DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Tamás Richárd Linkner

Comparative transcriptomic and proteomic analysis of HIV-1 and HIV-2 pseudovirion transduction in the early phase of viral infection

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND IMMUNE BIOLOGY

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List of Abbreviations

5'UTR	5'-untranslated region
AIDS	Acquired immunodeficiency syndrome
AP-1	activator protein 1
APOBEC3G	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BFV	Bovine foamy virus
BST-2	Bone marrow stromal antigen 2
BST-2	Bone Marrow Stromal Cell Antigen 2
Btas	Bovine foamy virus transactivator
CA	Capsid
cAMP	Cyclic adenosine monophosphate
CBF-β	Core-binding factor subunit beta
CCNT1	Cyclin T1
CCV	Clathrin-coated vesicle
CD317	Cluster of Differentiation 317
CD34	Cluster of Differentiation 34
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDK9	Cyclin-dependent kinase 9
cGAS	Cyclic GMP-AMP synthase
CID	Collision-induced dissociation
CMV	Cytomegalovirus
CRM1	Chromosomal maintenance 1
Ctrl	Control
CUL4A	Culling4A
DC	Dendritic cell
DCAF1	DDB1 and CUL4 Associated Factor 1
DDP1	Damage-specific DNA binding protein 1
DEG	Differentially expressed gene
DENV	Dengue virus
DEP	Differentially expressed protein
DET	Differentially expressed transcript
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
env	Envelope
FasL	Fas ligand
FBS	Fetal Bovine Serum
FDR	False discovery rate
FRK	Fyn related kinase
gag	Group specific antigen
GeLC–MS/MS	liquid chromatography–tandem mass spectrometry
GFP	Green fluorescent protein
Ghr	Growth hormone receptor
GO	Gene ontology
gp120	glycoprotein 120
gp41	glycoprotein 41
GRCh38	Genome Reference Consortium Human Build 38
GTP	Guanosine triphosphate
HCV	Hepatitis C virus
HEK293T	Human Embryonic kidney 293T
HIV-1	Human immunodeficiency virus 1
HIV-2	Human immunodeficiency virus 2
HPV	Human papillomavirus
HSV1	Herpes simplex virus 1
HTLV	Human T-lymphotropic virus 1
HUSH	Human silencing hub
ID	Identification
IFN	Interferons
IL12p40	Interleukin 12 subunit p40
IL2	Interleukin 2
IL6	Interleukin 6
IN	Integrase

IRF3	Interferon regulatory factor 3
IRF5	Interferon regulatory factor 5
ISG	Interferon-stimulated gene
ITIM	immunoreceptor tyrosine-based inhibitory motif
JAK/STAT	Januse kinase/signal transducer and activator of transcription
Lck	Lymphocyte-specific protein tyrosine kinase
LDL	Low density lipoprotein
LFC	Log-fold change
LTR	Long terminal repeat
MA	Matrix
MAPK	MAP kinase
MDM	Monocyte-derived macrophages
MHC-I class I	Major histocompatibility complex
MHC-II class II	Major histocompatibility complex
MLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
MxB	Myxovirus resistance protein
NC	Nucleocapsid
NCBI	National Center for Biotechnology Information
nef	negative regulatory factor
NES	Nuclear export signal
NF-AT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NK cell	Natural Killer Cell
OXOPHOS	Oxidative phosphorylation
Padj	Adjusted p-value
PBS	Phosphate Buffer Saline
PCA	Principal component analysis
pDC	Plasmacytoid dendritic cell
PEI	polyethylenimine
PGK	Phosphoglycerate kinase
PIC	Pre-integration complex
PKC	Protein kinase C
pol	Polymerase
PP2A	PPP2R5 family of protein phosphatase 2A
PR	Protease
P-TEFb	Positive transcription elongation factor b
rev	Regulator of expression of virion proteins
RNA	Ribonucleic acid
RRE	Rev response element
RT	Reverse transcriptase
SAMHD1	SAM domain and HD domain-containing protein 1
SDS	Sodium dodecyl sulfate
SERINC3	Serine incorporator 3
SERINC5	Serine incorporator 5
SHP-1	Src homology region 2 domain-containing phosphatase-1
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
snRNP	Small nuclear ribonucleoprotein
TAR	trans-activation response element
tat	Trans activator of transcription
Th1	T helper 1 cell
Th17	T helper 17 cell
TNFα	Tumor necrosis factor alpha
TNPO1	Transportin 1
TRIM5	Tripartite motif 5
tRNA	Transfer ribonucleic acid
TU	Transduction unit
UPLC	Ultra Performance Liquid Chromatography
vif	viral infectivity factor
vpr	viral protein r
vpu	viral protein u
vpx	viral protein x
VSV-G	Vesicular stomatitis virus G protein
WHO	World Health Organization

1. Introduction

The acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2). They are part of the *Lentivirus* genus within the family of *Retroviriade*. Both viruses are derived from simian immunodeficiency viruses (SIV), with HIV-1 originating from SIV strains found in chimpanzees and HIV-2 stemming from SIV strains in sooty mangabeys (Deeks et al., 2015) (German Advisory Committee Blood, 2016) (Reeves & Doms, 2002).

While HIV-1 is spread globally, HIV-2 remains largely confined in West-Africa and in nations with social and economic ties to this region such as Portugal and France. Additionally, regions with historical ties to Portugal such as southwest India, Angola, Mozambique and Brazil also reported cases with HIV-2 (Reeves & Doms, 2002) (Campbell-Yesufu & Gandhi, 2011).

Dual infection with both viruses is encountered where both viruses are endemic; such as in West-Africa (Esbjörnsson et al., 2014). Research has showed that HIV-2 possesses a potential protective effect against infection with HIV-1 (Travers et al., 1995**)**. Patients co-infected with both viruses show a delayed progression to AIDS, compared to people with HIV-1 mono-infection, moreover, higher number of $CD4^+$ and $CD8^+$ T cells are also a hallmark in dual infected patients (Esbjörnsson et al., 2012), although some studies casted doubt on the halting of diseases progression as a result of co-infection with HIV-2 **(**Norrgren et al., 1999) (Greenberg, 2001). Retroviruses, being members of the *Lentivirus* genus, have the unique ability to transcribe their genetic material from RNA into DNA which incorporates into the host cell's genome. This characteristic makes retroviruses a perfect vehicle for targeted gene delivery into cells. Retroviral vectors are frequently used in clinical studies and research for gene therapy (Milone & O'Doherty, 2018**)**, however, there are drawbacks and dangers associated with working with retroviral-derived vectors. Through insertional mutagenesis, gene therapy vectors can trigger oncogenesis, during which they deregulate the expression of proto-oncogenes found near the insertion sites (Cesana et al., 2012). Additionally, vectors may result in genomic dysregulation in target cells by integrating into coding sequences and regulatory elements (Moiani etl al., 2012).

As pathogens, HIV exerts a great effect on the cellular transcriptome and proteome. Research showed that genes related to the immune system, cytoskeleton and leukocyte migration were regulated differently in HIV-1 infected cells **(**Coelho et al., 2021**)**. Additionally, inflammation, immune response, cell cycle related genes as well as transcription factors were upregulated in infected macrophages (Mehla & Ayyavoo, 2012).

Studies also showed that in the early phases of viral infection (period from infection to proviral genome integration), significant proteomic changes were apparent, preceding the production of viral proteins. Functional analysis revealed that proteins related to protein synthesis, cell proliferation and T-cell activation pathways were enriched (Navare et al., 2012). Additionally, differentially expressed proteins (DEP) from HIV-1 infected $CD4^+$ T cells were found to be associated with ubiquitination, nucleocytoplasmic transport, cell cycle progression and citrate cycle pathways (Chan et al., 2007**).**

There are multiple studies focusing on HIV-1 and its effect on the host cell, however, the majority of these studies are focusing on the later phase of infection, and very few of them investigated the early-phase of the viral life cycle. Moreover, studies on HIV-2 are lacking, especially those aiming to characterize the proteo-transcriptomic changes and alteration of the cellular milieu as a result of infection with HIV-2. Our aim here was to investigate the early-phase alterations in the cellular transcriptome and proteome induced by HIV-2 infection and to compare these changes with those induced by HIV-1. While HIV-2 exhibits significant differences in viral replication dynamics and disease progression, generally associated with slower disease progression and lower transmissibility, the cellular mechanisms underlying these differences remain poorly understood. Despite extensive research on HIV-1, relatively little is known about the impact of HIV-2 on host cell pathways at the molecular level. By employing transcriptomic and proteomic analyses, this study seeks to elucidate the cellular responses triggered by HIV-2 in the initial stages of infection, providing insights into how these responses differ from those elicited by HIV-1. Also, we wanted to carry out transcriptomic analysis on Jurkat cells, to model the effect of HIV on T cells in the early phase of infection.

2. Theoretical background

2.1 Epidemiology of HIV

As of the time of writing this thesis, according to data from the World Health Organization (WHO), almost 39 million people worldwide were reported to be infected with HIV. The majority of infections were recorded in the Eastern and Southern Africa region (20.8 million), followed by Asia and the Pacific region (6.5 million), region of Western and Central Africa (4.8 million), Western and Central Europe and North America (2.3 million), Latin America (2.2 million), Eastern Europe and Central Asia (2 million), The Caribbean (0.33 million) and lastly the Middle East and North Africa region (0.19 million) (HIV.uw.edu 2024).

HIV-1 is responsible for the majority of those infections, however, infections caused by HIV-2, and dual HIV-1/2 infections are also thought to be included in the pooled data. To date, no accurate statistics on the prevalence of HIV-2 infection exist, with very outdated estimates of around 2 million infections majorly in the West African region, including cases that are infected with both HIV-1 and HIV-2 (Gottlieb et al., 2008). More recently, prevalence of HIV-2 infection was shown to extend to countries with significant colonial or social and economic connections with the West-African region, such as France, Spain and Portugal. Indeed, several former Portuguese colonies have reported infection with HIV-2, including Angola, Mozambique, Brazil and the Indian states of Goa and Maharashtra (HIV.uw.edu 2024), (Reeves & Doms, 2002)**,** Campbell-Yesufu & Gandhi, 2011).

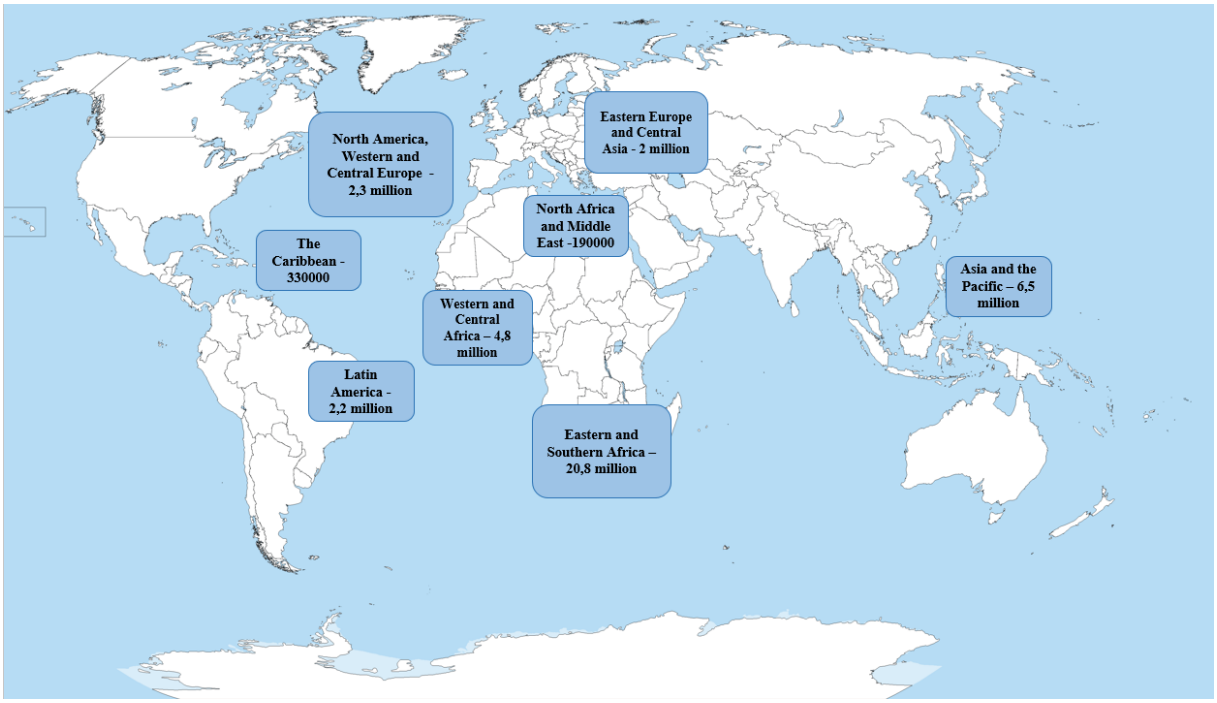


Figure 1. World map showing the number of the populations infected with HIV. Image was generated from data from https://www.hiv.uw.edu/go/screening-diagnosis/epidemiology/core-concept/all and WHO database (2024)

2.2 Genetic and protein structure of HIV-1 and HIV-2

The genome of HIV consists of two identical copies of single stranded RNA which is transcribed into DNA via the reverse transcriptase during infection. There is a long terminal repeat (LTR) in each end of the reverse transcribed HIV genome, and their transcriptional promoter is located on the 5' end. The *gag* gene whose reading frame is in the 5' to 3' direction encodes for the proteins of the outer core membrane, capsid protein, nucleocapsid and smaller, nucleic acid stabilizing protein. This is followed by the *pol* gene encoding for the enzymes

protease, reverse transcriptase and integrase. Adjacent to the *pol* gene is the *env* reading frame, which codes for the surface (SU) and transmembrane (TM) also known as the envelope glycoproteins. HIV genome encodes for several regulatory proteins in addition to genes encoding structural proteins such as Tat and Rev which are crucial for the initiation of HIV replication. Additionally, HIV genome codes for multiple auxiliary proteins such as Nef, Vif, Vpr and Vpu. HIV-2 encodes for the Vpx instead of Vpu (German Advisory Committee Blood, 2016) (Li et al., 2005). HIV-1 and HIV-2 contain only 50-55% identity in their nucleotide sequence of their genome. There is a 54% amino acid sequence similarity in their Gag, 55% in Pol and 35% in Env proteins (Li et al., 2015) **(**Motomura et al., 2008).

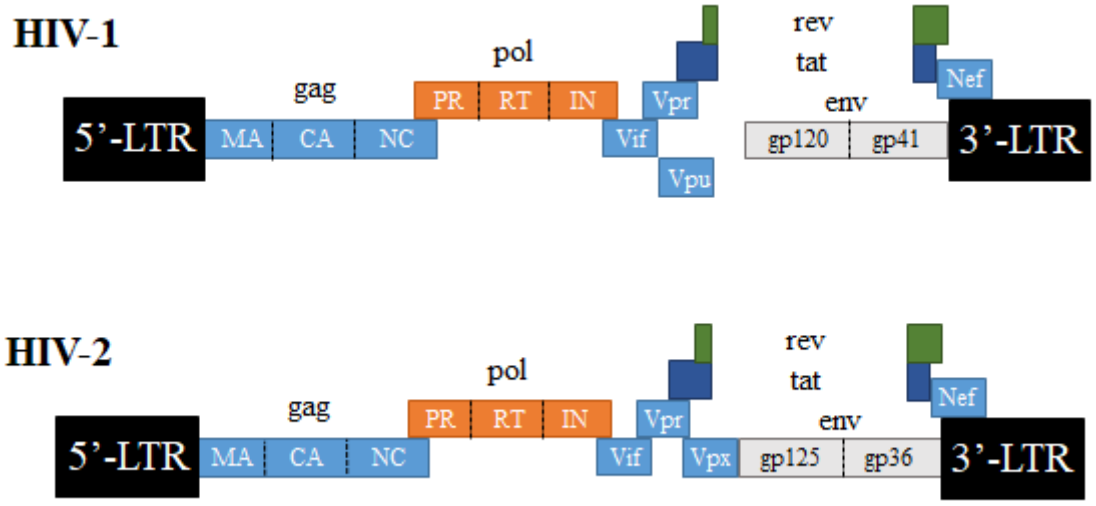


Figure 2. Structure of the HIV-1 and HIV-2 proviral DNA genome. Image was generated based on the work of Mahdi et al., 2018, van Heuvel et al., 2022 and Rey et al., 1989.

Structural proteins

Group specific antigen

A globular shell is constructed from Gag polyproteins which shows a conserved morphology in all retroviruses. Following budding from the host cell, the viral protease cleaves specific domains within Gag to generate structural proteins essential for mature infectious virion particles, including matrix (MA), capsid (CA), spacer p2, nucleocapsid (NC), spacer p1 and p6. A lipid bilayer originating from the host cells plasma membrane surrounds a shell composed of MA. Underneath this shell is an inner core formed from CA proteins which is followed by a ribonucleoprotein complex consisting of NC, diploid genomic RNA and replicative enzymes (Scarlata & Carter, 2003) **(**Freed, 1998**).**

Envelope

The *env* gene encodes for the surface expressed viral protein Env. The Env is a glycoprotein with a size of 160 kDa which is necessary for host cell binding and entry. After translation, the gp160 protein undergoes cleavage into gp120 and gp41in case of HIV-1. For HIV-2, the gp140 protein is cleaved into gp125 and gp36. After that, the gp proteins assemble into a non-covalently linked single subunit of a trimeric "spike" on the virion's surface. Gp41 and gp36 which is the C-terminal subunit, encompasses a cytoplasmic domain, a membrane spanning domain and an extracellular domain which facilitates the conformational change required for fusion. Gp120 and gp125 which are the N-terminal part localises entirely extracellularly and structured into five conserved regions (C1-C5) with five variable regions (V1-V5) distributed throughout.

Notably, the spike's exposed surface is characterised by the variable regions of gp120, which indicates that the high variability plays a dominant role in extracellular interactions; and an abundance of carbohydrates that aid in masking the protein's surface (Arrildt et al., 2012) (Rey et al., 1989) (Barroso et al., 2011) (Valadés-Alcaraz et al., 2022)

Polymerase

The three key enzymes required for HIV replication; protease (PR), reverse transcriptase (RT), and integrase (IN), are encoded by the *pol* gene. (Troyano-Hernáez et al., 2022**).**

Being an aspartyl protease, HIV PR functions as a homodimer with one active site. Each monomer contains an extended β-sheet region named flap, which is a glycine-rich loop that partially constitutes the substrate-binding site, and plays an important part in substrate binding (Brik & Wong, 2003**).** The viral PR cleaves the Gag protein at five positions, thus separating it into domains and peptides which leads to rearrangement of the virus into its mature infectious form. This happens concomitant with - or shortly after budding (Mattei et al., 2018). There is about 40% sequence identity between the amino acid sequences of HIV-1 and HIV-2 PRs (Weber et al., 2021).

The pol gene also encodes for the RT, which is translated as a Gag-Pol protein precursor that is then cleaved by PR into 66 kDa (p66) and 51 kDa (p51) subunits and together as a heterodimer, they form the active enzyme (Davis et al., 2008**)**. In all retroviruses, the RT is a characteristic enzyme that possesses two activities: First, a DNA polymerase activity which is capable of copying templates of RNA or DNA, and also an RNase H activity mediating RNA degradation.

Thanks to these abilities, RT is capable of converting the ssRNA genome of HIV into DNA, facilitating integration into the host cells chromosomes (Xaviet et al., 2020) **(**Boyer et al., 2012). Notably, RT activity of HIV-2 is lower than that of HIV-1, moreover, HIV-1 exhibits higher RNase H activity compared to HIV-2 (Boyer et al., 2012) **(**Boyer et al., 2006).

At the end of the *pol* gene, HIV integrase (IN) a 288-amino acid long protein, is encoded and released by the viral protease from a Gag-Pol polypeptide precursor. It has three independent domains: a HHCC motif containing N-terminal domain which is analogous to a zinc finger, a D, D-35, E motif encompassing central or catalytic domain which is required for the catalytic activity, and also the C-terminal domain which non-specifically binds to DNA, mainly contributing to the stability of the complex. All integrase activities are strictly dependent on the presence of metallic cationic cofactors coordinated by two residues of the catalytic triad (Delelis et al., 2008). Both HIV-1 and HIV-2 IN possess the same structural features, but at the nucleotide and amino acid sequence identity is 40 %, and 65 %, respectively (Bercoff et al., 2010**)**.

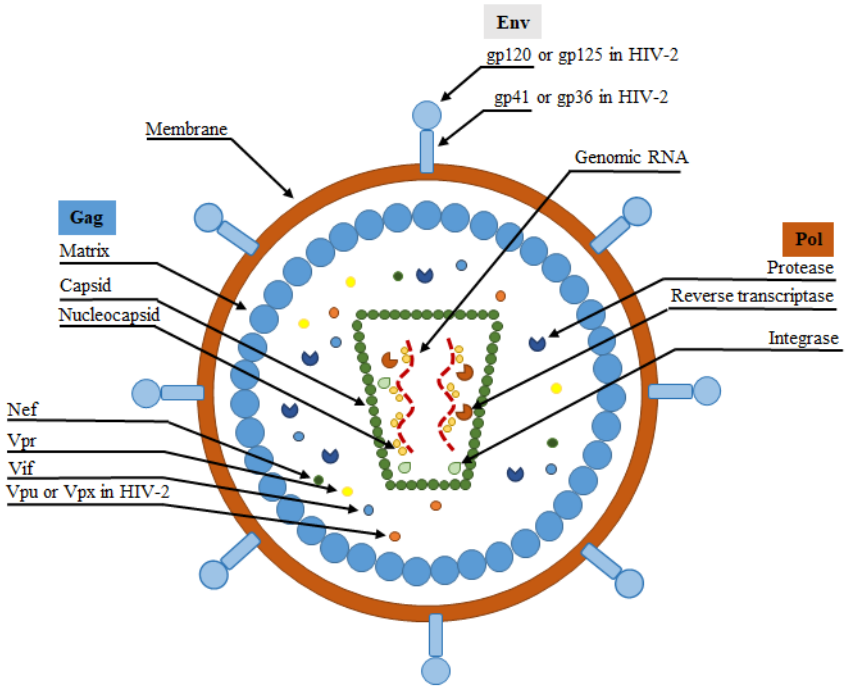


Figure 3. Structure of the HIV virion. Image was generated based on the work of van Heuvel et al., 2022, Frankel & Young, 1998, Mahdi et al., 2018 and Rey et al., 1989.

Regulatory genes

Trans-Activator of Transcription

The transcriptional activator Tat is one of the early expressed viral proteins which plays a crucial part in the replication of HIV through enhancement of proviral transcription. It achieves this via binding to the trans-activation response element (TAR) hairpin which is located at the 5' end of newly formed viral RNA transcripts. A 3-nucleotide pyrimidine bulge is an important feature in the TAR hairpin which binds the Tat protein. Also, there is the apical 6-nucleotide loop to which; in a Tat dependent manner, the transcriptional elongation factor pTEFb binds. After binding of TAR, the cyclin-dependent kinase 9 (CDK9) component of pTEFb phosphorylates the C-terminal domain of the RNA polymerase II, and thus enhances the activity of the polymerase. TATA box binding protein recruitment is also directed to the LTR promoter by pTEFb which promotes assembly of new transcription complex. Additionally, by recruiting chromatin-modifying proteins, Tat is able to remodel the promoter region (Das et al., 2011). Tat protein of HIV-2 differs from HIV-1 in terms of structure and function. HIV-1 Tat comprises 86 amino acids, meanwhile HIV-2 Tat has 130. Other than certain conserved regions, there is minimal homology between the two proteins. Moreover, the two molecules also differ in their ability to trans-activate gene expression directed by the LTRs of HIV-2 and HIV-1 (Arya, 1993**)**.

Regulator of expression of virion proteins

Rev is expressed from fully spliced viral RNA, and through binding to Rev response element in singly spliced, un-spliced and viral genomic RNA, promotes viral RNA transport. Moreover, Rev also plays a role in viral RNA splicing, stability and protein translation (Truman et al., 2020**)**. The structure of Rev consists of a nuclear localization signal and a leucine-rich nuclear export signal (NES). The former is recognized by importins/karyopherins, while the latter is recognized by chromosomal maintenance 1 or exportin 1 (CRM1). Through the nuclear pore complexes, Rev shuttles between the cytoplasm and the nucleus. During later stages of infection Rev binds to the rev response element (RRE) of viral RNA and facilitates transport of RNA via CRM1-dependent pathway. This process leads to the expression of distinct viral proteins and ultimately contributes to virus propagation (Taniguchi et al., 2014**)**. It is worth noting that while HIV-1 Rev is capable of exporting viral RNAs of HIV-2, HIV-2 Rev lacks the ability to export that of HIV-1 (Dillon et al., 1990).

Auxiliary proteins

Negative Factor

Nef is an accessory protein that localises to the cytosolic leaflet of various cell membranes through N-myristoylation at G2 of the Nef anchor domain. This interaction with the cell membrane is necessary for engaging the host cell's trafficking machinery, particularly the clathrin-coated vesicle (CCV) pathway. Nef utilises the CCV machinery to redirect selected transmembrane proteins from the surface of the infected cell which is done through sequestration or lysosomal degradation. By utilising these pathways, Nef effectively downregulates cell surface proteins involved in immune signalling antiviral restriction, and factors interfering with virion release (Buffalo et al., 2019). One of the main functions of Nef is the downregulation of CD4, as it can disrupt the processing of viral envelope glycoproteins and thus, inhibiting infectivity and interfering with the release of newly produced virions. The presence of CD4 also raises the possibility of superinfection, initiates premature cell death, and limits viral productivity (Kwon et al., 2020**)**. The rapid endocytosis of CD4 caused by Nef results in the degradation of molecules from the cell surface in lysosomes (Lundquist et al., 2002). Nef also has a prominent role in the deregulation of major histocompatibility complex class I (MHC-I) from the cell surface. Through interferingwith the recognition and destruction of infected cells, Nef-mediated downregulation of MHC-I molecules is beneficial to the pathogenesis of HIV (Pereira & daSilva, 2016) (Wonderlich et al., 2011**)**.

Viral Infectivity Factor

Vif is active in the later stages of HIV life cycle, and its main function is to enhance the infectivity of progeny virions (Lake et al., 2003**)**. The antagonization of restriction by Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G or A3G) is one of the main functions of Vif. Vif forms a complex with proteins such as Cullin 5, Elongin B and C. The complex functions as an E3 ligase and is able to facilitate poly-ubiquitination of APOBEC3G which leads to its proteasomal degradation (Takaori-Kondo & Shindo, 2013**)**. By utilizing the E3-ubiquitin ligase complex, Vif is also involved in the arrest of the cell cycle at the G2/M phase. Vif targets several members of the PPP2R5 family of protein phosphatase 2A (PP2A) regulators and thus, induces a halt in the cell cycle (Salamango & Harris, 2021) (Salamango et al., 2019). Vif also interacts with cellular tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (SHP-1) in an immunoreceptor tyrosine-based inhibitory motif (ITIM)-dependent manner to inhibit the production of type I interferons (IFN). This involves Vif being phosphorylated by host protein Fyn Related Kinase (FRK), followed by interaction with SHP-1. Activated SHP-1 then inhibits the ubiquitination of stimulator of

interferon genes (STING), preventing its oligomerization and resulting in the inhibition of production of type I IFN (Wang et al., 2022).

Viral Protein R

HIV Vpr is a multifunctional protein which plays multiple roles in the viral life cycle. Through interaction with the Pr55Gag-derived p6 protein, Vpr is incorporated into the viral particle becoming readily available after fusion and entry (Guenzel et al., 2014). Vpr has been shown to enhance viral DNA export into the nucleus of non-dividing cells via facilitating interactions with cellular machinery governing nucleo-cytoplasmic transport (Le Rouzic & Benichou, 2005). Vpr plays a prominent part in the regulation of cell cycle by inducing G2/M cell cycle arrest, however, the biological implications of the cell cycle arrest during the life cycle of HIV is not yet fully characterised. It was noted that HIV-1 LTR shows increased activity in the G2 phase, this implies that the halting of the cell at the G2 phase provides a preferred cellular milieu for efficient transcription of HIV (Guenzel et al., 2014).

Viral Protein U

Viral protein U (Vpu) is coded by the genome of HIV-1 and certain strains of simian immunodeficiency virus (SIV) (González, 2015). Vpu serves a dual role in the life cycle of HIV, firstly, it promotes the degradation of newly synthesized CD4 protein via the ubiquitin-proteasomal pathway. This downregulation of CD4 production not only blocks the superinfection of the infected cell, but also shields the host from immune surveillance and enhances viral replication fitness. Moreover, by counteracting the host restriction factor tetherin or bone marrow stromal antigen 2 **(**BST-2), Vpu increases the release of newly produced virions from the surface of the host cell. The effects of Vpu on BST-2 are multi-folded, including cell surface deregulation, inhibition of recycling, and reduction of its intracellular levels. Moreover, Vpu also plays a part in the regulation of host protein transport from endoplasmic reticulum to Golgi. It was also shown to alter MHC class II presentation and trigger apoptosis (Khan & Geiger, 2021).

Viral Protein X

Similarly, to Vpr, Vpx is involved in the early events of the viral life cycle, counteracting host innate defences. To enhance viral replication, Vpx targets the sterile alpha motif and histidine-aspartate domain containing protein 1 (SAMHD1) and the human silencing hub (HUSH)

complex via utilization of the damage-specific DNA binding protein 1 (DDB1)-Cullin4A (CUL4A)-associated factor 1 (DCAF1). Through these host proteins, Vpx promotes the assembly of the proteasome machinery in order to degrade target restriction factors (Fink et al., 2022). Moreover, in both dividing and non-dividing cells, Vpx is involved in the nuclear transport of viral DNA (Mahdi et al., 2018). Additionally, Vpx was found to interact with - and impede the interferon regulatory factor 5 (IRF5) mediated trans-activation, thus, reducing the production of IL6, IL12p40, and TNFα (Cheng & Ratner, 2014).

