INVESTIGATION OF THE IMPACT OF HUMAN IMMUNODEFICIENCY VIRUS ON CELLULAR SUMOYLATION

by

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ABSTRACT

INVESTIGATION OF THE IMPACT OF HUMAN IMMUNODEFICIENCY VIRUS ON CELLULAR SUMOYLATION

The manifestation of Human Immunodeficiency Virus type 1 (HIV-1) infection is acquired immunodeficiency syndrome (AIDS), one of the major health problems worldwide. From the first step of infection to the end, HIV-1 customizes numerous mechanisms of the host cell to optimize its replication, thus invading the immune system. Sumoylation is an essential regulator of the immune system through modulating different immune signaling pathways, including interferon signaling. SUMO proteins are the downstream effectors of interferon to antagonize bacterial and viral infections. Consecutively, various pathogens oppose sumoylation to neutralize immune responses. In this study, we investigated the interplay between host sumoylation and HIV-1. Our study demonstrates that HIV-1 diminishes cellular sumoylation by antagonizing the UBA2 protein, a subunit of the E1 SUMO-activating enzyme. HEK293 and Jurkat cells display abrogated sumoylation profiles by SUMO1 and SUMO2/3 when the HIV-1 genome is expressed. HIV-1 expression in HEK293 and Jurkat cells suppresses UBA2 protein levels as well. Therefore, HIV-1 targets cellular sumoylation by most probably antagonizing UBA2. Altogether, we demonstrated that HIV-1 impairs sumoylation, a cellular mechanism vital for immunity.

ÖZET

İNSAN BAĞIŞIKLIK YETMEZLİĞİ VİRÜSÜNÜN HÜCRESEL SUMOLASYON ÜZERİNDEKİ ETKİSİ

İnsan Bağışıklık Yetmezliği Virüsü tip 1 (HIV-1) enfeksiyonunun tezahürü, dünya çapında önemli sağlık sorunlarından biri olan kazanılmış bağışıklık yetmezliği sendromudur (AIDS). Enfeksiyonun ilk adımından sonuna kadar HIV-1, replikasyonunu optimal hâle getirmek ve böylece bağışıklık sistemini istila etmek için konakçı hücrenin sayısız mekanizmasını kendi lehine kullanır. Sumolasyon, interferon sinyali de dahil olmak üzere farklı bağışıklık sinyal yolaklarını modüle ederek bağışıklık sisteminin temel bir regülatörüdür. SUMO proteinleri, bakteriyel ve viral enfeksiyonlara karşı interferonun alt efektörleridir. Bunun yanında, çeşitli patojenler, bağışıklık tepkilerini nötralize etmek için sumolasyona saldırırlar. Bu çalışmada, konak sumolasyonu ile HIV-1 arasındaki etkileşimi araştırdık. Çalışmamız, HIV-1'in, E1 SUMO-aktive edici enzimin bir alt birimi olan UBA2 proteinini hedefleyerek hücresel sumolasyonu azalttığını göstermektedir. HEK293 ve Jurkat hücreleri, HIV-1 genomu ifade edildiğinde hem SUMO1 hem de SUMO2/3 ile sumolasyon profillerinde ciddi bir düşüş gösterdi. HEK293 ve Jurkat hücrelerinde HIV-1 ekspresyonu, UBA2 protein seviyesinde de azalmaya sebep oldu. Bu nedenle, HIV-1, büyük olasılıkla UBA2'ye saldırarak hücresel sumolasyonu hedeflemektedir. Tümüyle, HIV-1'in bağışıklık için hayati önem taşıyan hücresel bir mekanizma olan sumolasyonu tahrip ettiğini gösterdik.

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Cq	Chloroquine
D	Aspartic acid
E	Glutamic acid
G	Glycine
g	Gram
K	Lysine
L	Liter
M	Molar
mM	Milimolar
ng	Nanogram
V	Volt
x	Any amino acid
xg	g force or relative centrifugal force
α	Greek letter alpha representing different protein subunits
β	Greek letter beta representing different protein subunits
κ	Greek letter kappa representing different protein subunits
Ψ	Packaging signal
ψ	Hydrophobic residue
μg	Microlgram
μL	Microliter
μM	Micromolar
μm	Micrometer
℃	Degree Celsius

LIST OF ACRONYMS/ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Combinatorial Antiretroviral Therapy
BAF	Barrier-to-Autointegration Factor
CA	HIV Capsid Protein
DC	Dendritic Cell
DC-SIGN	DC-Specific Intercellular Adhesion Molecular 3-Grabbing Non-Integrin
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
EGFP	Enhanced Green Fluorescent Protein
ESCRT	Endosomal Sorting Complexes Required for Transport
ER	Endoplasmic Reticulum
HEK293	Human Embryonic Kidney 293 Cells
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type 1
HIV-2	Human Immunodeficiency Virus Type 2
HSV	Herpex Simplex Virus
IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	IκB Kinase
IRES	Internal Ribosome Entry Site
IRF	Interferon Regulatory Factor
LEDGF/p75	Lens Epithelium Derived Growth Factor
LLO	Listeriolysin O
LTR	Long Terminal Repeat
MA	HIV Matrix Protein
NC	HIV Nucleocapsid Protein
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

NPC	Nuclear Pore Complex
PBS	Primer Binding Site
PCP	*Pneumocystis carinii* Pneumonia
PIC	Pre-Integration Complex
PKA	Protein Kinase A
PKC	Protein Kinase C
PML	Promyelocytic Leukemia Protein
PPT	Poly-Purine Tract
Psi	Packaging Signal
PTM	Post-Translational Modification
RNA	Ribonucleic Acid
RNA Pol	RNA Polymerase
RRE	Rev Response Element
RT	HIV Reverse Transcriptase Protein
SIM	SUMO-Interacting Motif
SIV	Simian Immunodeficiency Virus
STUbL	SUMO-Targeted Ubiquitin Ligase
SUMO	Small Ubiquitin-like Modifier
SUMO1	Small ubiquitin-like Modifier 1
SUMO2/3	Small ubiquitin-like Modifiers 2 and 3
TAR	Transactivation Response Element
UPS	Ubiquitin-Proteasome Sysmtem

1. INTRODUCTION

"Life, although it may only be an accumulation of anguish, is dear to me, and I will defend it," says Mary Shelly in Frankenstein; or, The Modern Prometheus. Human societies have always experienced diseases and tried to grasp their base throughout history. Some interpreted disease as divine retribution, and some attempted to control it. They observed while dying. They survived while adapting. The history of disease manifests itself in the history of Homo sapiens. The human will to live concentrated the knowledge on diseases to perceive the foundation to overcome the condition. The developing biomedical knowledge established the Germ Theory as the link between disease and pathogens. The other environmental factors pursued the discovery of pathogens. Science has augmented, the genetics has emerged. The more we could master the nature of the disease, the more we would manipulate it.

1.1. Acquired Immunodeficiency Syndrome and Human Immunodeficiency Virus

Biomedical practices have been allowing us to construct a fundamental understanding of a disease. The growing biotechnology enables us to spawn new tools to examine and contemporary ways to wield diseases, yet another emerges. In 1981, a case study of 5 homosexual men with Pneumocystis carinii pneumonia (PCP) was reported in Los Angeles. Additional cases with PCP and Kaposi's sarcoma followed this report that year. By these increasing opportunistic infections, a new disease called "acquired immunodeficiency syndrome" (AIDS) was recognized (Sharp & Hahn, 2011). Human Immunodeficiency Virus (HIV), the infectious agent of AIDS, was isolated (Barré-Sinoussi et al., 1983) and characterized as transmitted via sexual, percutaneous, and perinatal routes (Hladik & McElrath, 2008).

AIDS patients were documented very carefully after the recognition of the disease. Although the first studies described AIDS as a "disease spread among homosexual men," this common belief was punctured by the studies reporting intravenous drug

users and hemophiliacs with AIDS independent of sexual orientation and behaviors. Clinical examination was the primary methodology to diagnose AIDS due to the lack of information on the causative agent. The main criteria outlining the disease were the immunosuppression with no unknown origin and an opportunistic infection or an infrequent neoplasm. The collective diagnostic methodology revealed rapidly increasing cases with more than 3000 patients with an approximately 50% mortality in 2 years. The clinical findings represented a high lethality rate with opportunistic infections and malignancies with the depletion of helper T cells. The autopsy findings acknowledged the presenting illness and the cause of death varying among patients, including pulmonary hemorrhage, cardiac arrythmia, and malnutrition. (Reichert et al., 1983). HIV infection also manifests in neurological disorders (Kaku & Simpson, 2014; Schütz & Robinson-Papp, 2013).

The descriptive, diagnostic, and autopsy reports assembled a library of knowledge on AIDS despite the unknown link between the causative agent and the disease. However, the isolation of a novel T-lymphotropic retrovirus based a breakthrough in AIDS research (Barré-Sinoussi et al., 1983). This retroviral invasion could develop in numerous anatomical locations, including genital tracts, intestinal tract, placenta, and bloodstream, depending on diverse transmission media, resulting in both horizontal and vertical transmission capability of HIV (Hladik & McElrath, 2008). The topologies of viral entry sites equip HIV with a vast amount of possible transmission routes. The cellular composition of epithelia, the endocytosis, exocytosis and transcytosis events of epithelial cells, and the abrasions of mucosal surface due to mechanical or chemical assaults could contribute to the viral invasion pathways (Hladik et al., 2007; Hladik & McElrath, 2008; Howell et al., 1997; Maher et al., 2005). Exposure to HIV manifests sequential progression patterns as 6 different stages despite the discrete transmission routes. Only the viral RNA can be detected in the early HIV infection, stage I. The viral capsid protein p24 presents itself in addition to the viral RNA in stage II. While the viral load grows exponentially, the viral RNA peaks at the stages II to III transition. Then, the viral load starts to decrease and stabilize, and new detection strategies arise (Cohen et al., 2011; Emau et al., 2006; Fiebig et al., 2003; Routy et al., 2015). Although the increasing viral load depletes the $CD4^+$ T cells, the stabilization of lower

RNA copy number preserves the T lymphocyte count. This balanced stage signifies the clinical latency of HIV infection, deviating from the acute infection phase. This latency is ruptured by an exponential growth in viremia from the latent HIV reservoirs and an exponential reduction in $CD4^+$ T cell count, resulting in AIDS (Cohen et al., 2011; Selinger & Katze, 2013).

HIV infection is still exceedingly widespread with an immense quantity of new cases annually (Gökengin et al., 2016; Taylor, 2018). Although contemporary strategies are unable to disintegrate the HIV genome from hosts and to eliminate HIV reservoirs, the viral replication is blocked with combinatorial antiretroviral therapy (ART) targeting reverse transcriptase, protease, and integrase. The application of ART augments the immune system and lowers the immunodeficiency-induced opportunistic syndrome and transmission risks in line with the reduced viral loads (Huot et al., 2018; Robbins et al., 2014; Saez-Cirion & Müller-Trutwin, 2019; Simon et al., 2006; Sutton et al., 2001), thus, increases the survival of patients.

The causative agent, HIV, is a retrovirus primarily infecting $CD4^+$ T cells. However, viral replication is not limited to helper T lymphocytes but develops in macrophages and dendritic cells as well (Cohen et al., 2011; Colomer-Lluch et al., 2020). This subgroup of retrovirus consists of two different viruses: HIV type 1 (HIV-1) and HIV type 2 (HIV-2) originated from two distinctly related primate lentiviruses, simian immunodeficiency virus (SIV) emerged in chimpanzees and sooty mangabey monkeys respectively (Hahn et al., 2000; Hirsch et al., 1989; Huet et al., 1990; Rambaut et al., 2004; Sharp & Hahn, 2011).

1.2. Replication Cycle of Human Immunodeficiency Virus

The replication cycle of HIV is a fundamental element in fathoming the molecular pathogenesis induced by HIV. The characterization of sequential events in HIV infection precipitates the knowledge of viral machinery, resulting in the developing strategies against the disease. Therefore, to perceive the cellular narrative, recognition of the viral genome is also pivotal. The genomes of HIV-1 and HIV-2 are positive-sense

single-stranded RNAs, comparable but not identical in sequence since they originated from two different SIV species. The genome architecture illustrates complex structures enclosed by two identical long terminal repeats (LTR). U3, R, and U5 regions reside within the LTRs. The packaging signal (Psi, Ψ) locates at the end of 5' LTR (Kuzembayeva et al., 2014), followed by *gag* gene. The *pol* gene pursues the *gag* gene with an overlapping region of approximately 200 bp (Jacks et al., 1988a; Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985). The composition of HIV-1 and HIV-2 genomes diverge at the end of *pol* gene and converges at the beginning of *env* gene at the downstream of 3' LTR. The *gag*, *pol* and *env* genes forms the essential/structural genes of both HIV species. The viral gene classification demonstrates two other classes in the HIV genome: regulatory and accessory genes. The regulatory genes of both HIV species are *tat* and *rev*, but the composition of accessory genes varies. The genomes of both species encompass 4 accessory genes, 3 of which overlap: *vif*, *vpr* and *nef*. However, *vpu* is only present in the HIV-1 genome and *vpx* exclusively dwells in the HIV-2 genome. The layout of accessory genes within these genomes has peculiar patterns with coinciding arrangements (Colomer-Lluch et al., 2020; Kuzembayeva et al., 2014; Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985).

