-based luciferase assay, it was experimentally confirmed that DDDX52 is a target of miR-197-3p. Now, our aim is to further characterize this target gene to find out its relevance with HIV-1 infection. For this, in the present work, we will clone DDX52 in a mammalian expression vector and express it to check its effect on HIV-1 replication and infectivity. So, it will provide new insights in the pathogenesis of HIV-1 infection.

Keywords: AIDS, pathogenesis, miR-197-3p, miRNA

Abbreviations

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HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
DDX52	DExD-box helicase 52
miRNA	Micro Ribonucleic Acid
CD4	Cluster of differenciation 4
gp	glycoprotein
PIC	Pre-Integration Complex
cART	Combination Antireteroviral Therapy
RISC	RNA-Induced Silencing Complex
GMO	Genetically Modified Organisms
pri-miRNA	primary miRNA
pre-miRNA	precursor miRNA
HDFs	HIV Dependency Factors
DGCR8	DiGeorge syndrome critical region 8
PCAF	P300/CBP-associated factor
LTR	Long Terminal Repeats
PCR	Polymerase Chain Reaction
HEK293T	Human Embryonic Kidney Cells 293 T
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

1 1. Introduction:

1.1 Background:

As the first step to understand the work done, it is important to learn about the basic studies done on HIV-1 and miRNA; and then we could relate on how they are interconnected. We then need to learn about miR-197-3p and DDX52 and their relevance in the study. Here, we will try to understand the basics first.

1.1.1 HIV-1 and AIDS:

HIV is a lentivirus and belongs to retroviridae family. It is an enveloped, positive sense, single strand RNA virus with two copies of RNA per virion. There are two kinds of HIV existing globally: HIV-1 and HIV-2. They deffer by more than 55% in sequences and are also antigenically distinct[1]. Globally HIV-1 is more prevalent and hence our main concern for the study. It is important to know about HIV-1, its genetic organization and life cycle to understand how it replicates. Later we can then understand interrelation of miRNA and HIV-1.

Organization of HIV-1 :

There are many studies done on the viral organization of HIV-1. These help us understand how different viral proteins play important role in infection and host-pathogen relationship.

The HIV-1 genome is encoded by an ~9kb RNA, and two genomic-length positive sense RNA molecules packaged in the particle. The genome encodes nine open reading frames Fig 1. Three of these encode the Gag, Pol, and Env polyproteins. These three polyproteins are further proteolyzed into individual proteins:

The four Gag proteins, MA (matrix), CA (capsid), NC (nucleocapsid), and p6.

The two Env proteins, SU (surface or gp120) and TM (transmembrane or gp41), are structural components that make up the core of the virion and outer membrane envelope.

The three Pol proteins, PR (protease), RT (reverse transcriptase), and IN (integrase), provide essential enzymatic functions and are also encapsulated within the particle.

HIV-1 encodes six additional proteins, often called accessory proteins, three of which (Vif, Vpr, and Nef) are found in the virion particle, Vif is important for the production of the infectious mature virions, Vpr is responsible for transport of nucleoprotein complexes across host cell

nucleus and Nef is responsible for the CD4 degradation. Two other accessory proteins, Tat and Rev, provide essential gene regulatory functions, and the last protein, Vpu, indirectly assists in assembly of the virion.[2] [3]

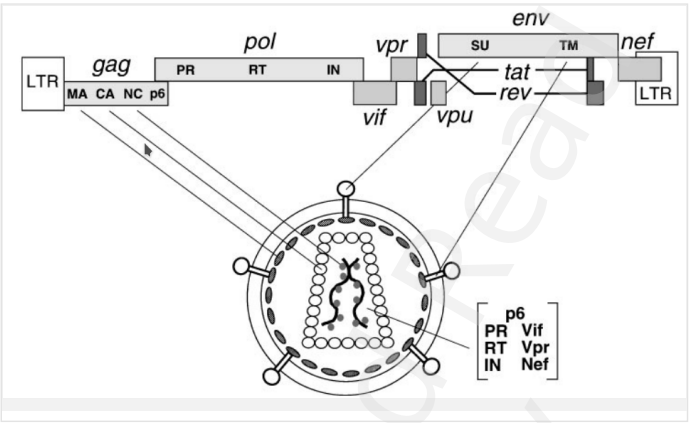


Fig 1 Organization of HIV-1 genome and virion (Frankel D. Alan & Young A. T. John 1998)

Life Cycle of HIV-1 :

It is essential for us to understand the stages of the life cycle of HIV-1 in order to interrelate how the infection and replication occurs; moreover it also helps us to understand the mechanism related to the therapeutic implications. The stages of the life cycle are Fig 2:

1. Binding and Entry : This is the first step of HIV-1 life cycle, here the HIV-1 attaches to the target cell. The HIV-1 envelope protein, gp120 binds to CD4 receptor present on the surface of the target cell and therefore induces conformational changes, resulting in the interaction of gp120 with co-receptors, CCR4 or CXCR5. Another glycoprotein, gp41 then induces the fusion of viral and cellular membrane leading to entry of the virus into the host cell.[2][3]

2. Reverse transcription and uncoating: After the entry into the host cell, HIV-1 then undergoes through the reverse transcription of the ssRNA into the viral dsDNA. Then uncoating occurs, which involves the disassembly of the viral core which results in the release of the viral genome into the cytoplasm. Though, the old literature suggests that uncoating occurs just after entry of virus and reverse transcription occurs later [2], recent studies done by Arhel[4] , suggests that reverse transcription occurs before uncoating and uncoating is considered as a transition between reverse transcription and pre-integration complex formation.