2.3 Life cycle of HIV

The life cycle of HIVs can be divided into two parts; an early, and a late phase. In the beginning of the early phase, the viral surface glycoprotein binds to the target receptor which triggers the fusion of the viral envelope and the cell membrane (Freed, 2015) (Chen, 2019**) (**Melikyan, 2014). Following entry, the viral core travels through the cytoskeleton to the nucleus. The viral core encompasses the capsid, nucleocapsid and viral proteins such as RT, PR and INT. During cytoskeletal travel the viral capsid disassembles in a process called uncoating which is required for the completion of the reverse transcrirption (Ambrose & Aiken, 2014). The reverse transcription starts inside the viral core during its transport through the cytoskeleton. The process is mediated by the RT enzyme during which the viral RNA genome is transcribed into double stranded DNA (Goff, 2001) **(**Xavier Ruiz & Arnold, 2020). Following the reaching of the nucleus, the pre-integration complex (PIC) assembles which contains the transcribed viral DNA as well as viral and host proteins (Goff, 2001) **(**Arhel, 2010**)** (Li, 2015). Moreover, recent data indicates that the uncoating and reverse transcription continues following the nuclear entry (Dharan et al., 2020) (Müller et al., 2022). The PIC enters into the nucleus via the nucleopore complex in an importin/karyophilin dependent manner. Host proteins which are involved in the nuclear entry are recruited through the nuclear localization signal of the Vpr and integrase proteins. Integrase interacts with members of the nuclear transport machinery and induces the translocation of the PIC into the nucleus (Jayappa et al., 2012) **(**Popov et al., 1998). The viral genome is then integrated into the host DNA, a process mediated by the viral IN (Hokello et al., 2024). The integration marks the transition from early to late phase of the HIV life cycle. In the late phase, the proviral transcription begins, during which new viral proteins are synthetized and new virons are assembled (Freed, 2015). In infected cells, the integrated viral DNA acts as a template for viral mRNA and genomic RNA synthesis. During the beginning of the late phase, proviral transcripion is dependent on cellular factors like nuclear factor kappa B

(NF-κB) until the expression of tat. Tat binds to the viral TAR region after which it recruits cellular proteins such as positive transcription elongation factor b (P-TEFb), comprising cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CCNT1). This allows the virus to synthetize full length viral RNA and different mRNA products (Liu et al., 2014). The transport of the viral mRNA from the nucleus to the cytoplasm is managed by the Rev protein (Hokello et al., 2024). HIV-1 virion assembly occurs at the cellular plasma membrane and the process is mediated by the Gag polyprotein. The Gag recruits the Env proteins and viral genomic RNA to the site of virus assembly. The virion acquires its lipid envelope and Env protein spikes during the budding from the plasma membrane. The Gag polyprotein first assembles into immature particles which following budding, is processed by the activated viral PR forming new infectious mature viral particles (Sundquist & Kräusslich, 2012) (Ganser et al., 2012).

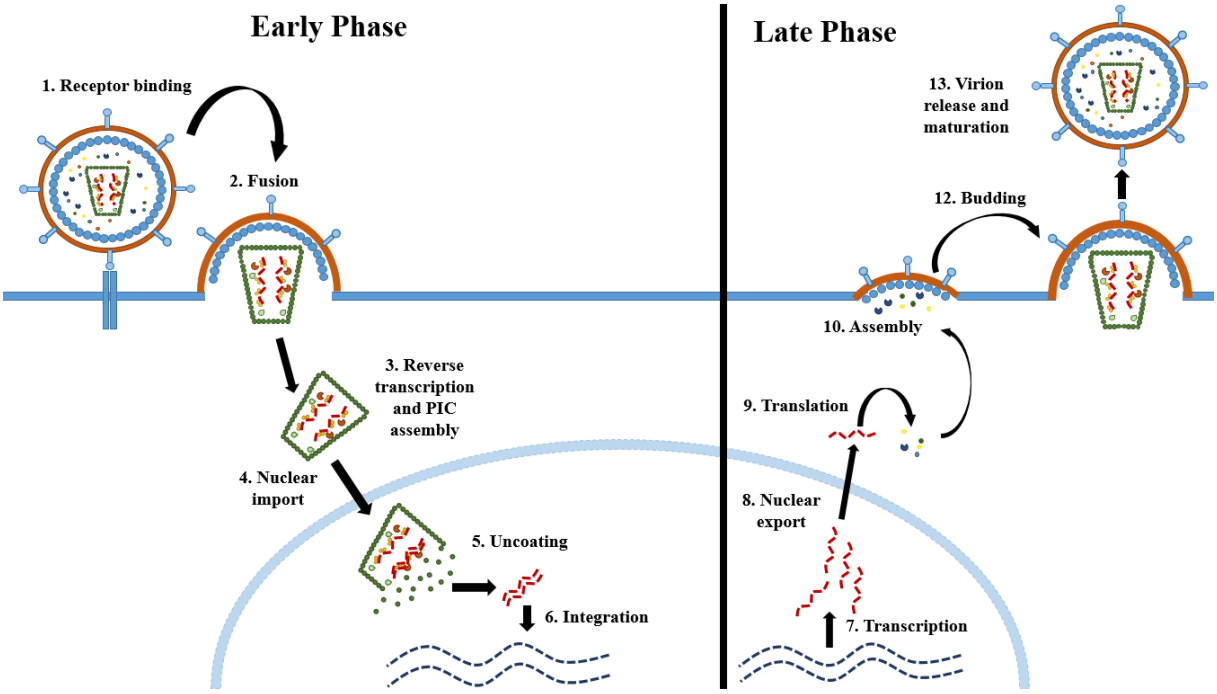


Figure 4. Overview of the life cycle of HIV viruses. The image was generated based on the work of Chou et al., 2024 **,** Engelman & Cherepanov., 2012, and Ramdas et al., 2020 **)**

2.4 Cellular restriction factors of HIV

The first line of defence against invading pathogens are the innate immune response, which relies on the sensing of pathogens through specific pattern recognition receptors. Signalling through these receptors initiate the production of cytokines, chemokines and interferons. The latter binds to receptors and through the Janus kinase/signal transducer and activator of

transcription (JAK/STAT) mediated signalization activities the production of interferon stimulated genes (ISG) (Schoggins & Rice., 2011). These genes help combat viral, bacterial and parasitic infections in the cell by targeting functions and pathways during the life cycle of the pathogen. Moreover they also aid neighboring cells resisting infection. The receptors and signalization proteins that are involved in the innate immune signalling are interferon stimulated themselves. This allows further amplification of these pathways upon pathogen sensing in the host cell (Crosse et al., 2018). ISGs inhibit pathogens through a variety of functions including oxidative molecule generation, autophagic machinery recruitment, inhibiting replication and depletion of molecules required for the microbial life cycle (MacMicking., 2012). Restriction factors represent a set of host proteins that discern and impede specific stages of the viral replication cycle. They are generally encoded by the aforementioned interferon inducible genes and are capable of rapid action and their activity is self-sufficient (Colomer-Lluch et al., 2018) **(**Boso & Kozak, 2020**)**. Some of these restriction factors are also able to stimulate a broad range of innate immune signaling in order to further suppress viral pathophysiology. Through its accessory proteins; such as Vif, Vpu, Nef and Vpx or by initiating mutations in its proteins, HIV is capable of counteracting many cellular restriction factors, some of which are mentioned below (Jia et al., 2015).

Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G

One of the cellular factors that restricts HIV is the APOBEC3GA3 proteins are cytidine deaminases that through interactions with the nucleocapsid domain of the Gag protein and/or viral RNA can be incorporated into progeny viruses. Following infection, the incorporated APOBEC3G deaminates cytidine to uridin in the viral minus-strand DNA during reverse transcription, leading to hypermutation in the viral genome, consequently, the transcribed viral DNA becomes non-functional and is degraded (Stupfler et al., 2021) **(**Jia et al., 2015) **(**Wang et al., 2012).

To counteract the inhibition imposed by A3 proteins, HIV utilizes Vif in order to initiate the degradation of A3 proteins via the host ubiquitin-proteasome machinery. (Jia et al., 2015) **(**Stupler et al., 2021). Vif is also able to inhibit APOBEC3G in a degradation-independent way. By interaction with the core-binding factor subunit beta (CBF-β), Vif is able to inhibit the transcription of A3 genes. Moreover, Vif is capable of inhibiting the A3G translation through interaction with the 5'UTR of A3G mRNAs. (Stupler et al., 2021).

Bone Marrow Stromal Cell Antigen 2

Bone Marrow Stromal Cell Antigen 2 (BST-2) also referred as tetherin or CD317 is a type II single-pass transmembrane protein (Perez-Caballero et al., 2009) (Malim & Bieniasz, 2012). In the presence of tetherin and in the absence of Vpu, HIV-1 particles undergo normal assembly, but encounter difficulties during their release as tetherin causes their entrapment at the cell surface of the host cell (Malim & Bieniasz, 2012). As viral budding progresses, tetherin integrates itself into the viral envelope and binds the viruses to the surface of the infected cell, subsequently leading to their internalization and degradation within lysosomes (Harris et al., 2012).

HIV-1 Vpu protein serves as a countermeasure against tetherin, with its antagonism beginning with a direct physical interaction between the antiparallel membrane-spanning helices of Vpu and tetherin. HIV-2 lacks Vpu and relies on alternative viral proteins in order to counter tetherin; such as the envelope glycoprotein which can exhibit a Vpu like activity through direct interaction with tetherin, which leads to the internalization of tetherins and subsequent degradation. (Malim & Bieniasz, 2012) (Harris et al., 2012).

Sterile alpha motif and HD-domain containing protein 1

Human sterile alpha motif and HD-domain containing protein 1 (SAMHD1) is a protein which possesses a nuclear localization signal on its N-terminus and a sterile-alpha motif and histidine/aspartic acid domain next to it. Tissue-resident macrophages, monocytes, dendritic cells (DCs), plasmacytoid DCs (pDCs), thymic T cells, natural kille cells (NK cells) and B cells all express SAMHD1. Through stimulation with dGTP or GTP, SAMHD1 manages the conversion of dNTPs into deoxynucleoside and inorganic triphosphate, thanks to its deoxynucleoside triphosphate triphosphohydrolase (dNTPase) activity. SAMHD1 function involves protection from the development of cancer and activation of the innate immune system (Antonucci et al., 2017). It is activated in response to elevated levels of dNTP and nucleic acid degradation in the absence of DNA replication. The main activity of SAMHD1 is to inhibit the replication of retroviruses and other DNA viruses via reduction of intracellular dNTP concentration. SAMHD1 also has a metal-dependent 3'-5' exonuclease activity, enabling it to degrade single stranded DNA and RNA, and can also act as a binding protein for single-strand nucleic acid (Antonucci et al., 2017) (Schmidt et al., 2015).

In human monocytes, monocyte-derived macrophages (MDMs) and $CD4^+$ T cells, SAMHD1 can initiate the degradation of HIV-1 RNA (Ryoo et al., 2014), Choi et al., 2015).

SAMHD1 is counteracted by HIV-2 Vpx through the recruitment of the cellular DDB1/DCAF1-dependent E3 ubiquitin ligase complex which initiates the ubiquitination and subsequent degradation of SAMHD1 (Mohamed et al., 2021).

Serine incorporator 3 and 5

Two members of the serine incorporator (SERINC) protein family, serine incorporator 3 (SERINC3) and 5 (SERINC5) contribute to the antiviral activity via enhancement of the type I interferon expression and also NF-κB (Cano-Ortiz et al., 2023). By incorporating themselves into virions, they possess the ability to inhibit retrovirus infectivity, however the mechanism of how these proteins are able to inhibit HIV is not yet well understood. As hypothesized, SERINC proteins are able to induce conformational changes in the viral envelope, thus preventing viral entry. Another idea suggests that thanks to its lipid-binding ability SERINC5 is able to alter the viral membrane structure, interfering with the function of Env (Pye et al., 2020) (Beitari et al., 2017) (Kirschman et al., 2022).

Research data show that in the absence of Nef, reverse transcription is hindered, suggesting that SERINC5 has some effect on the reverse transcription step of HIV-1 (Usami et al., 2015).

As a counteracting mechanism of SERINC, HIV encodes the Nef protein. Nef enhances the spreading of HIV in primary $CD4^+$ T cells, where it acts as a protein adaptor exploiting key host pathways of cellular transport and signal transduction (Usami et al., 2015**)** (Fackler, 2015). Myristoylated Nef forms homodimers at the plasma membrane where it interacts with SERINC5 and initiates its polyubiquitination which leads to lysosomal degradation of SERINC5 (Cano-Ortiz et al., 2023).

Tripartite Motif Containing 5

In a species specific manner, tripartite motif 5 (TRIM5) proteins which includes TRIM5α can recognize the capsid of retroviruses directly. One mechanism of how TRIM5 is capable of restricting HIV is that TRIM5 multimers interact with the capsid of the virus and recruit's proteasomes, which in turn degrade the virus particle. This results in the premature disassembly of the viral capsid (Jia et al., 2015) (Ganser-Pornillos & Pornillos, 2019) (Boso & Kozak, 2020). TRIM5 can also function as a pattern recognition receptor via its ability to bind the capsid of retroviruses. Capsid binding triggers a cascade which results in TRIM5α poly-ubiquitination which in turn activates activator protein 1 (AP-1) that leads to the activation of NF-κB pathway (Boso & Kozak, 2020).

Myxovirus resistance protein

As an interferon-inducible dynamin like GTPase, Myxovirus resistance protein (MxB) functions as an inhibitory molecule which can target HIV-1. MxB has no effect on the products of late reverse transcription, but its expression decreases the number of HIV-1 2-LTR circles and integrated viral nucleic sequences (Jia et al., 2015) (Boso & Kozak, 2020) (Liu et al., 2013). A suggested way how MxB inhibits HIV-1 is its ability to associate with the HIV-1 capsid to prevent uncoating (Fricke et al., 2014). Another proposed mechanism comes from MxBs localization at the nuclear pore complex, where MxB may influence the function of nucleoporins. Here, MxB can alter the transport of viral DNA. Additionally, MxB may have an effect on the activity of receptors involved in the nuclear transport and thus, influence the related cargos to the nuclear import. Also, MxB possesses the ability to block the nuclear import of HIV-1 Rev protein, thanks to its ability to impair the interaction between Rev and transportin 1 (TNPO1) (Wang et al., 2020).

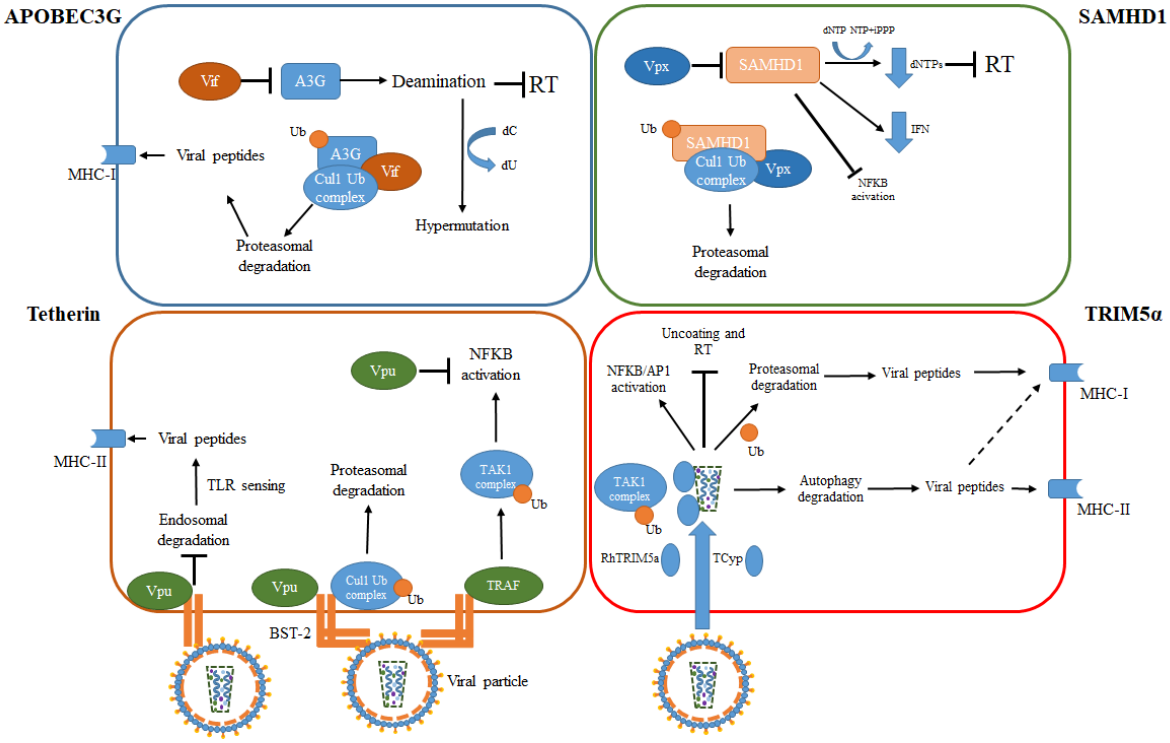


Figure 5. Interaction between HIV restriction factors and viral proteins. Image was generated based on the work of Colomer-Lluch et al., 2018.

2.5 Lentiviral Vectors

Replication incompetent lentiviral vectors serve as convenient and effective tools for stable and efficient transfer of genes into cells of human origin, making them attractive candidates for human gene therapy. Their main appeal is that they are stably integrated into the DNA of their target cells ensuring long-term gene expression. Lentivirus-based vectors offer a notable advantage over oncoretroviral delivery systems: they are able to introduce genes into non-dividing cells. Successful treatment was reported from patients with severe monogenic disorders; such as skin adhesion disorders, immunodeficiencies, neurometabolic and hemoglobinopathies diseases, based on somatic stem cells transduced with viral vectors (Zufferey et al., 1998) (Tucci et al., 2021).

The first lentivirus-based vectors contained large parts of the HIV genome. In addition to *pol* and *gag*, there were auxiliary genes such as *vpr, vif, nef* and *vpu* and also the regulators *rev* and *tat*. As for the envelope, G protein of the vesicular stomatitis virus (VSV-G) was utilized. The target receptors for VSV-G are low density lipoprotein (LDL) receptor, phosphatidylserine and perhaps other phospholipids, which allowed the lentivirus-based vectors to target a large variety of cells. In the next iteration of lentivectors, the design was modified to be safer. Accessory proteins were removed, as they were not found to exert an effect on the transfer of genetic material. To further improve vector safety, in third generation systems, the genome of the virus was divided into two plasmids which makes it less likely to generate recombinant viruses. The *pol* and *gag* genes are encoded on the same plasmid while the *env* and *rev* are on separate constructs which results in a vector made out of three plasmids. In these vectors, a promoter which is constitutively active was introduced into the upstream LTR region of the transgene, making the presence of *tat* gene obsolete. To further improve safety, deletions were introduced in the 3'LTR of the viral genome to create lentivirus vectors which are self-inactivating (SIN). During the process of reverse transcription, the deletion in the 3'LTR is transferred to the 5' LTR of the proviral DNA which results in the inactivation of the LTR in the proviruses (Milone & O'Doherty, 2018) (Dull el al., 1998) (Miyoshi et al., 1998) (Zufferey et al., 1998). There are also fourth generation lentiviral vectors, where they modified the packaging sequences in order to avoid replication competent particle formation. In these vectors, the HIV-1 ψ and RRE packaging sequences are located downstream of the self-inactivating LTR. Some viral components required for virus production, like structural and regulatory genes, are provided during vector manufacturing, but are excluded from the final viral genome. This overall further improves the safety of vectors (Vink et al., 2017) **(**Berkhout, 2017).

Lentivirus-based vectors became attractive from a clinical perspective due to their ability to transduce slowly or non-proliferating cells including CD34+ stem cells. Genetic diseases such as metachromatic leukodystrophy, Wiskot-Aldrich syndrome, β-thalassemia and X-linked adrenoleukodystrophy were successfully treated with lentivirus-based vectors (Milone & O'Doherty, 2018).

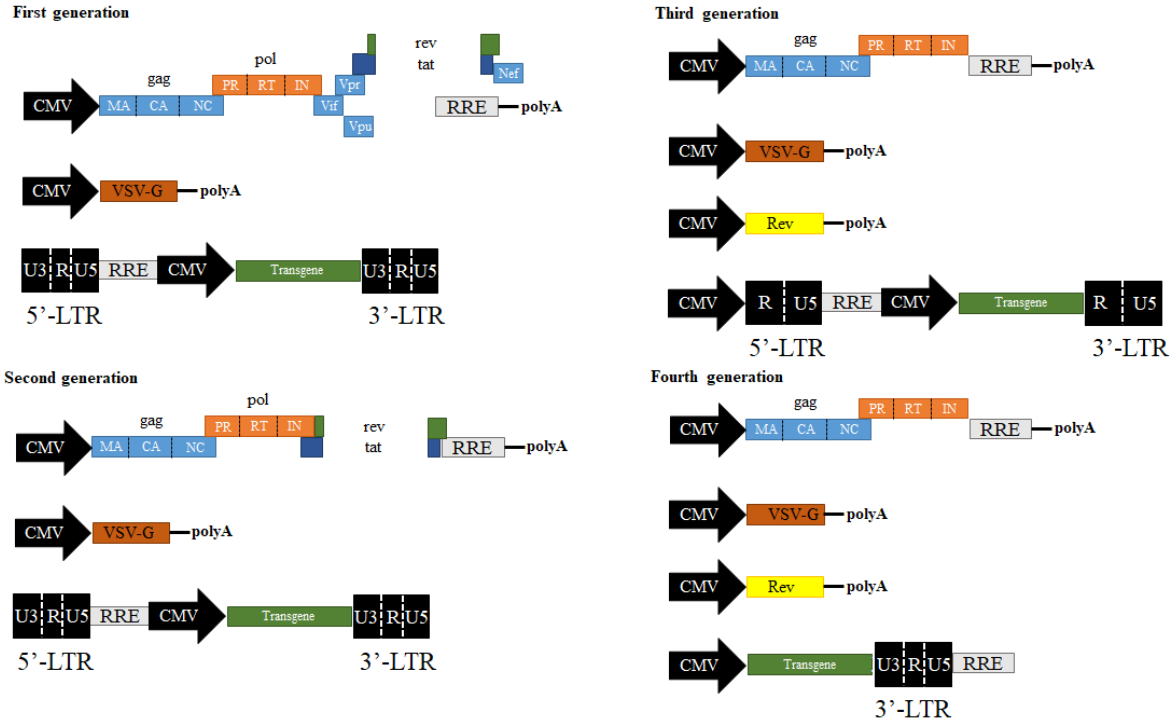


Figure 6. Main structure and plasmids of the four generations of lentivirus based vectors. Image was generated based on the work of Gutierrez-Guerrero et al., 2020, Berkhout, 2017, Vink et al., 2017, Sakuma et al., 2012.

2.6 Lentivector induced transcriptomic changes

Retroviral-dervied vectors used in gene therapy can trigger oncogenesis via proto-oncogene deregulation found near the insertion site. This process is called insertional mutagenesis. During the process, they are able to deregulate the expression of proto-oncogenes located near insertion sites through various molecular mechanisms; such as enhancer mediated activation, which involves interaction between the viral enhancer sequences and cellular promoter to increase the expression of proto-oncogene mRNA levels (Cesana et al., 2012). However, in the case of SIN

lentiviral vectors, they showed a lower risk of inducing insertional mutagenesis compared to other retroviral-based vectors such as gamma retroviruses (Milone & O'Doherty, 2018**)**.

Additionally, vectors could initiate dysregulation of gene expression in target cells through insertion into genes and regulatory elements. By integrating into transcribed genes, lentiviral vectors decrease the expression of genes through interfering with the polyadenylation and splicing of transcripts (Moiani et al., 2012).

Gammaretroviral vectors derived from mouse leukemia virus (MLV) integrate into active regulatory gene regions, thereby increasing the chance of deregulating gene expression. Proto-oncogene activation via insertion was noted in individuals treated with vectors derived from Moloney murine leukemia virus (MLV). However, lentiviral vectors derived from HIV were found to mostly integrate away from regulatory elements and thus, are less likely to induce transcriptional gene activation (Moiani et al., 2012). Moreover, it is more typical for lentivectors to randomly integrate into active transcribed genes which results in inactivation of the gene rather than activation (Wu & Dunbar, 2011).

Cesana et al carried out comprehensive transcriptomic analysis of chimeric lentiviral vector-cellular fusion transcripts in transduced primary hematopoietic stem/progenitor cells and human lymphoblastoid cells. Their data showed a notable excess of read-through transcription originating both inside and outside of the integrated virus. In addition, they also identified the vector sequences involved in the abnormal splicing process. They observed that self-inactivating lentiviral vectors possess a significantly lower tendency for aberrant splicing compared to vectors carrying active LTRs (Cesana et al., 2012).

In a different study, Cesana et al examined a range of lentiviral vectors, including those intended for clinical use. Their findings indicated that the primary mechanism driving early vector induced oncogenesis is the activation of oncogenes via promoter insertion. Reduced genotoxicity was observed in the case of self-inactivating lentiviral vectors specifically engineered to disable oncogene activation via promoter insertion. However, through enhancer mediated activation of oncogenes, these vectors induced tumors with the risk correlating with the strength of the promoter utilized in these vectors. Additionally, when using phosphoglycerate kinase (PGK) based promoters, the enhancer activity was reduced and oncogenesis was observed through inactivation of tumor suppressor genes (Cesana et al., 2014).

Bokhoven et al found that HIV-based vectors, through insertion into the growth hormone receptor (Ghr) locus, can generate mutant transcripts. In this locus, even in the absence of Tat protein, the HIV enhancer/promoter remains active, which results in an HIV/*Ghr* spliced

transcript which expresses GHR. This insertional mutagenesis is prevented with the use of self-inactivating HIV-based vectors where the enhancer/promoter is deleted (Bokhoven et al., 2009**)**. Moiani et al identified aberrantly spliced, chimeric transcripts in cells transduced with HIV-derived vectors in over half of the selected genes. Via utilizing constitutive and cryptic splice sites within the HIV 5' long terminal repeat, gag gene, β-globin gene and locus control region chimeric transcripts were generated. Transcripts which were aberrant accumulated at low level, partly due to nonsense-mediated degradation of mRNA, compared to constitutively spliced transcripts (Moiani et al., 2012).

2.7 Pathways induced by infection with HIV-1

Throughout its life cycle, HIV can initiate the activation of multiple signaling pathways inside the target cell. At the early phase of infection, Nef, gp120, Tat and Vpr possess T-cell signaling mimicking ability, which results in a sustained replication within the infected cells. Viral proteins block apoptotic pathways through inhibition of cell death-related signalling, and interaction with receptors on the cell surface such as chemokine co-receptors (Abbas & Herbein, 2013). Evidence shows that regulation of pathways such as inhibition of ERK/MAPK can suppress viral replication and minimize the cytopathic effects. This is probably achieved through impairment of reverse transcription and viral integration. Additionally, in infected T-cells, the Notch signalling pathway acts as a co-activator of the nuclear factor-κB (NF-κB) pathway. This in turn induces interleukin 2 (IL2) production which activates $CD4^+$ T cells and initiates their proliferation in parallel to initiating viral transcription (Coelho et al., 2021). Brooks et al showed in their study that numerous cellular pathways are able to activate the expression of latent HIV reservoirs. Moreover, pathways such as nuclear factor of activated T-cells (NF-AT) and protein kinase C (PKC) were found to be crucial inducers of replication of latent HIV in quiescent primary cells. Viral reactivation was almost completely abrogated by the inhibition of PKC, lymphocyte-specific protein tyrosine kinase (Lck) and NF-AT pathways, while viral expression was stimulated as a result of activation of PKC and NF-AT in the absence of other signals (Brooks et al., 2003**).**

In their study, Guo et al examined $CD4^+$ T cell transcriptome and showed that HIV-1 set-point viral load positively correlates with the gene expression of the oxidative phosphorylation (OXPHOS) pathway. OXPHOS is recognized as a signature of metabolism in the acute phase of viral infection (Guo et al., 2021).

Additionally, cell death related pathways; such as Fas/FasL may be utilized by HIV. HIV-generated immune activation can upregulate the expression of the cell death receptor Fas and its ligand FasL in infected cells. Bystander cells are also affected by this upregulation via secreted viral proteins such as Tat and Nef, or via interaction with HIV infected cells. This leads to elimination of not just the infected, but also uninfected bystander cells. (Poonia et al., 2009). HIV-1 proteins are also known to promote the replication of the virus via signaling pathways such as NF-κB, MAP kinase (MAPK) and JAK/STAT activation (Herbein et al., 2010).