HIV genome accessorizes virions with the polypeptide env, gp160, encoded by viral *env* gene. This polypeptide forms trimers, heavily glycosylated in the Endoplasmic Reticulum (ER) and go under proteolytic cleavage in the Golgi, resulting in mature spike proteins gp120 and gp41 (Fenouillet & Jones, 1995; Gu et al., 1995; Hallenberger et al., 1992; Reitter et al., 1998; Wei et al., 2003). The non-covalent interactions between gp120 and gp41 physically hold these two mature proteins together as well as their trimeric structure. In order to infect the cells, HIV requires a partially specific physical interaction with its host through various cellular factors including $\alpha 4\beta 7$ integrin, negatively charged cell-surface heparan sulfate proteoglycans or a DC-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN) and viral env protein (Arthos et al., 2008; Cicala et al., 2009; Geijtenbeek et al., 2000; Saphire et al., 2001; Wilen et al., 2012). This virion-host association starts the sequential interaction events. These begin with the viral spike protein env directly interacting with CD4 protein, the primary receptor of HIV on host cells. To discern the interplay, fathoming

the structural basis of gp120 is crucial. This glycosylated protein has 5 conserved and 5 variable domains. Variable domains 1-4 build loop structures due to disulfide bonds. The interaction of gp120 with CD4 displaces the variable loops 1 and 2, resulting in the rearrangement of the variable loop 3 by the formation of four-stranded β sheets (Chen et al., 2005; Hartley et al., 2005; Kwong et al., 1998; Maddon et al., 1986; Mcdougal et al., 1986; Wilen et al., 2012). Then, the sequential conformational changes in gp120 allow the spike protein to recognize a chemokine co-receptor, either CXCR4 or CCR5 (Berger et al., 1998). This interaction triggers further conformational changes in the spike protein, specifically in gp41. This hydrophobic protein exposes a fusion peptide to its host's plasma membrane, generating a six-helix bundle from amino-terminal helical regions and a carboxy-terminal helical region of gp41. Six-helix bundle, bridging the viral and host membranes, tethers energy to introduce a fusion pore (Chan et al., 1997; Melikyan, 2008; Weissenhorn et al., 1997; Wilen et al., 2012), delivering the viral particles into the host's cytoplasm.

The viral particles inserted into the cytoplasm are enclosed by the viral core, consisting of three proteins encoded by the *gag* gene: matrix (MA), capsid (CA), and nucleocapsid (NC). The fusion of the viral and host membranes exposes MA, which originally resides under the viral membrane in the virions, to the cytoplasm. The viral MA is phosphorylated at multiple residues depending on the temporal and spatial stages of viral replication. The MA phosphorylation pattern in the Pre-Integration Complex (PIC) suggests that the electrostatic interaction between the cell membrane and MA might be disrupted due to the overall charge alteration on this protein (Bukrinskaya, 2007; Kaushik & Ratner, 2004; Kiernan et al., 1998). Although how MA disassembles at the post-entry of HIV is not very well understood, it is known that the dissociation of the viral capsid is highly regulated. The viral core, composed of CA protein, has a canonical structure with a length of 100-120 nm and a width of 50-60 nm at the wide end. The CA proteins construct hexamers via associating with the N-terminal domains. Then, the CA hexamers interact thoroughly through their C-terminal domains with each other, leading to the canonical viral core (Arhel, 2010; Benjamin et al., 2005; Briggs et al., 2003; Ganser et al., 1999; Höglund et al., 2009; Li et al., 2000; Welker et al., 2000). The spatial and temporal regulation of the disassembly of the

viral core, the uncoating process, is modeled in three possible scenarios. The viral CA cone might start to dissociate immediately upon its injection into the cytoplasm (M. Bukrinsky, 2004; M. I. Bukrinsky et al., 1993; Dvorin & Malim, 2003; Nisole & Saïb, 2004; Suzuki & Craigie, 2007), gradually while moving along the nuclear pore complex (NPC) (Warrilow et al., 2009) or when it dissociates from the NPCs inside the nucleus (Aiken & Rousso, 2021; Arhel, 2010; Guedán et al., 2021; Klarmann et al., 1993; Müller et al., 2021, 2022; Shen et al., 2021; Zila et al., 2021).

The fusion of viral and host cell membranes leads to simultaneous events to the uncoating, including reverse transcription, a significant step in the HIV replication cycle. The single-stranded RNA genome of HIV needs to be used as a template to synthesize its double-stranded DNA genome to be integrated into the host genome. The conceptualization of the reverse transcription event in retroviral infection (Baltimore, 1970; Perevozchikov et al., 1970; Temin, 1964) provides a basal understanding of HIV infection. In a newly infected cell, the positive-sense RNA strand serves as the template for synthesizing the negative-sense DNA strand via priming *Lys3* tRNA of the host. 3' end of the *Lys3* tRNA base-pairs with the primer binding site (PBS) at downstream of the U5 region of 5' LTR. Then, the newly synthesized single-stranded DNA molecule, which includes R and U5 regions base-pairs with the same regions at the 3' end of the viral RNA, serves as a primer. Alongside, the 5' end of the viral RNA is being degraded by the RNAse H activity of the reverse transcriptase's (RT), generating a truncated viral RNA with PBS at the 5' end of this RNA (Hu & Hughes, 2012; Isel et al., 1996; Lanchy et al., 1998; Whitcomb et al., 1990). When the negative-sense DNA is synthesized, the RNA-DNA duplex is resolved through RNAse H activity of RT on the viral RNA. A degraded RNA fragment with poly-purine tract (PPT) in close proximity to the 3' LTR as well as the central PPT fragment, then, prime for the positive-sense DNA synthesis. The negative-sense DNA strand, while base-pairing to the newly synthesized DNA fragment, forms a loop structure along with the *Lys3* tRNA cleavage by RNAse H activity. The positive-sense strand adheres the 5' and 3' ends of the negative-sense stand by base-pairing with PPT and LTR at the 3' and PBS with the 5'. Therefore, this positive-sense strand serves as a primer, while the negative-sense strand act as a template (Charneau et al., 1992; Driscoll et al., 2001; Hu & Hughes,

2012; Hu & Temin, 1990; Julias et al., 2002; Lanchy et al., 1998; Panganiban & Fiore, 1988; Purohit et al., 2007; van Wamel & Berkhout, 1998; Whitcomb et al., 1990). Inositol phosphate IP6, a cellular metabolite packed in virions, increases the stability of the viral core and endorses reverse transcription through electrostatic interactions in the CA cone structure (Mallery et al., 2018, 2021). The HIV core might maintain the condensed RT presence (Aiken & Rousso, 2021; Huber et al., 1989), protect the viral RNA, newly synthesized DNA, and viral factors, or transport them toward the nucleus (Aiken & Rousso, 2021; Ambrose & Aiken, 2014; Müller et al., 2021).

The HIV replication cycle obligates the integration of newly synthesized viral DNA into the host's genome (Craigie & Bushman, 2012). To possess this process, the 5' and 3' LTRs of the reverse transcribed viral DNA associate with pre-integration complex (PIC) composed of a tetramer of viral protein integrase which additionally binds to several viral and cellular co-factors, including viral RT and MA in addition to the cellular factors LEDGF/p75, BAF and HRP (Taltynov et al., 2012; van Maele et al., 2006). The cleavage of 3' termini by endonuclease activity of integrase exposes 5'-CA-3'-carboxyl ends. The nucleophilic attack to the feasible regions (mostly transcribed genes) of the host genome through these 3'-carboxyl groups initiates the process called stand transfer, resulting in 5' overhang of the viral DNA and 3' overhang of the host's genomic DNA, which is being repaired via the host DNA repair machinery (Brégnard et al., 2014; Craigie & Bushman, 2012; Lusic & Siliciano, 2016; Marchand et al., 2006; Taltynov et al., 2012; van Maele et al., 2006). The roles of the cellular co-factors vary in viral integration. In viral integration, for example, BAF (barrier-to-autointegration factor) inhibits the autointegration resulting from the ligation of the 3' termini of the viral DNA to itself. Likewise, LEDGF/p75 (lens epithelium-derived growth factor) is a transcription factor that accelerates the nuclear localization of integrase and fastens the interaction of PIC and chromosomal DNA (Llano et al., 2004; Marchand et al., 2006; Taltynov et al., 2012; van Maele et al., 2006). The site and efficiency of integration are controlled spatially through viral and cellular factors, sequence specificity, and chromatin architecture and structure (Lusic & Siliciano, 2016). Altogether, these emphasize that HIV replication is highly regulated by manipulating various cellular machinery. The viral DNA inserted into the host genome is the provirus.

When integrated, the transcription of proviral DNA administers viral RNAs that translate into viral proteins and serve as viral genomic material packed into the new virions. To transcribe an RNA, a promoter is required. Therefore, HIV provirus employs 5' LTR as a promoter for viral RNA transcription (Duverger et al., 2013; Jeeninga et al., 2000; Karn & Stoltzfus, 2012; Schiralli Lester & Henderson, 2012; van Opijnen et al., 2004). The transcription of the HIV genome harnesses cellular RNA Polymerase (Pol) II (Nilson & Price, 2011). The RNA Pol II activity is negatively regulated through negative elongation factors, producing premature transcripts (Kao et al., 1987; Ping & Rana, 2001). The viral protein tat stimulates the transcriptional elongation through the recruitment of cellular elongation factors, including P-TEFb, to the 5' stem-loop transactivation response (TAR) element of viral RNAs. These cellular factors phosphorylate RNA Pol II to maintain the elongation of the transcription of viral RNAs (Asamitsu et al., 2018; Kao et al., 1987; Karn & Stoltzfus, 2012; Mancebo et al., 1997; Ping & Rana, 2001; Zhou et al., 1998). HIV transcripts can be singly or fully spliced to produce *env*, *vif*, *vpu* RNAs and *vpr*, *nef*, *rev*, *tat* RNAs respectively in addition to unspliced, full-length transcripts (Karn & Stoltzfus, 2012; Ohlmann et al., 2014). The viral protein rev binds to the rev response elements (RRE) on the viral RNAs to enhance their nucleus-to-cytoplasm translocation (Blissenbach et al., 2010; Karn & Stoltzfus, 2012; Shida, 2012; Taniguchi et al., 2014).

The protein synthesis through the viral transcripts compels the cellular translation machinery. The protein products are sorted into different cellular compartments. Thereby, the translation sites are insulated accordingly (Karn & Stoltzfus, 2012). The viral proteins env and vpu are synthesized by the ER-bound ribosomes from the singly spliced transcript of the HIV genome. The translation of either vpu or env from the single bicistronic transcript is specified via the start codon of vpu enclosed by a weak Kozak sequence. This feeble sequence allows efficient downstream env expression (Bolinger & Boris-Lawrie, 2009; de Breyne & Ohlmann, 2018; Karn & Stoltzfus, 2012; Krummheuer et al., 2007). Translation of HIV RNAs can be processed through cap-dependent and cap-independent mechanisms (Ohlmann et al., 2014). The 5' m7G cap of HIV transcript initiates the ribosomal scanning at the 5' UTR (Berkhout, 1996; Berkhout et al., 2011; Ricci et al., 2008; Soto-Rifo et al., 2012), or the structural RNA

elements called as internal ribosome entry sites (IRES) can recruit ribosomes for translation (Amorim et al., 2014; Brasey et al., 2003; Buck et al., 2001; Cochrane et al., 1991; Fernández-Miragall et al., 2009; Gendron et al., 2011; Vallejos et al., 2012; Wolff et al., 2003). The full-length HIV transcripts express either gag or gag-pol polyproteins. The gag and pol coding sequences are in different frames. Thus, ribosomal frameshifting enables the synthesis of the gag-pol polyprotein (Brierley & dos Ramos, 2006; Jacks et al., 1988b; Karn & Stoltzfus, 2012).

The expression of HIV proteins initiates the viral assembly. The NC in the gag and gag-pol polyproteins recognizes the Ψ at the downstream of the *gag* gene of the full-length viral RNAs. The gag and pol precursor polyproteins are positioned at the cytoplasmic region of the plasma membrane where the env protein accumulates while the interaction of MA and gp41 stabilizes this localization. The viral regulatory proteins nef, vpr and vpx also associate with the sites of gag-pol-env assembly. The HIV protease proteolytically processes the gag-pol polyprotein to produce MA, CA, NC, integrase, reverse transcriptase, and protease. The virions bud from the host cell via encapsulating the viral genome and proteins as well as the cellular factors by the plasma membrane (Colomer-Lluch et al., 2020; Ono & Freed, 2001; Sundquist & Kräusslich, 2012).

Overall, the knowledge of the HIV replication cycle enables the improvement of strategies to prevent lethality and transmission of HIV infection by the pharmaceutical treatments (ART) against HIV infection that inhibit the activity of reverse transcriptase, integrase, and protease. The blockage of the pol protein function constrains the basic but significant aspects of the HIV replication cycle, hence, the transmission and the disruption of cellular machinery as well as the HIV-induced cytopenia (Arts & Hazuda, 2012; Huot et al., 2018; Robbins et al., 2014; Saez-Cirion & Müller-Trutwin, 2019; Simon et al., 2006; Sutton et al., 2001).