3. Formation of pre-integration complex and nuclear export:

The duplex viral DNA genome interacts with various viral and cellular proteins and forms a pre-integration complex (PIC). This PIC is later translocated into the nucleus.[5]

4. Integration:

After nuclear entry of PIC, viral genome is integrated into the host chromosomal DNA with the help of viral proteins, integrase(IN).[2][3]

5. Transcription and translation:

The integrated virus, now called a pro-virus, can remain dormant within cell for years. But in an activated cell it actively transcribes like host genes using host transcription machinery. There are three kind of transcription generated, unspliced, single spliced and multiple spliced. Multiple spliced transcripts translocate to cytoplasm to generate early proteins like Tat, Rev, Nef. Tat and Rev then moves to nucleus, where Tat binds to LTR and activates transcription while Rev assists in export of unspliced and single spliced transcripts into cytoplasm. [3] [2]

6. Assembly and budding:

After the viral proteins are synthesized, they, along with HIV-1 full length RNA migrate to cell surface for the process called as assembly. The viral assembly is controlled by Gag polyproteins. Thereafter, viruses are pinched off from infected cells, by budding, which is also regulated by Gag. [3] [2]

7.Maturation:

This involves the cleavage of gag, gag-pol and env precursors into their individual components. This process is mediated by the viral and host proteases and results in mature infectious virions. [3] [2]

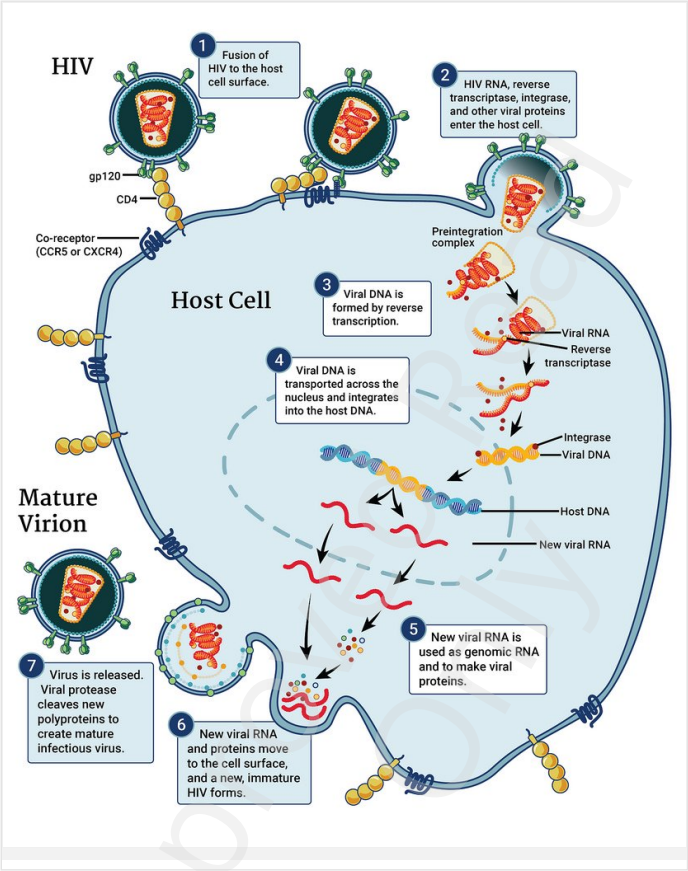


Fig 2 Schematic representation of stages of HIV-1 life cycle (NIH: National Institute of allergy and infectious diseases (NIAID),2018)

Acquired Immunodeficiency Syndrome :

Our main focus is on HIV-1, being more pandemic and widespread than HIV-2. HIV-1 infects the cell with CD4 receptors, which is expressed on many immune and non immune cells, mainly affecting the T helper lymphocytes and finally lead to AIDS, in which the CD4 cells decrease below 200 cells per mm^3 and HIV-1 count increases exponentially. There are mainly three stages of infection: acute, chronic and finally the symptomatic stage called as AIDS [6] Fig

3.The innate immune response to HIV is largely mediated by natural killer cells, and is also crucial for virus control. But viral escape mutants also emerge, and restrict the antiviral effects of natural killer cells. Even though due to the cART treatments the viral replication can now be controlled to a great extent, but still remain incurable and was cause of death for about 1 million people globally and about 1.8 million newly infected people (UNAIDS Report 2018)Fig 4 .

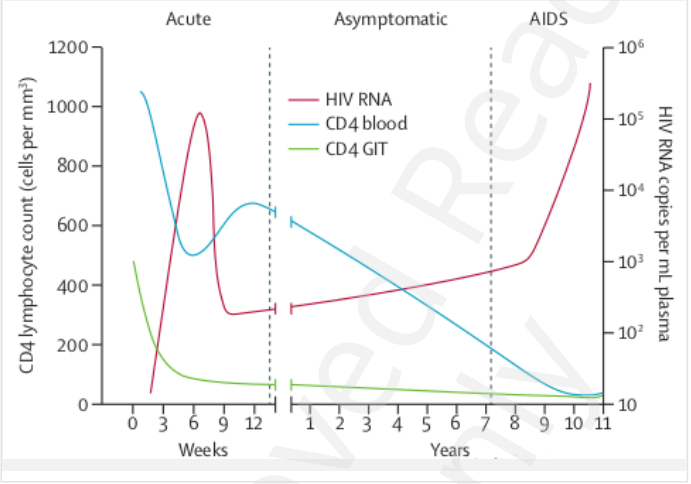


Fig 3 HIV-1 Stages of Infection (Maartens et al., 2014)

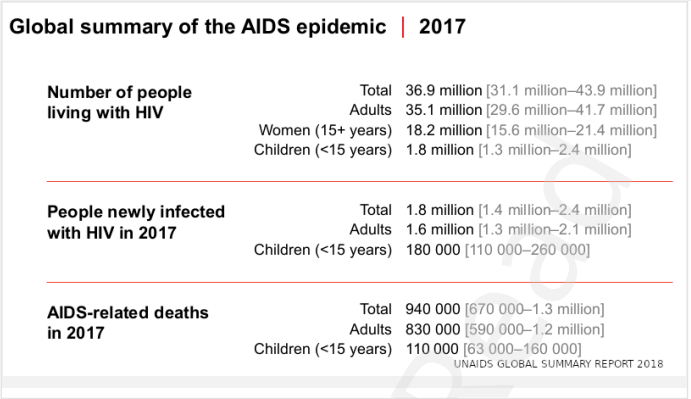


Fig 4 UNAIDS Global Summary Report 2018

1.1.2 **microRNA**

MicroRNAs (miRNAs) are endogenous ~ 22 nucleotides RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. miRNAs comprise one of the more abundant classes of gene regulatory molecules in multicellular organisms and likely influence the output of many protein-coding genes [7]. In the most recent release, 2578 mature human miRNA sequences have been identified (Sanger miRBase release 2010; http://www.mirbase.org/).