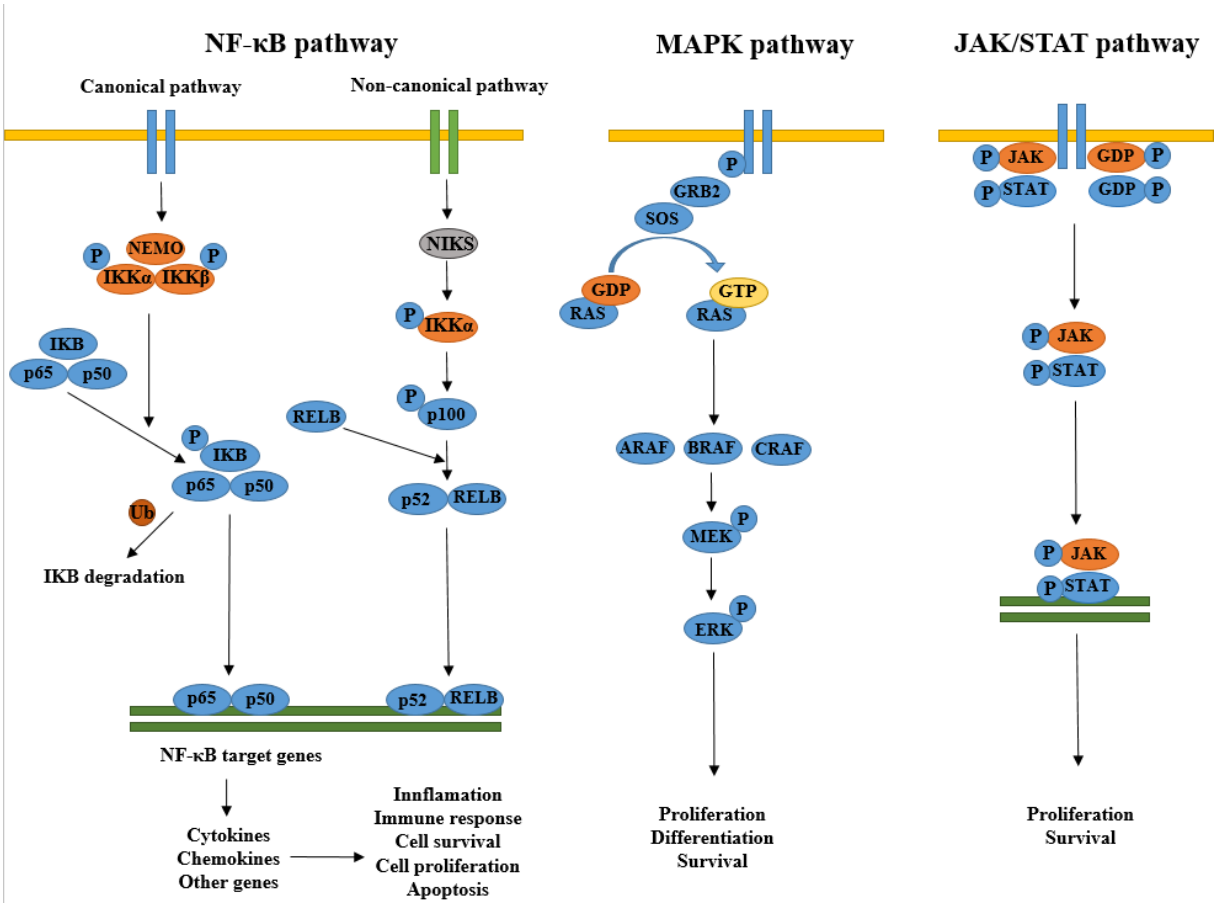


Figure 7. Three of the most common pathways influenced by HIV-1 infection. Image was generated based on the work of Peng et al., 2020, Schubert et al., 2023 and Hu et al., 2021.

2.8 Transcriptomic changes induced by HIV-1

In their analysis, Coelho et al revealed 208 differentially regulated genes by HIV-1 in $CD4^+$ infected T-cells. Among them, the pathogen associated molecular pattern receptor toll like receptor 7 (*TLR7*), interferon beta 1 (*IFNB1*) and TNF superfamily member 4 (*TNFSF4*) genes which were upregulated. Genes such as apolipoprotein D (*APOD*), syndecan 2 (*SDC2*), CXC motif chemokine ligand 11 (*CXCL11*) and vascular cell adhesion molecule (*VCAM1*) which are

involved in leukocyte migration were also differentially regulated. Genes involved in cytoskeletal maintenance; such as microtubule associated protein 1B (*MAP1B*), wnt family member 11 (*WNT11*) and others were also upregulated initially, and also at the final stages of the retroviral lifecycle (Coelho et al., 2021).

Wu et al focused on genes that are expressed differentially in $CD4^+$, $CD8^+$ T cells, and macrophages from patients infected with HIV. They found elevated levels of genes that regulate proteasome, proton transporting ATPase complex, actin filaments, and complement activation. Pathways such as mitochondrial signatures of disease progression and pathways linked to energy production, apoptosis, cell-cycle dysregulation and metabolism showed enrichment during the analysis (Wu et al., 2011).

In their study, Wen et al found that in infected monocytes and monocyte-derived macrophages (MDMs), genes involved in the cell cycle such as tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (*YWHAE*), RB binding protein 7, chromatin remodeling factor (*RBBP*), MCC regulator of WNT signaling pathway (*MCC*) and cyclin dependent kinase inhibitor 1A (*p21*) were upregulated. Additionally, genes such as protein phosphatase 2A (*PP2Aα*), minichromosome maintenance complex component 2 (*BM28*), ubiquitin-conjugating enzyme E2 L3 (*UbcH7*), ubiquitin conjugating enzyme E2 E1 (*UbcH6*) and serine/threonine kinase 38 (*Ndr*) were downregulated. (Wen et al., 2021**)**.

Deshiere et al showed that the expression of DNA methylases DNA methyltransferase 3 alpha (*DNMT3A*) and DNA methyltransferase 1 (*DNMT1*) was elevated together with histone acetyltransferase 1 (*HAT1*) and histone deacetylase 9 **(***HDAC9*) in HIV-1 infected macrophages. Additionally, in those cells, the expression of histone deacetylase 11 (*HDAC11*) was reduced.

GATA zinc finger domain containing 2B (GATAD2B); a transcriptional repressor and nuclear transporter, showed similar downregulation. GATAD2B is an important regulator of the nucleosome remodelling and histone deacetylation complex (NuRD) together with histone deacetylase 1 (HDAC1) and 2 (HDAC2). The remodelling of the host chromatin is important for HIV, in order to attain productive integration and transcription. Moreover, differential regulation of genes encoding for restriction factors such as *APOBEC3B*, ATM serine/threonine kinase (*ATM*) and lipoxygenase-3 (*LOX3*) was also detected in infected macrophages (Deshiere et al., 2017).

Pollara et al. employed single-cell transcriptome sequencing to elucidate the alterations and cellular dynamics linked to infection with HIV-1. They found that 45 genes were downregulated and 96 were upregulated in cells from un-treated HIV-1 infected patients. Notably, HIV-1 induced a proinflammatory state across all observed immune cell types, characterized by the

upregulation of interferon response genes. Additionally, compared to seronegative individuals, they found 83 upregulated and 31 downregulated genes in HIV-1 infected individuals under antiretroviral therapy (Pollara et al., 2022).

2.9 Proteomic changes induced by HIV-1

In the last decade, many studies were carried out in order to enrich our knowledge about proteomic changes generated by infection with HIV.

Navare et al in their experiment found 266, 60, and 22 differentially regulated proteins at 4, 8 and 20 hours post-infection, respectively, in $CD4^+$ SUP-T1 T cell line following infection with HIV-1. These proteomic changes happened at the early stage of the infection. Functional analysis of the DEPs revealed enrichment in pathways related to T-cell activation, cellular proliferation, and protein synthesis. (Navare et al., 2012).

In their work, Chan et al detected 3255 cellular proteins from HIV-1 infected $CD4^+$ T cell line. Out of those detected proteins, 344 were upregulated, and 343 were downregulated at 36 hours post-infection. This time-point denotes the peak of viral production. Pathway analysis showed that those proteins were involved in cell cycle progression, citrate cycle pathways, ubiquitination and nucleocytoplasmic transport. They also observed alterations in the level of proteins that known to interact with HIV-1 (Chan et al., 2007).

Chan et al have described in another of their work that over the course of infection, expression level of 168 proteins was altered in $CD4^+$ helper T cells infected with HIV-1. Their data suggest a highly dynamic cellular proteome during the progression of viral replication. At 8 hours post-infection, the abundance of tRNA synthetase and ribosomal proteins was evident in infected cells. Moreover, increased ATP production and flux through the glycolytic pathway were observed, suggesting elevated energy demands to sustain increased biosynthesis. This was further supported by increased abundance of ATP synthase components in the mitochondria. At 24 hours post-infection, they observed a decrease in the level of tRNA synthetases, translation initiation factors and ribosomal proteins. Moreover, at the same time-point, proteins involved in energetic, cell proliferation and cell survival pathways showed decreases expression. Additionally, the cellular level of proteins involved in the DNA repair and nucleosome assembly were upregulated (Chan et al., 2009).

In their study, Kraft-Terry et al found a total of 393 DEPs in HIV-1 infected MDMs in all of their observed time-points. The synthesis of IFN-induced proteins was upregulated according to their ingenuity pathway analysis conducted during the 7^{th} day of infection. This indicates an

increased antiviral response in macrophages as HIV-1 infection progressed. Virus production increased over time despite the upregulation of antiviral proteins such as signal transducers and activators of transcription 1 (STAT1), 2′-5′-oligadenylate sythetase 2 (2–5 OAS2), IFN-induced GTP-binding protein Mx1 (Mx1), IFN-induced guanylate-binding protein 1 and 2 (GBP1, GBP2), and BST2 (Kraft-Terry et al., 2011).

Al-Mozaini et al identified over 314 unique peripheral blood plasma proteins, out of which 100 were significantly differentially regulated between HIV-1, HIV-2 and HIV-1 elite controller samples. Between the two HIV strains, the protein profiles were remarkably similar. However, despite these similarities, 6 proteins showed significant differences between HIV-1 and HIV-2 infected samples including prohibitin-2 (PHB2), calcineurin subunit B type 1 (PPP3R1), electron transfer flavoprotein subunit beta (ETFB), rhombotin-2 (LMO2), protein S100-A94s (S100A9) and also viral Vif. Their conclusion was that these differentially regulated proteins could be utilized as diagnosis and prognostic molecules of HIV infected individuals. Hypothesizing that using single markers might not provide accurate prognostic data, they suggested that it is better to use disease-specific protein panel markers which provide more reliable information (Al-Mozaini et al., 2021**)**.

3. Aims

Despite the almost 40 years of HIV research so far, very few studies aimed to characterize the early phase of the HIV lifecycle in different cell types. Moreover, few articles aimed to characterize HIV-2 generated cellular changes in comparison to HIV-1. Additionally, the use of lentiviral-derived vectors for gene therapy is continuously being adopted, and great efforts to improve their safety and stability are being spent in their research and development, yet their effects on the host cell transcriptome and proteome is understudied.

In order to enrich our knowledge of HIV-1 and HIV-2 generated proteomic and transcriptomic changes, we set the following aims for our study:

1, Analysis of the proteo-transcriptomic changes in HEK-293T cells induced by HIV-1 and 2-based lentiviral vectors in the early-phase of lentiviral transduction

2, Comparative analysis of the proteo-transcriptomic changes between HIV-1 and HIV-2 highlighting the similarities and differences in the pathways involved in HEK-293T and Jurkat cells

The study was carried out between 2018 and 2022, conducted at the department of Biochemistry and Molecular Biology, utilizing services from the Proteomics Core Facility and the Center for Clinical Genomics and Personalized Medicine at the University of Debrecen.

4. Materials and methods

4.1 Utilized plasmids

We used a 2nd generation lentiviral vector system to generate HIV-1, HIV-2, and control pseudovirions. For the generation of HIV-1 virions we utilized the following plasmids: the transfer vector pWOX-CMV-GFP, which was adapted to express mCherry instead of green fluorescent protein (GFP), the packaging plasmid psPAX2 (a kindly gift from Dr. D. Trono at the University of Geneva Medical School), and pMD.G coding for the vesicular stomatitis virus G protein. For HIV-2 pseudovirion production, the subsequent vectors were utilized: a ROD-based HIV-2 protein expression vector named CGP, CRU5SINCGW transfer vector featuring a GFP expression cassette under the CMV promoter (both are a kindly gift from Joseph P. Dougherty at the Robert Wood Johnson Medical School), and the pMD.G plasmid. In the case of control pseudovirion generation, the vector pTY-EFeGFP, a lentiviral transducing vector containing a GFP expression cassette under an EF1α promoter, and the pMD.G vectors were utilized.

4.2 Cell lines and maintenance:

Human embryonic kidney (HEK-293T) cell line were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. For the passage of HEK-293T cells, first the culture medium was discarded, and cells were rinsed with 1x Phosphate Buffer Saline (PBS). This was followed by the detachment of cells from the flask surface using 0.25% (w/v) Trypsin-EDTA solution. This was followed by centrifugation at 100 g, 24°C, for 5 minutes. Next, the cells were resuspended in fresh DMEM, and seeded back into the flask with a seeding density ranging from $0.5x10^6$ to $1.5x10^6$ cells. In the case of Jurkat cell line, the cells first were centrifuged at 100 g, 24°C, for 5 minutes. This was followed by resuspending the cell pellet into fresh RPMI (Sigma-Aldrich, St. Louis, MO, USA) medium and $1x10^6$ cells were seeded back into the flask.

4.3 Production of HIV-1, HIV-2 and control pseudovirions

For generation of HIV-1 pseudovirions, pWOX-CMV-mCherry, psPAX2, and pMD.G plasmids in a 3:2:1 ratio were utilized. CGP, CRU5SINCGW and pMD.G plasmids were employed in a 1:1:1 ratio for the generation of HIV-2 virions. pTY-EFeGFP and p.MDG in a 1:1 ratio were utilized for control pseudovirion production. The day before transfection, HEK-293T cells were passaged in order to get an approximate confluence of 70% (~$3x10^6$ cells/flask (growth surface was 75cm^2/flask)) on the following day. HEK-293T cells were transfected with the above mentioned plasmid ratios, for the procedure we utilized the polyethylenimine (PEI) method. After the process the cells were incubated for five hours at 37°C with 5% CO2 in antibiotic-free, 1% FBS containing DMEM. Five hours later, the medium was replaced with DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% glutamine. At 24, 48, 72 hours post transfection, the medium containing the pseudovirions were collected and filtered through a 0.45-µm polyvinylidene fluoride filter (Merck Millipore, Darmstadt, Germany). The supernatants were pooled together, and concentrated via ultracentrifugation (100,000 × g for 2 hours and 10 minutes at 4°C). Next, the pellet containing the virions was resuspended into PBS and stored at -70°C. A colorimetric assay based on enzyme-linked immunosorbent assay (ELISA) was utilized for the measurement of the reverse transcriptase activity (Roche Applied Science, Mannheim, Germany). To determine the transduction units/ml (TU/ml) of the produced pseudovirions, transduction experiment were carried out on HEK-293T cells. The quantity of the control pseudovirions was subsequently calibrated to the reverse transcriptase (RT) equivalence of HIV-1 pseudovirions, ensuring equal virion quantities for the transduction experiments.

4.4 Transduction of- and RNA isolation from HEK-293T cells for transcriptomic analysis

The day before the transduction, HEK-293T cells were seeded into 6-well plates ($5x10^5$ cells/well (growth surface was 9,6cm^2/well)) in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. On the day of transduction, the medium was removed, and the cells were transduced with 5 ng RT-equivalent of HIV-1/HIV-2 or control pseudovirions in serum and antibiotic-free media, supplemented with 8 µg/ml polybrene. At zero, two, eight, 12 and 26 hours post transduction the medium was discarded, and cells were washed with PBS before suspension into TRIzol reagent (Thermo Fisher Scientific, MA, USA). RNA isolation was carried out following the manufacturer's instructions. The quality of the

RNA was assessed using the Agilent RNA 6000 Nano kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). High-throughput sequencing was carried out on the MGI DNBSEQ G400 (MGI tech, Shenzhen, China) sequencer using the MGIEasy RNA Library Prep Set at the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen.

4.5 Transduction of HEK-293T cells for proteomic analysis

The day before transduction, HEK293T cells were seeded into T-25 flasks at a density of 0.7-1x10^6 cells per flask (growth surface was 25cm^2/flask), in 5 ml of DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. On the next day, the medium was removed, and the cells were transduced with 15 ng RT-equivalent of HIV-1/HIV-2 or control pseudovirions in serum and antibiotic-free media supplemented with 8 µg/ml polybrene. This was followed by an incubation at 37 °C with 5% CO2 for zero, two, eight, 12, and 26 hours. Next, the medium was discarded, and the cells were mechanically detached and suspended in PBS. After a brief centrifugation, the pellet was stored at -20°C for subsequent analysis.

4.6 Transduction of- and RNA isolation from Jurkat cells for transcriptomic analysis

On the day of the transduction Jurkat cells were seeded in 24-well plates at a density of 1.25 × 10^5 cells/well (working volume was 0.5-1.0 ml/well) in RPMI supplemented with 10% FBS and 1% L-glutamine, along with 8 µg/ml polybrene. This was followed by transduction with HIV-1, HIV-2 or control pseudovirions corresponding to 250,000 transduction units/ml (TU/ml). Jurkat cells were collected at zero, two, eight, 12, and 26 hours post-transduction, washed with PBS, and then lysed using TRIzol reagent (Thermo Fisher Scientific, MA, USA). RNA isolation was carried out following the manufacturer's instructions. The RNA quality was assessed using the Agilent RNA 6000 Nano kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Subsequently, high-throughput sequencing was conducted on the NextSeq 500 sequencer (Illumina, CA, USA) at the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen.

4.7 Transcriptomic data analysis of HEK-293T cells from the immediate and early phase of lentiviral transduction

The statistical and bioinformatic analysis of the early phase data was carried out by Viktor Ambrus (University of Debrecen, Department of Biochemistry and Molecular Biology).

RNA-seq raw fastq data underwent adaptor removal and quality trimming (phred score 30) utilizing Trimmomatic v0.36 (Bolger et al., 2014). The minimal trimmed read length was set to 36 bp. Quality check was conducted using FastQC v0.11.9 (Andrews S. Fastqc: A Quality Control Tool for High Throughput Sequence Data). The reads were mapped to the GRCh38 Human Genome Assembly reference genome using HISAT2 v2.1.0 (Langmead et al., 2009, Kim et al., 2015). The reference genome index files were created with Bowtie v.1.2.2 (Langmead et al., 2009). Aligned reads were quantified using FeatureCounts v2.0.1 (Liao et al., 2014, Yates et al., 2016). The obtained count matrix was used for analysis with R v4.2.3 (The R Development Core Team. R: A Language and Environment for Statistical Computing). Normalized counts, obtained through the median-of-ratios method were used for estimating differential expression (DE) with DESeq2 v1.38.3 (Love et al., 2014). To improve the accuracy of fold change estimates, we applied the "Adaptive Shrinkage" package version 2.2-63 (Stephens, 2017). Transcripts from the immediate early phase time points (0 and 2 hours) with an adjusted p-value lower than 0.05 and absolute log2 fold changes (abs(LFC)) >0.58 were considered as differentially expressed. In case of transcripts from the later time points (8, 12 and 26 hours) the adjusted p-value were the same but the (abs(LFC)) were set to >1. Gene ontology (GO) enrichment analysis of the DE genes was conducted using the clusterProfiler package (Yu et al., 2012).

4.8 GeLC-MS/MS analysis

The GeLC-MS/MS analysis of the transduced samples were carried out by Dr. Gergő Kalló and the collegues of the Proteomics Core Facility of the University of Debrecen. Lysis of the HIV-1, HIV-2 or control pseudovirion transduced cells was done in 100 µL lysis buffer (50 mM Tris pH 8.3, 1 mM EDTA, 17 mM β-mercaptoethanol, 0.5% (v/v) Triton-X100) using three freeze–thaw cycles. Bradford method was utilized to determine the protein concentration, and 100 µg of protein for each case underwent in-gel digestion followed by liquid chromatography–tandem mass spectrometry (GeLC–MS/MS) analysis (Dzieciatkowska et al., 2014). Briefly, samples were run into a 5% SDS-polyacrylamide gel using a 100 V current for 20 min. The proteins

were stained with PageBlue Protein Staining solution (Thermo Scientific, Waltham, MA, USA), and the stained gel slice was excised, separated into three equal portions and submitted to in-gel trypsin digestion. Reduction was performed with 20 mM dithiothreitol (Bio-Rad, Hercules, CA, USA) for 1 h at 56 °C, followed by alkylation with 55 mM iodoacetamide (Bio-Rad, Hercules, CA, USA) for 45 min at room temperature in the dark. Overnight trypsin digestion was performed at 37 °C utilizing stabilized MS-grade TPCK-treated bovine trypsin (ABSciex, Framingham, MA, USA). The digested peptides were extracted and dried in a speed-vac (Thermo Scientific, Waltham, MA, USA). The peptides were re-dissolved in 33 µL 1% formic acid (VWR Ltd., Radnor, PA, USA) before LC–MS/MS analysis. BCA method was used to determine the peptide concentration of the samples. Before mass spectrometry analyses, the samples were spiked with equal amounts of indexed retention time (iRT) peptide mixtures (Biognosys, Schlieren, Switzerland), and the samples were analysed in duplicate.

Prior to the mass spectrometric analysis, peptides were separated in a 180 min water/acetonitrile gradient using an Easy nLC 1200 nano UPLC (Thermo Scientific, Waltham, MA, USA). The peptide mixtures were desalted in an ACQUITY UPLC Symmetry C18 trap column (20 mm × 180 µm, 5 µm particle size, 100 Å pore size, Waters, Milford, MA, USA), followed by separation in Acclaim PepMap RSLC C18 analytical columns (150 mm × 50 µm × 2 µm particle size, 100 Å pore size, Thermo Scientific, Waltham, MA, USA). Chromatographic separation was performed utilizing a gradient of 5–7% solvent B over 5 min, followed by a rise to 15% of solvent B over 50 min and then to 35% solvent B over 60 min. Thereafter, solvent B was increased to 40% over 28 min and then to 85% over 5 min, followed by a 10 min rise to 85% of solvent B, after which the system returned to 5% solvent B in 1 min for a 16 min hold-on. Solvent A was 0.1% formic acid in LC water (Sigma, St. Louis, MO, USA); solvent B was 95% acetonitrile (Sigma, St. Louis, MO, USA) containing 0.1% formic acid. The flow rate was set to 300 nL/min.

Data-dependent analyses were conducted using an Orbitrap Fusion mass spectrometer (Thermo Scientific, Waltham, MA, USA). The 14 most abundant multiply charged positive ions were selected from each survey MS scan using a scan range of 350–1600 m/z for MS/MS analyses (Orbitrap analyzer resolution: 60,000, AGC target: 4.0 × 105, acquired in profile mode). Collision-induced dissociation (CID) fragmentation was performed in the linear ion trap with 35% normalized collision energy (AGC target: 2.0 × 103, acquired in centroid mode). Dynamic exclusion was enabled during the cycles (exclusion time: 45 s).

4.9 Data analysis of mass spectrometry

The analysis of the mass spectometry data was carried out by Ajneesh Kumar (Proteomics Core Facility of the University of Debrecen), Miklós Emri (University of Debrecen, Department of Nuclear Medicine and Translational Imaging) and Balázs Kunkli (University of Debrecen, Department of Biochemistry and Molecular Biology). The acquired LC-MS/MS data were used for protein identification with the utilization of MaxQuant 1.6.2.10 software (Cox & Mann, 2008) searching against the Human SwissProt database (release: 2020.02, 20394 sequence entries), the HIV-1 and HIV-2 SwissProt databases (release: 2020.02, 381 sequence entries for HIV-1 and 109 sequence entries for HIV-2), and against the contaminants database provided by the MaxQuant software. Cys carbamidomethylation, Met oxidation, and N-terminal acetylation were set as variable modifications with a maximum of 2 allowed missed cleavage sites. Results were imported into Scaffold 4.8.9 software (ProteomeSoftware Inc., Portland, OR, USA). Proteins were considered acceptable with at least 3 identified peptides using 1% protein false discovery rate (FDR) and 0.1% peptide FDR. For label-free quantification, the normalized total precursor intensities were utilized, and quantitative values of the identified proteins were normalized to the quantitative values of the iRT mixture. Data were further normalized to the concentration of the peptides in the samples determined by BCA method after the digestion.

A mass of mixed effects of ANOVA models were applied, one for each protein, to select the significantly different protein quantities between the investigated groups. Sample and measurement repetitions were modelled as random effects, while the transduced cell groups were modelled as fixed effects (Oberg & Vitek, 2009). Following linear model fitting, post-hoc tests were applied to determine the p-values of group differences, and significant results with an FDR < 0.05 criteria were retained.

4.10 Proteomic data analysis of HEK-293T cells from the early phase of lentiviral transduction

The analysis of the proteomic data was carried out by Miklós Emri (University of Debrecen, Department of Nuclear Medicine and Translational Imaging) and Balázs Kunkli (University of Debrecen, Department of Biochemistry and Molecular Biology). The compilation of identified proteins with intensity values was exported from Scaffold and further processed in the R environment (v4.3.1) (The R Development Core Team. R: A Language and Environment for

Statistical Computing). Normalized values were calculated accounting for variations in overall sample protein concentrations (determined post-digestion using the BCA method), injection volumes and the sum of detected protein intensities per sample. A series of mixed-effects ANOVA models were employed, one for each protein, to discern statistically significant differences in protein abundances between the investigated groups. Sample and measurement replicates were treated as random effects, meanwhile the different transduced cell groups were modelled as fixed effects (Oberg & Vitek, 2009). We ran the computations using the emmeans (v1.8.8) and lme4 (1.1.34) packages (Bates et al., 2015). Following linear model fitting, post-hoc tests were applied to determine the p-values of group differences and significant results with an FDR < 0.05 criteria were retained. The protein coding transcript annotation was obtained from the daily updated 'gene_info', 'gene2go' files available on the NCBI FTP site (https://ftp.ncbi.nlm.nih.gov/gene/DATA/, accessed on 15 November 2023) and reorganized using in-house shell scripts. GO enrichment analysis was carried out with the topGO R package (v2.52.0) using a custom-generated gene-to-GOs mapping file (Alexa et al., 2006). The protein universe consisted of the entire set of proteins (807) detected in all 26 h post-transduction samples; significant proteins were filtered according to the FDR < 0.05 threshold. In the topGO algorithm, the default 'weight01' method was chosen with the Kolmogorov–Smirnov statistical test and selected the enriched GO terms having FDR-adjusted p-values < 0.05. Data visualization was performed with ggplot2 (v3.4.3), ggrepel (v0.9.3), ggpubr (v0.6.0), GOplot (v1.0.2) (Elio Campitelli Ggnewscale: Multiple Fill and Colour Scales in 'ggplot2'. R Package Version 0.4.9.), ggnewscale (v0.4.9), VennDiagram (v1.7.3) R packages.

4.11 Transcriptomic data analysis of Jurkat cells from the early phase of lentiviral transduction

The analysis of the transcriptomic data from transduced Jurkat cells was carried out by Szilárd Póliska and collegues of the Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen. The reads were mapped to the GRCh38 Human Genome Assembly reference genome using Hisat2 v2.1.0 (Langmead et al., 2009). The BAM files generated during alignment were imported into StrandNGS software for analysis. The quantification of the reads were done with the integrated DESeq algorithm. Moderated T-test were utilized for the determination of the DETs. The downstream analysis consisted of transcripts passing significance filters with adjusted p-value (padj) < 0.05 and absolute log2 fold-change

(abs(LFC)) > 1. For the correction of errors originating from the multiple testing Benjamini-Hochberg FDR analysis were utilized.

GO analysis were carried out in CytoScape 3.4.0 with the utilization of the ClueGO v2.3.5 application. The GO biological process categories were determined with the use of two sided hypergeometric test and Bonferroni step down correction.

5 Results

5.1 Analysis of transcriptomic changes in the early phase of HIV-1 transduction in HEK-293T cells

Compared to cells transduced with the control vector, at the 0-hour time point, HIV-1 influenced the expression of a total of 2676 transcripts from which 1533 were up-, and 1143 were downregulated. From the upregulated ones, 1256 encoded proteins, 226 non-coding RNAs, 1 mitochondrial RNA, 42 pseudogenes and 8 were the products of unknown genes. From the downregulated transcripts, 710 were protein-coding, 294 non-coding RNAs, 11 were mitochondrial RNAs, 94 were pseudogenes, 2 were ribozymes, and 32 were unknown transcripts.

Two hours after transduction, HIV-1 induced significant changes on 4551 transcripts. Among these, the expression of 2366 transcripts were upregulated, from which 1916 were encoding for proteins, 98 coding for pseudogenes, and 334 were non-coding RNAs. Additionally, 18 gene products were categorized as unknown transcripts. Moreover, 2185 genes were downregulated by HIV-1, among these were 1634 transcripts coding for proteins, 153 for pseudogenes, 363 for non-coding RNA transcripts, 18 for mitochondrial RNA, one for ribozyme, and 16 were products of unknown genes.

Compared to the control , 8 hours post-transduction, HIV-1 altered the cellular level of 158 transcripts, out of which 115 were increased and 43 were decreased. Amongst the increased ones; 71 were protein-coding, 23 were non-coding RNAs, 5 were mitochondrial RNAs, 12 pseudogene, and 4 derived from uncategorized genes. Amongst the decreased ones, 32 encoded proteins, 7 non-coding RNAs and 4 pseudogenes.

In comparison to cells transduced with the control l, at the 12-hour mark, HIV-1 altered the expression of a total of 114 transcripts. Out of these, 18 were positively-, and 96 negatively regulated. Of the positively regulated ones, 11 coded for proteins, 6 non-coding RNAs and 1 was the product of pseudogene. Of the downregulated transcripts, 59 were products of protein

-coding transcript, 27 were non-coding RNAs, 5 were mitochondrial RNAs, 1 was a pseudogene and 4 were unknown gene products.