1.3. Post-Translational Modifications in HIV Infection

HIV exploits various cellular processes and escapes from cellular defense mechanisms from the entry to the host cell. The cellular GTPase Dynamin, known for its function in clathrin-mediated endocytosis, stabilizes the HIV entry pore in the canonical infection model (Aggarwal et al., 2017; Jones et al., 2017), or drives the non-canonical infection (Miyauchi et al., 2009). The viral env protein rearranges the cytoskeleton through P2Y2 signaling to degrade NLRP3, which inhibits F-actin remodeling (Paoletti et al., 2019). HIV hijacks the cellular transport system to mobilize the HIV core and translocate it into the nucleus (Ramdas et al., 2020; Tavares et al., 2021). The viral protein vif destabilizes APOBEC3, which promotes viral hypermutation (R. S. Harris et al., 2002; Marin et al., 2003; Sheehy et al., 2003). HIV also recruits endosomal sorting complexes required for transport (ESCRT) machinery to promote the assembly of new virions and budding (Ahmed et al., 2019; Strack et al., 2003). Additionally, the HIV-1 tat protein, stimulating the viral transcription through the TAR element, also alters the epigenetic landmark of the host genome to regulate the host transcriptional program (Boehm et al., 2013; Lusic et al., 2003; Reeder et al., 2015). Considering the diverse repertoire of HIV to induce cellular reprograming and to manipulate the cellular factors to enhance the infectivity as well as the viral factors processing diverse events, it is not surprising but very interesting to expect that the cellular post-translational modification (PTM) machineries are modulated by HIV.

PTMs are the covalent modifications that alter the biochemical properties of a protein, resulting in the augmented protein repertoire of the cells. The diversification of a single protein through PTMs orchestrates numerous cellular events, leading to various physiological regulations. These biochemical characteristics, including activity, stability, and localization, govern the fate, function, and interaction network of a protein via the addition or the removal of a chemical group (Duan & Walther, 2015; Prabakaran et al., 2012; Walsh et al., 2005; Wang et al., 2022). A vast quantity of PTMs was reported to dictate the HIV replication cycle through viral or cellular factors. One of the significant PTMs in HIV replication is the processing of its spike protein env. The viral protein env is heavily N-linked glycosylated in the ER. The glycosylation

of env promotes its interaction to form a trimeric state. Maturation of env protein is not limited to the addition of sugars but extends to the proteolytic cleavage in the Golgi by Furin and Furin-like proteases before its transport to the plasma membrane (Fenouillet & Jones, 1995; Gu et al., 1995; Hallenberger et al., 1992; Ji et al., 2005; Pritchard et al., 2015; Reitter et al., 1998; Wei et al., 2003). Another major PTM is the myristoylation of the viral gag, gag-pol, and nef proteins. N-myristyl transferases covalently attach myristic acid to those proteins, targeting them to the plasma membrane (Bentham et al., 2006; M. P. G. Harris & Neil, 1994; Matsubara et al., 2005; Resh, 2004). This PTM promotes the gag-gag interaction as well (H. Li et al., 2007). The myristoylation of nef exposes the protein to an additional PTM, phosphorylation by protein kinases A and C (PKA and PKC). The phosphorylation of nef is required to enhance viral infectivity (Coates et al., 1997; Guy et al., 1987; P. L. Li et al., 2005); however, the mechanism needs to be further investigated. The different functions of reverse transcriptase (RNA-dependent DNA polymerization and RNAse H activities) could be triggered by its phosphorylation by Cdk2 or casein kinase II that is activated by HIV protein rev (Chen et al., 2018; Harada et al., 1999; Idriss et al., 1999; Leng et al., 2014; Ohtsuki et al., 1998). HIV protein integrase is subjected to numerous PTMs as well. Acetylation of the viral integrase by p300 and GCN5 intensifies the DNA binding affinity of integrase (Cereseto et al., 2005; di Fenza et al., 2009; Puras Lutzke et al., 1994; Zheng & Yao, 2013). This protein is also phosphorylated by JNK. This phosphorylation recruits Pin1 to stabilize the viral protein (Manganaro et al., 2010; Zheng & Yao, 2013). Sumoylation of integrase also provokes the infectivity of HIV (Zamborlini et al., 2011).

HIV manipulates the modification of cellular proteins as well during infection. As mentioned, the viral entry obligates the rearrangements of the cytoskeleton. The viral spike protein env triggers the MAPK cascade through PTMs, resulting in the reorganization of the cytoskeleton as well as the T cell survival (Abbas & Herbein, 2014; Chen et al., 2018; Kinet et al., 2002; Viard et al., 2003; Wu & Yoder, 2009). The activation of NF-κB via the ubiquitylation of IκB, promoted by the phosphorylation of IκB by the IκB kinase (IKK), is also achieved through env signaling, contributing to the viral gene expression. (Chen et al., 2018; Deng et al., 2016; Flory et al., 1998;

Juszczak et al., 1991). Likewise, CD4, the receptor for HIV, is also ubiquitylated. This process is stimulated by the viral factor vpu to suppress the hyper-infection, leading to efficient infection (Fujita et al., 1997; Margottin et al., 1998; Schubert et al., 1998). As mentioned, APOBEC3 is destabilized by the ubiquitylation promoted by vif (R. S. Harris et al., 2002; Marin et al., 2003; Sheehy et al., 2003). Additionally, a recent study showed that the HIV factor vpr drastically alters the histone H1 ubiquitylation profile for the efficient infection through impairment of DNA repair (Johnson et al., 2022).

PTMs do not always progress the HIV replication. The ubiquitylation of integrase diminishes the stability of the protein despite the fact that its acetylation, phosphorylation, and sumoylation intensify its function and viral infectivity (Ali et al., 2019; Z. Zhang et al., 2021; Zheng & Yao, 2013). The ubiquitylation of viral CA by TRIM5α sequesters the viral core to the proteasome, designating premature uncoating. The N-terminal RING zinc-binding domain of TRIM5α has an E3 ubiquitin ligase activity, whereas B-Box2 and coiled-coil (RBCC) domains are required for the oligomerization. The oligomerization of TRIM5α reinforces the activity of the RING domain. The C-terminal SPRY domain, on the other hand, contributes to the interaction with the nuclear capsid. The auto-ubiquitylation of TRIM5α also enhances its antiviral activity. TRIM5α also activates signaling cascades to promote antiviral defense mechanisms through NF-κB (Anderson et al., 2006; Diaz-Griffero et al., 2006; Imam et al., 2019; Javanbakht et al., 2005, 2006; Langelier et al., 2008; Nisole et al., 2005; Pertel et al., 2011; Rold & Aiken, 2008; Stremlau et al., 2004, 2006; Tareen & Emerman, 2011; Uchil et al., 2013; X. Wu et al., 2006). Sumoylation of TRIM5α also regulates the NF-κB signaling and antiviral mechanism of the protein through SUMO-interacting motif (SIM) interaction networks (Arriagada et al., 2011; Lukic et al., 2013). The cellular protease Furin, while establishing the infectivity by processing the HIV spike protein env, also proteolytically cleaves another viral factor, tat. This irreversible PTM on the tat protein obstructs its transactivation activity, precipitating the premature viral nascent RNAs (Tikhonov et al., 2004). This viral factor also undergoes ubiquitylation. However, this PTM has a versatile function in the regulation of the tat. On the one hand, the K48-linked polyubiquitylation of the tat accelerates its proteasomal degra-

dation (Zhang et al., 2014). On the other hand, the viral protein tat is subjected to the other modes of ubiquitylation stimulates the viral transcription competently (Brès et al., 2003; Faust et al., 2017). Furthermore, sumoylation of the viral protein p6 hampers the viral budding by inhibiting the mono-ubiquitylation of p6, which is required for the viral assembly and release (Friedrich et al., 2016; Gottwein & Kräusslich, 2005; Gurer et al., 2005; Ott et al., 1998).

Altogether, these studies demonstrate that HIV is able to utilize the host PTM machinery to increase infectivity. However, PTMs, regulating the antiviral defense mechanisms, could also restrict the HIV replication cycle through various cellular and viral factors. The reprograming of cellular PTM machinery, either specifically or globally, by pathogens is still an emerging field. Therefore, the viral strategies to overcome the immunity and the immune strategies against pathogens through the PTMs need to be further characterized.

1.4. Sumoylation and Innate Immunity

Sumoylation is a peptide-based PTM in eukaryotic cells via the covalent attachment of Small Ubiquitin-like Modifiers (SUMOs) onto the target proteins. This reversible modification governs the stability, solubility, localization, and interaction patterns of proteins, indicating an expanded repertoire of the proteome (Celen & Sahin, 2020; Gareau & Lima, 2010; Hay, 2005; Sahin et al., 2022). The first evidence of SUMO proteins was reported in *Saccharomyces cerevisiae* (Meluh & Koshland, 1995). The evidence of SUMO proteins started to accumulate in the literature, indicating the similarities of SUMOs with ubiquitin (Celen & Sahin, 2020) as well as the covalent attachment to modify its substrates (Mahajan et al., 1997; Matunis et al., 1996). Encoding the conserved SUMO proteins is limited to eukaryotic genomes; thus, sumoylation is a eukaryotic PTM (Celen & Sahin, 2020; Hay, 2005; Sahin et al., 2022). 4 different SUMO paralogs have been reported in the human genome: SUMO1-5. Among these, the sequences of SUMO2 and SUMO3 exceptionally overlap, leading to the collective classification of SUMO2/3 (Celen & Sahin, 2020). SUMO1 and SUMO2/3 are ubiquitously expressed in all eukaryotic cells, whereas the expression pattern of SUMO4

and SUMO5 are restrained to a limited number of tissues (Sahin et al., 2022). SUMO4 was reported to lack the ability to maturate, thus, to modify the substrates (Owerbach et al., 2005). Sumoylation is a fundamental process in eukaryotic cells, and the impairments in sumoylation machinery cause deficiencies in cell survival and organismal development (Celen & Sahin, 2020; Nacerddine et al., 2005; Sahin et al., 2022).

The sumoylation cascade is akin to ubiquitylation machinery (Celen & Sahin, 2020). Upon the maturation of SUMO proteins by the proteolytic cleavage by sentrin-specific proteases (SENPs), they are unmasking a C-terminal diglycine (-GG) motif. Maturation of SUMOs enables their ATP-dependent activation through heterodimeric E1 SUMO-activating enzyme (SAE1/UBA2). Then, UBC9, the universal E2 SUMO-conjugating enzyme, accepts the activated SUMO proteins. The SUMO conjugation to the substrates can be achieved directly by the UBC9 or with the help of E3 SUMO ligases. The SUMO acceptor on the target protein is the lysine (Lys, K) residue, primarily residing in the consensus motif ψKxD/E by the isopeptide bond (Celen & Sahin, 2020; Gareau & Lima, 2010; Hay, 2005; Sahin et al., 2022). Consequently, sumoylation reshapes the biochemical characteristics and function of its substrates, materializing the regulation of cellular events in which these proteins are involved. The consensus sumoylation motif dwells in the SUMO2/3 protein sequences, allowing the sumoylation of SUMO2/3 as well. The sumoylation of SUMO proteins facilitates the poly-SUMO chains, comparable to ubiquitin. Additional non-consensus sumoylation motifs can also participate in the chain formation, resulting in branched chains. The lack of consensus sumoylation motif on SUMO1, au contraire, disables the ability of poly-sumoylation of SUMO1. Nonetheless, the non-consensus motifs can cooperate to form poly-SUMO chains under stress conditions (Celen & Sahin, 2020; Gareau & Lima, 2010; Hay, 2005; Hendriks et al., 2014; Matic et al., 2008; Sahin et al., 2022). Although SUMO1 and SUMO2/3 share the competence of attachment to the target Lys residues, the physiological sumoylation patterns differ. SUMO1 is predominantly conjugated to its substrates under normal physiological conditions, yet SUMO2/3 is essentially pooled in the unconjugated form. The expeditious conjugation profile is programmed upon the presence of cellular stress (Golebiowski et al., 2009; Sahin Umut et al., 2014; Saitoh & Hinchey, 2000). These suggest distinct roles of sumoylation by SUMO1 and SUMO2/3.

Moreover, sumoylation is reversible by the covalent detachment of SUMO proteins from the substrates by SENPs (Celen & Sahin, 2020; Jentsch & Psakhye, 2013; Kunz et al., 2018; S. J. Li & Hochstrasser, 1999; S.-J. Li & Hochstrasser, 2000; Psakhye & Jentsch, 2012). Sumoylation can provide or restrain the interaction profile of proteins by the SUMO-SIM interactions (Celen & Sahin, 2020; Sahin et al., 2022). A cellular PTM machinery often regulates the other. Sumoylation is also capable of monitoring other PTMs, majorly ubiquitylation through SUMO-targeted ubiquitin ligases (STUbLs). STUbLs are E3 ubiquitin ligases that specifically identify the poly-SUMO chains. This recognition stimulates the covalent attachments of ubiquitin to the sumoylated protein, resulting in the proteasomal degradation of the substrate (Miteva et al., 2010; Praefcke et al., 2012).