For us to understand the miRNA inter-connection with HIV-1, we must first study about the miRNA biogenesis and mechanism of action in breif.

Biogenesis:

Transcription : RNA polymerase II and III are responsible for the transcription of primary miRNA (pri-miRNA). In 2004, Ohler et al. [8] , gave observations which provided indirect evidence that many of the other miRNAs also are pol II products. Although these observations indicate that many miRNAs are pol II transcripts, others might still be pol III transcripts [8] .

Maturation: Although the biogenesis appears different in plants, there are several steps involved in maturation of mammalian miRNA, here we have given a full model from transcription to the targetting of mRNA [9]: Fig 5

1. Transcription of miRNA gene leading to formation of pri-miRNA, as discussed above.

2. Nuclear cleavage of pri-miRNA by Drosha RNase III endonuclease leading to formation of precursor miRNA(pre-miRNA).

3. Active transport of pre-miRNA from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin-5.

4. The other end is then processed in the cytoplasm by the enzyme Dicer, which is also a RNase III endonuclease forming a duplex.

5. The duplex is then acted upon by helicase which releases miRNA.

6. At last they eventually become incorporated as single-stranded RNAs into a ribonucleoprotein complex, known as the RNA-induced silencing complex (RISC).

7. The RISC targets the mRNA to either perform cleavage or translational repression, depending upon the sufficient complimentarity of mRNA to miRNA.

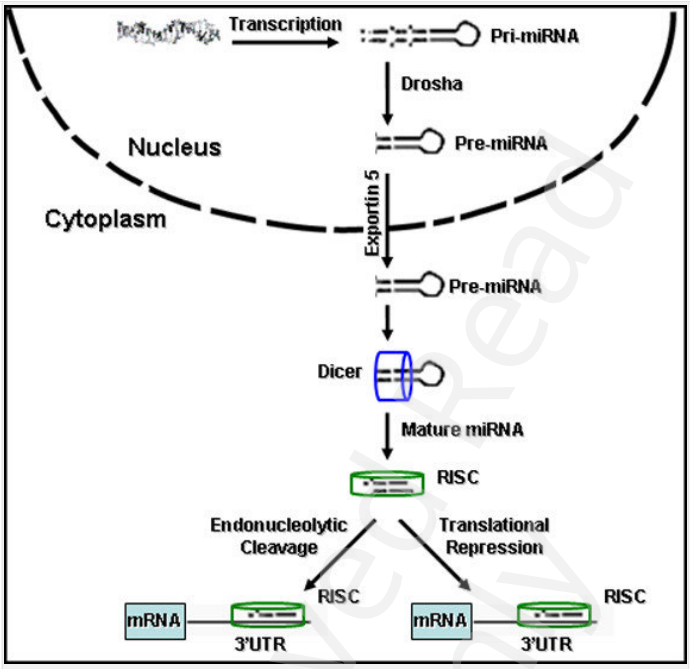


Fig 5 Biogenesis of miRNA (Papagiannakopoulos and Kosik 2008)

Now, after understanding the basic concepts behind HIV-1 and miRNA, we can further investigate their interrelationship.

1.2 What do we want to know?

i)Why is it important to study HIV-1 and what are the targets of HIV-1?

ii)How does HIV-1 causes AIDS?

iii)How does HIV-1 replicates? What are the different life-stages of HIV-1?

iv)What are microRNAs? How are miRNAs and HIV-1 interconnected?

v)What is miR-197-3p? How can it be used to study host-pathogen relationship?

vi)What is DDX52? How does it affect HIV-1 replication and infectivity?

1.3 Objectives:

i)To successfully clone DDX52 in a mammalian expression vector.

ii)To find out the effect of DDX52 on the replication and infectivity of HIV-1.

2 Literature Review:

2.1 How are miRNA and HIV-1 interconnected?

In the last decade, there have studies done on the HIV Dependency Factors (HDFs) and the essentiality of certain HDFs in the regulation of viral replication[10] [11] [12]. This opens up ways for studying not only the pathways, but also for many therapeutic approaches directed towards HDFs to be a potential strategy to combat HIV-1. Furthermore, the inhibition of these proteins by RNAi strategies such as siRNA mediated knockdowns have shown to influence the HIV-1 replication [13] [14]. Thus, manipulating the proteins at translation step by RNAi strategies can be very useful in further studies.

Understanding the role of miRNAs in HIV-1 infection is an emerging area of interest. Few cellular miRNAs have been shown to directly bind to the viral RNA and inhibit HIV-1 replication. However, a growing number of cellular miRNAs can indirectly affect HIV-1 infection by targeting HDFs. As an ever-evolving strategy, HIV-1 seems to be able not only to modulate cellular miRNA profiles but also to interfere with the overall biogenesis of miRNAs [15].