26 hours after transduction, HIV-1 significantly altered the expression of 380 transcripts, from which 48 were upregulated and 332 were downregulated. From the upregulated transcripts 26 encoded for proteins, 14 for non-coding RNAs, 7 products of pseudogenes and 1 coded for an unknown gene product. From the 332 downregulated transcripts, 264 were protein-coding, 56 were non-coding RNAs, 8 were pseudogenes and 4 were transcripts derived from unknown genes. The number of the differentially regulated transcripts is listed in Table 1.

Upregulated by HIV-1					
	0h	**2h**	**8h**	**12h**	**26h**
Protein	1256	1916	71	11	26
Non-Coding RNA	226	334	23	6	14
Mithocondrial RNA	1	0	5	0	0
Pseudogenes	42	98	12	1	7
Ribozymes	0	0	0	0	0
Ig-V gene	0	0	0	0	0
Unknown gene product	8	18	4	0	1
Total	**1533**	**2366**	**115**	**18**	**48**
Downregulated by HIV-1					
	0h	**2h**	**8h**	**12h**	**26h**
Protein	710	1634	32	59	264
Non-Coding RNA	294	363	7	27	56
Mithocondrial RNA	11	18	0	5	0
Pseudogenes	94	153	4	1	8
Ribozymes	2	1	0	0	0
Ig-V gene	0	0	0	0	0
Unknown gene product	32	16	0	4	4
Total	**1143**	**2185**	**43**	**96**	**332**

Upregulated by HIV-2					
	0h	**2h**	**8h**	**12h**	**26h**
Protein	1672	1716	99	107	35
Non-Coding RNA	240	261	31	26	8
Mithocondrial RNA	0	0	3	0	0
Pseudogenes	92	128	75	91	78
Ribozymes	0	0	0	0	0
Ig-V gene	1	0	0	0	0
Unknown gene product	10	9	3	1	0
Total	**2015**	**2114**	**211**	**225**	**121**

Downregulated by HIV-2					
	0h	**2h**	**8h**	**12h**	**26h**
Protein	1646	875	43	46	35
Non-Coding RNA	273	306	27	24	21
Mithocondrial RNA	12	16	0	1	4
Pseudogenes	106	83	2	2	0
Ribozymes	2	1	0	0	0
Ig-V gene	0	0	0	0	0
Unknown gene product	21	27	0	1	1
Total	**2060**	**1308**	**72**	**74**	**61**

Table 1. Number of differentially regulated transcripts at 0, 2, 8, 12 and 26 hours post transduction from HIV-1 (top) and HIV-2 (bottom) transduced HEK294T cells.

5.2 Differentially induced transcripts by HIV-1 in the immediate early phase of transduction in HEK-293T cells

The ten most significantly DETs encoding proteins across all observed time-points in HIV-1 transduced cells are chosen and depicted in Figure 8.

At zero-hour cells treated with HIV-1 showed upregulation of *IRS4*, *HCFC1*, *HSPG2*, *CD109*, *OAS3*, *NID1*, *KMT2D*, *SOX5*, *POLR2A*, and *FRY*.

H4C3, *ASS1IGSF6*, *PHLDA3*, *IL3RA*, *APOE*, *ANKRD18A*, *CHRNG*, *MFSD4B* and *MAN1A2* were downregulated.

At the 2-hour time-point, HIV-1 resulted in the up-regulation of *SLC7A11*, *ASPM*, *SACS*, *CD109*, *ZBTB41*, *BRCA2*, *TEX15*, *ZKSCAN8*, *CHAC1* and *PTAR1*, respectively. On the other hand, *H4C3 EMP3*, *PHLDA3*, *ASS1*, *ID1/3*, *RPL39L*, *SHC2*, *STX8*, and *RGS16* were downregulated. The full list of top 10 differentially down- and upregulated transcripts at the different time points were listed in Table 2.

5.3 Differentially induced transcripts by HIV-1 in the early phase of transduction in HEK-293T cells

The DETs that were regulated significantly by HIV-1 are selected and presented in Figure 8.

At the eight hour time-point, HIV-1 upregulated the expression of the *LRRIQ1*, *CENPE*, *AKAP9*, *ANKRD12*, *CCDC88A*, *BOD1L1*, *GCC2*, *ANKRD26*, *SMC4* and *DRD4*. In the same

time-point, *WDR38*, *FOS*, *BOLA2B*, *CDKN1A*, *ID3*, *H4C3*, *COL11A1*, *EMP3*, *RELB* and *PHLDA3* were downregulated.

In decreasing order of magnitude, the top 10 upregulated protein-coding transcripts induced by HIV-1 at the 12-hour time-point were the *SCG3*, *FOXD4*, *NRG4*, *LRRCC1*, *NAP1L2*, *BRCA2*, *NBN*, *TAS2R20*, *COX20* and *TMEM145*. Meanwhile, at the same time-point, *H4C3*, *COL11A1*, *TMEM132E*, *CAMK1D*, *SH2D3C*, *TSPAN11*, *PDCD11*, *NOP9EGR1* and *KIF1A* were downregulated.

At the 26 hours post-transduction, the expression of *HMOX1*, *OSGIN1*, *VGF*, *NQO1*, *NAP1L2*, *HSPA1A*, *DHRS2*, *ETV4*, *MT2A* and *GCLM* were upregulated; while *H4C3*, *KLHDC7B*, *INPP5D*, *GRIK3*, *TMEM132E*, *ABCG1*, *AKNA*, *DHRS3*, and *ABI3BP* were found to be downregulated by HIV-1. The full list of top 10 differentially down- and upregulated transcripts at the different time points were listed in Table 2.

Differentially regulated by HIV-1 at 0h post-transduction			
Upregulated		**Downregulated**	
Insulin receptor substrate 4	IRS4	H4 clustered histone 3	H4C3
Host cell factor C1	HCFC1	Argininosuccinate synthase 1	ASS1
Heparan sulfate proteoglycan 2	HSPG2	Immunoglobulin superfamily member 6	IGSF6
CD109 molecule	CD109	Pleckstrin homology like domain family A member 3	PHLDA3
2'-5'-oligoadenylate synthetase 3	OAS3	Interleukin 3 receptor subunit alpha	IL3RA
Nidogen 1	NID1	Apolipoprotein E	APOE
Lysine methyltransferase	KMT2D	Ankyrin repeat domain 18A	ANKRD18A
SRY-box transcription factor 5	SOX5	Cholinergic receptor nicotinic gamma subunit	CHRNG
RNA polymerase II subunit A	POLR2A	Major facilitator superfamily domain containing 4B	MFSD4B
FRY microtubule binding protein	FRY	Mannosidase alpha class 1A member 2	MAN1A2

Differentially regulated by HIV-1 at 2h post-transduction			
Upregulated		**Downregulated**	
Solute carrier family 7 member 11	SLC7A11	H4 clustered histone 3	H4C3
Assembly factor for spindle microtubules	ASPM	Epithelial membrane protein 3	EMP3
Sacsin molecular chaperone	SACS	Plecstrin homology like domain family A member 3	PHLDA3
CD109 molecule	CD109	Argininosuccinate synthase 1	ASS1
Zinc finger and BTB domain containing 41	ZBTB41	Inhibitor of DNA binding 1 HLH protein	IID1
BRCA2 DNA repair associated	BRCA2	Inhibitor of DNA binding 3 HLH protein	ID3
Testis expressed 15, meiosis and synapsis associated	TEX15	Ribosomal protein L39 like	RPL39L
Zinc finger with KRAB and SCAN domains 8	ZKSCAN8	SHC adaptor protein 2	SHC2
ChaC glutathione specific gamma-glutamylcyclotransferase 1	CHAC1	Syntaxin 8	STX8
Protein prenyltransferase alpha subunit repeat containing 1	PTAR1	Regulator of G protein signaling 16	RGS16
Differentially regulated by HIV-1 at 8h post-transduction			
Upregulated		**Downregulated**	
Leucine rich repeats and IQ motif containing 1	LRRIQ1	WD repeat domain 38	WDR38
Centromere protein E	CENPE	Fos proto-oncogene AP-1 transcription factor subunit	FOS

A-kinase anchoring protein 9	AKAP 9	BolA family member 2B	BOLA2B
Ankyrin repeat domain 12	ANKR D12	Cyclin dependent kinase inhibitor 1A	CDKN1A
Coiled-coil domain containing 88A	CCDC 88A	Inhibitor of DNA binding 3, HLH protein	ID3
Biorientation of chromosomes in cell division 1 like 1	BOD1 L1	H4 clustered histone 3	H4C3
GRIP and coiled domain containing 2	GCC2	Collagen type XI alpha 1 chain	COL11A1
Ankyrin repeat domain 26	ANKR D26	Epithelial membrane protein 3	EMP3
Structural maintenance of chromosomes 4	SMC4	RELB proto oncogene NF-kB subunit	RELB
Dopamine receptor D4	DRD4	Pleckstrin homology like domain family A member 3	PHLDA3
Differentially regulated by HIV-1 at 12h post-transduction			
Upregulated		**Downregulated**	
secretogranin III	SCG3	H4 clustered histone 3	H4C3
forkhead box D4	FOXD 4	collagen type XI alpha 1 chain	COL11A1
neuregulin 4	NRG4	transmembrane protein 132E	TMEM12E
leucine rich repeat and coiled-coil and coiled centrosomal protein 1	LRRC C1	calcium/calmodulin dependent protein kinase ID	CAMK1D
nucleosome assembly protein 1 like 2	NAP1L 2	SH2 domain containing 3C	SH2D3C
BRCA2 DNA repair associated	BRCA 2	tetraspanin 11	TSPAN11
nibrin	NBN	programed cell death 11	PDCD11

taste 2 receptor member 20	TAS2R 20	NOP9 nucleolar protein	NOP9
cytochrome c oxidase assembly factor COX20	COX20	early growth response 1	EGR1
transmembrane protein 145	TMEM 145	kinesin family member 1A	KIF1A
Differentially regulated by HIV-1 at 12h post-transduction			
Upregulated		**Downregulated**	
secretogranin III	SCG3	H4 clustered histone 3	H4C3
forkhead box D4	FOXD 4	collagen type XI alpha 1 chain	COL11A1
neuregulin 4	NRG4	transmembrane protein 132E	TMEM12E
leucine rich repeat and coiled-coil and coiled centrosomal protein 1	LRRC C1	calcium/calmodulin dependent protein kinase ID	CAMK1D
nucleosome assembly protein 1 like 2	NAP1L 2	SH2 domain containing 3C	SH2D3C
BRCA2 DNA repair associated	BRCA 2	tetraspanin 11	TSPAN11
nibrin	NBN	programed cell death 11	PDCD11
taste 2 receptor member 20	TAS2R 20	NOP9 nucleolar protein	NOP9
cytochrome c oxidase assembly factor COX20	COX20	early growth response 1	EGR1
transmembrane protein 145	TMEM 145	kinesin family member 1A	KIF1A
Differentially regulated by HIV-1 at 26h post-transduction			
Upregulated		**Downregulated**	
heme oxygenase 1	HMOX 1	H4 clustered histone 3	H4C3
oxidative stress induced growth inhibitor 1	OSGIN 1	kelch domain containing 7B	KLHDC7B
VGF nerve growth factor inducible	VGF	inositol polyphosphate-5-phosphatase D	INPP5D

NAD(P)H quinone dehydrogenase 1	NQO1	glutamate ionotropic receptor kainate type subunit 3	GRIK3
nucleosome assembly protein 1 like	NAP1L2	transmembrane protein 132E	TMEM132E
heat shock protein family A (Hsp70) member 1A	HSPA1A	ATP binding cassette subfamily G member 1	ABCG1
dehydrogenase/reductase 2	DHRS2	AT-hook transcription factor	AKNA
ETS variant transcription factor 4	ETV4	dehydrogenase/reductase 3	DHRS3
metallotioenin 2A	MT2A	hes family bHLH transcription factor 5	HES5
glutamate-cysteine ligase modifier subunit	GCLM	ABI family member 3 binding protein	ABI3BP

Table 2. Top 10 differentially up and downregulated transcripts from HIV-1 transduced cells at different time points.

5.4 Analysis of transcriptomic changes in the early phase of HIV-2 transduction in HEK-293T cells

At 0 hours post-transduction, HIV-2 significantly altered the expression of a total of 4075 transcripts. Out of these the level of 2015 were increased while 2060 were decreased. Amongst the upregulated transcripts, 1672 were found to be coding for proteins, 240 were non-coding RNAs, 92 products of pseudogenes, 1 product of an Ig-V gene and 10 were products of unknown genes. Out of the downregulated transcripts, 1646 encoded for proteins, 273 non-coding RNAs, 12 mitochondrial RNAs, 106 pseudogenes, 2 ribozymes and 21 were products of uncategorized genes.

Compared to the control, the expression of 3422 genes were differentially altered in cells transduced with HIV-2 at two hours after transduction. 2114 transcripts were upregulated, amongst these were 1716 transcripts which encoded for proteins, 128 for pseudogenes, 261 for non-coding RNAs and nine were products of uncharacterized genes. On the other hand, HIV-2

downregulated the expression of 1308 genes, consisting of 875 protein-coding transcripts, 306 non-coding RNAs, and 16 genes encoding mitochondrial RNAs. Additionally, 83 were products of pseudogenes, one ribozyme transcript, and 27 transcripts were categorized as unknown genes. A summary of the DETs at the different time points are depicted on Figure 7.

At 8 hours, HIV-2 managed to alter the expression of 283 transcripts from which 211 were up-, and 72 were downregulated. Of the upregulated ones 99 encoded for proteins, 31 non-coding RNAs, 3 mitochondrial RNAs, 75 pseudogenes and 3 were products of unknown genes. Amongst the downregulated transcripts, 43 encoded for proteins, 27 were non-coding RNAs and 2 pseudogenes.

12 hours post transduction, HIV-2 differently regulated the expression of 299 transcripts from which 225 were up-, and 74 were downregulated. Of them 107 were protein-coding, 26 were non-coding RNAs, 91 were pseudogenes, and 1 coded for uncategorized gene product. From the downregulated transcripts, 46 were protein coding, 24 were non-coding RNAs, 1 was mitochondrial RNA, 2 were products of pseudogene, and 1 was an unknown gene product.

At the 26 hours time-point, HIV-2 altered the expression of a total of 182 transcripts from which 121 were up- and 61 were downregulated. Out of the upregulated transcripts 35 coded for proteins, 8 non-coding RNAs and 78 were products of pseudogenes. Of the downregulated transcripts 35 encoded proteins, 21 non-coding RNAs, 4 mitochondrial RNAs and 1 was a product of unknown genes. The number of the differentially regulated transcripts is listed in Table 1.

5.5 Differentially induced transcripts by HIV-2 in the immediate early phase of transduction in HEK-293T cells

The ten most significantly DETs encoding for proteins across all observed time points in HIV-2 transduced cells are chosen and depicted in Figure 9.

HIV-2, at the zero-hour time-point upregulated the expression of *OL1A2*, *KRT5*, *COL6A3*, *KRT7*, *DCN*, *S100A2*, *COL3A*1, *THBS1*, *KRT14* and *S100A6*. Meanwhile HIV-2 downregulated the expression of *H4C3*, *ASS1*, *PHLDA3*, *ANKRD36B*, *ANKRD36C*, *SKAP1*, *SDSL*, *EMP3*, *MAN1A2* and *FAM133B*. At the 2 hour time-point, HIV-2 increased the expression of *COL1A2*, *COL6A3*, *THBS1*, *KRT7*, *KRT5*, *COL3A1*, *KRT14*, *CHAC1*, *S100A2*, and *IRS4*. At the same time the expression of *H4C3*, *ANKRD36C*, *CHRNG*, *ANKRD36B*, *ANKRD18A*, *ANKRD36*, *IGSF6*, *FAM133B*, *WDR38*, and *KNOP1* were downregulated. The full list of top 10

differentially down- and upregulated transcripts at the different time points were listed in Table 3.

5.6 Differentially induced transcripts by HIV-2 in the early phase of transduction in HEK-293T cells

The top 10 DETs that were significantly regulated by HIV-1 are selected and depicted in Figure 9.

At the eight-hour time-point HIV-2 upregulated the expression of *COL1A2*, *KRT5*, *SERPINE1*, *KRT14*, *S100A6*, *KRT7*, *DCN*, *THBS1*, *ETV5* and *COL6A3*. *H4C3*, *ID3*, *EDA2R*, *AHNAK*, *TMEM132E*, *ID1*, *AHNAK2*, *PALM3*, *HES5* and *ADAP1* were downregulated in the same time-point.

12 hours post-transduction, the most upregulated protein-coding transcripts were the *COL1A2*, *KRT7*, *DCN*, *ANPEP*, *DPT*, *ITGBL1*, *KRT5*, *TGFBI*, *S100A6* and *FABP4*. Additionally, the expression level of *H4C3*, *ABCG1*, *ID3*, *ANKRD36C*, *TMEM132E*, *SPAN11*, *ANKRD18A*, *RIMS3*, *PHLDA3* and *CAMK1D* were downregulated by HIV-2.

At the 26-hour time-point, HIV-2 increased the cellular transcription of *COL1A2*, *KRT7*, *KRT5*, *KRT14*, *DCN*, *S100A6*, *SERPINE1*, *S100A2*, *SLX1B*, and *THBS1* were upregulated by HIV-2, while *H4C3*, *EGR1*, *KLHDC7B*, *RELB*, *ABCG1*, *TMEM132E*, *SHC2*, *DHRS3*, *TSPAN11* and *AKNA* were found to be downregulated. The full list of top 10 differentially down- and upregulated transcripts at the different time points were listed in Table 3.

Differentially regulated by HIV-2 at 0h post-transduction			
Upregulated		**Downregulated**	
Collagen type I alpha 2 chain	COL1A2	H4 clustered histone 3	H4C3
Keratin 5	KRT5	Argininosuccinate synthase 1	ASS1
Collagen type VI alpha 3 chain	COL6A3	Pleckstrin homology like domain family A member 3	PHLDA3
Keratin 7	KRT7	Ankyrin repeat domain 36B	AKNRD36B
Decorin	DCN	Ankyrin repeat domain 36C	ANKRD36C
S100 calcium binding protein A2	S100A2	Src kinase associated phosphoprotein 1	SKAP1
Collagen type III alpha 1 chain	COL3A1	Serine dehydratase like	SDSL
Thrombospondin 1	THBS1	Epithelial membrane protein 3	EMP3
Keratin 14	KRT14	Mannosidase alpha class 1A member 2	MAN1A2
S100 calcium binding protein A6	S100A6	Family with sequence similarity 133 member B	FAM133B
Differentially regulated by HIV-2 at 2h post-transduction			
Upregulated		**Downregulated**	
Collagen type I alpha 2 chain	COL1A2	H4 clustered histone 3	H4C3
Collagen type VI alpha 3 chain	COL6A3	Ankyrin repeat domain 36C	ANKRD36C
Thrombospondin 1	THBS1	Cholinergic receptor nicotinic gamma subunit	CHRNG
Keratin 7	KRT7	Ankyrin repeat domain 36B	ANKRD36B
Keratin 5	KRT5	Ankyrin repeat domain 18A	ANKRD18A
Collagen type III alpha 1 chain	COL3A1	Ankyrin repeat domain 36	ANKRD36

Keratin 14	KRT14	Immunoglobulin superfamily member 6	IGSF6
ChaC glutathione specific gamma-glutamylcyclotransferase 1	CHAC1	Family with sequence similarity 133 member B	FAM133B
S100 calcium binding protein A2	S100A2	WD repeat domain 38	WDR38
Insulin receptor substrate 4	IRS4	Lysine rich nucleolar protein 1	KNOP1
Differentially regulated by HIV-2 at 8h post-transduction			
Upregulated		**Downregulated**	
Collagen type I alpha 2 chain	COL1A2	H4 clustered histone 3	H4C3
Keratin 5	KRT5	Inhibitor of DNA binding 3, HLH protein	ID3
Serpin family E member 1	SERPINE1	Ectodysplasin A2 receptor (EDA2R	EDA2R
Keratin 14	KRT14	AHNAK nucleoprotein	AHNAK
S100 calcium binding protein A6	S100A6	Transmembrane protein 132E	TMEM132E
Keratin 7	KRT7	Inhibitor of DNA binding 1, HLH protein	ID1
Decorin	DCN	AHNAK nucleoprotein 2	AHNAK2
Thrombospondin 1	THBS1	Paralemmin 3	PALM3
ETS variant transcription factor 5	ETV5	Hes family bHLH transcription factor 5	HES5
Collagen type VI alpha 3 chain	COL6A3	ArfGAP with dual PH domains 1	ADAP1
Differentially regulated by HIV-2 at 8h post-transduction			
Upregulated		**Downregulated**	
Collagen type I alpha 2 chain	COL1A2	H4 clustered histone 3	H4C3

Keratin 5	KRT5	Inhibitor of DNA binding 3, HLH protein	ID3
Serpin family E member 1	SERPINE1	Ectodysplasin A2 receptor (EDA2R	EDA2R
Keratin 14	KRT14	AHNAK nucleoprotein	AHNAK
S100 calcium binding protein A6	S100A6	Transmembrane protein 132E	TMEM132E
Keratin 7	KRT7	Inhibitor of DNA binding 1, HLH protein	ID1
Decorin	DCN	AHNAK nucleoprotein 2	AHNAK2
Thrombospondin 1	THBS1	Paralemmin 3	PALM3
ETS variant transcription factor 5	ETV5	Hes family bHLH transcription factor 5	HES5
Collagen type VI alpha 3 chain	COL6A3	ArfGAP with dual PH domains 1	ADAP1
Differentially regulated by HIV-2 at 12h post-transduction			
Upregulated		**Downregulated**	
Collagen type I alpha 2 chain	COL1A2	H4 clustered histone 3	H4C3
Keratin 7	KRT7	ATP binding cassette subfamily G member 1	ABCG1
Decorin	DCN	Inhibitor of DNA binding 3, HLH protein	ID3
Alanyl aminopeptidase, membrane	ANPEP	Ankyrin repeat domain 36C	ANKRD36C
Dermatopontin	DPT	Transmembrane protein 132E	TMEM132E
Integrin subunit beta like 1	ITGBL1	Tetraspanin 11	TSPAN11
Keratin 5	KRT5	Ankyrin repeat domain 18A	ANKRD18A

Transforming growth factor beta induced	TGFBI	Regulating synaptic membrane exocytosis 3	RIMS3
S100 calcium binding protein A6	S100A6	Pleckstrin homology like domain family A member 3	PHLDA3
Fatty acid binding protein 4	FABP4	Calcium/calmodulin dependent protein kinase ID	CAMK1D
Differentially regulated by HIV-2 at 26h post-transduction			
Upregulated		**Downregulated**	
Collagen type I alpha 2 chain	COL1A2	H4 clustered histone 3	H4C3
Keratin 7 (KRT7)	KRT7	Early growth response 1 (EGR1	EGR1
Keratin 5	KRT5	Kelch domain containing 7B	KLHDC7B
Keratin 14	KRT14	RELB proto-oncogene NF-κB subunit	RELB
Decorin	DCN	ATP binding cassette subfamily G member 1	ABCG1
S100 calcium binding protein A6	S100A6	Transmembrane protein 132E	TMEM132E
Serpin family E member 1	SERPINE1	SHC adaptor protein 2	SHC2
S100 calcium binding protein A2	S100A2	Dehydrogenase/reductase 3	DHRS3
SLX1 homolog B, structure-specific endonuclease subunit	SLX1B	Tetraspanin 11	TSPAN11
Thrombospondin 1	THBS1	AT-hook transcription factor	AKNA

Table 3. Top 10 differentially up and downregulated transcripts from HIV-2 transduced cells at different time points.

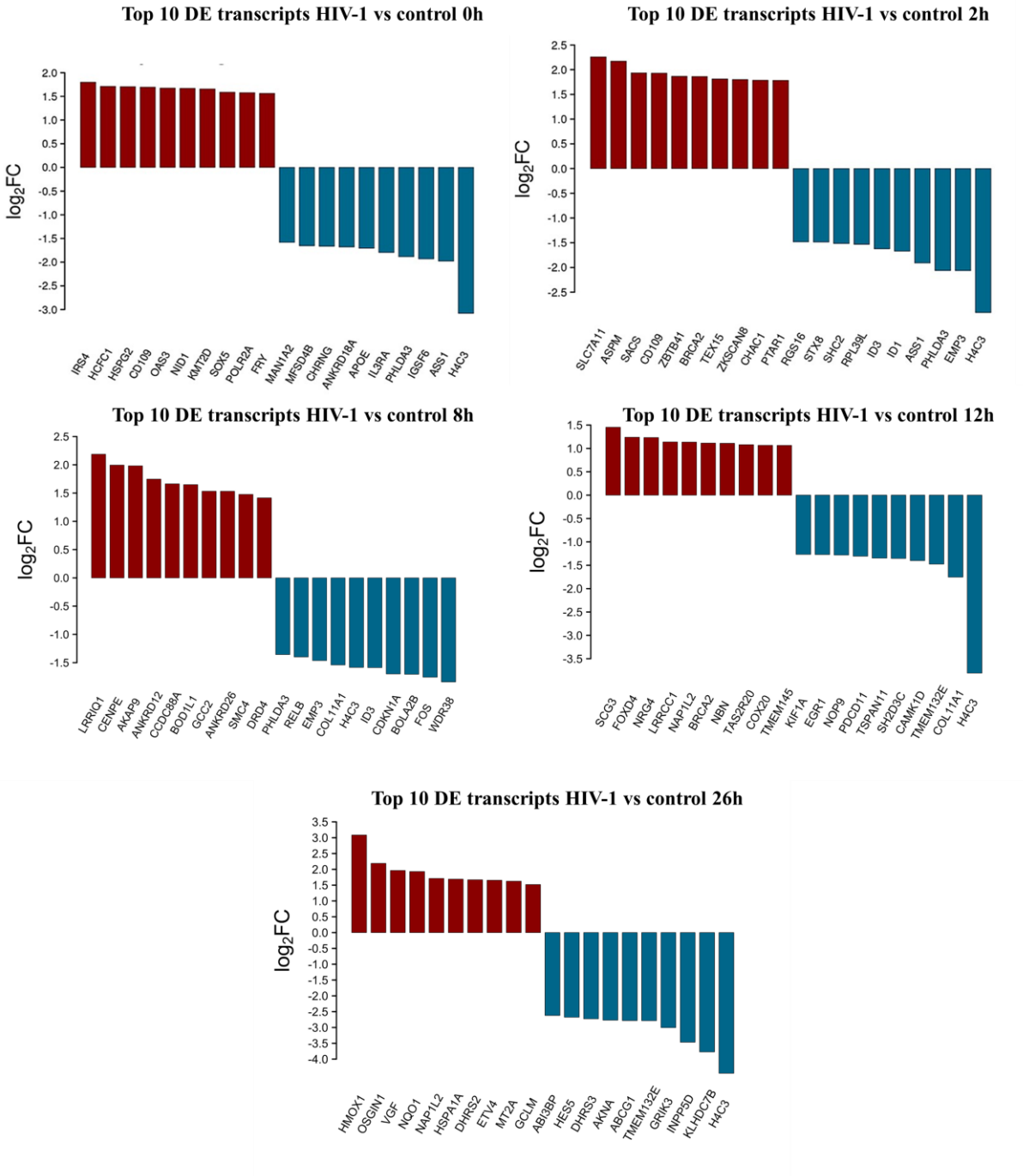


Figure 8. Top 10 selected up- and downregulated DETs at 0, 2, 8, 12 and 26-hour time-points following transduction with HIV-1 compared to the control. The log_2FC of the DETs is on the y axis while the name of the DETs are on the x axis.

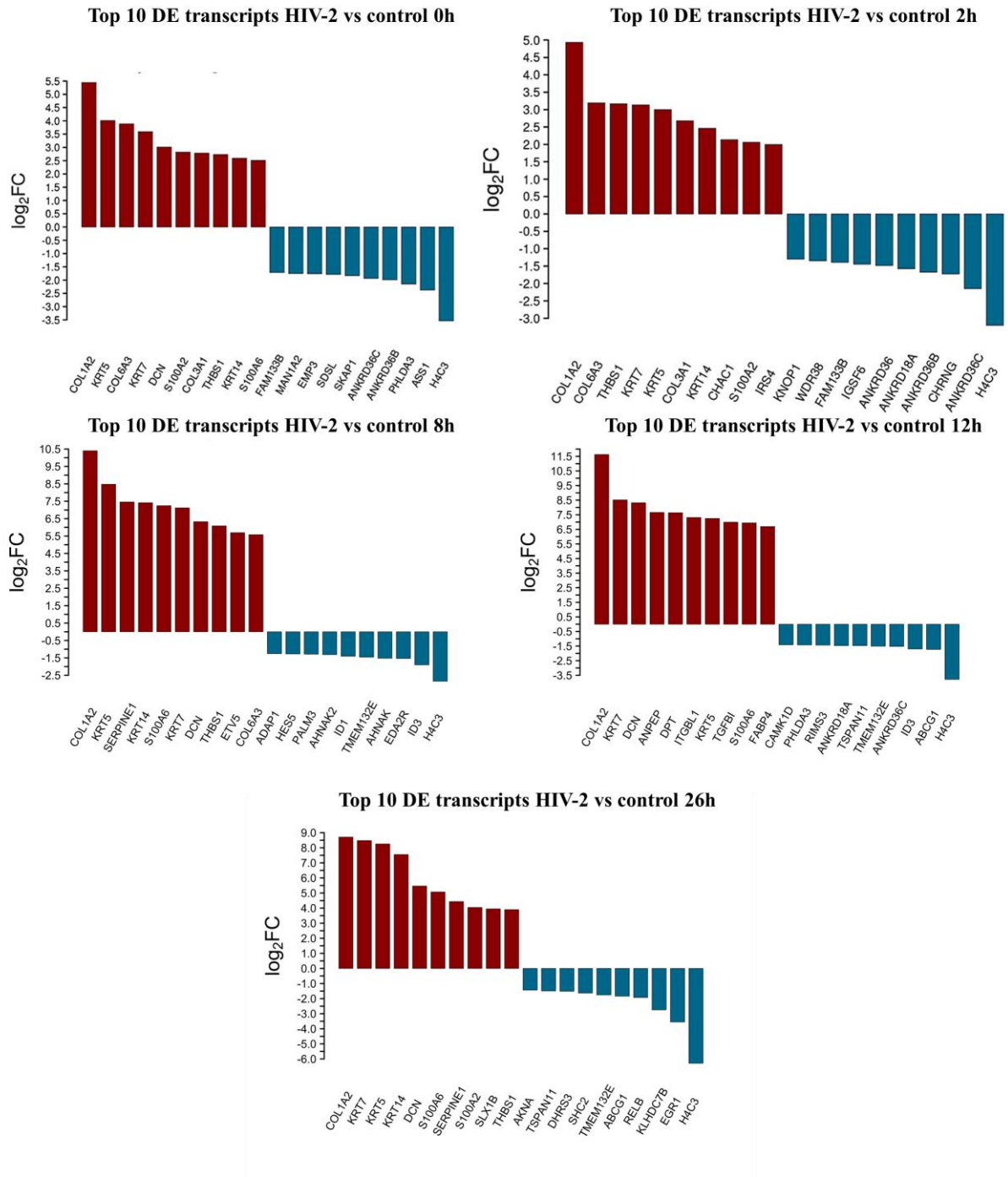


Figure 9. Top 10 selected up- and downregulated DETs at 0, 2, 8, 12 and 26 hour time-points following transduction with HIV-2 compared to the control. The log_2FC of the DETs is on the y axis while the name of the DETs are on the x axis.