Sumoylation orchestrates various cellular and physiological events, including stress responses, signaling, and nuclear integrity. Among those events, the role of sumoylation in innate immunity is critical for viral infections. Sumoylation modulates innate immune response by governing the biochemical features of the proteins included in the signaling cascades, including NF-κB and interferon signaling (Adorisio et al., 2017; Hannoun et al., 2016).

Sumoylation of NEMO, the regulatory subunit of IKK, leads to the activation of IKK, phosphorylating the IκB. This progressive PTM modulation causes the ubiquitylation of IκB, thus, its proteasomal degradation. Collectively, the sumoylation of NEMO activates the NF-κB, generating an antiviral response, whereas the desumoylation of NEMO by SENP2 incapacitates the NF-κB activation cascade (Huang et al., 2003; Lee et al., 2011; Mabb et al., 2006; Wuerzberger-Davis et al., 2006). IκB, the inhibitor of NF-κB, is also sumoylated by SUMO1. This mode of sumoylation enhances the stability of IκB, maintaining the inactive NF-κB (Perkins, 2013; Tsai et al., 2016). However, the sumoylation of IκB by SUMO2/3 induces its ubiquitylation and degradation (Aillet et al., 2012). Altogether, these demonstrate that sumoylation finely adjusts the NF-κB signaling through acting on different steps in the cascade, even via the different modes of sumoylation at the same level.

Sumoylation finetunes the interferon (IFN) signaling pathway as well. Interferon Regulatory Factor 3 (IRF3) sumoylation impairs the transcriptional activity of IRF3 (Kubota et al., 2008). SENP2 also desumoylates IRF3, resulting in the reinforcement of the IFN transcription in addition to Promyelocytic Leukemia protein (PML) induced activation of IRF3 (Adorisio et al., 2017; Chen et al., 2015; Ran et al., 2011). Those exemplify sumoylation's importance and different regulatory roles in IFN signaling, yet they are not limited to IRF activity. SUMO proteins mediate the IFN response as well. SUMO expression is regulated through microRNAs stimulated by IFN (Sahin et al., 2014). SUMO is an important restriction factor in bacterial and viral infections (Ribet et al., 2010; Sahin et al., 2014).

As exampled, the regulation of immune signaling by this PTM emphasizes the significance of sumoylation. Sumoylation modulates T cell expansion and function, indicating its role in diverse mechanisms of the immune system (Cammann et al., 2022; Ding et al., 2016; Friend et al., 2014; Hannoun et al., 2016).

Over the course of evolution, pathogens developed strategies to interfere with their hosts' sumoylation machinery to target central signaling pathways for efficient replication and escape from immune responses in addition to hijacking the viral protein modifications (Everett et al., 2013; Lowrey et al., 2017; Ribet & Cossart, 2010, 2018; Wimmer et al., 2012). Pathogenic manipulation of the host sumoylation system implements infectivity. Herpes Simplex Virus type 1 (HSV-1) reprograms the host sumoylome via bridging the SUMO-targeted ubiquitylation. The viral infectivity factor ICP0 serves as a STUbL, tagging the sumoylated proteins of its host by ubiquitin. This modification precipitates the proteasomal targeting of the proteins. The destabilized sumoylome promotes the HSV-1 infection via furnishing a cellular environment free from the antiviral activities of sumoylation (Boutell et al., 2002, 2011; Sloan et al., 2015). The alterations in cellular sumoylation machinery are not limited to the SUMO-targeted ubiquitylation. Epstein-Barr Virus (EBV) protein kinase BGLF4 accumulates in the nucleus through SUMO-SIM interactions, diminishing the cellular sumoylome in the lytic cycle (R. Li et al., 2012). EBV also strategizes the expression of microRNAs antagonizing PML and RNF4 to impede this cellular machinery

(J. Li et al., 2017; Skalsky et al., 2012). Pathogens can rearrange the cellular sumoylome not only by targeting the sumoylated proteins directly but also by interrupting the SUMO conjugation. *Listeria monocytogenes* degrades UBC9, the universal E2 SUMO-conjugating enzyme via the bacterial virulence factor listeriolysin O (LLO) in a proteasome-independent manner (Ribet et al., 2010), whereas *Klebsiella pneumoniae* suppresses the translation of SUMO transcripts through let-7 microRNA as well as endorses desumoylation by SENP2 (Sá-Pessoa et al., 2020). The reprograming strategies for cellular sumoylation are not narrowed down to the decrease of sumoylation. EBV, while impairing the cellular sumoylation in the lytic cycle, expands the cellular sumoylation in the latent phase through latent membrane protein-1 to administer the oncogenicity of LMP1 and the maintenance of latency (Bentz et al., 2011, 2012, 2015). Additionally, the influenza virus reconstructs the topology of the cellular sumoylome by combining specific sumoylation and desumoylation patterns, leading to immune escape (Domingues et al., 2015; Schmidt et al., 2019).

Overall, these display that sumoylation is a critical regulator of innate immunity. Therefore, pathogens develop strategies to overcome this cellular defense mechanism for infection. The interplay between sumoylation and pathogens is not only limited to the reprograming of the host sumoylome but also extends to the exploitation of this system to advance the infectivity, including the sumoylation of HIV protein integrase. Hence, the characterization of host sumoylation machinery in HIV infection is particularly compelling (Adorisio et al., 2017; Celen & Sahin, 2020; Hannoun et al., 2016; Sahin et al., 2022).

2. AIM

Sumoylation is a post-translational modification regulating various cellular and physiological events, including immunity. Additionally, several pathogens are known to invade their hosts' sumoylation machinery to disarm the immune responses, resulting in enhanced infectivity. Human Immunodeficiency Virus is a retrovirus interrupting various cellular events and hijacking numerous cellular machineries to invest efficient infection. To date, there has been no study to dissect the interplay between host sumoylation and HIV at a global level despite certain viral factors are known to be sumoylated. Therefore, we aimed to elucidate whether HIV-1 computes an alteration in cellular sumoylation machinery overall. In order to decode this interplay *in vitro*, we expressed the HIV-1 genome in HEK293 and Jurkat cell lines. We maintained this study via purposing to grasp this reprograming mechanism. Therefore, we investigated possible machineries, including SUMO-targeted ubiquitylation and the impairments in the SUMO conjugation pathway in the HIV-1 genome expressing HEK293 and Jurkat cells.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals, Reagents and Kits

Table 3.1 Chemicals used in this study.

Chemical	Supplier
2-mercaptoethanol	Merck, Germany
4'6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Germany
Acrylamide/Bis-acrylamidel	Bio-Rad, USA
Ammonium persulfate (APS)	AppliChem, Germany
Ampicillin	Merck, Germany
Bromophenol blue	Sigma-Aldrich, Germany
Calcium chloride dehydrate	Sigma-Aldrich, Germany
Chloroform	Sigma-Aldrich, Germany
Chloroquine	Sigma-Aldrich, Germanyck
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
Ethanol	Merck, Germany
Ethylenediaminetetraacetic acid (EDTA)	Wisent Bioproducts, Canada
Glycerol	MP Biomedicals, USA
Glycine	NeoFroxx, Germany
HEPES	Sigma-Aldrich, Germany
Hydrochloric acid	Sigma-Aldrich, Germany
Isopropanol	Sigma-Aldrich, Germany
Luria-Bertani (LB) Agar	Caisson Laboratories, USA
Luria-Bertani (LB) Broth	Caisson Laboratories, USA
Methanol	Merck, Germany
MG132	Calbiochem, Germany

Table 3.1 Chemicals used in this study. (cont.)

Chemical	**Supplier**
Paraformaldehyde (PFA)	Santa Cruz Biotechnology, USA
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate (SDS)	Merck, Germany
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Germany
Tris-base	Sigma-Aldrich, Germany
Tween 20	Merck, Germany

Table 3.2 Reagents and kits used in this study.

Reagent or kit	**Supplier**
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche, Switzerland
Direct-zol™ RNA MiniPrep Plus	Zymo Research, USA
ECL	Advansta, USA
Neon™ Transfection System 100 μL Kit	Thermo Fisher Scientific, USA
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific, USA
SensiFAST™ cDNA Synthesis Kit	Bioline, UK
SensiFAST™ SYBRR No-ROX Kit	Bioline, UK
Sirius	Advansta, USA
ZymoPURE™ MidiPrep Kits	Zymo Research, USA

3.1.2. Antibodies, Buffers and Solutions

Table 3.3 Antibodies used in this study.

Antibodies	**Catalog No**	**Supplier**
α-β-actin	MA1115	BosterBio, USA
α-GFP	sc-9996	Santa Cruz Biotechnology, USA
α-integrase	sc-69721	Santa Cruz Biotechnology, USA

Table 3.3 Antibodies used in this study. (cont.)

Antibodies	**Catalog No**	**Supplier**
α-LC3B	L7543	Sigma-Aldrich, Germany
α-mouse	7076S)	CST, USA
α-rabbit	7074S)	CST, USA
α-rev	sc-69729	Santa Cruz Biotechnology, USA
α-SAE1	sc-398080	Santa Cruz Biotechnology, USA
α-SUMO1	M00631-1	BosterBio, USA
α-SUMO1	33-2400	Thermo Fisher Scientific, USA
α-SUMO2/3	ab3742	Abcam, UK
α-tubulin	sc-23948	Santa Cruz Biotechnology, USA
α-UBA2	sc-376305	Santa Cruz Biotechnology, USA
α-UBC9	ab75854	Abcam, UK
α-ubiquitin	A-106	R&D Systems, USA

Table 3.4 Buffers and solutions used in this study.

Buffer or Solution	**Recipe**	**Application**
2X Laemmli Buffer	100 mM Tris-HCl (pH 6.8) 4% (w/v) SDS 0.2% (w/v) bromophenol blue 20% (v/v) glycerol 200 mM *beta*-mercaptoethanol in ddH_2O	Western Blot
Blocking Solution	5% (w/v) milk powder in TBST	Western Blot
HEPES-buffered Saline (2X) (2X HBS)	50 mM HEPES 280 mM NaCl 12 mM D-glucose 10 mM KCl 1.5 mM Na_2HPO_4 in ddH_2O pH 7.05	Transfection

Table 3.4 Buffers and solutions used in this study (cont.)

Buffer or Solution	**Recipe**	**Application**
Phosphate-buffered Saline (PBS)	80 mM $NaHPO_4$ 1.5 M NaCl 20 mM KH_2PO_4 30 mM KCl in ddH_2O pH 7.4	Transfection
SDS Gel Running Buffer	1% (w/v) SDS 3.03% (w/v)Tris base 14.4% (w/v)glycine in ddH_2O	Western Blot
Stripping Buffer	1.5% (w/v) glycine 0.1% (w/v) SDS 1% (v/v) Tween 20 in ddH_2O pH 2.2	Western Blot
Tris-buffered Saline (TBS)	20 mM Tris base 150 mM NaCl in ddH_2O pH 7.4	Western Blot
Tris-buffered Saline with Tween 20 (TBST)	20 mM Tris base 150 mM NaCl 0.1% (v/v) Tween 20 in ddH_2O pH 7.4	Western Blot
Western Blot Transfer Buffer	3.03% (w/v) Tris base 14.4% (w/v) glycine 20% (v/v) Methanol in ddH_2O	Western Blot

Table 3.5 SDS gel recipes.

Application	**Percentage**	**Recipe**
Stacking gel	4%	0.25 M Tris-HCl (pH 6.8) 0.01% (w/v) SDS 4% (w/v) acrylamide:bisacrylamide 0.01% (w/v) APS 0.001% (w/v) TEMED in ddH_2O
Resolving gel	8%	0.375 M Tris-HCl (pH 8.8), 0.01% (w/v) SDS 8% (w/v) acrylamide:bisacrylamide 0.01% (w/v) APS 0.001% (w/v) TEMED in ddH_2O
Resolving gel	15%	0.375 M Tris-HCl (pH 8.8), 0.01% (w/v) SDS 15% (w/v) acrylamide:bisacrylamide 0.01% (w/v) APS 0.001% (w/v) TEMED in ddH_2O

3.1.3. Cell Culture Reagents and Media

Table 3.6 Cell culture reagents.

Reagent	**Supplier**
DMEM	Gibco, USA
Fetal Bovine Serum (FBS)	Gibco, USA
Penicillin/Streptomycin (100X)	Lonza, Switzerland
RPMI-1640	Cytiva, UK
Trypsin (0.05%)	Gibco, USA

Table 3.7 Cell culture media used in this study.

Medium	Recipe
Complete DMEM	10% (v/v) FBS 1X Pen/Strep in DMEM
Complete RPMI-1640	10% (v/v) FBS 1X Pen/Strep in RPMI-1640

3.1.4. Plasmids and Oligos

pfNL-43-dE-EGFP (36865) was purchased from Addgene. Plasmid map is in the Appendix A.

Table 3.8 Oligos used in this study for RT-qPCR.

Primer Name	Sequence (5' to 3')	Amplicon Size
UBA2-ExpF	CCCGAAAGCTAATATCGTTGCC	221 bp
UBA2-ExpR	ACTCGGTCACACCCTTTTTGA	
Gapdh-ExpF	GGAGCGAGATCCCTCCAAAAT	197 bp
Gapdh-ExpR	GGCTGTTGTCATACTTCTCATGG	

3.1.5. Equipment and Software

Table 3.9 Disposables used in this study.