The miRNA are known to be possessed by both HIV-1 as vmiRNA [16]; although the studies are going on for vmiRNA, existence is not yet fully accepted by the scientists; and the host, which then results in the translational regulations[17]. miRNAs are known to alter in the replication of HIV-1, though it may be positive or negative depending on the targets of the miRNA and their interconnection with the HIV-1 infectivity and replication. On the other

hand, HIV-1 is known to interfere in the biogenesis of different miRNAs which may affect HIV-1 replication.Fig 6

As we have earlier discussed the biogenesis of miRNA, it makes it simpler for us to understand various studies done on the interaction of HIV-1 in the miRNA biogenesis by interfering with proteins involved in biogenesis such as DiGeorge syndrome critical region 8 (DGCR8),Dicer and Drosha. On the other hand there are many studies done on the impact of miRNA on the HIV-1 replication and infectivity by many means including direct targeting the HIV-1 RNA, avoiding the nuclear export, inhibiting the transcription and translation of HIV-1 viral dsDNA by targeting the proteins responsible such as PCAF and Pur-alpha and inhibiting integration Fig 7[15].

This helps us to understand how the HIV-1 can alter the expression of miRNA.

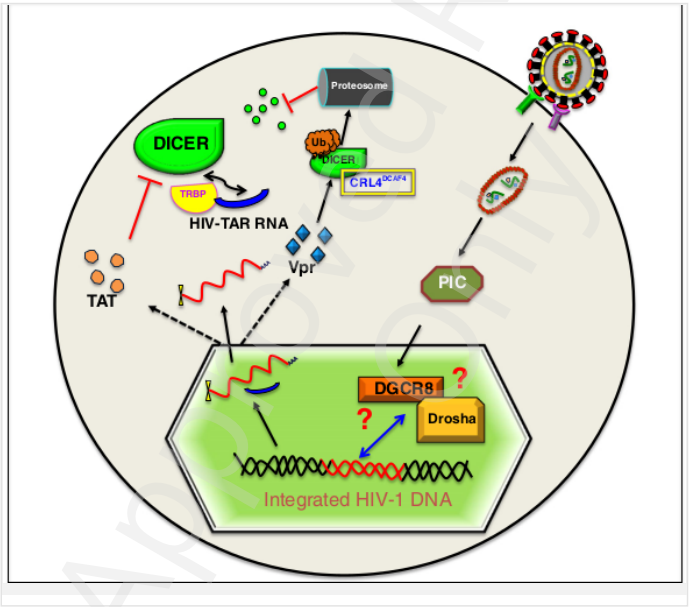


Fig 6 **Affect of HIV-1 in the proteins involved in miRNA biogenesis (Swaminathan G et al., 2013)**

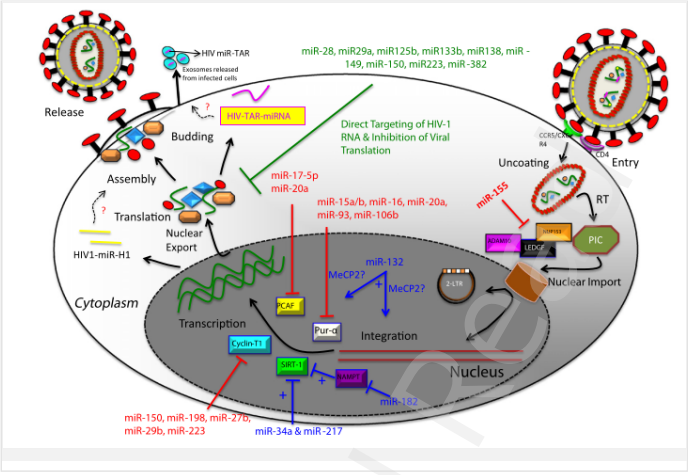


Fig 7 **Impact of cellular miRNA on HIV-1 replication and infection. (Swaminathan G et al., 2013)**

2.2 Why studying miR-197-3p and DDX52 is important?

miR-197-3p have already been studied and is associated with a wide range of pathologic conditions, which range from type 2 diabetes mellitus, myocardial infarction, metabolic syndrome, multiple sclerosis, preeclampsia, Hepatitis B virus infection to major human malignancies such as lung cancer, hepatocellular carcinoma, pancreatic cancer, thyroid cancer or cervical carcinoma [18] [19] [20].

As we discussed earlier, miR-197-3p had already been studied in the lab where by comparing micro arrays from NCBI- GEO database, they found miRNAs which significantly modulated upon HIV-1 infection. Out of them, miR-197-3p showed consistent pattern of expression, when validated by Real-Time PCR.

Sequence of miR-197-3p : CGGGUAGAGAGGGCAGUGGGAGG

Further, by target prediction it was determined that DDX52 (DExD-box helicase 52) was one of the target genes of miR-197-3p, which is known to code for the protein probable ATP-dependent RNA helicase. Thus the 3' untranslated region of DDX52 was cloned in a mammalian expression vector and by reporter-based luciferase assay, it was experimentally

confirmed that DDX52 is a target of miR-197-3p. It was also seen that over-expression of this miRNA causes an increase in the viral infectivity.[21]

Thus, studying miR197-3p and DDX52 and the host-pathogen relationship with HIV-1 is important, for which we must first do molecular cloning of DDX52 and then study its affects on HIV-1 replication and infectivity.

3 Methodology

3.1 Cloning of DDX52 In Mammalian Expression Vector:

DDX52 was cloned in mammalian expression vector pcDNA 6/His C (Fig 8)tagged vector. For this, CDS sequence of DDX52 was amplified from cDNA by polymerase chain reaction (PCR) using gene specific primers with restriction sites. Then the amplified product was digested with restriction enzymes (Bam H1 and Xho1). Then digested fragments were ligated into digested pcDNA 6/His C vector using T4 DNA ligase at 4°C overnight. Transformation of ligation mixture was carried out in *E. coli* DH5α competent cells. Plasmid screening for the positive clone was done by restriction digestion, and later confirmed by DNA sequencing.