5.7 Gene ontology analysis of the significantly altered transcripts in the immediate early-phase of HIV transduction in HEK-293T cells

To further categorize the biological functions of the detected transcripts Gene ontology (GO) enrichment analysis was carried out. We utilized the protein-coding transcripts that showed statistically significant differential expression ($p > 0.05$, $log2FC > 0.58$) at the two-hour time-point from HIV-1 and HIV-2 transduced cells in comparison to control pseudovirion treated ones (Figure 10). The results revealed that both HIVs similarly affected GO terms such as protein serine/threonine kinase activity, structural constituent of ribosome, GTPase activator activity, nucleoside triphosphatase regulator and ubiquitin-like protein transferase activity. Amongst the GO terms influenced differentially by HIV-1 were rRNA and tau protein binding, tau-protein kinase activity, and catalytic activity, acting on DNA. Conversely, HIV-2 affected protein coding genes were involved in Rho GTPase binding, DNA-binding transcription factor binding, transcription coactivator activity, and RNA polymerase II-specific DNA-binding transcription factor binding.

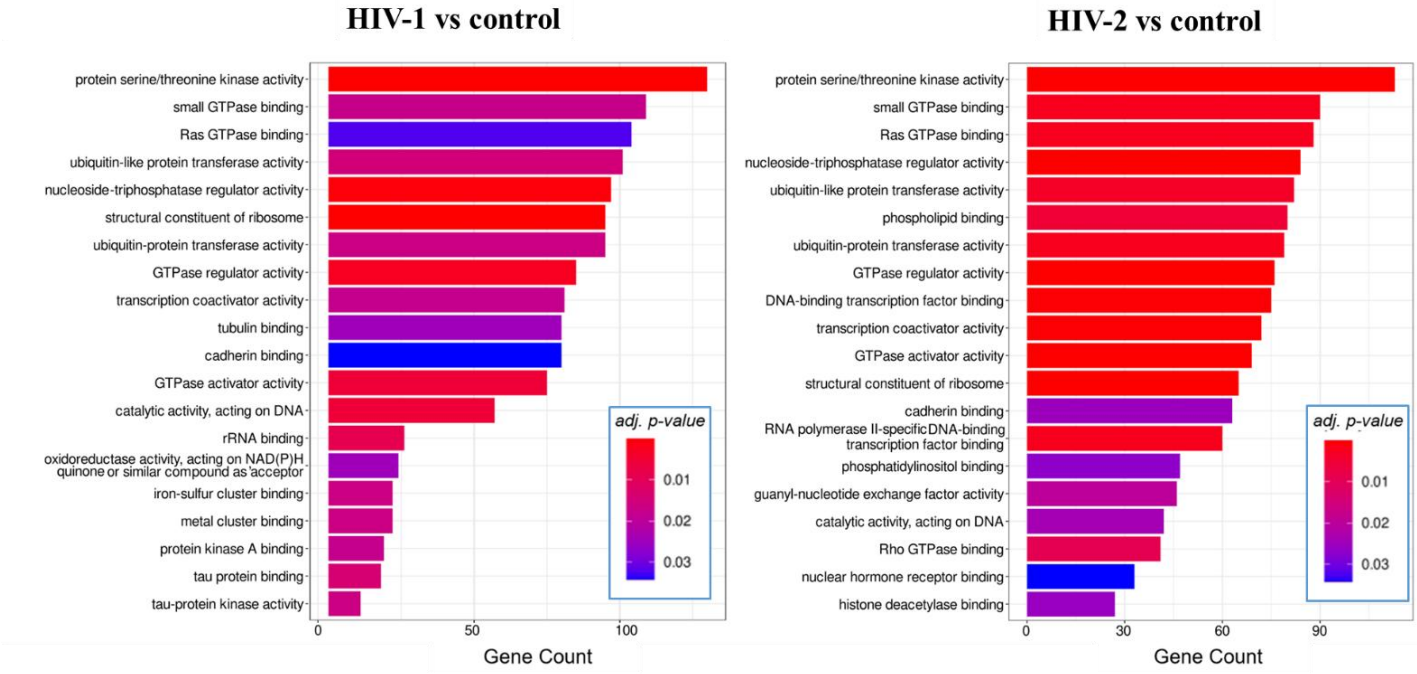


Figure 10. GO analysis of the differentially expressed genes (DEGs) from HIV-1 (left) and HIV-2 (right) transduced cells at the two-hour time-point. The top genes were categorized based on significant enrichment terms, with colour intensity indicating the significance of each term. Counts corresponds to the number of DEGs linked to the given gene ontology ID in the analysis.

5.8 Gene ontology analysis of the significantly altered transcripts in the early phase of HIV transduction in HEK-293T cells

Similarly, in immediate early-phase of transduction, GO analysis was carried out on identified transcripts using the most significant differentially expressed ($p < 0.05$, log2FC > 1) protein-coding transcripts at the 8, 12, and 26-hour time points from both HIV-1 and HIV-2 transduced cells, compared to control pseudovirion transduced ones. This analysis revealed further differences between HIV-1 and HIV-2 transduced cells in comparison to the control.

At the 8-hour mark, HIV-1 affected transcripts which are associated with the cell cycle, positive regulation of RNA metabolic processes and positive regulation of metabolic processes. Meanwhile, HIV-2 influenced the expression of genes involved in tissue development, extracellular matrix organization and cell adhesion (Figure 11).

At the 12-hour mark, no significantly altered GO terms were detected in HIV-1 transduced HEK-293T cells. In contrast, HIV-2 affected the expression of transcripts involved in cell differentiation, cell adhesion, and cell migration (Figure 12).

At 26 hours post-transduction, HIV-1 altered transcripts involved in homeostatic processes, response to toxic substances and response to oxidative stress. In comparison, HIV-2 influenced transcripts associated with peptide cross-linking, negative regulation of cell migration and negative regulation of cell motility (Figure 13).

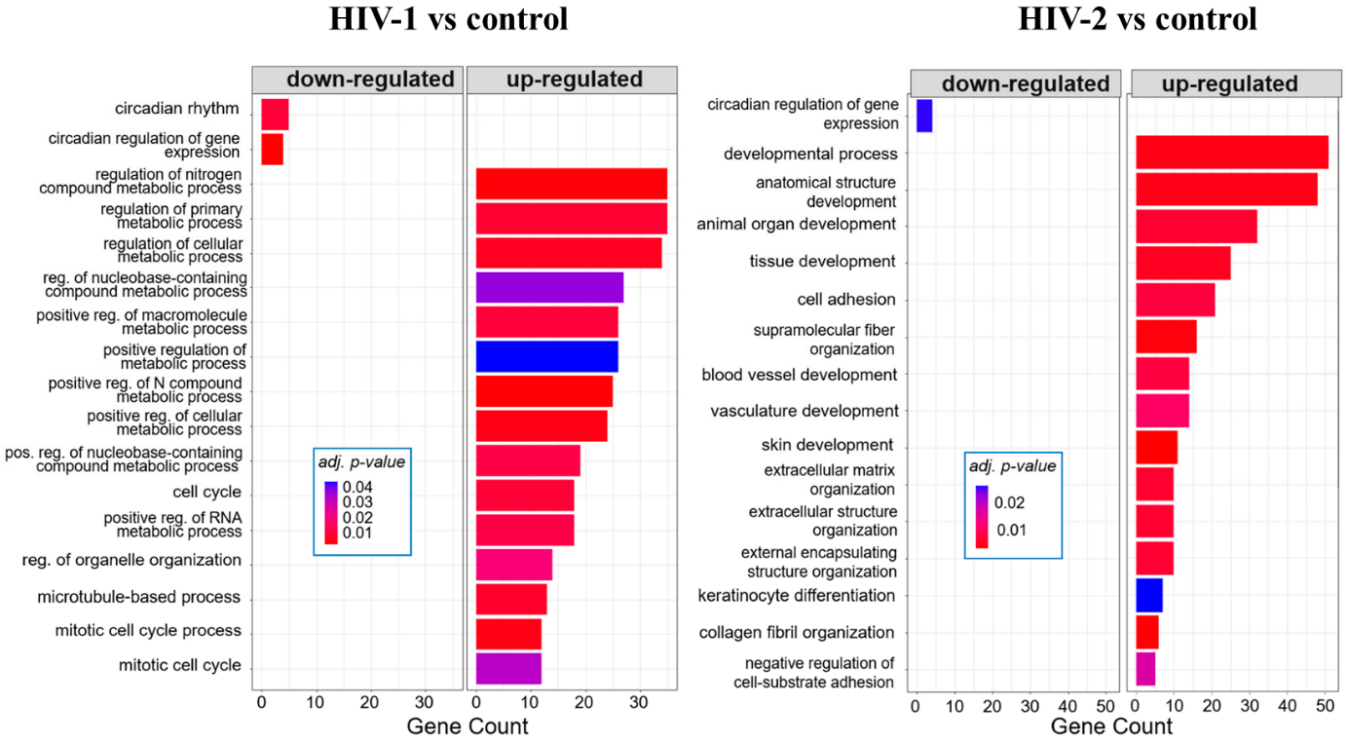


Figure 11. GO analysis of the DEGs from HIV-1 (left) and HIV-2 (right) transduced cells at the eight hour time-point. The top genes were categorized based on significant enrichment terms, with colour intensity indicating the significance of each term. Counts correspond to the number of DEGs linked to the given gene ontology ID in the analysis.

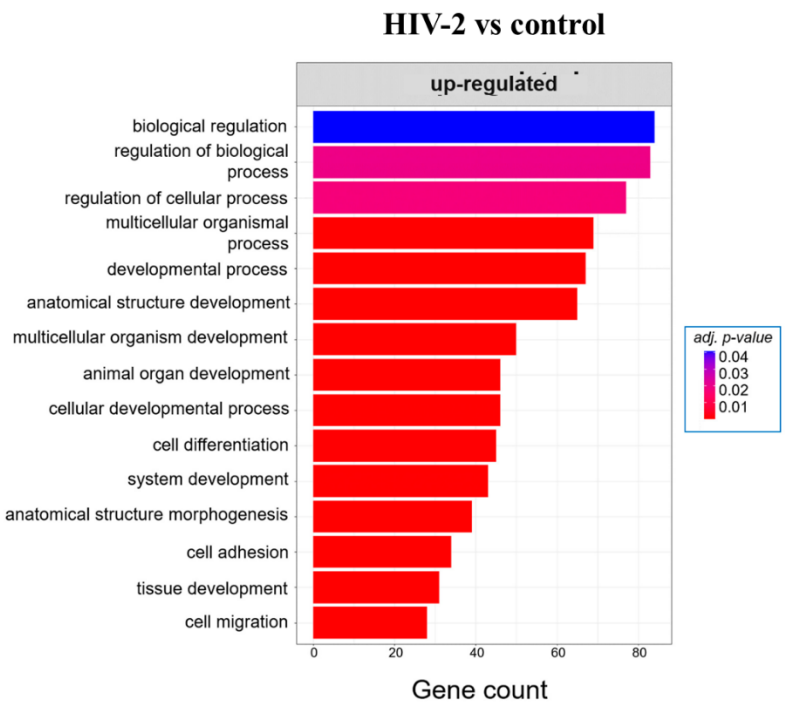


Figure 12. GO analysis of the DEGs from HIV-2 transduced cells at the 12-hour time-point. The top genes were categorized based on significant enrichment terms, with colour intensity showing the significance of each term. Counts correspond to the number of DEGs linked to the given gene ontology ID in the analysis.

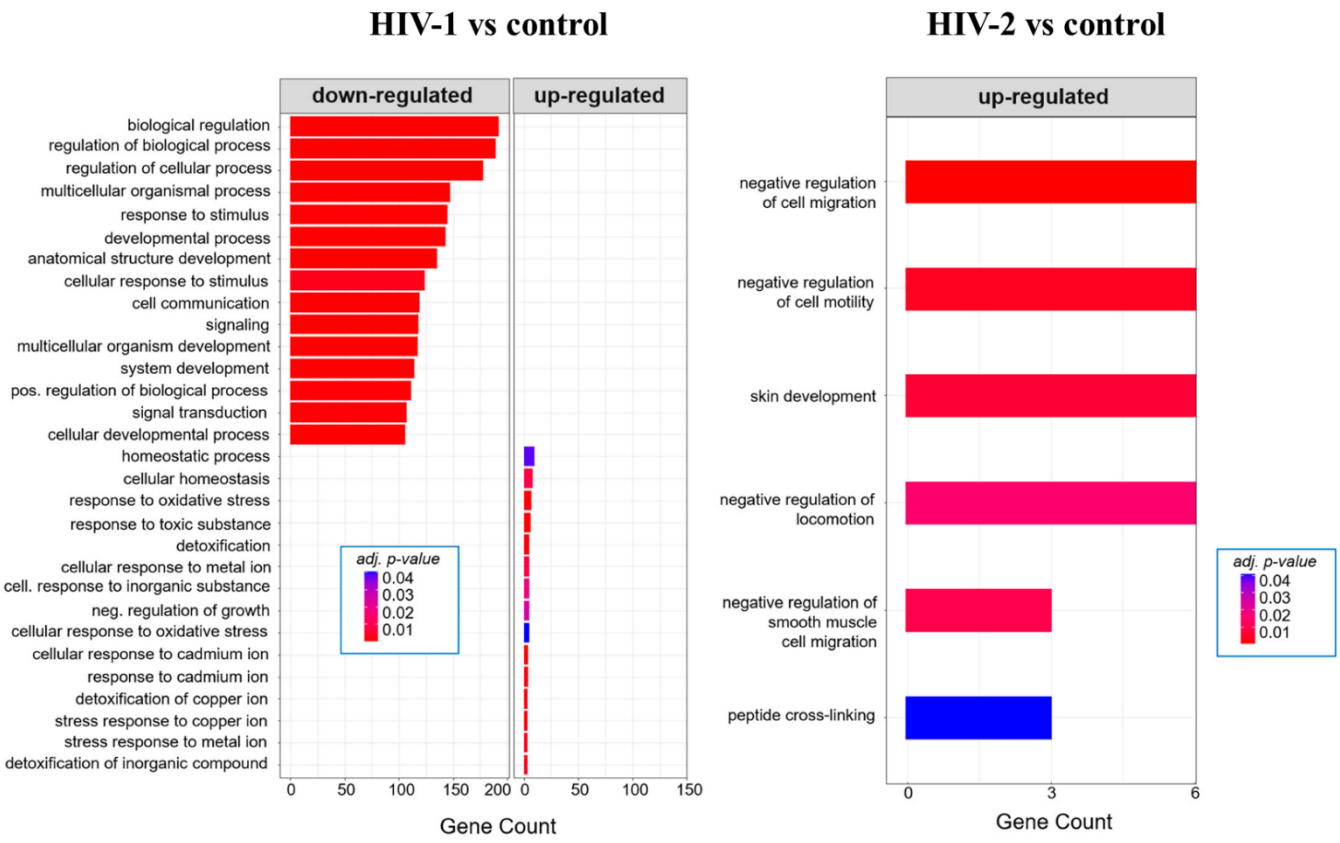


Figure 13. GO analysis of the DEGs from HIV-1 (left) and HIV-2 (right) transduced cells at the 26-hour time-point. The top genes were categorized based on significant enrichment terms, with colour intensity showing the significance of each term. Counts corresponds to the number of DEGs linked to the given gene ontology ID in the analysis.

5.9 Proteomic changes in the immediate early-phase in HEK-293T cells

Over 1000 protein were identified by analysis of transduced host cell proteome. Following false discovery rate analysis, seven proteins exhibited statistically significant downregulation in comparison to the control. Notably, five proteins were found to be downregulated in HIV-1 transduced cells, and an additional two proteins showed decreased expression in cells transduced with HIV-2 compared to the control (Figure 14).

Both HIV-1 and HIV-2 downregulated the expression of mitochondrial 60kDa heat shock protein (HSPD1), non-POU domain-containing octamer binding protein (NONO), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), serine/arginine-rich splicing factor 6 (SRSF6) and histone H1.4 (H1-4). Furthermore, complex protein 1 subunit theta (CCT8) and

FK506-binding protein (FKBP4) showed significant decrease only in HIV-2 transduced cells compared to HIV-1.

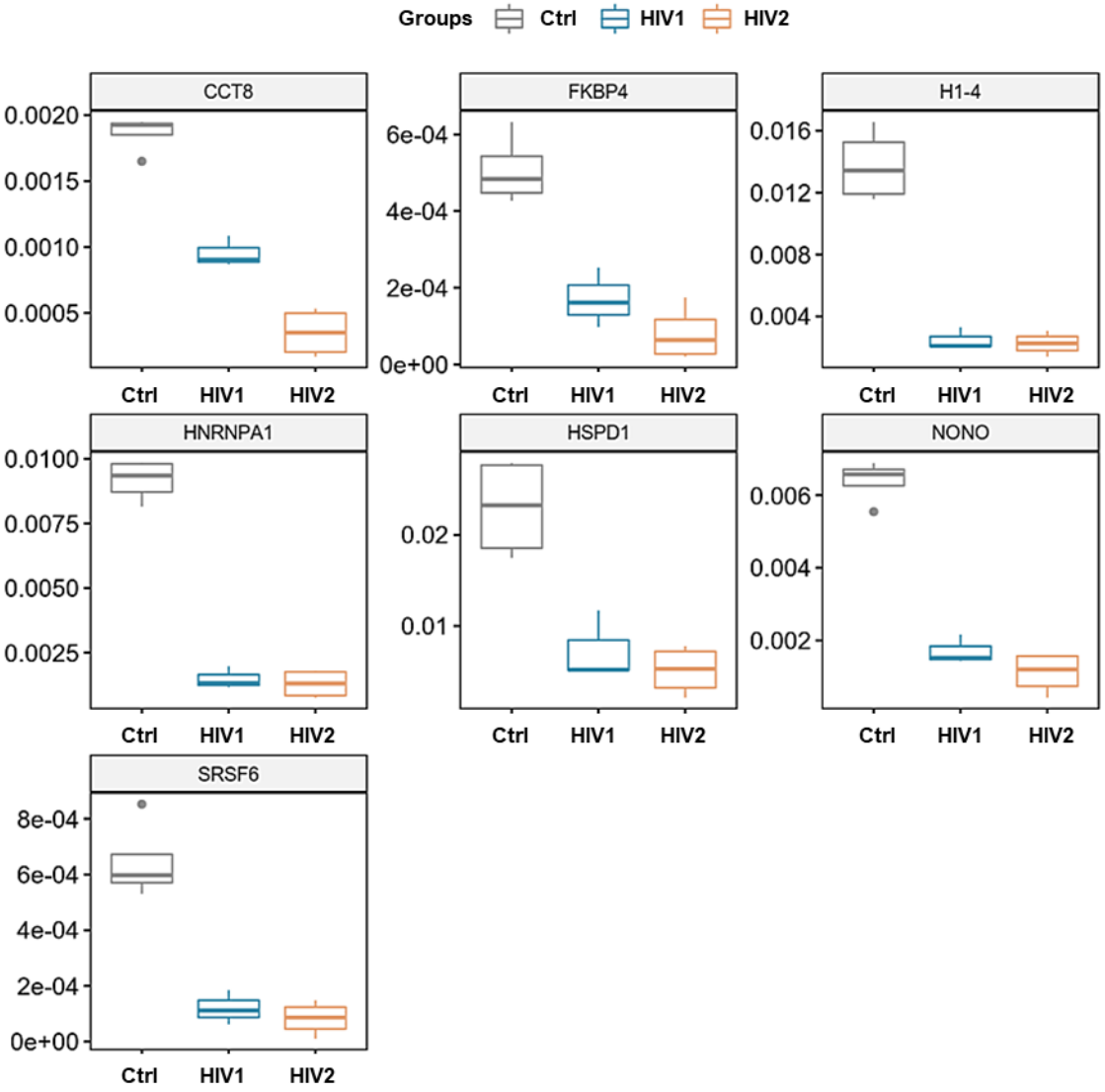
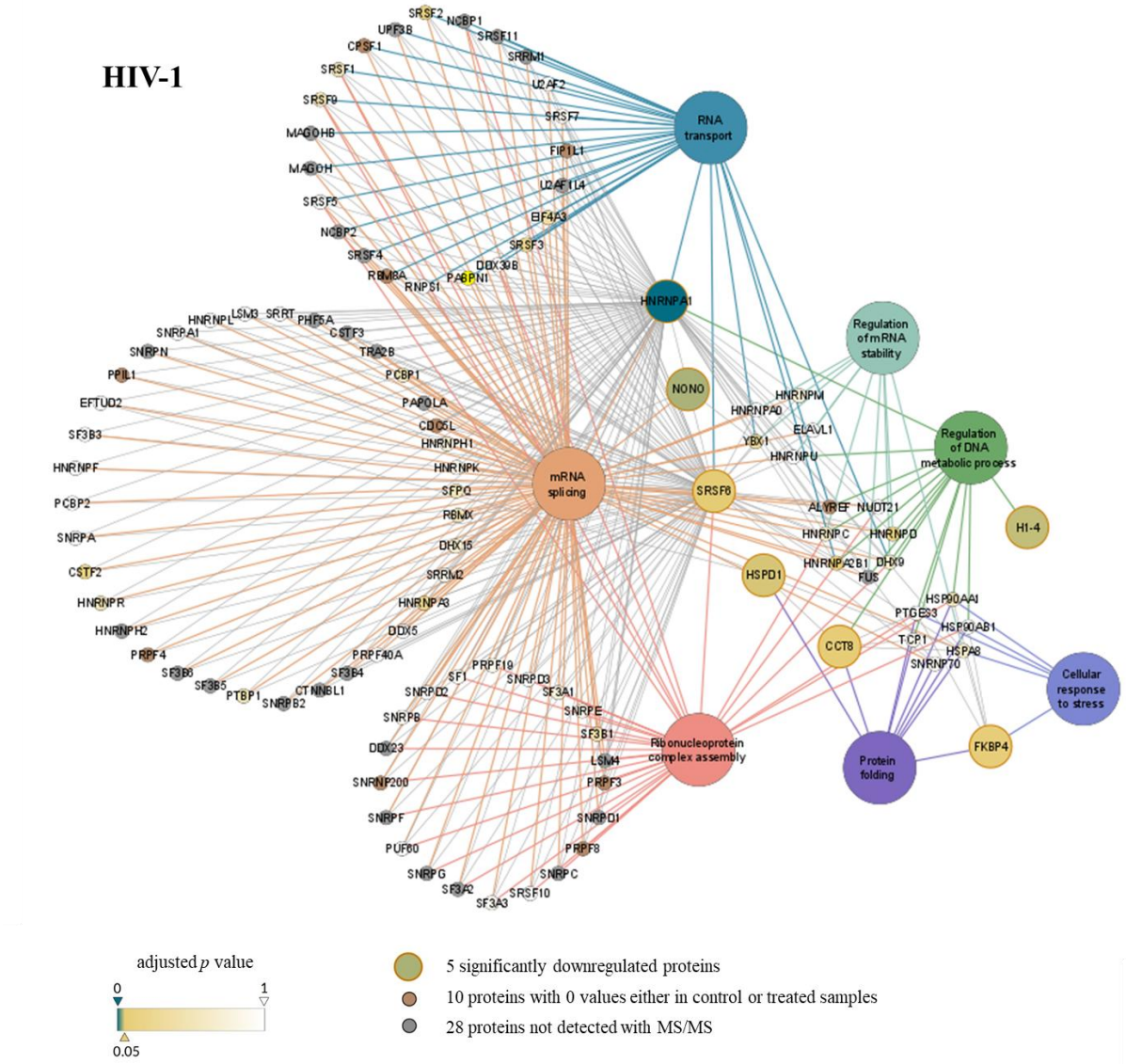


Figure 14. Downregulated proteins at the two-hour time-point. The boxplot illustrates statistically significant changes in the downregulated proteins following HIV-1 and HIV-2 transduction, with a p-value < 0.05. Moreover, CCT8 and FKBP4 were significant only in cells transduced with HIV-2. The abbreviation "ctrl" corresponds to the control.

Following the identification of the seven significantly downregulated proteins, interaction network and biological process enrichment analyses were carried out using Cytoscape 3.8.1 **(**Shannon et al., 2003). The integrated STRING database was used to generate the physical interaction network, with a confidence level of 0.7–100 first shell interactors was queried, and

for the gene ontology enrichment Cytoscape's ClueGO v2.5.7. plugin was used **(**Bindea et al., 2009). In the gene ontology analysis of the enriched network compromising 107 proteins altogether, functional clusters of GO terms were first generated. ClueGO utilizes term similarity to define functional groups of multiple terms. In our analysis, initial group size was set to three terms (two being the default value), and the percentage for group merge was left at the default value of 50%. Further, 33 of the 72 resulting GO terms were grouped into six functional clusters, the rest of them, 39 in total, did not reach the group merge threshold. However, among these was the GO term mRNA splicing (GO:00000398) including 97 proteins in the enriched network. Due to the redundancy of GO terms in the clusters, we selected seven biological processes that were the most representative and significant according to their *p*-values corrected with the Bonferroni step-down method ($p < 0.05$). Altogether, 105 proteins were covered by these biological processes in the interaction network (Figure 15). Moreover, 77 of these 105 proteins were also detected by MS/MS, indicating a wider interaction network centred around the significantly downregulated proteins. However, only 67 of these 77 proteins had corresponding quantitative data of sufficient quality to enable statistical analysis, as they could not be quantified in some of the replicate measurements.