Disposables	Supplier
6-well plates	TPP, Switzerland
12-well plates	TPP, Switzerland
96-well qPCR plates	Thermo Fisher Scientific, USA

Table 3.9 Disposables used in this study (cont.)

Disposables	**Supplier**
Centrifuge tubes, 15 mL	Capp, Denmark
Centrifuge tubes, 50 mL	Capp, Denmark
Coverslips (18-mm)	Merck, USA
Microcentrifuge tubes, 0.2 mL	Axygen Scientific, USA
Microcentrifuge tubes, 1.5 mL	Axygen Scientific, USA
Microcentrifuge tubes, 2 mL	Axygen Scientific, USA
Micropipette Tips (10 uL)	Capp, Denmark
Micropipette Tips (200 uL)	Capp, Denmark
Micropipette Tips (1000 uL)	Capp, Denmark
Microscope slides	Thermo Fisher Scientific, USA
Nitrocellulose Membrane (0.2 mm)	GE Healthcare, USA
Pasteur pipettes	Isolab, Germany
Serological Pipettes (5 mL)	Capp, Denmark
Serological Pipettes (10 mL)	Capp, Denmark
Serological Pipettes (25 mL)	Capp, Denmark
T25 tissue culture flasks	TPP, Switzerland
T75 tissue culture flasks	TPP, Switzerland

Table 3.10 Devices used in this study.

Device	**Supplier**
Autoclave	AS260T, Astell, UK
Centrifuge	Allegra X-22, Beckman Culture, USA
CO_2 incubator	WTC, Binder, Germany
Confocal Microscopy System	SP5-AOBS,Leica Microsystems, Germany
Deep Freezers	-20°C, Ugur, UFR 370 SD, Turkey -80°C, ULT deep freezer, Thermo, UK -150°C, Thermo Fisher Scientific, USA

Table 3.10 Devices used in this study (cont.)

Device	**Supplier**
Fluorescent microscope	Axio Observer.Z1, Zeiss, Germany
Heat block	Block Heater Analog, VWR, USA
Ice machine	AF20, Scotsman Inc., Italy
Laminar flow	Class IIB, Tezsan, Turkey
Microcentrifuge Centrifuge	CT 15RE, VWR, USA
Micropipettes	Finnpipette, Thermo Fisher Scientific, USA
Nanodrop	ND-1000, Thermo Fisher Scientific, USA
pH meter	Hanna Instruments, USA
Pipettor	S1 Pipet Filler, Thermo Fisher Scientific, USA
Power supply	EC XL 300, Thermo Fisher Scientific, USA
Real-Time PCR system	PikoReal, Thermo Fisher Scientific, USA
Refrigerator	Uğur, USS 374 DTKY, Turkey
Vortex	Silverline, VWR, USA
Western blot documentation system	G-BOX Chemi XX6, Syngene, UK

Table 3.11. Software used in this study.

Software	**Supplier**
Graphpad Prism 8	GraphPad Software, USA
ImageJ	NIH, USA
LAS X	Leica Microsystems, Germany
Syngene	Genetools, UK

3.2. Methods

3.2.1. Mammalian Cell Culture

The maintenance of Human Embryonic Kidney (HEK293) cells was obtained through complete DMEM containing 10% Fetal Bovine Serum (FBS) and 1% Peni-

cillin/Streptomycin (Pen/Strep) as explained in Table 3.7. The standard growth conditions of 37℃ and 5% CO_2 were preserved throughout this study. HEK293 cells with approximately 80% confluency were to be split to maintain the growth. HEK293 cells were washed with Phosphate-buffered saline (PBS) to remove residual FBS after the medium was discarded. 1 volume (V) 0.05% Trypsin-EDTA was added onto the cells and incubated for 5 minutes at 37℃ to dissociate HEK293 cells. 2 V complete DMEM was added onto the cells to deactivate Trypsin. HEK293 cells were centrifuged at 300 xg for 5 minutes to remove residual Trypsin. The pellet, containing cells, was resuspended in fresh complete DMEM and a 1:10 ratio of the cells was seeded into a new flask, to be maintained approximately for 2 days. The pellet of HEK293 cells was also resuspended in DMEM containing 20% FBS and 10% DMSO to freeze the cells. After resuspension, cells directly moved to -80℃ for storage.

Jurkat cell maintenance was conducted in a complete RPMI-1640 medium containing 10% FBS and 1% Pen/Strep, as explained in Table 3.7. The growth conditions are 37℃ and 5% CO_2. When the concentration of Jurkat cells reached to 500,000 cells/mL, the cell suspension was directly centrifuged at 300 xg for 5 minutes to remove the old medium. The fresh medium was added to the pellet containing Jurkat cells in accordance with the density of 300,000 cells/mL, and the pellet was resuspended. In order to freeze Jurkat cells, the pellet was resuspended in 1 V FBS in the concentration of 8,000,000 cells/mL and incubated at 4℃ for 30 minutes. Then, 1 V FBS containing 12% DMSO was added to the FBS-containing cells to obtain 4,000,000 cells/mL. The suspension was stored -80℃.

Both cell lines were thawed by mixing 5 V FBS and 1 V frozen cells. The suspension was centrifuged at 300 xg for 5 minutes. The pellets were resuspended in fresh complete media accordingly.

3.2.2. Transfection

The transfection reagent recipe for a well in a 12-well or 6-well plate is explained in Table 3.12. Firstly, plasmid DNA was diluted in ddH_2O. Then, 2M $CaCl_2$ was added

in a dropwise manner. The mix was incubated at room temperature for 5 minutes. After incubation, 2X HBS was added to the transfection reagent. The reagent was mixed well and incubated for 10 minutes. The incubation is followed by the dropwise addition of the transfection reagent onto the HEK293 cells in a dropwise fashion. In order to maximize the transfection, each drop was placed on a different area of the well. Then, the wells are mixed gently and incubated at 37℃ with 5% CO_2 for the required time for the experiment.

Table 3.12 Transfection reagents.

Ingredient	**12-well)**	**6-well**
DNA	1μg	2μg
2 M $CaCl_2$	6.25 μL	12.5 μL
2X HBS	62.5 μL	125 μL
ddH_2O	up to 125 μL	up to 250 μL

The plasmid DNA delivery to Jurkat cells was conducted through electroporation. 1,000,000 Jurkat cells were resuspended in 100 μL buffer R of the commercial Neon Electroporation System kit. 5 μg of plasmid DNA was added onto the cells and mixed. The electroporation chamber was filled with a minimum of 4 mL buffer E2 of the system to reach the electrode. 100 μL cell suspension was taken by 100 μL Neon pipette tip, and the tip was placed into the chamber. 3 pulses of 1350 Volt electrical current was conducted for 10 milliseconds each. 100 μL of electroporated cells were mixed with fresh complete RPMI-1640 and incubated at 37℃ with 5% CO_2 for the required time for the experiment.

3.2.3. Treatments

Both cell lines were treated with pharmaceutical agents for 24 hours before the lysis. The same volume of vehicle (ddH_2O or DMSO) was delivered to another well as a negative control. Table 3.13 explains the final concentration and vehicle of each agent.

Table 3.13 Pharmaceutical agents.

Pharmaceutical agent	**Vehicle**	**Final concentration**
Cq	H_2O	30 μM
MG132	DMSO	2 μM

3.2.4. SDS-PAGE and Western Blot

Both HEK293 and Jurkat cells were lysed in Laemmli buffer for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The lysates were boiled at 95℃ for 10 minutes, followed by 10 minutes of centrifuge at maximum speed to remove residual DNA and insoluble cell debris. The soluble lysates were loaded as a minimum of 10 μL to SDS-PAGs whose percentages were determined according to the proteins of interest. The protein ladder was loaded into a well of each gel to determine the protein sizes. The SDS-PAGE is conducted at constant voltage. 80V was used for the stacking of proteins, followed by 100V for resolving gel. SDS-PAGE was performed in SDS Gel Running Buffer, explained in Table 3.4.

After the SDS-PAGE, the gel was transferred into a sandwich of filter papers and faced to nitrocellulose membrane. The gel was placed in the cathode, whereas the nitrocellulose membrane was in the anode. Therefore, the proteins (charged negatively due to the SDS) were transferred to the membrane under the constant voltage of 100V for at least 3 hours. The transfer was conducted in ice cold Western Blot Transfer Buffer explained in Table 3.4. Additionally, this procedure was performed in 4℃ to prevent overheating, whereby voltage changes.

The nitrocellulose membranes were blocked in Blocking Solution (Table 3.4) for 1 hour at room temperature. Then, they were incubated with appropriate antibody solutions (1:1000 dilution each in blocking solution) for 16 hours at 4℃ÀAfter incubation, membranes were washed 3 times with TBST for 5 minutes each to remove unbound antibodies. Then, the membranes were incubated with appropriate 2°antibodies (anti-

mouse or anti-rabbit antibodies) for 1 hour at room temperature, followed by 3 times washing with TBST for 5 minutes each. The membranes were transferred to TBS to preserve the antibodies for long-term storage at 4℃. The membranes were visualized with ECL or Sirius (Table 3.2) via Syngene System.

After visualization, the membranes were stripped by Stripping Buffer (Table 3.4) if required. The protocol was to incubate the membrane with Stripping Buffer 2 times for 10 minutes each. Stripping was followed by washing the membrane with TBS 2 times for 10 minutes and with TBST 2 times for 10 minutes. After the washes, the membrane was blocked for 1 hour at room temperature and incubated with appropriate antibodies.

3.2.5. Imaging of EGFP Signal

The HEK293 cells were seeded on coverslips. A well of HEK293 cells was transfected with pfNL-43-dE-EGFP plasmid. Another well remained untransfected as a negative control. Both wells were washed with PBS 3 times for 5 minutes each at 24 hours post-transfection. 4% PFA was added onto the cells and incubated at 37℃ for 15 minutes for fixation. Then, PFA was discarded, and the cells were washed 3 times for 5 minutes each. The coverslip was flipped, and the cells were placed onto the DAPI mounting medium on the microscope slide. The coverslip was sealed and visualized via confocal microscopy (Leica Microsystems). The samples were stored at -20℃. Images were processed through LasX software.

3.2.6. RNA isolation, cDNA synthesis, and RT-qPCR

HEK293 and Jurkat cells were lysed with ice-cold Direct-Zol reagent. RNAs were isolated according to the manufacturer's protocol. RNA concentrations were measured by NanoDrop. RNAs were stored at -80℃.

cDNA synthesis was conducted via SensiFAST^{TM} cDNA Synthesis Kit according to the manufacturer's protocol. The mix was prepared as in Table 3.14 on ice. The

reactions were conducted by the thermal cycler with adjusted temperature and time as explained in Table 3.15, followed by a hold step at 4℃. The cDNAs were stored at -20℃.

Table 3.14 cDNA synthesis mix.

Ingredient	**Volume**
RNA	1 μg
Buffer	4 μL
Enzyme	1μL
ddH_2O	up to 20 μL

Table 3.15 cDNA synthesis

Temperature	**Time**	**Purpose**
25℃	10 min	primer annealing
42℃	15 min	reverse transcription
48℃	15 min	for highly structured RNAs
85℃	5 min	inactivation

RT-qPCR was conducted via Thermo PikoReal RT PCR Systems. Primers in Table 3.8 used for *UBA2* mRNA polymerization and for *gapdh* mRNA as an internal control. cDNAs were diluted in a 1:5 ratio before use. 0.25 μM of each primer (forward and reverse) were mixed with 2 μL of cDNA. The volume of the mix was adjusted to 5 μL, and 5 μL 2X SYBR green was added. Relative expressions were calculated by $2^{-\Delta\Delta Ct}$ method. The cycles are as in Table 3.16.

Table 3.16 RT-qPCR cycles.

Cycles	Temperature	Time	Purpose
1	95℃	2 min	Polymerase activation
40	95℃	5 sec	denaturation
	65℃	10 sec	annealing
	72℃	5 sec	extension
1	melting curve		

3.2.7. Bacterial Culture and Plasmid DNA Isolation

Bacterial cells transformed with plasmids were grown in an LB broth medium containing ampicillin at 30℃ for 18 hours. Bacterial cells were pelleted, and plasmid DNA isolation was performed via ZymoPURE^{TM} MidiPrep Kits according to the manufacturer's instructions. The plasmid DNA concentrations were measured by NanoDrop.

3.2.8. Data Processing

All quantifications of western blots were performed via ImageJ. The Gray density of western blot signals was calculated, and actin signals were used as internal controls for normalization.

3.2.9. Statistical Analysis

All statistical analyses were performed via GraphPad Prism 8. All tests were performed for a minimum of n=3 sample group. P¿0.05 indicates non-significant (ns). Asterisks indicates the levels of significance: * is P≤0.05, ** is P≤0.01, and *** is P≤0.001.

4. RESULTS

4.1. HIV-1 Impairs Cellular Sumoylation *in vitro*

pfNL43-dE-EGFP is a lentiviral plasmid encoding the Human Immunodeficiency Virus type 1 (HIV-1) genome whose expression is driven by long terminal repeats. This plasmid is able to express HIV-1 gag and pol polypeptides as well as viral regulatory elements; however, insertion of an EGFP cassette into *env* gene disables viral particle formation. Therefore, this vector was introduced into Human Embryonic Kidney (HEK293) and Jurkat cells to mimic HIV-1 infection *in vitro*. Studies on temporal dynamics of HIV-1 expression revealed that the HIV-1 life cycle is accomplished within 24 hours upon infection (Mohammadi et al., 2013). Hence, the experimental examination was carried out in 24-hour time intervals.