The cloning was DDX52 was confirmed by DNA sequencing, later screening was done by Real Time PCR and immunoblotting for RNA and protein respectively.

In breif the cloning consists of several steps:

1. HEK 293T cells ➔ RNA isolation ➔ Reverse transcription PCR ➔ cDNA ➔ PCR ➔ DDX52 amplification ➔ PCR Purification ➔Restriction digestion (Bam H1(HF) and Xho1)➔PCR Purification

2. pcDNA 6 His C ➔ plasmid isolation by miniprep (APS Lifetech.) ➔ Restriction digestion (Bam H1(HF) and Xho1)➔PCR Purification

3. Digested products of pcDNA 6 His C and DDX52 ➔ Ligation ➔ Transformation ➔ Plating ➔ Screening by double restriction digestion

4.Checking for Over-expression ➔Transfection of clone DDX52 in HEK293T cells➔ Harvesting➔

i) For RNA ➔ RNA isolation ➔Reverse transcription PCR ➔ cDNA ➔ Real time PCR

i) For Protein➔preparation of protein lysate➔ Immunoblotting

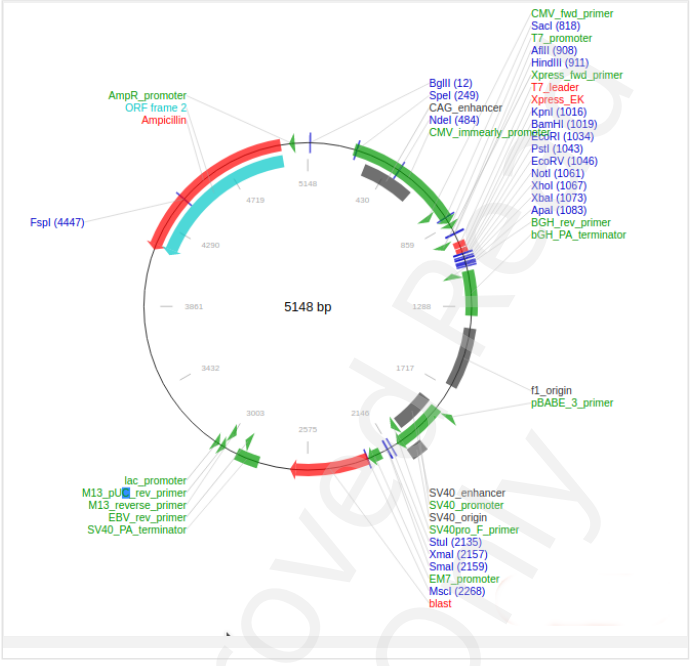


Fig 8 Plasmid map of pcDNA 6/His C

3.1.1 RNA Isolation and Reverse Transcription:

RNA was prepared from transfected HEK 293T cells using TRIzol Reagent (Invitrogen). Then cDNA was made from 2µg of RNA using MMLV reverse transcriptase (Invitrogen) as per manufacturer's instructions. Briefly, the reaction mixture contained 1x first strand synthesis buffer, 10 mM DTT, 5 ng random primers, 0.5 mM dNTPs, RNase inhibitor and 200 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase enzmye (MMLV RT) in 20 µl reaction volume. RNA was first denatured at 58°C for 10 minutes. cDNA synthesis was performed at 37°C for one hour followed by inactivation at 70°C for 10 minutes. These were done at both time, while cloning and while screening.

3.1.2 PCR For Cloning:

Primers used:

Forward Primer-

5' TTC**GGATCC**ATGGACGTCCACGATCTC 3'

GGATCC: Restriction site for Bam H1

Reverse Primer-

5' CGG**CTCGAG**TTAACTTTTGTCTTCAAG3'

CTCGAG: Restriction site for Xho1

Table 1 Reaction Mixture for PCR

Content	Volume
NEB Phusion Master Mix	25µl
Template (DDX52) (100 ng/µl)	1µl
Forward Primer	2.5µl
Reverse Primer	2.5µl
H_2O(nuclease free)	18µl
Total Volume	50µl

Table 2 Program for thermocycler

Temperature	Time
5 cycles:	
98°C	2 min
98°C	15 sec
70Δ-5°C	30 sec
72°C	1min 30 sec
30 cycles	

98°C	15 sec
55°C	30 sec
72°C	1min 30 sec
Final extension:	
70°C	10 min

Gel extraction was done according to the QIAgen PCR purification kit.

3.1.3 Restriction Digestion:

Table 3 Reaction mixture ofor restriction digestion

Content	Volume
For DDX52 : at 37°C for 4 hours	
Template	20µl (1µg)
Bam H1(HF)-NEB	1µl
Xho1-NEB	1µl
Cut Smart buffer-NEB	2.5µl
H_2O(nuclease free)	0.5µl
Total volume	25µl
For pcDNA 6/His C at 37°C for 1.5 hours	
Template	6.6µl (2µg)
Bam H1(HF)-NEB	1.5µl
Xho1-NEB	1.5µl
Cut Smart buffer-NEB	2µl
H_2O(nuclease free)	8.4µl
Total Volume	20µl

Column purification for both the insert and the empty vector was done with QIAgen PCR purification kit according to manufacturer's protocol.

3.1.4 Ligation

After the restriction digestion of DDX52 and pcDNA 6/His C, they were then ligated using T4 DNA ligase and ligation buffer and was left at 4°C for overnight.

To calculate ng of insert to include in ligation reaction, the formula used was:

{[ng of vector]*[kb size of insert]}/{kb size of vector}*(insert vector ratio) = ng of insert required

Table 4 Reaction Mixture for Ligation

Content	Volume
Vector (pcDNA 6/His C) (30ng/µl)	2µl
Insert (DDX52) (60ng/µl)	1µl
T4 DNA ligase(NEB)	1µl
Ligase Buffer (NEB)	1µl
H_2O(Nuclease free)	5µl
Total volume	10µl

3.1.5 Screening:

After ligation, transformation was carried out in *E.coli* DH5α competent cells and was plated on ampicillin(100µg/ml) containing agar plates. Later, from the colonies plasmid DNA was extracted and screened by double restriction digestion (Bam H1(HF)/Xho1). The positive clone was sent for sequencing for confirmation.