HIV-1
RNA transport
mRNA splicing
Ribonucleoprotein complex assembly
Regulation of mRNA stability
Regulation of DNA metabolic process
Protein folding
Cellular response to stress
HNRNPA1
NONO
SRSF8
HSPD1
CCT8
FKBP4
H1-4
SRSF2
NCBP1
UPF3B
SRSF11
CPSF1
SRRM1
SRSF1
U2AF2
SRSF9
SRSF7
MAGOHB
FIP1L1
MAGOH
U2AF1L4
SRSF5
EIF4A3
NCBP2
SRSF3
SRSF4
DDX39B
RBM8A
PA2G4
RNPS1
HNRNPL
LSM3
SRRT
PHF5A
SNRPA1
CSTF3
SNRPN
TRA2B
PPIL1
PCBP1
EFTUD2
PAPOLA
SF3B3
CDC5L
HNRNPH1
HNRNPF
HNRNPK
PCBP2
SFPQ
RBMX
SNRPA
DHX15
CSTF2
SRRM2
HNRNPR
HNRNPA3
HNRNPH2
DDX5
PRPF4
PRPF40A
SF3B6
SF3B4
SF3B5
PRPF19
SF1
SNRPD3
PTBP1
CTNNBL1
SNRPB2
SNRPD2
SF3A1
SNRPB
SNRPE
SF3B1
DDX23
LSM4
SNRNP200
PRPF3
SNRPF
SNRPD1
PUF60
PRPF8
SNRPG
SNRPC
SF3A2
SRSF10
SF3A3
HNRNPM
HNRNPA0
ELAVL1
YBX1
HNRNPU
ALYREF
NUDT21
HNRNPC
HNRNPD
HNRNPA2B1
DHX9
FUS
HSP90AA1
PTGES3
HSP90AB1
TCP1
HSPA8
SNRNP70
adjusted p value
0
1
0.05
5 significantly downregulated proteins
10 proteins with 0 values either in control or treated samples
28 proteins not detected with MS/MS

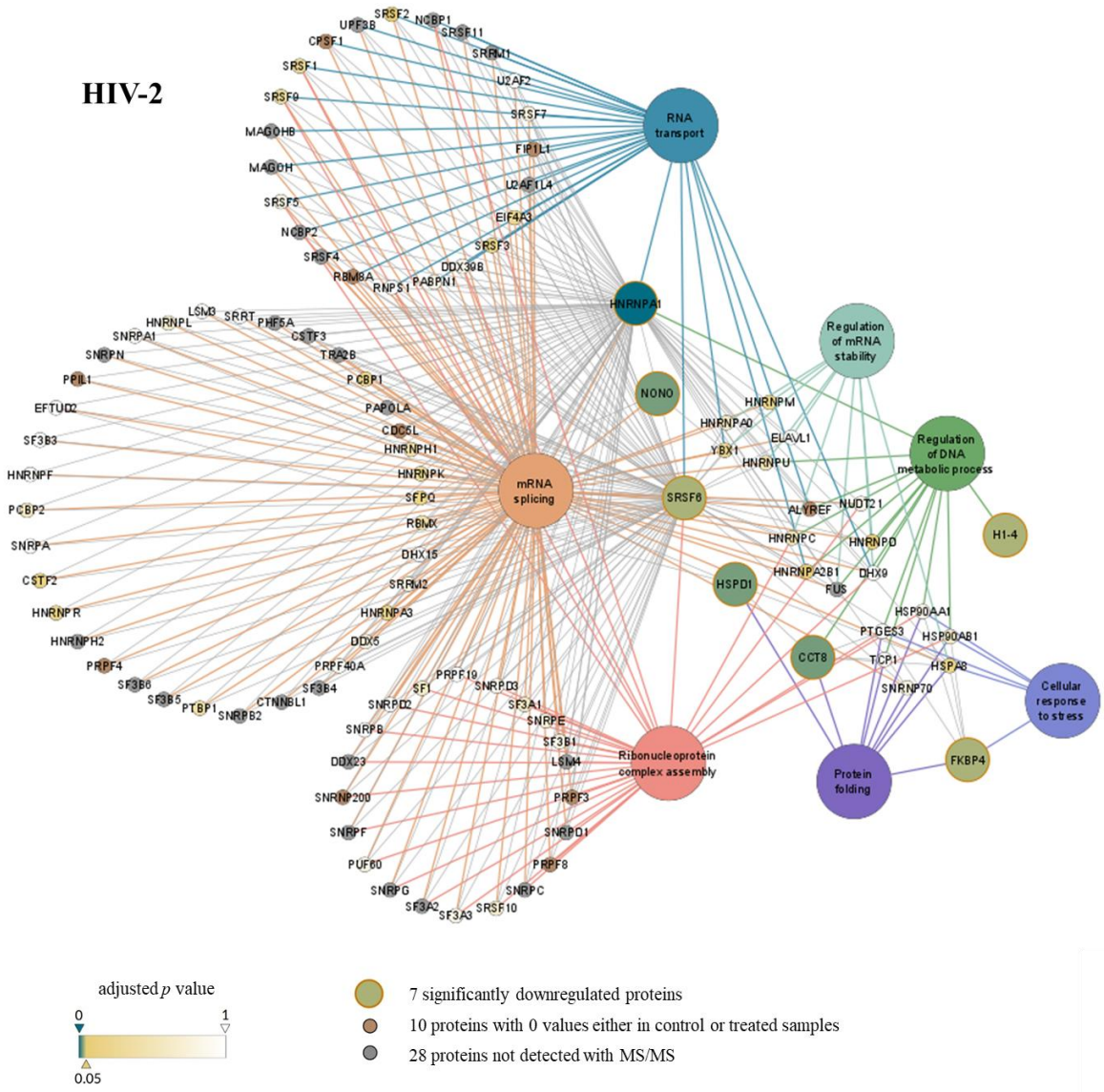
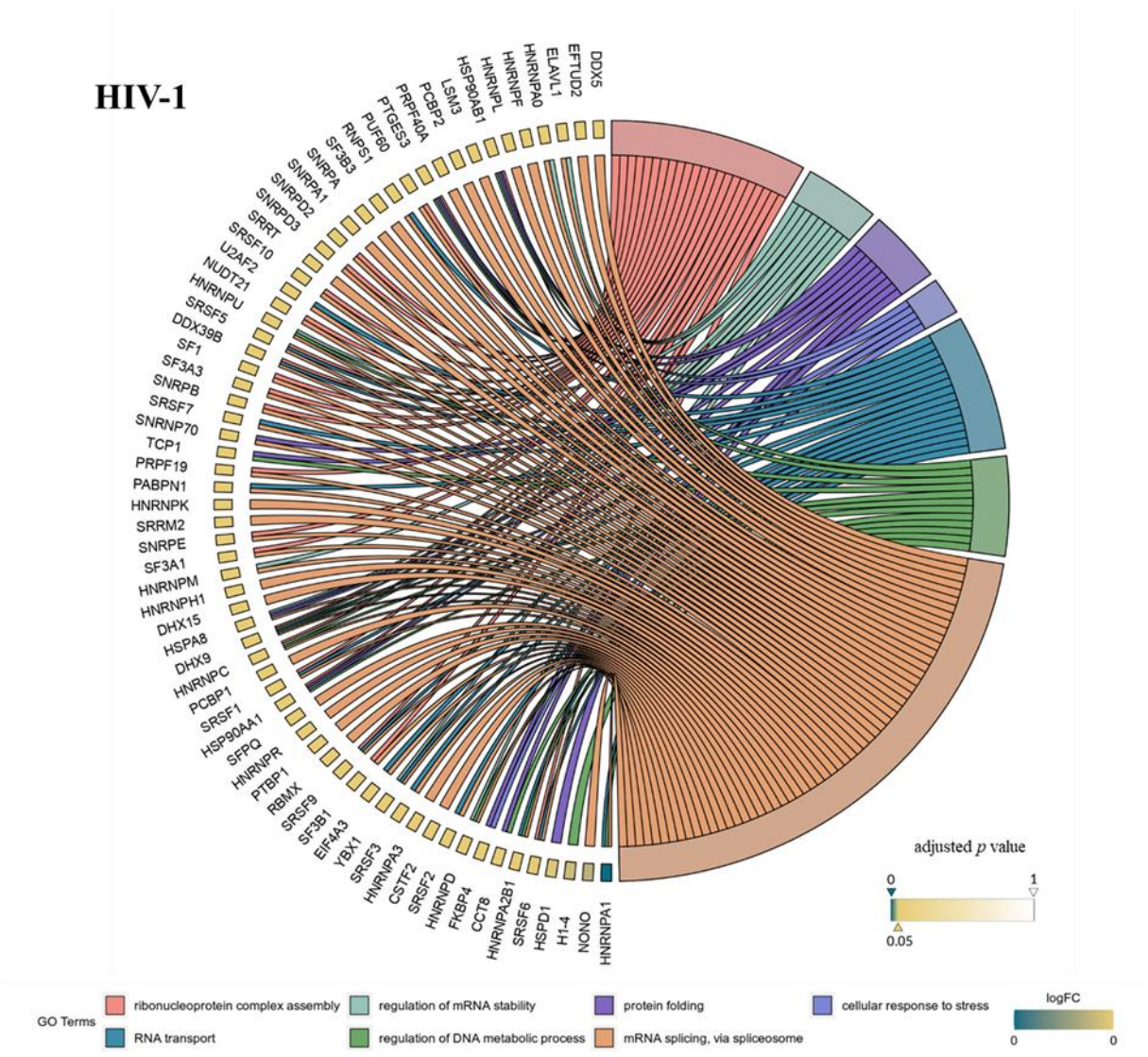


Figure 15. The protein interaction network of proteins downregulated by HIV-1 (top panel) and HIV-2 (bottom panel) are shown. Downregulated proteins are depicted as medium-sized circles, while the larger circles represent the associated biological process Gene Ontology (GO) term pathways. Smaller circles indicate interacting proteins, with colour and intensity reflecting the significance of change in their regulation compared to the control ($\alpha = 0.05$; colour scale: p = 0-marine blue, p = 0.05-gold, p = 1-white). Physical protein-protein interactions within the members of the enriched, extended network are not displayed.

The terms we employed for matching, encompassing processes such as ribonucleoprotein complex assembly (GO:0022618), RNA transport (GO:0050658), regulation of mRNA stability

(GO:0043488), regulation of DNA metabolic processes (GO:0051052), protein folding (GO:0006457), mRNA splicing via spliceosome (GO:0000398), and cellular response to stress (GO:0033554), are depicted alongside their respective proteins in Figure 16.



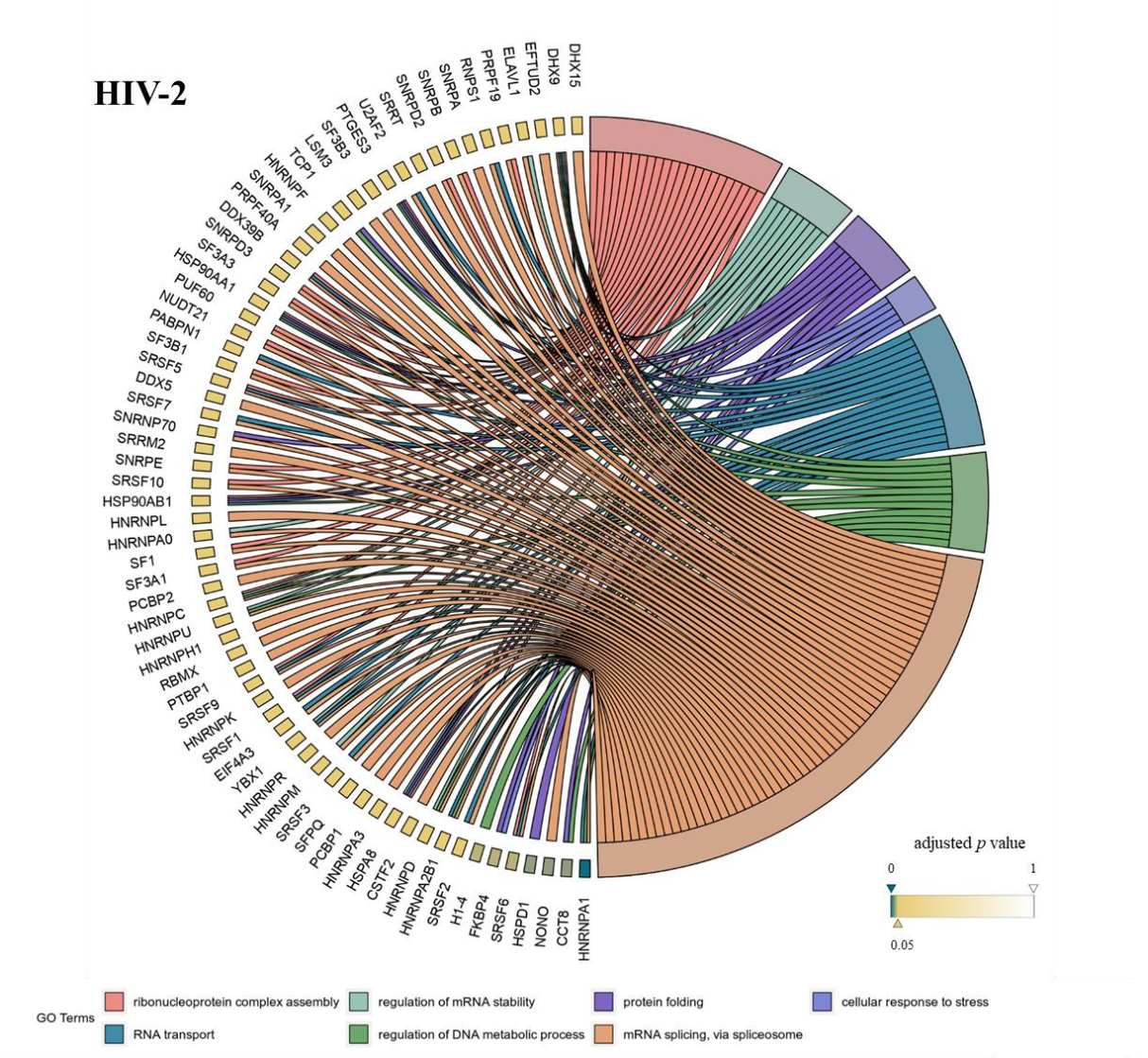


Figure 16. Associations between the seven chosen GO terms (ribonucleoprotein complex assembly (GO:0022618), RNA transport (GO:0050658), regulation of mRNA stability (GO:0043488), regulation of DNA metabolic processes (GO:0051052), protein folding (GO:0006457), mRNA splicing via spliceosome (GO:0000398), and cellular response to stress (GO:0033554)) and proteins from the enriched interaction network detected through tandem mass spectrometry (MS/MS) analysis. The colour intensities of the rectangles on the figure shows the significance of changes in the corresponding protein levels compared to the control ($\alpha = 0.05$; colour scale: p = 0—marine blue, p=0.05—gold, p = 1—white). The chord plots were generated using the GOplot R package (Walter et al., 2015).

5.10 Proteomic changes in the early phase in HEK-293T cells

Through mass spectrometry analysis a total of 871 proteins were detected at eight, 817 at 12, and 810 at 26 hours post-transduction. At eight hours, proteomic analysis revealed no significant changes in the cellular proteome. However, at 12 hours, we detected 17 proteins which showed difference in their expression, with 5 altered by HIV-1, 4 by HIV-2, and 8 by both viruses compared to the control. Among the proteins influenced by HIV-1 were the ATP synthase F1 subunit beta (ATP5F1B), valosin-containing protein (VCP) and synaptotagmin-binding cytoplasmic RNA interacting protein (SYNCRIP). HIV-2 significantly altered the expression of non-SMC condensin I complex subunit H (NCAPH), phosphoribosylformylglycinamidine synthase (PFAS), valyl-tRNA synthetase 1 (VARS1) and proline-rich coiled-coil 2A (PRRC2A). Altered proteins by both viruses included Zyxin (ZYX), arginyl-tRNA synthetase 1 (RARS1), heat shock protein family A member 1A (HSPA1A), nucleophosmin 1 (NPM1), and deoxyuridine triphosphatase (DUT). At 26 hours, a total of 117 proteins were differentially regulated following transduction with HIVs. HIV-1 significantly altered the expression of 25 proteins, meanwhile HIV-2 influenced 39 proteins. Moreover, both HIVs altered the expression of 48 proteins respectively, compared to the control. Additionally, 5 proteins were exclusively regulated by HIV-2 in comparison to HIV-1. HIV-1 altered proteins included insulin-like growth factor 2 receptor (IGF2R), DEAD-box helicase 3 X-linked (DDX3X), protein disulfide isomerase family A member 3 (PDIA3), HIV-1 Tat Specific Factor 1 (HTASF1), lysyl-tRNA synthetase 1 (KARS1), proteasome 20S subunit alpha 2 (PSMA2), and ATPase Family AAA Domain Containing 3A (ATAD3A). HIV-2 managed to influence the cellular level of nucleolin (NCL), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) and serine and arginine-rich splicing factor 1 (SRSF1). Both viruses affected the expression of heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), heterogeneous nuclear ribonucleoprotein K (HNRNPK), serine and arginine-rich splicing factor 2 (SRSF2), dynein cytoplasmic 1 light intermediate chain 1 (DYNC1LI1) and eukaryotic translation initiation factor 2B subunit delta (EIF2B4). When HIV-2 was compared to HIV-1, 17 proteins had showed altered expression, including transferrin receptor (TFRC), heat shock protein family A members, ribosomal protein L23a (RPL23A) and RAN binding protein 1 (RANBP1). The differential pattern in the protein expression at 12 and 26 hours are depicted on Figures 17 and 18, while Figure 19 shows proteins that were differentially regulated across all observed time-points.

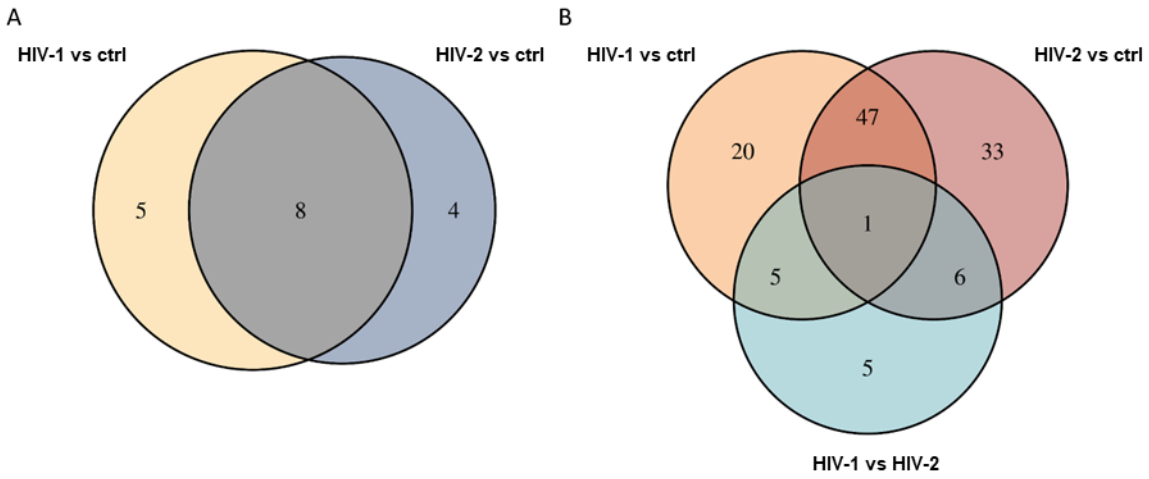


Figure 17. Venn diagram showing the differential protein expression between HIV-1 vs. control or HIV-2 vs. control transduced samples and also between HIV-1 vs. HIV-2 at 12 (**A**) and at 26 (**B**) hours post-transduction.

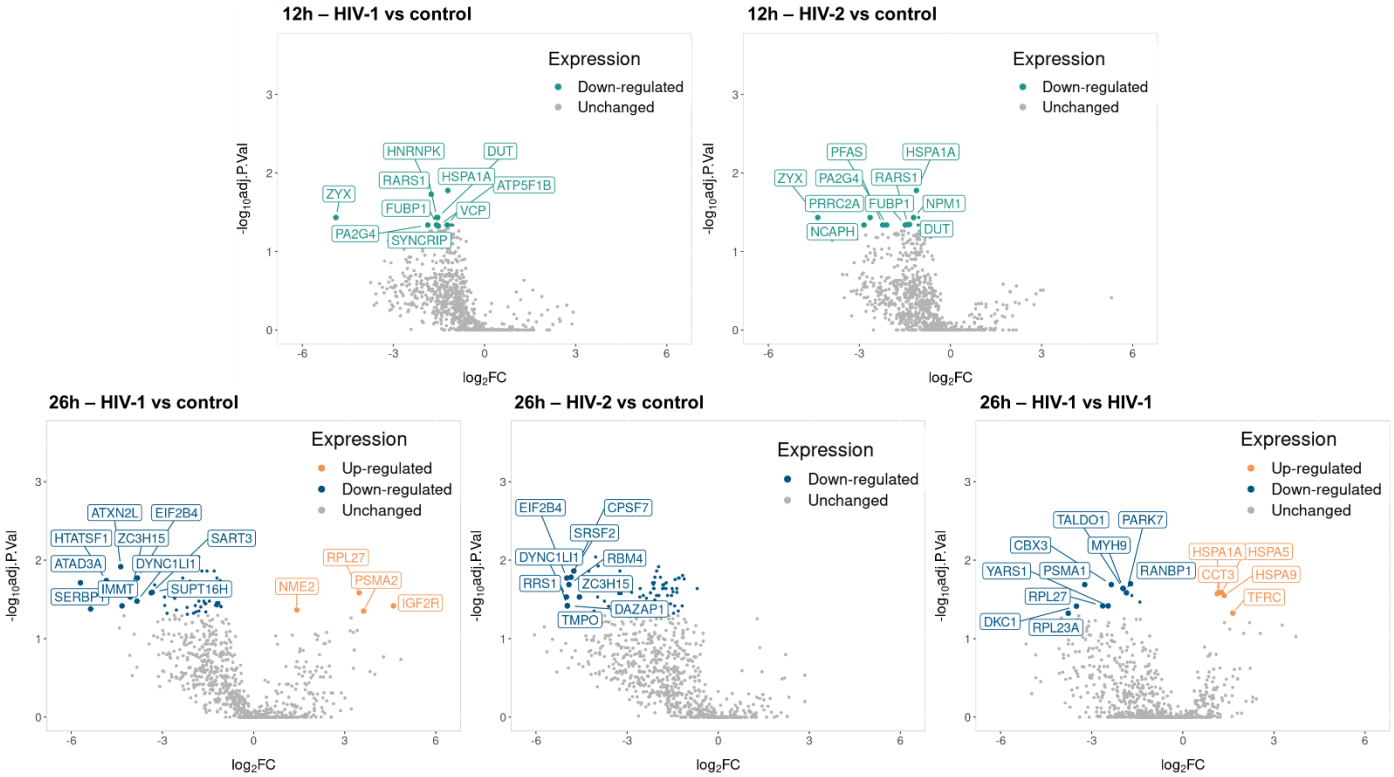


Figure 18. Volcano plots showing the changes in protein expression in HIV-1 and HIV-2 transduced cells at 12 (top) and 26 h (bottom) post-transduction. The x-axis shows the log2 fold change (log2FC) of detected proteins, while the y-axis displays the log10 adjusted *p*-value for each protein. Each dot represents a detected protein, with color indicating significant upregulation (light orange) or downregulation (green, blue), and grey dots representing non-

significantly altered proteins. It's important to note that only the top ten significantly regulated proteins, either up or downregulated, are labelled in the plot for each category.

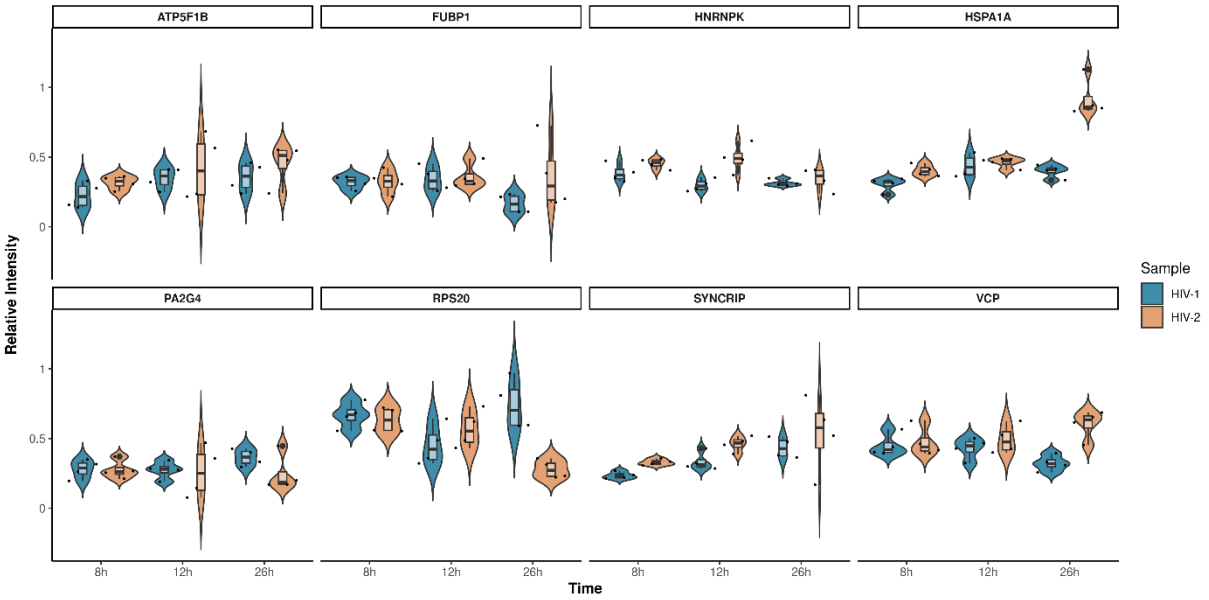


Figure 19. Violin plots depicting the intensity distribution of differentially regulated proteins across all of the observed time-points for HIV-1 (blue) and HIV-2 (light orange) transduced cells. The x-axis shows the selected time-points, while the y-axis corresponds to the intensity of the proteins.

5.11 Gene ontology analysis of proteins in the 12 and 26 hours time-points in HEK-293T cells

To better understand the proteomic changes in transduced cells, GO enrichment analysis was carried out on the HIV-1 and HIV-2 transduced samples at 12 and 26 hours post-transduction (Figure 20). In comparison to the control, at 12 hours, HIV-1 altered the expression of proteins involved in ubiquitin protein ligase binding, ATP metabolic processes and mRNA splicing via spliceosome. Meanwhile, HIV-2 affected the expression of proteins associated with regulation of localization, regulation of cellular response to stress and negative regulation of macromolecule biosynthetic processes. At the 26-hour time-point, HIV-1 influenced the cellular level of proteins related to the RNA polymerase II transcription regulator complex, viral genome replication and response to virus. Moreover, HIV-2 altered proteins associated

with viral genome replication, regulation of viral genome replication, and the spliceosomal small nuclear ribonucleoprotein (snRNP) complex.

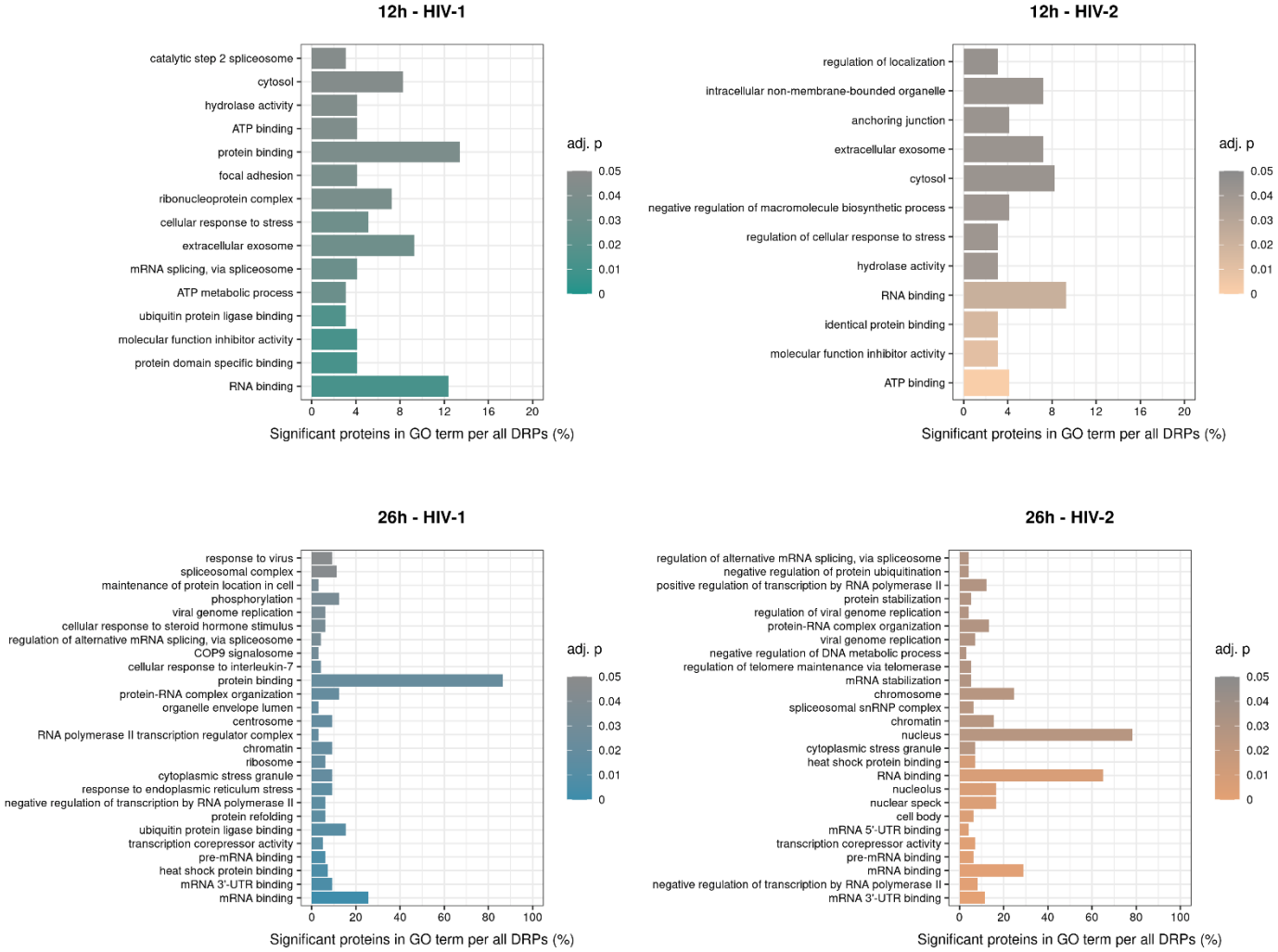


Figure 20. The GO term classification shows the enriched, significant proteins identified in HIV-1 (left) and HIV-2 (right) transduced cells at 12 (top) and 26 (bottom) hours post-transduction, compared to the control. The colour intensity indicates the adjusted p-value for each term, while the x-axis reflects the percentage of significant proteins within each GO term relative to all DEPs. The enriched GO terms are listed on the y-axis.

In order to further enrich the associations between the DEPs and GO terms, we selected six GO terms from samples transduced with HIV-1 and HIV-2 at both 12 and 26 hours. Figure 21

illustrates the connections between the DEPs and the chosen GO terms, along with the logFC of the DEPs.