HEK293 cells transfected with pfNL43-dE-EGFP were subjected to confocal microscopy imaging to verify the viral expression. EGFP signal was detected at 24 hours post-transfection (Figure 4.1). After the preliminary verification, EGFP expression was confirmed biochemically via Western Blot by probing anti-GFP antibodies. EGFP protein is expressed in the HEK293 cells transfected with pfNL43-dE-EGFP, but not in the cells transfected with a control construct. HIV-1 integrase and rev proteins were detected in line with the EGFP signal (Figure 4.2a), suggesting that the plasmid is successfully delivered into the HEK293 cells. Since HIV-1 predominantly infects $CD4^+$ T cells due to viral tropism (Wilen et al., 2012), pfNL43-dE-EGFP was introduced into Jurkat cells by electroporation to maintain a physiologically relevant system because Jurkat cells are immortalized cell line of human T lymphocytes (Schneider et al., 1977). Western Blot analyses of Jurkat cells by probing against EGFP, HIV-1 integrase, and rev proteins displayed a complementary pattern to HEK293 cells (Figure 4.2b), indicating that electroporation with pfNL43-dE-EGFP was successful.

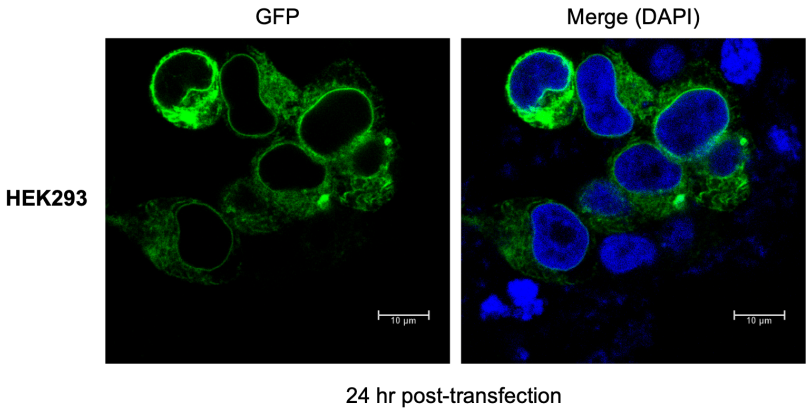

Figure 4.1. Confocal imaging of EGFP expression in pfNL43-dE-EGFP transfected HEK293 cells.

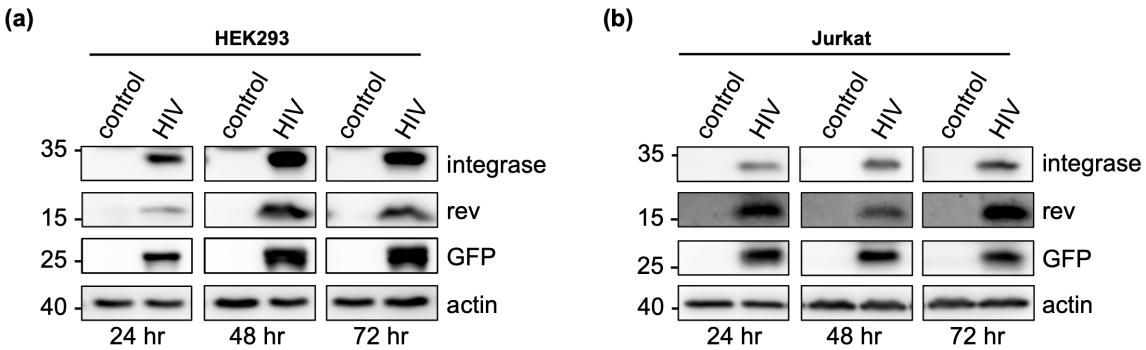

Figure 4.2. Confirmation of HIV-1 expression in HEK293 and Jurkat cells.

In order to characterize the cellular sumoylation dynamics, levels of SUMO1- and SUMO2/3-conjugated proteins were investigated in both HEK293 and Jurkat cells via Western Blot by probing anti-SUMO1 and anti-SUMO2/3 antibodies. Although cellular sumoylation by SUMO1 did not show any significant alterations at 24 hours post-transfection, levels of proteins modified with SUMO2/3 in HEK293 cells transfected with pfNL43-dE-EGFP deviated from the HEK293 cells transfected with control vector. Remarkably, levels of SUMO1- and SUMO2/3-conjugated proteins were significantly reduced at 48 hours post-transfection. While the reduction in the SUMO1-conjugated protein levels was maintained at 72 hours post-transfection, impairment in SUMO2/3 modification was established more dramatically (Figure 4.3a). Physiologically more

relevant Jurkat cells were also investigated for SUMO1 and SUMO2/3-conjugated proteins in 24-hour time intervals. Defects in cellular sumoylation by both SUMO1 and SUMO2/3 manifested in a similar pattern (Figure 4.3b), suggesting that HIV-1 expression diminishes cellular sumoylation mechanism *in vitro*.

Although a control plasmid was delivered to HEK293 and Jurkat cells in order to eliminate the artifacts of transfection and electroporation, we wanted to understand whether this reduction in cellular sumoylation is directly caused by HIV-1 or an artifact of a lentiviral expression system. Therefore, a lentiviral construct encoding only EGFP was introduced into HEK293 and Jurkat cells instead of pfNL-dE-EGFP. EGFP signal was examined by Western Blot by probing anti-GFP antibodies in both cell lines. Levels of SUMO1- and SUMO2/3-conjugated proteins did not deviate from the control vector as expected (Figure 4.4), indicating that the impairment in cellular sumoylation is not a consequence of lentiviral expression system but explicitly induced by HIV-1.

Overall, these data affirm that expression of the derivative of the HIV-1 genome, which is unable to form viral particles but able to produce viral proteins, triggers a drastic reprogramming of global sumoylation *in vitro*. Therefore, this post-translational modification (PTM) could be a potential target of HIV-1 infection.

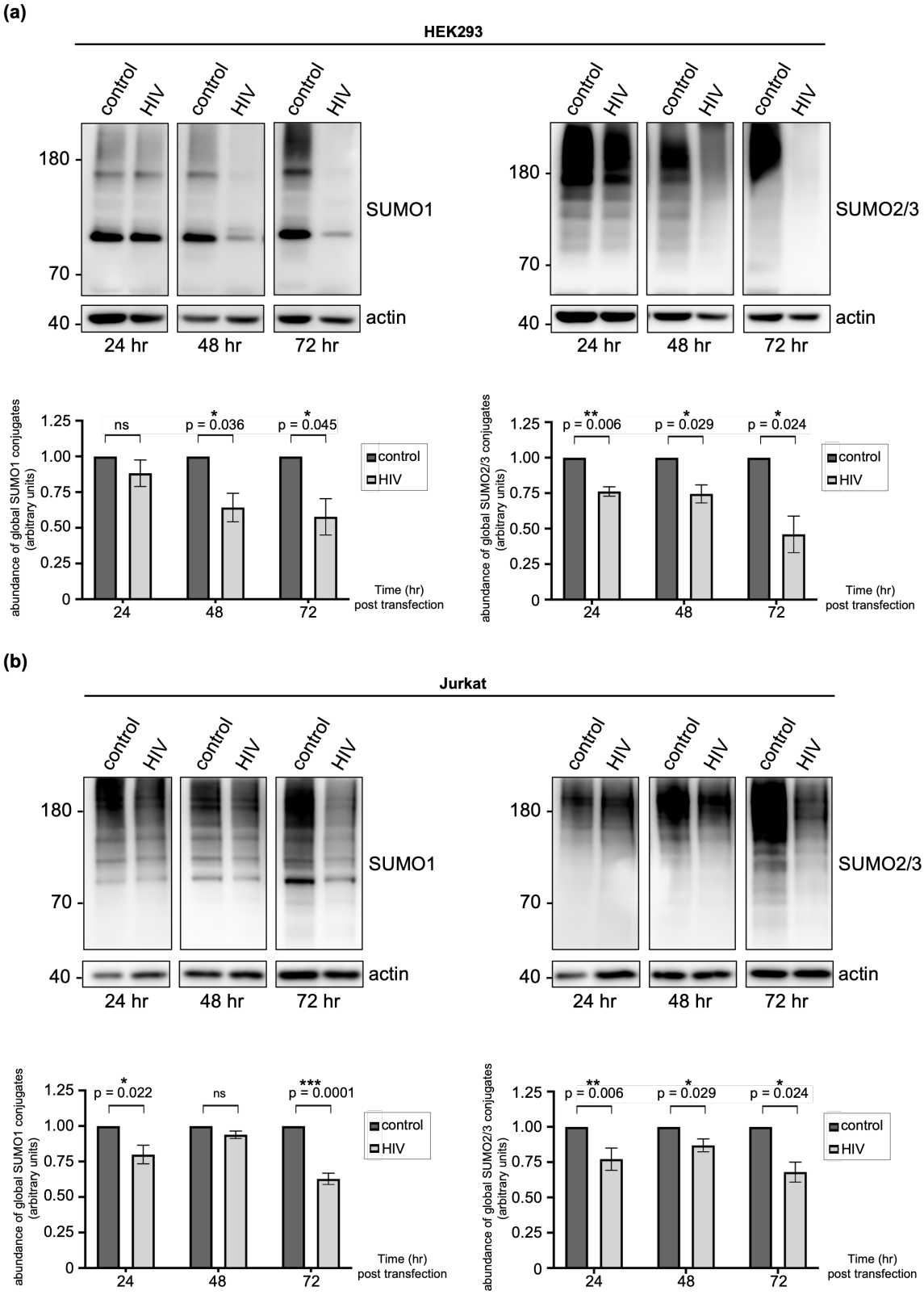

Figure 4.3. Cellular sumoylation profile of HIV-1 genome-expressing HEK293 and Jurkat cells.

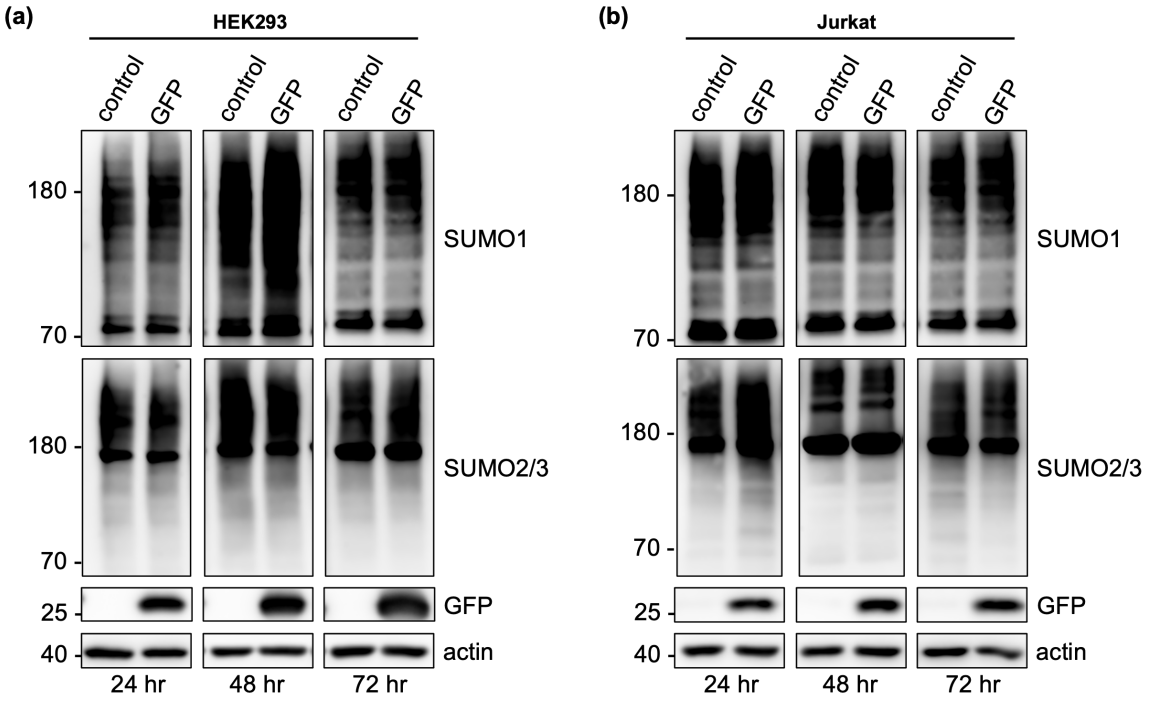

Figure 4.4. Cellular sumoylation profile of EGFP-expressing HEK293 and Jurkat cells.