3.2 Checking Over-expression :

The cloned DDX52 was then transfected into HEK-293T cells. Briefly, the transfection mixture contained DDX52 cloned plasmid DNA, transfection reagent PEI, incomplete DMEM media and pEGFP plasmid DNA; empty vector pcDNA 6/His C plasmid DNA served as the control. After about 36 hours, The cells were harvested using 1x PBS and TRIzol Reagent (Invitrogen) was used for RNA isolation and TNN lysis buffer was used for protein extraction which were then used to check over-expression of DDX52.

3.2.1 Quantitative Real Time PCR:

Expression profile of DDX52 was analyzed by quantitative real-time PCR in a 10µl reaction mixture containing SYBR Green iTaq supermix (Bio-Rad, USA) and 10 pmol concentration of each of the human β-actin and gene specific oligonucleotide primer pairs using the Realplex^4 Mastercycler (Eppendorf, Germany). The changes in the threshold cycle (C_T) values were calculated by the equation:

$\Delta C_T = C_{T,target} - C_{T,input}$.

The fold difference was calculated as follows:

Fold difference= $2^{(-\Delta\Delta ct)}$

Primers for DDX52 for Real Time PCR

Forward Primer-

GCACCTTCTTCTTGGGAAGC

Reverse Primer-

ACATCAGAAGCCCCAAAATG

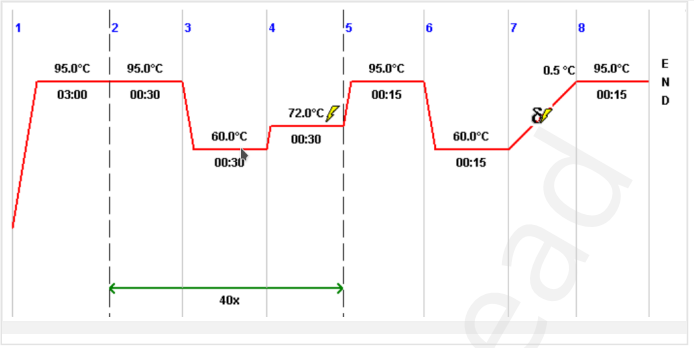


Fig 9 Program for Real time PCR

Table 5 Reaction Mixture For Real time PCR

Contents	Volume
For DDX52:	
SYBR Green iTaq supermix	5µl
Forward Primer:	0.2µl
Reverse Primer:	0.2µl
DDX52(5ng/ µl)	2µl
H_2O(nuclease free)	2.6µl
For β-actin:	
SYBR Green iTaq supermix	5µl
Forward Primer:	0.2µl
Reverse Primer:	0.2µl
DDX52 (5 ng/µl)	2µl
H_2O(nuclease free)	2.6µl

3.2.2 Immunoblotting:

As discussed above, after 36 hours of transfection, cells were harvested. Protein lysate was prepared by using TNN lysis buffer [(50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.12 M NaCl, 0.5% NP40, 0.5 mM NaF, 1 mM DTT, 0.5 mM PMSF, (phenyl methyl sulfonyl fluoride)] on ice for 45 min with intermittent vortexing. Protein concentration was determined using Bradford assay and then loaded on SDS-PAGE. The proteins were transferred on to PVDF membrane and the

membrane was probed with anti-DDX52 and anti-GAPDH antibodies. The blots were developed using the ECL Plus system (GE Healthcare, USA).

3.3 Checking the effect of DDX52 on HIV-1 Replication and Infection:

3.3.1 LTR Driven Gene Expression:

HEK293T cells were co-transfected with pEGFP-N1 (control reporter plasmid), pLTR-luc, pNL4-3 (molecular clone of HIV-1) and DDX52 or pcDNA 6/His C; empty vector(Control); using Polyethylenimine (Polysciences, Inc., USA). Cells were harvested at 36 hrs post transfection and lysed using Cell Culture Lysis Reagent (Promega, USA). The supernatant was collected in a 96 well white, polystyrene plate. Fluorescence reading was taken for normalization, followed by luminescence reading, by adding Steady Glo luciferase substrate (Promega, USA) in a M5 microplate multimode reader (Molecular Devices, USA). Fold change upon addition of DDX52 was calculated.

3.3.2 Virus quantification by HIV-1 p24 Antigen Capture Assay:

HEK293T cells were co-transfected with pNL4-3 and DDX52 and another set with pNL4-3 and pcDNA 6/His C empty vector(control). Briefly, cells were incubated with virus for 5 h in CO_2 incubator, the media was then changed with complete media and incubated at 37°C in CO_2 incubator for 36h. The culture supernatants of the transfected cells were then used to determine virus production by p24 antigen capture assay(ABL inc.) according to the given protocol.

3.3.3 β-galactosidase staining assay:

First TZM-bl cells were seeded in a 24 well plate at a density of 0.125 million cells/ well. Based on p24 absorbance results, 100pg of virus supernatant was added to the cells for infection. After 36 hours, staining procedure was done. The staining procedure is as follows:-

TZM-bl cells were washed twice with 1X PBS 36h post-infection. They were then fixed with 1% glutaraldehyde in 1X PBS for 10 min at room temperature. The cells were washed twice with 1X PBS and then incubated at 37°C with the X-gal reaction mixture containing 5mM potassium ferricyanide , 5mM potassium ferrocyanide, 2mM $MgCl_2$ and 50 mg/ml X-Gal (made in Dimethyl formamide) in 1X PBS. The reaction was stopped with 3% DMSO in PBS after the development of blue color. The cells that stained with β-gal were counted in 5 random fields and the number of infectious virions were calculated in the viral stock.