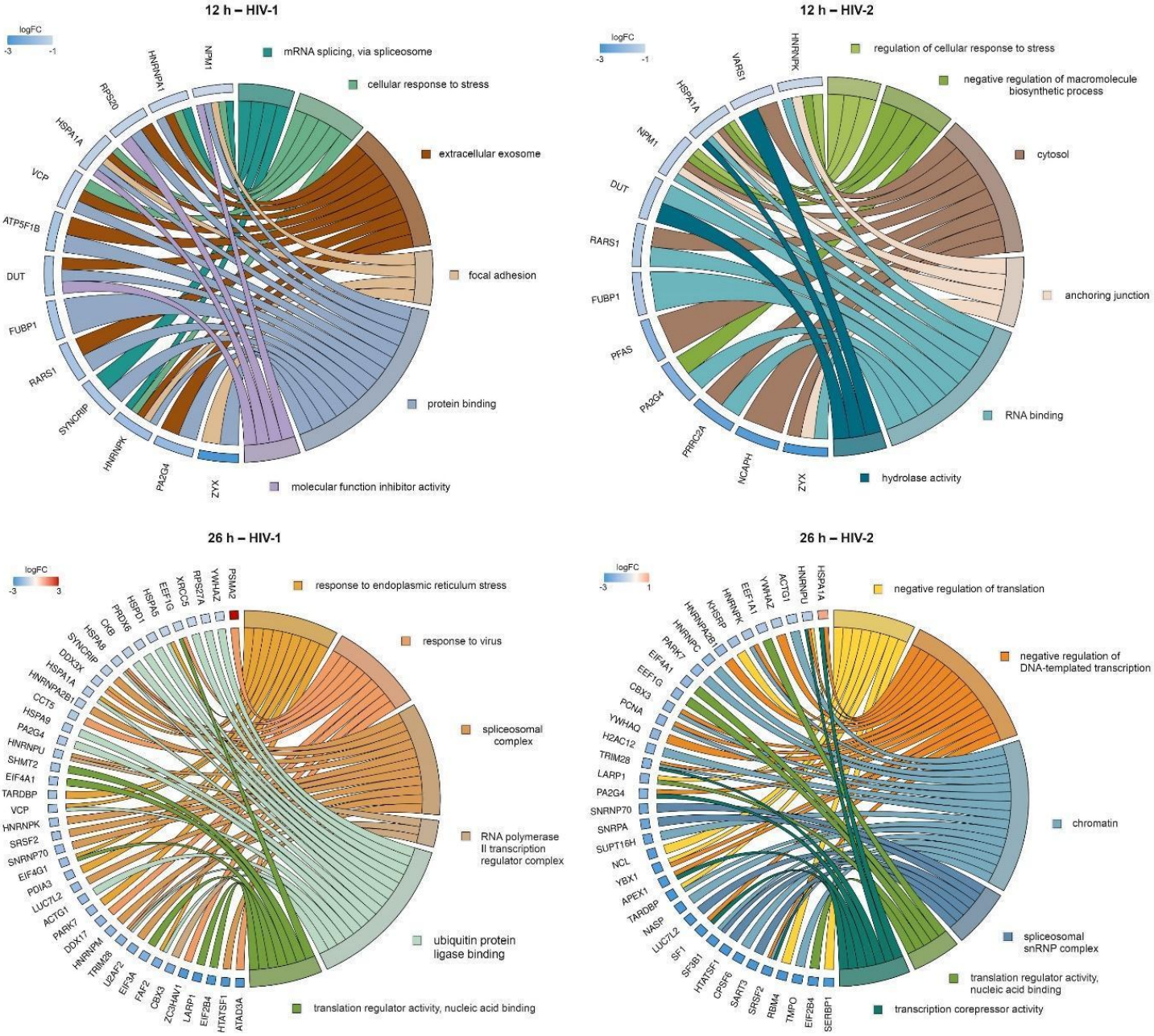


Figure 21. Protein association analysis is illustrated in this figure, showcasing the relationships between six selected Gene Ontology (GO) terms and DEPs by HIV-1 (left) and HIV-2 (right) at 12 (top) and 26 (bottom) hours post-transduction. The chosen GO terms for HIV-1 were mRNA splicing via spliceosome, cellular response to stress, extracellular exosome, focal adhesion, protein binding, and molecular function inhibitor activity. For HIV-2 at 12 hours post-transduction, the selected terms were regulation of cellular response to stress, negative regulation of macromolecule biosynthetic processes, cytosol, anchoring junction, RNA binding,

and hydrolase activity. At 26 hours post-transduction, the selected terms for HIV-1 included response to endoplasmic reticulum stress, response to virus, spliceosomal complex, RNA polymerase II transcription regulator complex, ubiquitin protein ligase binding, and translation regulator activity, nucleic acid binding. For HIV-2, the terms were negative regulation of translation, negative regulation of DNA-templated transcription, chromatin, spliceosomal small nuclear ribonucleoprotein (snRNP) complex, translation regulator activity, nucleic acid binding, and transcription corepressor activity. The logarithm of the fold change values are represented by the color intensities of the rectangles, indicating the extent of changes in corresponding protein levels relative to the control. It is important to note that, at 26 hours post-transduction, the majority of DEPs are observed in the HIV-2 vs. control comparison. However, some DEPs were selectively identified from the HIV-2 vs. HIV-1 comparison, exemplified by instances such as HSPA1A, which is upregulated compared to HIV-1 (shown here) but downregulated compared to the control.

5.12 Differentially induced transcripts in the early-phase of transduction in Jurkat cells

We detected no significantly altered transcript at the zero-, and two-hour time-points. Eight hours post-transduction, HIV-1 altered the expression of 39 transcripts. Out of the 39, 38 were downregulated while only 1, a non-coding RNA transcript was upregulated. Of the downregulated transcripts, 29 coded for proteins, 3 for pseudogenes and 6 for various RNA transcripts. Among the differentially downregulated transcripts were the argininosuccinate synthase 1 (*ASS1*), cyclin G2 (*CCNG2*) and protein phosphatase, Mg2+/Mn2+ dependent 1K (*PPM1K*). At twelve hours, HIV-1 differentially regulated 45 transcripts compared to the control transduced Jurkat cells. From the 45, 43 were down- and 2 were upregulated. Amongst the downregulated transcripts, 35 coded for proteins, 2 for pseudogenes and 6 were coding for various RNA products. Meanwhile, two non-coding RNA transcripts were upregulated. The differentially downregulated transcripts include methylenetetrahydrofolate reductase (*MTHFR*) and spondin 2 (*SPON2*). At the 26-hour time- point, the expression of 42 transcripts were altered by HIV-1 with 40 down- and 2 upregulated gene products. Amongst the differentially downregulated transcripts, 36 coded for proteins, 1 for pseudogenes and 3 for RNA products. Additionally, 1 protein and 1 pseudogene encoding transcript were upregulated. The protein-coding downregulated transcripts included cluster of differentiation 300a (*CD300a*), laminin subunit gamma 2 (*LAMC2*) and serine peptidase inhibitor, Kazal type 2 (*SPINK2*).

Eight hours post-transduction, HIV-2 altered the expression of 15 transcripts with 12 being down-, and 3 upregulated. Amongst the differentially downregulated transcripts 10 coded for proteins, 1 for a pseudogene and 1 for an RNA product. Moreover, 1 pseudogene and 2 assorted RNA products were upregulated. There were no unique genes regulated only by HIV-2 at the 8-hour time-point other than a pseudogene and an RNA product compared to control transduced cells. 12 hours post-transduction, HIV-2 affected the expression of 23 genes out of which 21 were down-, and 2 were upregulated. Of the downregulated ones, 19 coded for proteins and 2 for different RNA transcripts. Additionally, 1 protein coding and 1 non-coding RNA transcript were upregulated. The differentially regulated genes by HIV-2 at the 12-hour time point included cysteine rich angiogenic inducer 61 (*CYR61*) and ubiquinol-cytochrome c reductase complex assembly factor 3 (*UQCC3*). At the 26-hour time point, HIV-2 altered the expression of 36 transcripts, with 33 being down-, and 3 being upregulated. Of the downregulated ones 31 coded for proteins and 2 for different RNA products. Of the upregulated ones, 1 coded for protein and 2 for pseudogenes. The downregulated transcripts included the early growth response 1 (*EGR1*) and TOX high mobility group box family member 2 (*TOX2*).

The differential pattern in the gene expression by the two viruses at the three time points are depicted on Figure 22 and Table 4.

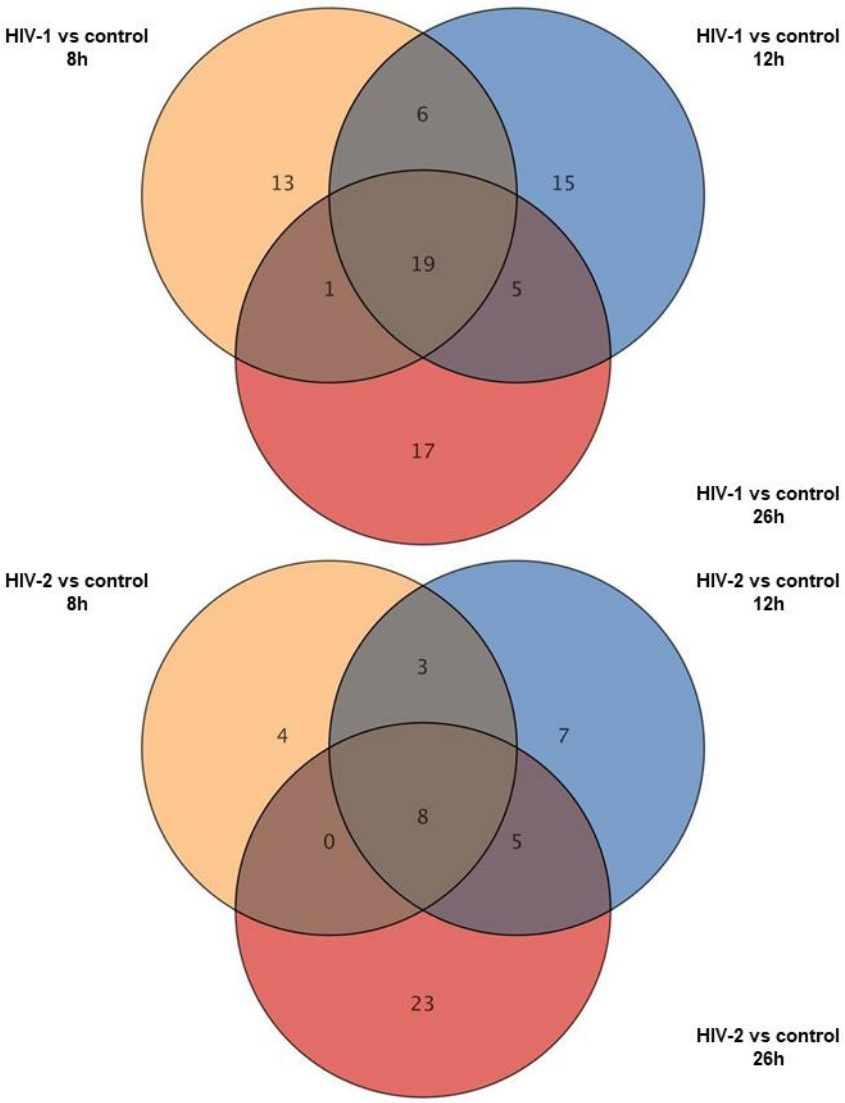


Figure 22. Venn diagram showing the differential gene expression between HIV-1 vs. control (top) or HIV-2 vs. control (bottom) transduced samples at the 8, 12 and 26-hour time-points.

Upregulated by HIV-1			
	8h	**12h**	**26h**
Protein	0	0	1
Pseudogenes	0	0	1
Non-coding RNA	1	2	0
Total	**1**	**2**	**2**
Downtegulated by HIV-1			
	8h	**12h**	**26h**
Protein	29	35	36
Pseudogenes	3	2	1
Non-coding RNA	6	6	3
Total	**38**	**43**	**40**

Upregulated by HIV-2

	8h	12h	26h
Protein	0	1	1
Pseudogenes	1	0	2
Non-coding RNA	2	1	0
Total	**3**	**2**	**3**
Downtegulated by HIV-2			
	8h	**12h**	**26h**
Protein	10	19	31
Pseudogenes	1	0	0
Non-coding RNA	1	2	2
Total	**12**	**21**	**33**

Table 4. Number of DETs at 8, 12 and 26 hours post transduction from HIV-1 (top) and HIV-2 (bottom) transduced Jurkat cells.

5.13 Gene ontology analysis of the significantly altered transcripts in the early phase of HIV transduction in Jurkat cells

GO analysis was carried out on the significantly altered transcripts 26 hours post-transduction. Our analysis revealed that HIV-1 altered the expression of transcripts related to steroid biosynthetic process, cholesterol biosynthetic process and isoprenoid biosynthetic process. Meanwhile, HIV-2 only affected transcripts which are involved in the cholesterol biosynthetic process. This indicates that HIV-1 has a greater effect on the host cell 26 hours post-transduction compared to HIV-2 at the same time-point. The different GO terms altered by the two virus are listed on Table 5.

Go Terms influenced by HIV-1	GO Terms influenced by HIV-2
Cholesterol biosynthetic process (GO:0006695)	Cholesterol biosynthetic process (GO:0006695)
Isoprenoid biosynthetic process (GO:0008299)	
Regulation of cholesterol biosynthetic process (GO:0045540)	
Organic hydroxy compound biosynthetic process (GO:1901617)	
Steroid biosynthetic process (GO:0006694)	

Table 5. GO terms of the DETs from HIV-1 (top) and HIV-2 (bottom) transduced Jurkat cells at the 26 hour time-point.

6. Discussion

HIV has affected more than 40 million people at the time of writing this dissertation. The majority of infections are caused by HIV-1, however, a significant number of cases are caused by HIV-2. There are multiple similarities between the two HIV viruses such as their method of transmission and pathogenesis. However, there are also numerous differences in their replication dynamics and course of infection. There is an initial increased viral production phase in the early stage in case of HIV-2 infection, followed by an extended latency period. Additionally, compared to HIV-1, HIV-2 exhibits reduced viral replication and a slowed progression of infection towards AIDS (Campbell-Yesufu & Gandhi, 2011**)** (MacNeil et al., 2007**)** (Bock & Markovitz, 2001**)**. The variables contributing to these unique replication dynamics and the mechanism influencing the preference for an extended latency period of HIV-2 infection are not fully understood.

There are multiple studies focusing on the cellular effects of HIV-1, however there are very few studies in regards to HIV-2. Additionally, lentivirusbased vectors are valuable clinical and research and thereby, understanding the intracellular effects of the vectors on the host cell is important for their safe and targeted application. We set the goal to analyse the effect of HIV-1 and 2-based lentiviral vectors on the cellular transcriptome and proteome during the early phase of transduction, focusing on 5 distinct time-points.

It is important to note that, despite using two different platforms for transcriptomic analysis (Illumina and MGI), studies have shown that the gene expression profiles of the analyzed cells are comparable between the two technologies (Póliska et al., 2024).

Immideate Early-Phase Transcriptomic Changes

Firstly, in order to define the changes in the gene expression of the HIV-1 and HIV-2 transduced cells, RNA-seq analysis was carried out. In the immediate early-phase of HIV transduction, both viruses influenced the expression profile o thousands of transcripts. 4551 transcripts were regulated differentially by HIV-1, and 3422 by HIV-2. We detected significant differences between the top 10 most up and downregulated transcripts between HIV-1 and HIV-2 transduced cells. Compared to HIV-2, HIV-1 regulated transcripts were much more diverse. Differentially altered transcripts by HIV-1 include transporters (*SLC711A*), DNA repair proteins (*TEX15, BRCA2*) and chaperones (*SACS*). The upregulation of the cysteine/glutamate antiporter *SLC711A* was indeed a significant find. HIV-1 Tat protein has been shown to increase the export of glutamate from the cell via SLC117A. The increased glutamate export results in cellular glutamate depletion, leading to oxidative stress and exo-cytotoxicity in microglia cells

(Gupta et al., 2010). The balance in the level of glutamate/cysteine also has a prominent part in the differentiation of inflammatory dendritic cells (DC). Disruption of this transport mechanism could potentially lead to a decreased number of functional DCs and thereby limitation of the effective activation of T helper 1 cells (Th1) during HIV infection (D'Angelo et al., 2010).

Interestingly, most of the top 10 differentially regulated transcripts by HIV-2 were related to the intra- and extracellular matrix. Protein-coding transcripts such as *COL1A2*, *COL6A3*, *COL3A1*, *KRT14*, *KRT7* and *KRT5* were upregulated by HIV-2. This pattern was observed at later time-points as well. Viruses, such as HIV and human papillomavirus (HPV), can impact the cellular levels of cytokeratins, however there are no specific interactions between HIV proteins and collagens or cytokeratins (Nittayananta et al., 2012) (Kellokoski et al., 1991). HIV-1 Tat protein was shown to impact the regulation of collagens, as well as other extracellular matrix proteins such as fibronectin and laminin, particularly in the later stages of infection (McArthur et al., 2001). Additionally, in neuroblastoma cells, extracellular Tat was shown to disrupt neuronal differentiation by competing with type I collagens within the extracellular matrix (Cornaglia-Ferraris et al., 2001**)**.

Fluctuations in the integrity and composition of the extracellular matrix are crucial for an effective immune response against pathogens, and for the successful communication between infected and effector cells. Disruption of these processes significantly impair the capacity of immune cells to effectively respond to infections (Tomlin & Piccinini, 2018). Additionally, the remodelling of the intracellular matrix is necessary for the efficient transport and assembly of viral particles (Matarrese & Malorni, 2005) (Stolp & Fackler, 2011).

An interesting find was the upregulation of *THBS1* by HIV-2. The transcript was amongst the top 10 most upregulated protein-coding genes in all of the observed time-points with the exception of the 12 hours time-point. THBS1 is an extracellular matrix glycoprotein with the ability to block HIV-1 LTR transactivation by extracellular Tat and cell proliferation. Moreover, it is also able to block viral entry via binding to the HIV envelope glycoprotein gp120 (Rusnati et al., 2000) (Crombie et al., 1998).

Rather surprisingly, in case of HIV-2, we observed little to no difference between the top up- and downregulated genes at the 0 and 2 hour time-point. This might suggest that the pathogenic impact of HIV-2 on cells may manifest at a later stage compared to HIV-1. Additionally, there is limited knowledge on the effects of VSV.G proteins on cells, especially in the early stages. Furthermore, the effect that was observed at the 0-hour time point, induced by both HIVs, might be attributed to the varying concentrations of VSV.G. As stated in the methodology, the virus

quantity was assessed based on the reverse transcriptase (RT) activity rather than the quantity of virions.

In order to gather more information about the affected transcriptome, GO analysis was carried out. Noteworthy variations were observed in the molecular functions of the impacted transcripts. Both viruses induced changes in the expression of genes related to GTPase regulator activity, GTPase activator activity, protein serine/threonine kinase activity, protein processing, structural constituent of ribosome and nucleoside-triphosphatase regulator activity. Our hypothesis suggest that the alterations observed in these transcripts come from the viral entry and response to the transduction, as proteins with serine/threonine kinase, GTPase activation activity, and GPTase regulation activity are typically linked to proliferation, transport and signalling. It is important to note that certain protein kinases play important roles in the life cycle of specific viruses, such as HIV, HPV, Ebola, and Influenza A, where phosphorylation of viral proteins is crucial for their functionality (Pagano et al., 2013) (Keating & Striker, 2012), **(**Francis et al., 2011). For viral protein trafficking and maturation, HIV uses the small GTPase Rab proteins during later stages of viral replication (Spearman, 2018). We observed changes in transcripts coding for proteins with iron-sulfur cluster binding, metal cluster binding, tau protein binding, rRNA binding ability and tau-protein kinase activity in case of HIV-1 transduced cells. Moreover, protein-coding transcripts from HIV-2 transduced cells were associated with RNA polymerase II-specific DNA binding transcription factor binding, DNA-binding transcription factor binding, phosphatidylinositol binding, Rho GTPase binding ability and guanyl-nucleotide exchange factor activity. Both viruses affected the expression of transcripts, products of which are involved in ubiquitination activity, a post-translational modification implicated in the regulation and degradation of various host proteins. This mechanism is crucial for the antiviral response, modulating innate RIG-like and Toll-like receptor signalling (Davis & Gack, 2015). Several HIV accessory proteins exploit the host cell ubiquitin system to initiate degradation of cellular restriction factors and other targets, such as targeting SAMHD1 by HIV-2 Vpx, APOBEC3G by Vif, and Tetherin by HIV-1 Vpu (Calistri et al., 2014) (Strebel, 2013).

Early-Phase Transcriptomic Changes

To further outline the changes in the cellular transcriptome caused by HIV-based vectors, we conducted examination of later time-points, 8, 12 and 26 hours to be exact. Following transduction with HIV-1, HEK-293T cells showed a dynamic fluctuation in the count of DETs, transitioning from 158 at 8 hours, to 114 at 12 hours, and eventually reaching 380 at 26 hours post-transduction. In contrast, at the 8-hour time point, HIV-2 exhibited a differential regulation

of 283 transcripts, a significantly higher number compared to HIV-1 at the same time point. By 12 hours, HIV-2 induced alterations in the expression of 299 transcripts, which decreased to 182 at 26 hours. The increased number of transcripts influenced by HIV-2 in the initial time points suggests a more varied impact on the transduced cell, followed by a plateau at 26 hours, in contrast to HIV-1, which consistently altered the transcriptome of the transduced cell.

At the eight-hour time point, HIV-1 upregulated the expression of the AKAP9 coding transcripts amongst others. The AKAP family of proteins have been identified as contributors to cyclic adenosine monophosphate (cAMP)-mediated signaling. Alterations in the cellular lever of AKAP proteins plays a role in various health conditions, such as immune deficiencies, cancers and chronic heart failure (Tröger et al., 2012). Altering the regulation of the cellular cAMP levels has been linked to T-cell dysfunction, which aids HIV by allowing the virus to evade host immune response (Moreno-Fernandez et al., 2012).

Furthermore, the cellular level of group and coiled-coil domain containing 2 (GCC2) encoding transcript was increased at 8 hours following transduction with HIV-1. GCC2 was shown to be involved in the downregulation of MHCI by Nef, and thus, ensures the protection of the host cell from CD8+ T-cell-mediated killing (Kumari et al., 2019).

The expression of cyclin-dependent kinase inhibitor 1 A (*CDKN1A*) was deregulated following HIV-1 transduction. Previous studies have shown that CDKN1A; via formation of complexes with the viral integrase, can inhibit the integration of the provirus into stem cells of hematopoietic origin (Zhang et al., 2007). Additionally, it has been reported that HIV-1 Vpr has the ability to activate the cellular expression of CDKNA1A, inducing cell-cycle arrest in macrophages and T cells, thus facilitating the integration of the pre-integration complex (PIC) (Vázquez et al., 2005) (Chowdhury et al., 2003).

In conclusion, the modulation of the cellular expression of CDKNA1A can positively impact the life cycle of HIV, particularly the events of reverse transcription and integration. However, this regulatory mechanism may be tied to other cellular factors, and could exhibit variability depending on the type of infected cell.

Transcripts which encode for proteins that play a role in the DNS structure maintenance were expected to be detected around 15 hours following transduction, as genome integration typically begins around that time–point (Murray et al., 2011). Indeed, we detected the upregulation of protein coding transcripts related to the repair of DNA breaks such as *NBN* and *BRCA2* at 12 hours post-transduction (Roy et al., 2011) (Varon et al., 1998).

HIV-1, at 12 hours post-transduction also increased the cellular level of *COX20*, a cytochrome C oxidase member and an integral contributor to the mitochondrial respiratory chain complex

IV (Li et al., 2022). During HIV-1 uncoating the p2 peptide is released which activates the cytochrome C oxidase thus increasing ATP production which is required for efficient reverse transcription and nuclear import of the PIC (Ogawa et al., 2015). During HIV replication, Tat protein hinders the activity of cytochrome C oxidase, resulting in Tat-mediated apoptosis (Lecoeur et al., 2012).

26 hours after transduction, HIV-1 induced the upregulation of protein coding transcripts that are associated with the oxidative stress response. HIV-1 is able to increase the production of reactive oxygen species which leads to the disruption of the oxidative stress pathways and the induction of mitochondrial dysfunction (Ivanov et al., 2016). The cellular level of transcripts encoding for the NQO1, HMOX1 and OSGIN1 were increased by HIV-1, which indicates an active defence against the generated oxidative stress.

Similar to the previous time-points, alterations induced by transduction with HIV-2 pseudovirions at later time-points differed significantly from those caused by HIV-1.

Transduction with HIV-2 significantly increased the cellular level of a transcripts encoding for the DCN, a protein involved in cell proliferation and intracellular communication in all of our observed time-points. Increased expression of DCN can initiate apoptosis via caspase-3 and initiate cell cycle arrest at the G0-G1 phase through the upregulation of p21 (Wu et al., 2008) (Ständer et al., 1999).

HIV-2 also increased the cellular level of *S100A6* transcript, which encodes a protein with a calcium-binding ability that is involved in stress response, cytoskeletal functions and cell proliferation. Increased expression of S100A6 was associated with cell proliferation and migration in cancer cells (Li et al., 2014) (Duan et al., 2014). The alteration of transcripts associated with cell proliferation are important for the integration of the viral DNA. Furthermore, the modulation of cell proliferation is crucial for the expansion of the viral reservoir and it is an important part of HIV's impact on the host cell (Virgilio & Collins, 2020) (Sieg et al., 2001).

Among the transcripts differentially regulated by HIV-2 at 12 hours post-transduction, were transcripts encoding lipid homeostasis involved proteins. Individuals with HIV infection and with subsequent therapy tend to develop abnormalities in their lipid homeostasis, a condition termed lipodystrophy (Baril et al., 2005) (Giralt et al., 2006). The cellular transcriptomic level of a robust biomarker of HIV-induced metabolic syndrome and lipodystrophy; FABP4, was elevated by HIV-2 but not by HIV-1 even at later time-points (Coll et al., 2008).

The downregulation of ABCG1, a pivotal transporter involved in cholesterol efflux was indeed an important finding. It is known that HIV-1 Nef can inhibit the cholesterol efflux by

downregulating ATP binding cassette subfamily a member 1 (ABCA1), with little impact on ABCG1. Dysregulation of cholesterol homeostasis in infected cells plays a critical role in the replication of HIV, as adequate supply of cholesterol is important for the generation of virions. Through the induction of Toll-like receptor (TLR) signalling, inhibition of cholesterol efflux induces the differentiation of inflammatory macrophages (Mujawar et al., 2006) (Yvan-Charvet et al., 2010). Given that HIV-2 didn't exert an effect on *ABCA1*; even at later time-points, but had an effect on *ABCG1*, it appears that HIV-2 has a limited impact on cellular cholesterol levels. Furthermore, it is possible that the early dysregulation of cellular cholesterol during the life cycle of the virus may induce an early immune response against HIV-2-infected cells through the induction of inflammation.

Just as observed at earlier time-points, transduction with HIV-2 increased the expression of transcripts coding for components of the intra- and extracellular matrix, including *KRT4, 5, 8, COL1A2* and *COL6A3*. At the same time, HIV-1 only managed to affect the cellular level of collagens; such as *COL11A1*.

Both HIVs managed to influence the cellular level of *EGR1*, a transcription factor involved in the Tat-dependent HIV gene expression. EGR1 plays a prominent part in the therapeutic strategy called "kick and kill" wherein cells which are latently infected are subjected to latency reversal agents to reactivate dormant HIV reservoirs. Following treatment with various agents, EGR1 was shown to be upregulated, and it has been established that EGR1 directly interacts with the HIV-1 promoter, thereby inducing proviral transcription (Woodson & Kehn-Hall, 2022) (Wong et al., 2022).

Furthermore, both HIVs were observed to decrease the expression of *RelB*. Previous studies have demonstrated that viral components, such as human T-lymphotropic virus 1 (HTLV1) Tax1, bovine foamy virus (BFV) transactivator (Btas) and HIV-1 Vpr, can interact with RelB and in the end facilitate virus replication (Wang et al., 2018). Silencing of RelB was revealed to initiate the arrest of the cell cycle at the G1 phase, which promotes HIV gene expression. Intriguingly, HIV-1 Tat protein, is able to induce G1 arrest, and was also shown to interact with RelB to enhance the gene expression of the virus (Wang et al., 2018) (Ge et al., 2016) (Kundi et al., 1998). Furthermore, through the generation of a G1-like state in infected macrophages, HIV-1 is able to bypass SAMHD1 mediated restriction and facilitate viral replication (Mlcochova et al., 2017). In summary, it is possible that Tat protein of HIV-1; through the downregulation of RelB, can initiate G1 arrest. Meanwhile, the virus might use the available RelB in order to bypass cellular restrictions in the early-phase, and enhance gene expression during the later stages of infection.

The cellular level of *CAMK1D* was decreased by both HIVs. In correlation with our findings, the activity of CAMK1D is known to be downregulated by infection with HIV-1 (De Martini et al., 2019). Moreover, previous research showed that siRNA knockdown of CAMK1D negatively regulates infection with HIV-1 (Zhou et al., 2008).