4.2. HIV-1 Induces a Specific Loss of UBA2 Protein and Interferes with SUMOs' Conjugation

Manipulation of cellular sumoylation by pathogens is a heterogenous phenomenon. Herpes Simplex Virus type 1 (HSV-1) protein ICP0 is a SUMO-targeted Ubiquitin ligase (STUbL), which ubiquitylates explicitly SUMO-conjugated proteins. Proteins modified with SUMO are sent to the proteasome and degraded by it when ubiquitylated by ICP0, indicating that HSV-1 induces rearrangement of cellular sumoylation through proteasomal degradation of sumoylated proteins (Boutell et al., 2011). Therefore, with the aim to grasp whether the mechanism of HIV-1-induced cellular sumoylation loss is to harness the Ubiquitin-proteasome system (UPS), levels of SUMO-conjugated proteins were investigated when proteasomal degradation was interfered with by MG132, a pharmacological inhibitor of the proteasome. HEK293 cells transfected with either the control vector or pfNL43-dE-EGFP were treated with 2 μM MG132 for the last 24 hours before lysis. To address the effects of MG132, another set of HEK293 cells was

incubated with the same amount of DMSO, the vehicle of MG132. Levels of Ubiquitin-conjugated proteins were analyzed via Western Blot using an anti-Ubiquitin antibody. The ubiquitylated proteins were heavily accumulated in the presence of MG132 (Figure 4.5a). Jurkat cells were treated with 2 μM MG132 for the last 24 hours as well. A similar accumulation of Ubiquitin-conjugated proteins was observed when MG132 was added (Figure 4.5b). Therefore, these data, illustrating the concentrated ubiquitylated proteins, suggest successful pharmacological inhibition of the proteasome. To contemplate whether HIV-1 instrumentalizes proteasomal degradation to deplete cellular sumoylation, levels of SUMO1- and SUMO2/3-conjugated proteins were examined in both HEK293 and Jurkat cells. Although proteasome was inhibited, hence, cannot degrade ubiquitylated proteins, cellular sumoylation by both SUMO1 and SUMO2/3 significantly deviated from HEK293 cells transfected with control vector (Figure 4.6a). Jurkat cells, treated with MG132, also displayed a similar pattern of SUMO1- and SUMO2/3-conjugated protein levels (Figure 4.6b). Levels of sumoylated proteins were comparable in the absence or presence of M132, whereas ubiquitylated proteins were accumulated by pharmacological proteasome inhibition. Thereby, these data indicate that HIV-1 does not exploit UPS to antagonize cellular sumoylation.

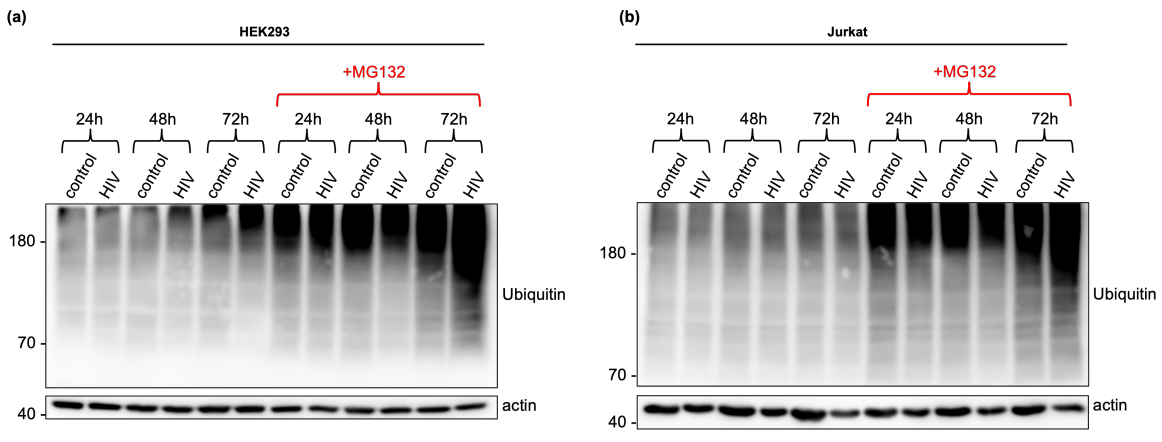

Figure 4.5. Ubiquitylation profile of MG132-treated HEK293 and Jurkat cells.

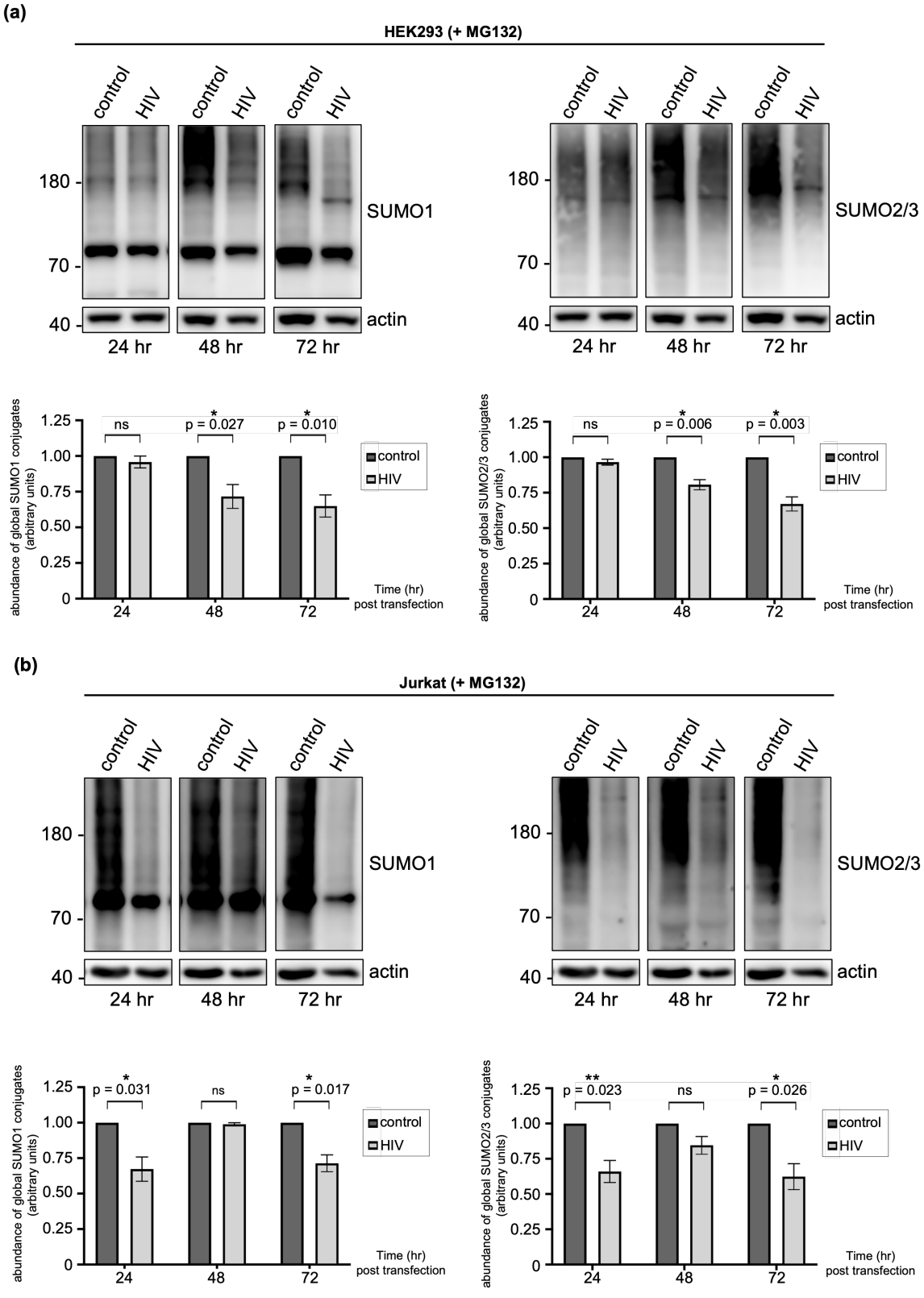

Figure 4.6. Cellular sumoylation profile of MG132-treated HEK293 and Jurkat cells.

Miscellaneous quality of reorganization of cellular sumoylation by pathogens acknowledges other possible schemes for the revision of sumoylated protein abundance by HIV-1. On the one hand, *Listeria monocytogenes* utilizes the bacterial virulence factor listeriolysin O (LLO) to establish insufficient cellular sumoylation by degrading UBC9, SUMO-conjugating enzyme in a proteasome-independent fashion (Ribet et al., 2010). On the other hand, *Klebsiella pneumoniae* limits cellular sumoylation via stimulating desumoylation by SENP2, a SUMO deconjugating enzyme, or via translational repression of SUMO transcripts by let-7 microRNA (Sá-Pessoa et al., 2020). Hence, HIV-1 could possibly impede SUMO conjugation or endorse SUMO deconjugation. With the intention of disclosing the possible mechanism of HIV-1 to govern cellular sumoylation, unconjugated levels of SUMO1 and SUMO2/3 proteins were analyzed by Western Blot in both HEK293 and Jurkat cells. Although SUMO2/3 protein levels did not alter significantly, SUMO1 protein amasses through time while SUMO1-conjugated protein levels abate (Figure 4.7), suggesting HIV-1 readjusts either SUMO conjugation or deconjugation. To dissect the mechanism hijacked by HIV-1, enzymes in the SUMO conjugation pathway were examined via Western Blot. Proteolytic cleavage of SUMOs unmasks a C-terminal diglycine (-GG) motif supplying mature proteins. Heterodimeric SUMO-activating enzyme (SAE1/UBA2) activates SUMOs in ATP-dependent manner. Activation of SUMOs is followed by their transfer onto UBC9, the only SUMO-conjugating enzyme. Although this cascade is very similar to ubiquitylation pathway, sumoylation does not predominantly rely on the presence of E3 SUMO ligases as UBC9 directly or through E3 SUMO ligases (Celen & Sahin, 2020). Therefore, sumoylation fundamentally depends on E1 and E2 enzymes. Western Blot characterization of E1 and E2 enzymes essentially addresses the aim of whether HIV-1 exploits the SUMO conjugation cascade. Surprisingly, HEK293 cells transfected with pfNL43-dE-EGFP featured similar levels of UBC9 compared to HEK293 cells transfected with the control vector, contradicting Listeria's mechanism. Likewise, SAE1 levels, a subunit of the SUMO E1 enzyme, demonstrated similar patterns. Strikingly, HIV-1 genome introduction deployed a deterioration in UBA2 levels at 48 hours post-transfection. This decline cumulated at 72 hours post-transfection in line with the devaluation in cellular sumoylation (Figure 4.8a). UBC9 and SAE1 levels in Jurkat cells did not deviate from

the controls when the HIV-1 genome was expressed, either. Notably, time-dependent decay of UBA2 was manifested by HIV-1 genome expression (Figure 4.8b), suggesting that HIV-1 employs an insult to SUMO conjugation pathway via promoting a specific UBA2 deficiency.

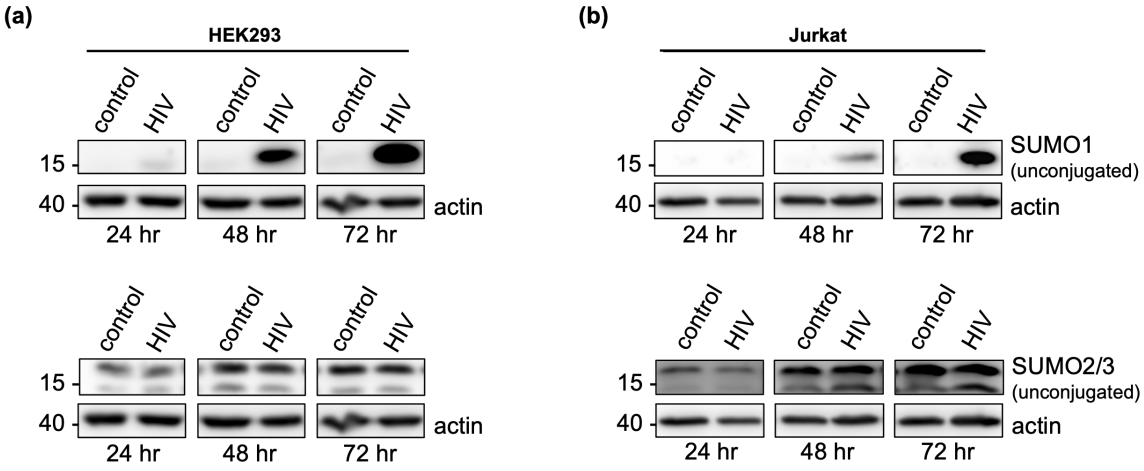

Figure 4.7. Unconjugated SUMO1 and SUMO2/3 proteins in HIV-1-expressing HEK293 and Jurkat cells.