4 Results and Discussion:

4.1 PCR amplification of DDX52:

After the PCR amplification of DDX52, it was run on a 1% agarose gel in 1x TAE. The band of DDX52 was observed at 1.8kb, signifying that DDX52 was amplified.

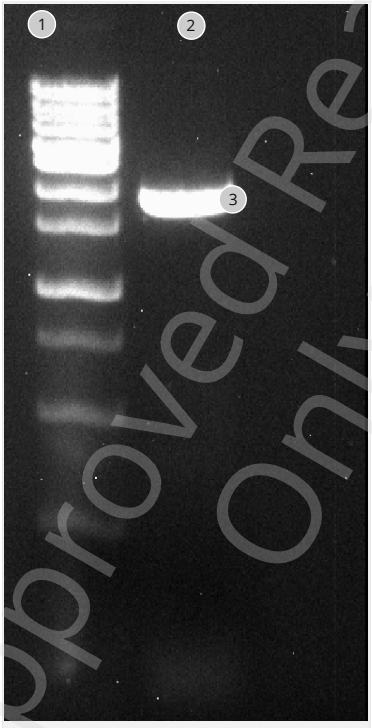
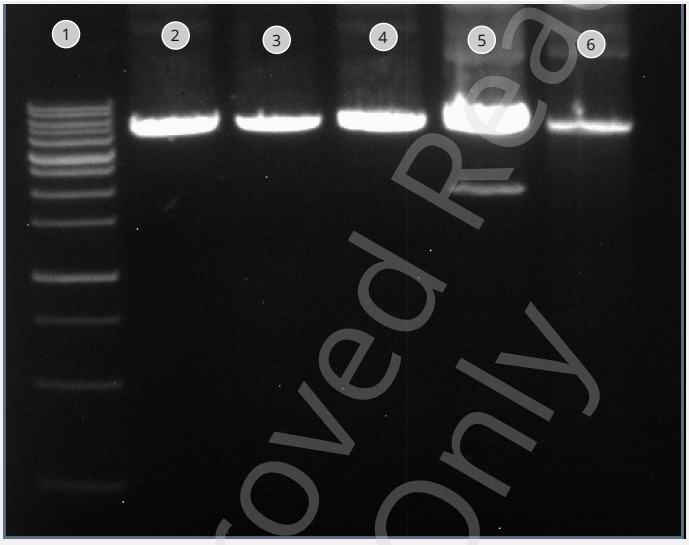


Fig 10 Verification for DDX52 in 1% Agarose gel in 1x TAE

4.2 Screening of clones by restriction digestion and sequecing:

After plating, the colonies were inoculated in LB broth containing Ampicillin (100µg/ml) and plasmid isolation was done by alkaline lysis method and then screened by double restriction digestion by Bam H1(HF) and Xho1. A positive result was observed, which was then sent for sequencing. The sequencing results came positive for DDX52 without any mutation.



1 1kb Ladder 2 colony no. 1 (-ve) 3 colony no. 2 (-ve) 4 colony no. 3 (-ve)
5 colony no. 4 (+ve) 6 colony no. 5 (-ve)

Fig 11 Screeningby double digestion in 1% agarose gel in 1x TAE

4.3 Checking over-expression by Real time PCR and Immunoblotting:

The over expression of DDX52 was then checked at gene level by Real time PCR(Fig 12, Fig 13) and at protein level by Immunoblotting(Fig 14,Fig 15).

Real-Time PCR showed an increased expression for the cloned DDX52 with a fold change of ~160 and ~140 respectively. Later, by immunoblotting, again over-expression of DDX52 was observed at 67kDa.

Thus, the cloning and expression of DDX52 was successfully completed.

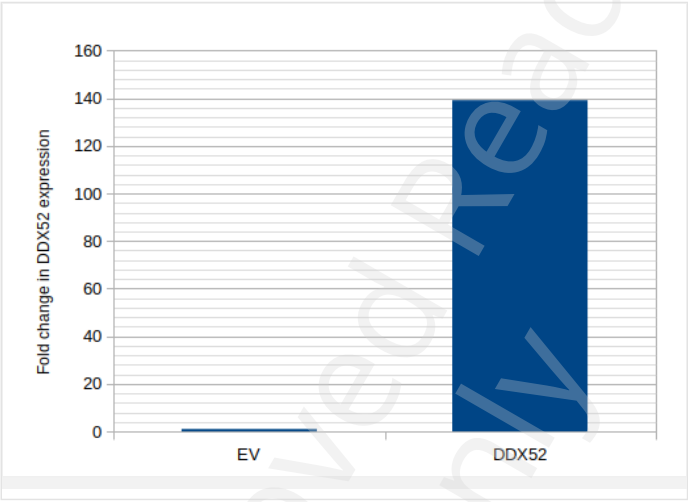


Fig 12 Graph for RT PCR shows over-expression of DDX52

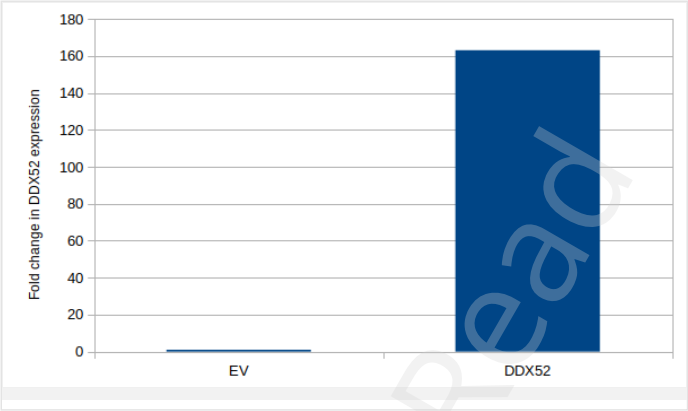


Fig 13 Graph for RT PCR shows over-expression of DDX52

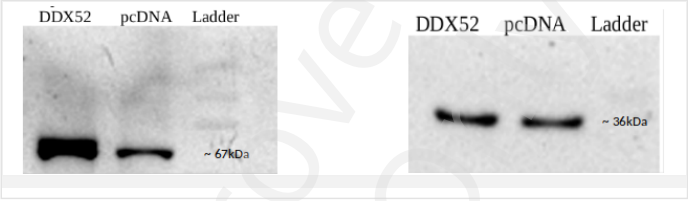


Fig 14 Set 1: Checking of DDX52 over-expression(left) by immunoblotting and its GAPDH(Right)