Our data showed the differential regulation of pseudogenes in all of the observed time-points by both HIVs which is indeed a notable find. Traditionally considered non-functional DNA resulting from gene duplication events, frame-shifts and mutations, pseudogenes have recently been redefined due to their role in cellular homeostasis, showing the ability to influence the cellular level of their parent genes (Gupta et al., 2015). Emerging evidence suggests that self-derived mRNAs, including pseudogenes, have a crucial part in regulating immune responses against viruses and tumours. As an example, 15S and 5S ribosomal RNA derived pseudogenes, have been implicated in enhancing the expression of proinflammatory cytokines (Han et al., 2021)

Cells transduced with HIV-1 showed miniscule changes in the number of pseudogenes compared to control-transduced samples, while HIV-2 transduced cells displayed a significantly higher number of differentially regulated pseudogenes compared to HIV-1 and control. At 8 hours post-transduction in HIV-2 transduced cells, 77 pseudogenes showed differential regulation, this number increased to 93 at 12 hours and decreased to 78 pseudogenes at 26 hours. Amongst the HIV-2 affected pseudogenes were those originating from eukaryotic translation elongation factor alpha 1 (*eEF1a1*), protein disulfide isomerase family A member 3 (*PDIA3*), and nucleophosmin 1 (*NPM1*). eEF1a1 has been identified as a reverse transicpriton cofactor, playing a prominent role in the stabilization of the reverse transcriptase complex (Warren et al., 2012) (Rawle et al., 2018). Disulfide isomerases like PDIA3 facilitate the rearrangement of disulfide bonds in gp120 during its interaction with CD4, leading to the fusion of viral surface glycoprotein with the host cell membrane (Ou & Silver, 2006) (Fenouillet et al., 2001). NPM1 has been shown to interact with HIV-1 Tat, also, HIV-1 infection initiates the acetylation of NPM1, a mechanism crucial for Tat-mediated transcription and Tat nuclear localization (Gadad et al., 2011).

As pseudogenes traditionally known as negative regulators of gene expression, the differential regulation we observed in the expression of several pseudogenes might contribute to the unique effect HIV-2 has on the host cell.

Similar to earlier time points, we employed GO analysis to elucidate differences between cells transduced with HIV-1 and HIV-2. At 8 hours, HIV-1 had a pronounced influence on cellular metabolism, as shown by the significant GO terms such as positive regulation of RNA

metabolic processes, regulation of primary metabolic processes and positive regulation of metabolic processes. In contrast, HIV-2 primarily influenced transcripts associated with keratinocyte differentiation, extracellular matrix organization or cell adhesion. Increased metabolic activity just as the one induced by HIV-1 increased viral replication and infectivity, as it is known that the inhibition of glycolysis leads to a concurrent inhibition of reverse transcription (Taylor & Palmer, 2020) (Kang & Tang, 2020).

Surprisingly, no significant GO terms were detected at the 12-hour time point from cells transduced with HIV-1. We detected the lowest number of altered transcripts at 12 hours compared to other time points from HIV-1-infected HEK-293T cells. Meanwhile, at the 12-hour mark, HIV-2 affected animal organ development, system development and cell differentiation related genes. At the 26 hour time-point, HIV-1 differentially regulated transcripts which are associated with the response to detoxification, cellular response to toxic substances, and oxidative stress. In contrast, HIV-2 showed less impactful influence on the cellular environment, affecting genes primarily related to the skin development, negative regulation of cell migration, and negative regulation of cell motility.

Proteomic Analysis: Key Findings

To further elucidate the changes detected from the transcriptomic analysis, proteomic investigation was also carried out. It was revealed by our analysis that in the first two hours of transduction, both virus managed to alter the expression of hnRNPA1, NONO, H1-4, HSPD1 and SRSF6. Moreover, HIV-2 also downregulated the expression of FKBP4 and CCT8.

hnRNPA1 belongs to a complex family of ribonucleoproteins and had an important role in the transcription, stability, and transport of newly synthesized cellular mRNAs. It has been shown that through binding to specific sites on the newly synthesized transcript, it can function as a splice inhibitor of HIV-1 *tat* mRNA upon expression from the integrated proviral genome (Tange et al., 2001). Tat is known for its highly apoptotic nature, so this inhibition proves advantageous for the virus through the prevention of overexpression of Tat. Furthermore, through Rev stimulation, hnRNPA1 facilitates the transport of viral mRNA to the cytoplasm from the nucleus (Hallay et al., 2006). It is known that hnRNPA1 has a crucial functional and regulatory role in viral infections. It is able to elevate viral infections such as in case of sindbis virus, enteroviruses and rhinoviruses. However, it shows an inhibitory effect in case of hepatitis C virus (HCV) and Human T cell lymphotropic virus (HTLV-1) infections (Kaur & Lal, 2020).

In the latter stages of infection, HIV-1 increases the expression and cytoplasmic relocation of hnRNPA1, aiming to optimize viral protein expression before the budding process, as demonstrated in previous research (Monette et al., 2009).

However, our finding shows a notable downregulation of hnRNPA1 levels by both HIV strains during the immediate early phase of transduction. This may perhaps mean that there is a cellular defence mechanism actively limiting the viral replication at this early stage.

Nuclear protein NONO has a role in transcriptional regulation and RNA splicing. It was shown to associate with reverse transcription and PIC during HIV infection (Schweitzer et al., 2013) (St Gelais et al., 2015). Overexpression of NONO leads to a decrease in the infectivity of HIV-1, which negatively impacts reverse transcription and viral gene expression in Jurkat cells. It has been postulated that the downregulation of reverse transcription is achieved either through direct contact with the reverse transcriptase complex or through indirect interaction with other components of the complex. However, the exact mechanism remains unclear (St Gelais et al., 2015). Additionally, NONO has a crucial part in the innate immune response against foreign DNA as part of the HEXIM1-DNAPK-Paraspeckles components-ribonucleoprotein complex (HDP-RNP) operating through the cGAS-STING-IRF3 pathway (Morchikh et al., 2017).

SRSF6 was an another protein downregulated by both HIVs. SRSF6 functions as an alternative splicing regulator which is able to interact with the splicing sites 3'ss A3 in Tat mRNA. Moreover, it may also be needed for the adequate activation of splicing. It was shown in a study that overexpression of the protein impairs viral gene expression by a not yet fully uncovered mechanism (Erkelenz et al., 2015).

Both HIVs managed to downregulate the cellular level of HSPD1 which through interaction with viral gp41 protein, is incorporated into newly formed virions (Speth et al., 1999). HSPD1 is also a part of the PIC, as during early phase of viral life cycle, the protein interacts with the HIV-1 integrase, safeguarding it against denaturation (Parissi et al., 2001). The inclusion of HPSD1 in the virions serves to ensure the stability and proper folding of the HIV integrase, particularly when cellular heat shock protein levels are diminished. Notably, previous investigations have shown that there is a correlation between the level of HPSD1 in serum and viral load, the levels of which show reduction during anti-retroviral therapy (Anraku et al., 2012). Not just HIV but other viruses are known to utilize HSPD1 for replication, such as influenza A and hepatitis B (Wyżewski et al., 2018) (Park et al., 2003).

There is little information about the involvement of H-1.4 histone protein in the life cycle of HIV. It was mentioned that it is detected at the site of latent viral LTR and may play a role in repressing Tat-mediated transcription (Gadad et al., 2011) (O'Brien et al., 2010).

As an important mediator of nuclear translocation, FKBP4 demonstrated antiviral effect against herpes simplex virus 1 (HSV-1) (Haas et al., 2018). Furthermore, it was discovered that in the brain, during HIV-generated inflammation and astrocyte activation, its levels are increased (Tatro et al., 2009). Additionally, studies have revealed an increase in the level of FKBP4 upon HIV-1 Tat expression in infected Jurkat cells (Jarboui et al., 2012).

The discovery of CCT8 downregulation, an important indicator of disease progression towards AIDS, was particularly intriguing. Levels of CCT8 were shown to correlate with progression towards AIDS (Spadoni et al., 2015). It is able to interact with Vif, but the significance of the interaction is not yet understood (Luo et al., 2016). The interesting observation that HIV-2 was able to downregulate the cellular level of CCT8 beyond the levels found in HIV-1 transduced cell raises questions about its involvement in the decreased pathogenicity of HIV-2.

Using STRING and GO terms, an intricate network of cellular processes in which the differentially regulated proteins are involved was visualized. The GO terms included cellular response to stress, protein folding, mRNA stability and mRNA splicing. It is noteworthy to mention that the initial low level of proteins may be overridden at later time-points later as the infection progresses.

Similarly, to the transcriptomic analysis, we conducted analysis of proteomic data from later time-points as well. It was revealed from our data that a significant portion of the altered proteins are connected to mRNA processing, proteasome and chaperon function. We did not detect any significantly regulated protein at 8 hours post-transduction, however, our analysis showed that both HIVs influenced the expression of 17 and 117 different proteins at 12 and 26 post-transduction, respectively.

HSPA1A, hnRNPK, PA2G4 and Zyx were altered at 12 hours post-transduction by both HIVs. In addition to actin polymerization and focal adhesion, Zyx also takes part in the intracellular signalling (Rauskolb et al., 2011). Besides its involvement in cell growth, apoptosis and differentiation, PA2G4 is also downregulated by HIV-1 Vpr, resulting in G2 arrest and apoptosis in U87MG cells (Zhang et al., 2014).

Only HIV-1 downregulated the expression of hnRNPQ, a member of a protein family involved in mRNA transcription, splicing, export, stability and translation. Through interaction with Rev protein, hnRNPQ also takes part in the proviral transcription of HIV-1. It is involved in the mRNA stability, splicing and RNA replication of Hepatitis C virus (Vincendeau et al., 2013).

At 26 hours, both viruses altered the cellular level of SRSF2 and TRIM28. SRSF2 proteins were found to be involved in the downregulation of HIV-1 replication through splicing of *tat* and *rev* transcripts (Jacquenet et al., 2005).

TRIM28 promotes HIV-1 latency through inhibition of the P-TEFb and initiating the SUMOylation of CDK9 along with SUMO4 (Ma et al., 2019).

At the 12-hour mark, HIV-2 downregulated the cellular level of NCAPH and PRRC2A, both of which play a prominent part in cell proliferation and migration (Sun et al., 2019) (Tan et al., 2023). To support viral replication and ensure survival, viruses; including retroviruses, manipulate the cell cycle and cellular proliferation (Bagga & Bouchard, 2014). The differential impact of HIV-2 on the expression of these two proteins warrants closer investigation.

In comparison to HIV-1, at 26 hours post-transduction, HIV-2 altered the cellular level of a slightly higher number of proteins. Amongst the differentially regulated proteins by HIV-2 were members of the HSP70 (1A, 5 and 9) and TFRC. With functions such as mediating folding, translocation and degradation of target proteins, HSP70 acts as a chaperone protein. It is also involved in HIV infection as it was shown to be capable of dose-dependent inhibition of HIV-1 in $CD4^+$ T cells (Babaahmady et al., 2007). It was also revealed that HPS70 may inhibit the ubiquitination and degradation of APOBEC3G by Vif (Sugiyama et al., 2011). HPS70 also played a role in facilitating the nuclear import of HIV-1 PIC by enhancing the interaction between the HIV-1 matrix and karyopherin alpha (Agostini et al., 2000).

TFRC is a membrane glycoprotein that mediates the uptake of transferrin-iron complex in a receptor-mediated endocytosis manner (Ponka & Lok, 1999). In a study, it was revealed that HIV infection correlates with induced cellular levels of iron, which increases the replication of HIV (Chang et al., 2015). In a T cell line, it was shown that HIV-1 Nef aids in diminishing the recycling pace of TFRC, leading to its build up in early endosomes and consequently decreasing its presence on the cell surface (Madrid et al., 2005). Notably, in another study, Nef protein of HIV-1 and its simian precursors showed no impact on the surface level of TFRC. Furthermore, in myeloid and T cell line, Nef protein from other lentiviruses showed an ability to reduce the internalization of the receptor (Koppensteiner et al., 2014). This different alteration of the levels of TFRC might be related to the stage of the viral life-cycle, and the type of the host cell. Additionally, TFRC has been found as a potential port of entry for the Influenza A virus (Mazel-Sanchez et al., 2023). Considering the broad range of receptors utilized by HIV-2, there is a possibility that the virus may also utilize TFRC. All in all, the different regulation of TFRC by HIV-2 is indeed a notable finding and warrants further investigation.

Moreover, HIV-2 was able to decrease the cellular level of RANBP1 compared to HIV-1. RANBP1 possesses a nuclear export signal (NES) domain which is analogous in both function and structure to the one present in HIV Rev, this may indicate that RANBP1 might share a step with Rev in the post-transcriptional pathway (Audia et al., 2023). The downregulation of

RANBP1 by HIV-2 prompts inquiries about the proteins role in hindering the transport of proviral transcripts.

Similarly, to the very early time point, GO term analysis was carried out from the differentially regulated proteins to further outline the differences between the two pseudovirions. In case of both HIVs, only a low number of GO terms were affected at 12 hours post-transduction. However, at the 26-hour time-point, both virus altered the expression of proteins related to post-transcriptional regulation of gene expression, regulation of DNA-templated transcription elongation and regulation of alternative mRNA splicing via the spliceosome. Moreover, differences were observed between the two viruses at the 26-hour time point. In case of HIV-1, GO terms like positive regulation of apoptotic processes, regulation of gene expression and cellular response to unfolded protein were present. Meanwhile, HIV-2 altered the expression of proteins related to positive regulation of translation, negative regulation of protein ubiquitination and negative regulation of DNA metabolic processes.

Jurkat Cell Transcriptomic Analysis

In order to gather more information about the effect of HIV-based pseudovirions on the host cells, we also carried out transcriptomic analysis on Jurkat T cells. As $CD4^+$ T-cells are the primary target of HIV, we wanted to reveal the effects of the viruses on T cells. We noted that similarly to HEK-293T cells, HIV-1 exerted a larger effect on the cells, represented by the higher number of DETs compared to HIV-2. We detected 39 at eight, 45 at 12, and 42 transcripts at the 26 hours time-point from HIV-1 transduced Jurkat. In contrast, HIV-2 only altered the expression of 15 at eight, 23 at 12 and 36 transcripts at 26 hours post transduction. Moreover, the majority of transcripts regulated differentially by HIV-2 were also altered by HIV-1. Transcripts such as, acyl-CoA synthetase short-chain family member 2 (*ACSS2*), 5-oxoprolinase (*OPLAH*) and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*) were similarly regulated by both virus at the different time points.

Eight hours post-transduction HIV-1 differentially regulated the expression of the insulin induced gene 1 (*INSIG1*). Research showed that INSIG1 is upregulated during HIV-1 infection and it acts as a sentinel responding to HIV replication and inhibits via the acceleration of HIV-1 gag protein degradation (Zhang et al., 2019). As we detected the downregulation of INSIG1 at 8 hours post-transduction, it is possible that during the early phase of infection, it has a yet undiscovered role in the viral life cycle.

HIV-1 managed to alter the expression of the fatty acid synthase (*FASN*) 12 hours post-transduction. Research showed that the replication of HIV-1 increases the cellular level of FASN and its activity is required for the successful life cycle of the virus. In the same study, Kulkarni et al. used FASN inhibitors to effectively block HIV replication. Moreover, FASN is also required for the replication of other viruses including cytomegalovirus (CMV), Dengue virus (DENV) and Epstein-Barr virus (EBV) (Kulkarni et al., 2017). We detected the downregulation of FASN instead of the upregulation observed in the previously mentions study. It's possible that the downregulation of FASN is an early effect of HIV-1 which is later reverted during replication. Additionally, FASN plays an important role in the differentiation, function and survival of various immune cells including T helper 17 (Th17) and regulatory T cells (Treg) (Xiao et al., 2024). In light of this, it is plausible that the early downregulation of FASN by HIV-1 may contribute to the immune dysfunction observed during HIV infection, aiding the virus to avoid immune surveillance (Mu et al., 2024).

Our data showed that both HIV-1 and HIV-2 are able to downregulate the expression of the aldolase, fructose bisphosphate C (*ALDOC*) 26 hours post transduction compared to control transduced cells. Downregulation of the metabolic pathways by HIV is a sign of HIV latency, and during viral reactivation, the negative effect on metabolism is reverted as elevated glycolysis is required for efficient viral replication (Shytaj et al., 2021) (Barrero et al., 2013).

An interesting finding was the differential regulation of transcripts, products of which are related to the mevalonate pathway. Transcripts such as, 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR1*), *HMGCS1* and mevalonate diphosphate decarboxylase (*MVD*) were altered in almost all of the observed time point by both viruses. HIV-1 further altered the expression of mevalonate kinase (*MVK*) at 12 hours post-transduction. The mevalonate pathway which is part of the cholesterol biosynthesis plays an important role in the proliferation, survival and effector functions of T cells (Thurnher & Gruenbacher, 2015) **(**Kennewick & Bensinger, 2023). Moreover, the mevalonate pathway also has a role in the activation of trained immunity, and the inhibition of the pathway reverts the increased cytokine production observed in activated immune cells (Bekkering et al., 2018)). Additionally, the upregulation of the cholesterol biosynthesis is noted in HIV-1 infected T cells which is only observed in the presence of a functioning Nef. The increased cholesterol synthesis is required for the efficient budding of the HIV virions from the cell surface (Zheng et al., 2003) **(**van't Wout et al., 2005). We observed the downregulation of mevalonate synthesis associated genes as an effect of HIV vectors in the early phase of transduction. As previously mentioned, the

mevalonate pathway contributes greatly to immunity, so we hypothesize that the observed reduction in the expression of mevalonate synthesis genes contributes to the HIV generated immune dysfunction (Mu et al., 2024). Inhibitors against the HMG-CoA reductase enzyme called statins are known to be decrease HIV-related co-morbidities such as HIV-associated cardiovascular disease (CVD) and non-Hodgkin lymphoma (Grinspoon et al., 2019) (Grinspoon et al., 2023) (Chao et al., 2011).

Similarly to the transduced HEK-293T cells, we carried out GO analysis to further reveal the affected biological processes. Both HIVs significantly altered genes that are part of the cholesterol biosynthetic process.

There were very few transcripts that were regulated simmilalry in both HEK293T and Jurkat cells by HIV-1 and HIV-2. Most of the detected transcripts were unique to the given cell line. One exception is the arginosuccinate synthase 1 (*ASS1*) which was downregulated by both HIV-1 and HIV-2 at 8 hours in HEK293T cells and also by HIV-1 at 8 hours in Jurkat cells. The product of this transcript is involved in the de novo synthesis of arginine, and also described in the context of Herpes simplex virus 1 (HSV1) infection as the knockdown of ASS1 enhances viral replication (Grady et al., 2013). However, there is no information about the involvement of ASS1 in the life cycle of HIV. Additionally, the previously described *CDKN1A* was also differentially regulated at 26 hours post transduction by HIV-1 in both HEK293T and Jurkat cells. Moreover, HIV-2 at 26 hours post transduction significantly downregulated the expression of the earlier discussed *EGR1* and *HMGCS1* in both cell type.

Conclusion and Future Perspective

In conclusion, the analysis of very early and early time points of HIV-1 and HIV-2 transduction revealed significant differences between the two HIV based pseudovirions. These observations may contribute to a better understanding of the pathomechanisms associated with lentiviral vector transduction. Additionally, our results offer potential insights into the distinctive features of HIV-2's replication cycle, an area that needs future investigations. For future research involving other cell lines such as monocyte based ones might be a good addition to the knowledge pool about HIV generated proteo-transcriptomic changes. Moreover, a study involving primary cells such as $CD4^+$ T cells might provide a further information about the early phase of lentiviral transduction.

It is important to note that most of the findings from the proteomic analysis did not correlate with those from the transcriptomic analysis. Changes observed at the transcriptomic level are not always reflected at the proteomic level due to post-transcriptional and post-translational regulatory mechanisms that influence protein expression. Furthermore, the cellular concentration of certain proteins may already be sufficient, eliminating the need for additional synthesis. Additionally, the experimental methodologies and the relatively long intervals between the time points likely limited the potential for direct correlation between the two data sets at corresponding time points (Zapalsa-Sozoniuk et al., 2019).

Summary

Our goal was to analyse the effect of HIV-1 and HIV-2 pseudovirions on the cellular transcriptome and proteome focusing on the early phase of transduction. Moreover, we wanted to shed light on the unique characteristics of HIV-2 as research on this virus remains limited.

In the very early phase of HIV transduction, significant changes are detected between HIV-1 and HIV-2 transduced HEK-293T cells. HIV-1 managed to alter the expression of a diverse array of transcripts including DNA repair proteins, transporters and chaperons. Meanwhile at the same time-point, changes generated by HIV-2 were less diverse and mainly affected transcripts coding for members of the extra and intracellular matrix. At later time-points, this pattern was also observed as HIV-1 continuously modified the cellular transcriptome, whereas HIV-2 initially had a varied effect but reached a plateau at 26 hours post-transduction. Product of transcripts altered by HIV-1 function as signalling, transport and oxidative stress involved molecules. While HIV-2 influenced the expression of transcripts involved in lipid homeostasis. As observed in early time points, HIV-2 had a significant effect on transcripts coding for proteins involved in the remodeling of extra and intracellular matrix, including keratins and collagens. Utilizing the altered protein coding transcripts, GO analysis was carried out, which revealed multiple common pathways influenced by both HIV strains. Moreover, significant differences were also observed between the two viruses; such as the differential regulationof oxidative stress-related genes by HIV-1 at 26 hours post-transduction.

A notable find was the differential alteration of the cellular level of pseudogenes by both HIV strains. Compared to HIV-1, HIV-2 exhibited a greater capacityto alter the cellular level of pseudogenic RNA. As pseudogenes are described as negative regulators of gene expression, the impact of HIV-2 on pseudogene expression may provide insights into its unique pathophysiology.

To further delineate the differences caused by the HIV transduction, proteomic analysis was carried out alongside the transcriptomic investigation. Analysis of the very early phase of HIV transduction revealed five proteins that were differentially regulated by both HIV strains, and an additional two which were influenced only by HIV-2. Amongst the altered proteins were molecules with role in mRNA splicing and protein folding. We did not detect any significantly altered protein at eight hours post-transduction. However, at the 12 hour time- point, 17 different proteins were altered by HIV with roles in intracellular signalling, apoptosis and mRNA splicing. 26 hours post–transduction, a total of 117 protein were differentially regulated by HIV-1 and HIV-2. The functions of the altered proteins were diverse with roles in mRNA splicing, cell proliferation and protein folding.

GO analysis of the differentially regulated proteins revealed further similarities and significant differences between the two HIV types. Both viruses managed to alter proteins with roles in post-translational regulation of gene expression and regulation of alternative mRNA splicing via the spliceosome. Meanwhile, only HIV-1 influenced the expression of proteins taking part in cellular response to unfolded protein, while HIV-2 influenced the expression of negative regulation of protein ubiquitination involved proteins.

Analysis of transduced Jurkat cells revealed further differences between the two virusess. HIV-1 altered a greater number and diversity of transcripts compared to HIV-2. Both virus altered the expression of transcripts involved in the cholesterol biosynthetic processes including key genes such asHMGCR and MVD.

In conclusion, we hope that our study helps researchers better understand the complex cellular changes induced by HIV-1 and HIV-2 infection, as well as transduction with lentivirus-based vectors. Furthermore, we aim to contribute to a deeper understanding of the unique characteristics of HIV-2.

Összefoglalás

Kutatásunk célja az volt, hogy elemezzük HIV-1 és HIV-2 pszeudovirionok a sejt transzkriptomjára és proteomjára gyakorolt hatását a fertőzés korai fázisára összpontosítva. Továbbá szerettük volna tanulmányozni a HIV-2 fertőzés karakterisztikáját mivel nem sok erre fókuszáló kutatás létezik.

A HIV transzdukció nagyon korai fázisában jelentős változásokat észleltünk HIV-1 és HIV-2 fertőzött HEK293T sejtek között. A HIV-1 számos transzkriptum expresszióját képes volt megváltoztatni beleértve DNS javító fehérjéket, transzportereket és chaperonokat. Ezzel szemben a HIV-2 okozta változások kevésbe voltak sokfélék és főleg az extra és intracelluláris mátrix tagjait kódoló transzkriptumokat érintették. Ezeket a változásokat későbbi időpontokban is megfigyeltük, ahogy a HIV-1 folyamatosan befolyásolta a sejt transzkriptomját a HIV-2-vel szemben, ami a korai időpontokban változatos hatást gyakorolt a transzkriptumokra de a transzdukció után 26 órával elért egy platót. A HIV-1 által befolyásolt transzkriptumok termékei többek között részt vesznek a szignalizációban, transzportban és oxidatív stresszben. Ezzel szemben a HIV-2 által megváltoztatott expressziójú transzkriptumok fontos szerepet játszanak a lipid homeosztázisban. Továbbá ahogy a korai időpontokban is észleltük a HIV-2 szignifikáns hatást gyakorolt az extra és intracelluláris mátrixot alkotó keratinokra és kollagénekre. A fehérje kódoló transzkriptumokat felhasználva gén ontológiai elemzést végeztünk, ami felfedett számtalan HIV-1 és HIV-2 által egyaránt befolyásolt útvonalat. Továbbá jelentős különbségeket is felfedeztünk a két vírus között, mint például az oxidatív stressz szabályozásában szerepet játszó fehérje kódoló transzkriptumok HIV-1 általi eltérő szabályozását.

Egy érdekes felfedezés volt a HIV vírusok hatása a pszeudogének szabályozására. HIV-1-hez hasonlítva a HIV-2 nagyobb hatást gyakorolt a pszeudogéneket kódoló transzkriptumok sejt béli szintjére. A pszeudogéneket a gén expresszió negatív szabályozóinak tartják, szóval a hatás amit a HIV-2 mutatott a kifejeződésükre talán hozzájárulhat a HIV-2 fertőzés egyedi sajátosságainak megértéséhez.

Hogy további adatokra tegyünk szert a HIV vírusok sejtre gyakorolt hatásáról proteomikai elemzést végeztünk a transzkriptomikai analízis mellett. A nagyon korai fázisban detektált fehérjék közül öt proteint mind a HIV-1 és HIV-2 képes volt szabályozni, további kettőt csak a HIV-2 befolyásolt. Az eltérő szabályozású fehérjék többek között részt vettek az mRNS splicingban és fehérje foldingban. Nyolc órával a transzdukció után nem detektáltunk további szignifikánsan befolyásolt fehérjéket. Azonban 12 órával a HIV-1 és HIV-2 transzdukció után összesen 17 fehérje kifejeződése változott. Ezen fehérjék szerepet játszanak az intracelluláris jelátvitelben, apoptózisban és mRNS splicingban. Továbbá az 26 órás időpontnál mindkét vírus szignifikánsan befolyásolta 117 fehérje kifejeződését, többek között mRNS spilcingban, sejt proliferációban és fehérje foldingban szerepet játszó fehérjék szintje változott.

A szignifikánsan változott fehérjék gén ontológiai elemzése további hasonlóságokat és jelentős különbségeket fedett fel a két HIV vírus között. Mindkét vírus képes volt befolyásolni a post translational regulation of gene expression és regulation of alternative mRNA splicing via the spliceosome funkciókban szerepet játszó fehérjék expresszióját. Azonban csak a HIV-1 volt hatással a cellular response to unfolded protein-ben részt vevő fehérjék kifejeződésére. Ezzel szemben a HIV-2 szignifikánsan befolyásolta a negative regulation of protein ubiquitination-ben szerepet játszó fehérjék expresszióját.

A transzduktált Jurkat sejtek elemzése további különbségeket tárt fel a két HIV között. Megállapítottuk, hogy a HIV-1 a HIV-2-höz képest több és változatosabb transzkriptumokat tudott megváltoztatni a fertőzött sejtekben. Mindkét vírus hatással volt a koleszterin bioszintézis folyamataiban részt vevő transzkriptumok expresszióját olyan érintett génekkel, mint a HMGCR és az MVD.

Mindent összevetve reméljük, hogy az eredményeinkkel segíthetünk megérteni a HIV-1 és HIV-2 és a belőlük létrehozott lentivirálsi vektorok által generált komplex változásokat. Továbbá reméljük, hogy tanulmányunkkal közelebb kerülhetünk ahhoz, hogy megértsük a HIV-2 egyedi tulajdonságait.

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Registry number: DEENK/10/2025.PL
Subject: PhD Publication List

Candidate: Tamás Richárd Linkner
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Linkner, T. R.**, Ambrus, V. A., Kunkli, B., Szojka, Z., Kalló, G., Csősz, É., Kumar, A., Emri, M., Tőzsér, J., Mahdi, M.: Comparative Analysis of Differential Cellular Transcriptome and Proteome Regulation by HIV-1 and HIV-2 Pseudovirions in the Early Phase of Infection.
   Int. J. Mol. Sci. 25 (1), 1-26, 2024.
   DOI: http://dx.doi.org/10.3390/ijms25010380
   IF: 4.9 (2023)
2. **Linkner, T. R.**, Ambrus, V. A., Kunkli, B., Szojka, Z., Kalló, G., Csősz, É., Kumar, A., Emri, M., Tőzsér, J., Mahdi, M.: Cellular Proteo-Transcriptomic Changes in the Immediate Early-Phase of Lentiviral Transduction.
   Microorganisms. 9 (11), 1-21, 2021.
   DOI: http://dx.doi.org/10.3390/microorganisms9112207
   IF: 4.926

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List of other publications

3. Miltner, N., **Linkner, T. R.**, Ambrus, V. A., Al-Muffti, A. S., Ahmad, H., Mótyán, J. A., Benkő, S., Tőzsér, J., Mahdi, M.: Early suppression of antiviral host response and protocadherins by SARS-CoV-2 Spike protein in THP-1-derived macrophage-like cells.
Front. Immunol. 13, 999233, 2022.
DOI: http://dx.doi.org/10.3389/fimmu.2022.999233
IF: 7.3

Total IF of journals (all publications): 17,126
Total IF of journals (publications related to the dissertation): 9,826

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

16 January, 2025

DEBRECENI EGYETEM
Egyetemi és Nemzeti Könyvtár

Keywords
HIV-1, HIV-2, transcriptomic analysis, proteomic analysis, lentivirus, viruses, viral infection, cell biology, host response

Kulcsszavak
HIV-1, HIV-2, transzkriptomikai analízis, proteomikai analízis, lentivírusok, vírusok, virus ferzőtés, sejt biológia, szervezet válasz

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Appendix