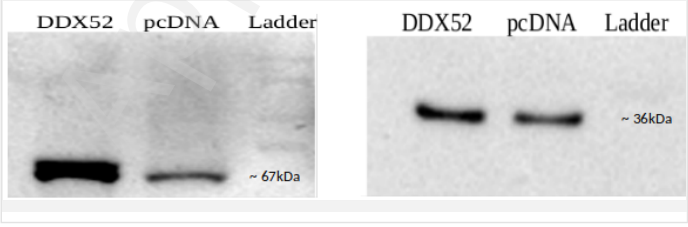


Fig 15 Set 2: Checking of DDX52 over-expression(left) by immunoblotting and its GAPDH(Right)

4.4 Effect Of DDX52 On LTR Driven Gene Expression:

To check whether DDX52 has any effect on the LTR driven gene expression of HIV-1, HEK-293t cells were transfected with DDX52, along with pNL4-3 and LTR-luc plasmids. It was then observed that there was an average fold change of ~0.5 (i.e. 50% decrease)Fig 16 in the luminescence activity of the LTR upon over expression of DDX52. Thus, it can be concluded that DDX52 causes a decrease in the viral replication process of HIV-1.

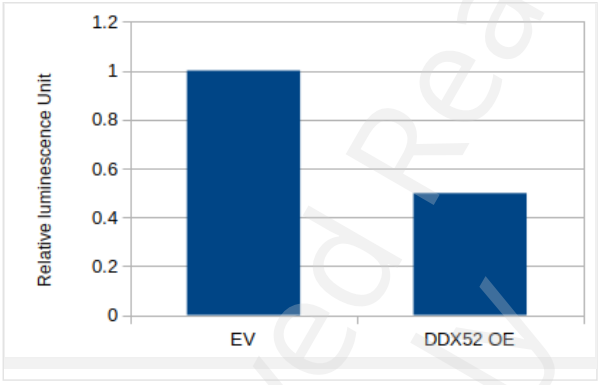


Fig 16 Graph showing a decrease in the luminescence for DDX52 over-expressed cells.

4.5 Effect of DDX52 on virus replication by HIV-1 p24 Antigen Capture ELISA:

To confirm the decrease in the viral replication, quantification of HIV-1 was done by p24 antigen capture ELISA (ABL inc.). The p24 levels were observed having an average fold change of about 0.4 (Fig 17)when compared with control. This confirms DDX52 causes decrease in the viral replication of HIV-1 by about 60%.

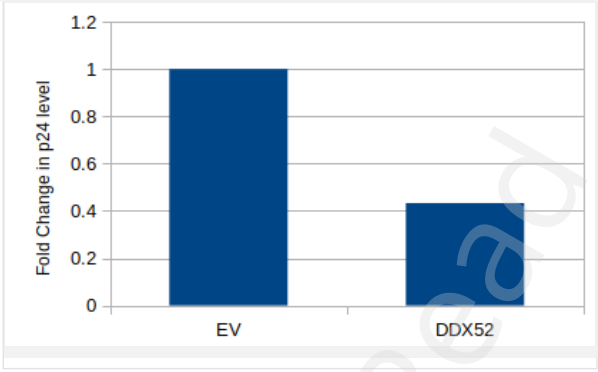


Fig 17 Graph depicting decrease in the p24 level

4.6 Effect of DDX52 on viral infectivity by β-Galactosidase assay:

The infectivity of HIV-1 was checked by infecting TZM-bl cells with virus in duplicates and then staining by β-Galactosidase assay. The preliminary results for infectivity assay indicate that it probably downregulates infectivity.

5 Conclusions:

DDX52 was successfully cloned in the mammalian expression vector, pcDNA 6/His C and was checked for it's over-expression by Real-Time PCR and Immunoblotting. Furthermore, DDX52 was then used to check its effect on the HIV-1 replication and infectivity, which was done by performing LTR based luciferase assay, HIV-1 p24 Antigen Capture ELISA and TZM-bl β-Galactosidase assay. It was clearly observed that DDX52 causes decrease in the HIV-1 replication by ~55%, as observed in LTR based luciferase assay and ~60% decrease was observed in the HIV-1 p24 Antigen Capture ELISA. Thus, DDX52 is confirmed to negatively affect the HIV-1 replication, also the preliminary results for infectivity assay indicate that it probably downregulates infectivity. Furthermore, this study provides an insight of how miR-197-3p and DDX52 are interlinked, adding up to the studies done on miRNA and HIV-1. It also showed a clear case of how the host's miRNA can affect the HIV-1 host-pathogen relationship.

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Source

1. Fig 1: Frankel D. Alan & Young A. T. John 1998
2. Fig 2: NIH: National Institute of allergy and infectious diseases (NIAID),2018
3. Fig 3: Maartens et al., 2014
4. Fig 4: UNAIDS Survey 2018
5. Fig 5: Papagiannakopoulos and Kosik 2008
6. Fig 6: Swaminathan G et al., 2013
7. Fig 7: Swaminathan G et al., 2013
8. Fig 8: https://www.addgene.org/vector-database/2145/