Although UBA2 deteriorates upon simulation of HIV-1 infection, the strategy of this virus to diminish UBA2 was not clearly understood. There are disparate approaches that could be administered by HIV-1 to destabilize UBA2 levels. HIV-1 tat protein is able to differentiate the transcriptional program of this virus by recruiting cellular factors to the 5' stem-loop transactivation response (TAR) element of nascent viral RNAs (Mancebo et al., 1997). HIV-1 protein tat also instrumentalizes the topology of epigenetic markers as well as the RNA Polymerase II (Pol II) to orchestrate cellular transcriptional program (Reeder et al., 2015). Considering the multitasking competence of the tat in transcriptional regulation, transcriptional downregulation of *UBA2* mRNA is one of the possible hypotheses. Thereupon, relative UBA2 transcript levels were measured in both HEK293 and Jurkat cells. Total RNA was isolated from HEK293 cells transfected with either the control plasmid or pfNL43-dE-EGFP. Then, cDNA was synthesized by reverse transcription. RT-qPCR analyses revealed nonsignificant fluctuation in UBA2 RNA levels (Figure 4.9a). Jurkat cells subjected to electroporation with the control or pfNL43-dE-EGFP vectors demonstrated a similar

pattern except for a significant decline in UBA2 RNA abundance at 48 hours post-transfection (Figure 4.9b), indicating that HIV-1 does not regulate UBA2 transcription substantially.

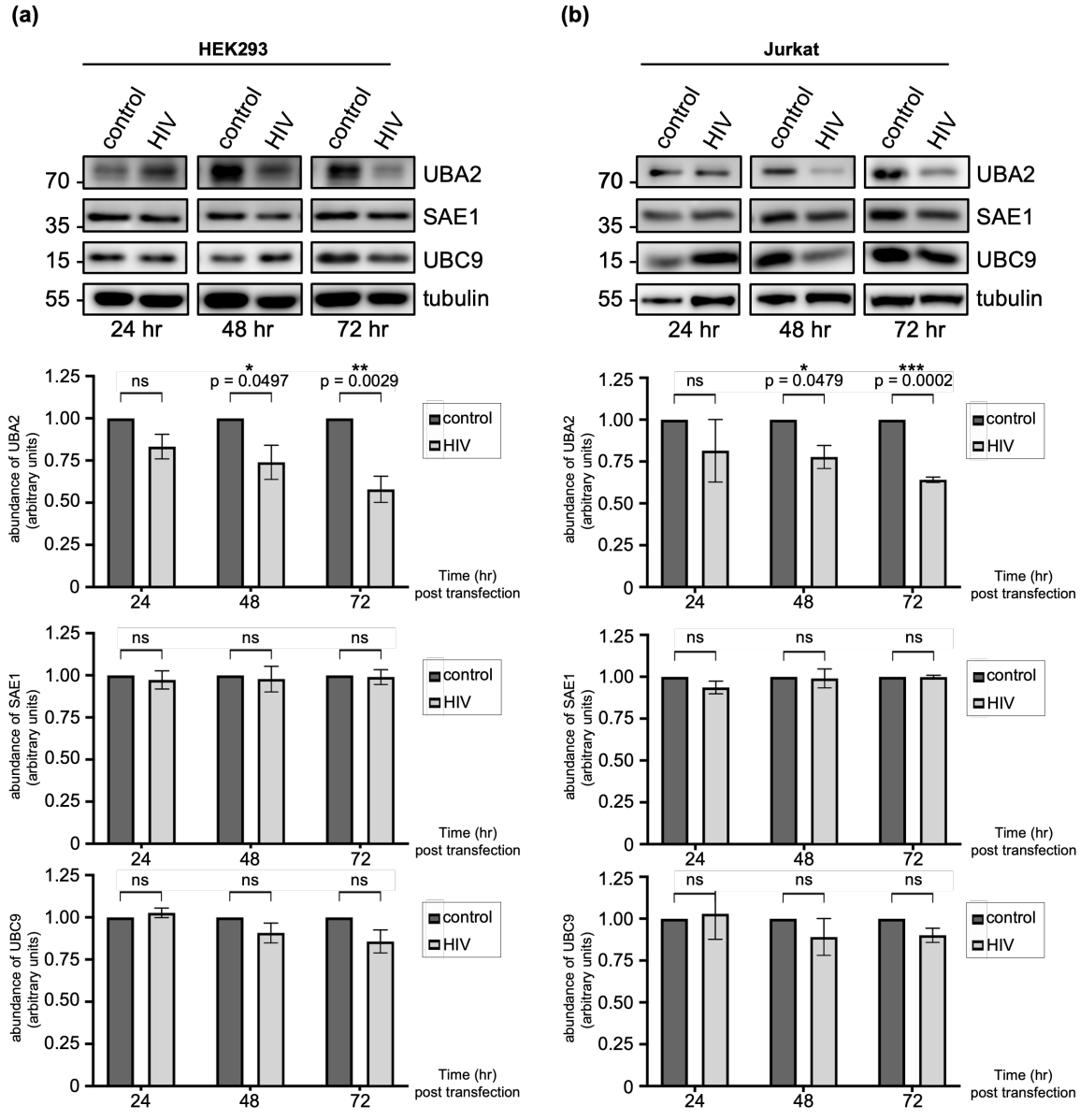

Figure 4.8. Sumoylation cascade of HIV-1-expressing HEK293 and Jurkat cells.

HIV-1 could extensively strategize to remodel cellular sumoylation via ravaging UBA2 protein itself, including translational repression of UBA2 transcript and depleted protein stability rather than transcriptional regulation. With the aim of discerning whether the UBA2 protein is destabilized by HIV-1, the possibility of proteasomal degradation of UBA2 was assessed. HEK293 and Jurkat cells expressing the HIV-1 genome were treated with either the vehicle or MG132 for the last 24 hours, as explained. Both HEK293 and Jurkat cells treated with MG132 showed an accumulation of ubiquitylated proteins; however, proteasomal inhibition did not rescue the UBA2 decay (Figure 4.10). These data imply the proteasomal-independent mechanism of UBA2 decay.

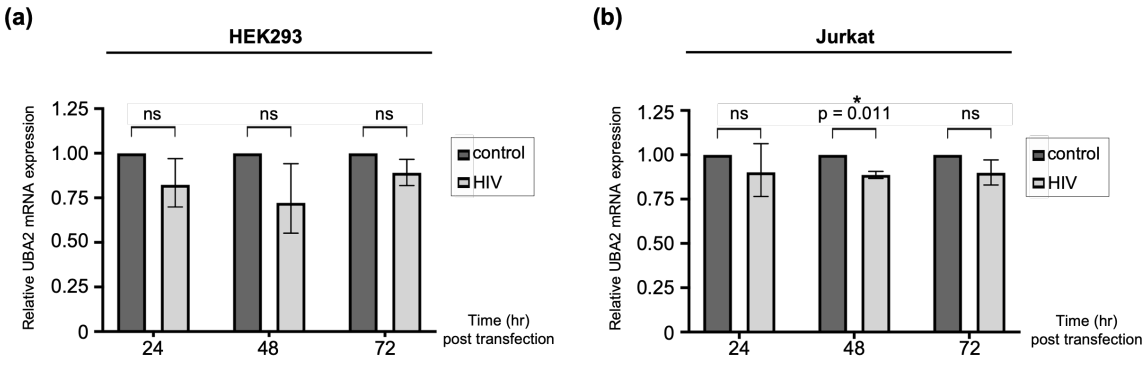

Figure 4.9. Relative *UBA2* mRNA levels of HIV-1-expressing HEK293 and Jurkat cells.

HIV-1 skillfully dictates diverse autophagic regulations (Killian, 2012; Santerre et al., 2021; Wang et al., 2012), hinting that UBA2 might be sequestered in autophagosomes and degraded. Thereby, the autophagic blockade was introduced by 30 μM chloroquine (Cq), a pharmaceutic agent, for the 24 hours before lysis. LC3B-II accumulated in the presence of Cq (Figure 4.11), suggesting successful inhibition of autophagy. Heretofore, our data established an HIV-1-induced conjugation loss of SUMO proteins due to defects in physiological UBA2 levels. Therefore, we firstly examined cellular sumoylation. The abundance of SUMO1- and SUMO2/3-conjugated proteins was investigated by Western Blot in HEK293 cells transfected with pfNL43-dE-EGFP to address the cellular sumoylation. When these cells were treated with Cq, despite

the concentrated levels of LC3B-II, SUMO1- and SUMO2/3-conjugated protein levels remained reduced in HIV-1 genome-expressing cells (Figure 4.12). Then, UBA2 protein levels were assessed via Western Blot to understand the consequence of autophagy inhibition. UBA2 protein displayed lower levels in HEK293 cells expressing HIV-1 genome (Figure 4.13). Together, these data entailed that inhibition of autophagy does not rescue the restrained UBA2 protein, thus, the restricted cellular sumoylation. Hereby, HIV-1 does not utilize autophagy to debilitate SUMO conjugation.

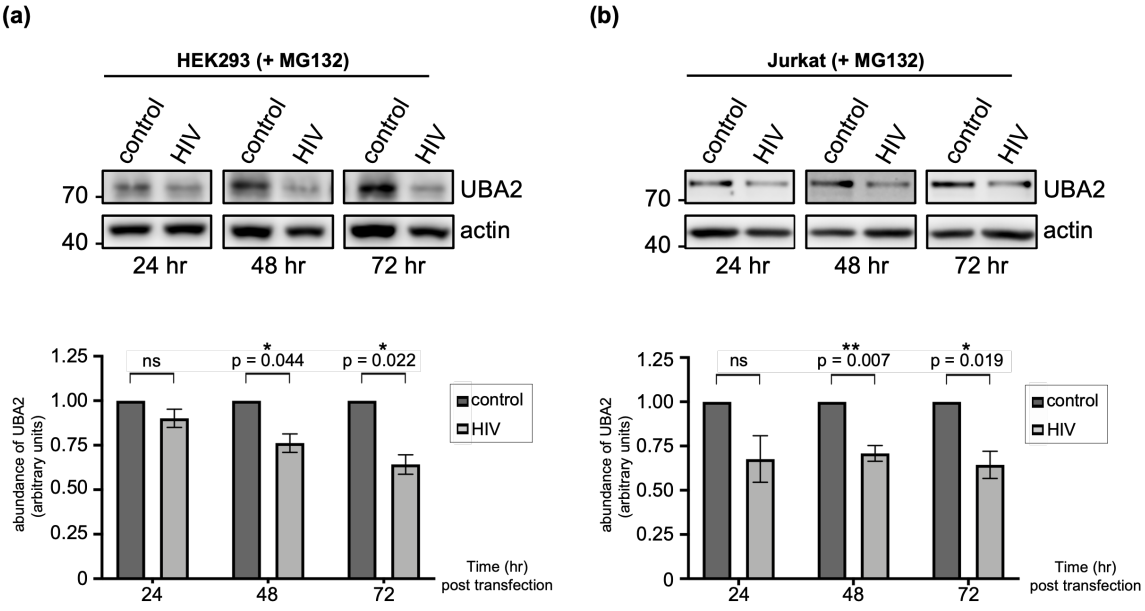

Figure 4.10. UBA2 protein levels of MG132-treated HEK293 and Jurkat cells.

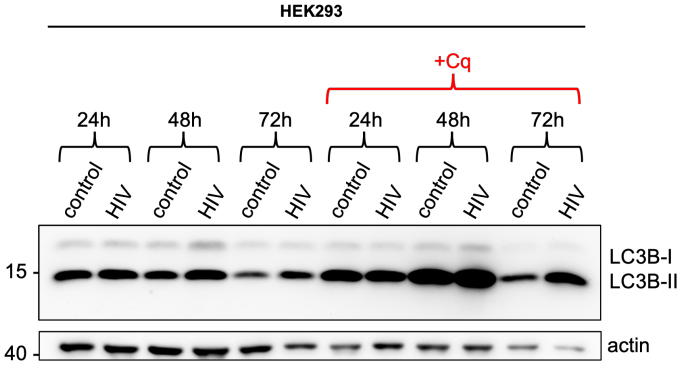

Figure 4.11. LC3B protein levels of Cq-treated HEK293 cells.

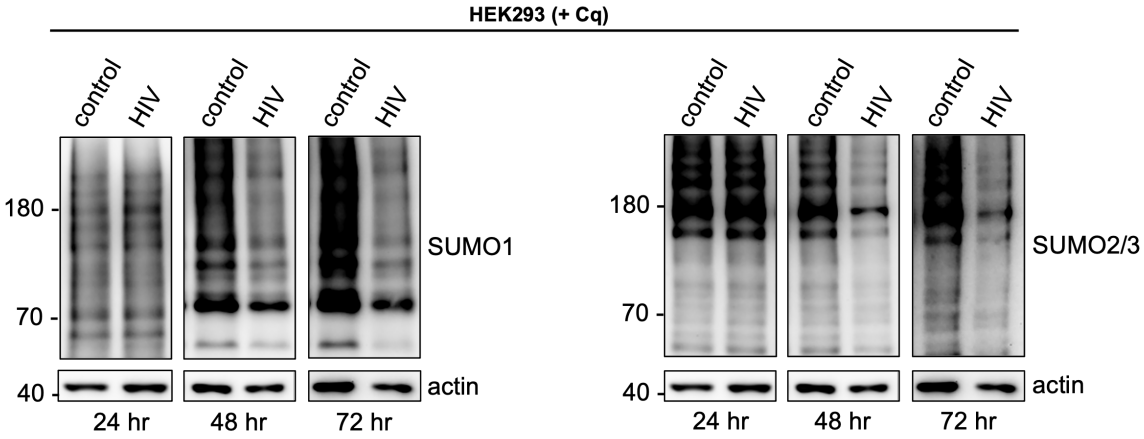

Figure 4.12. Sumoylation profile of Cq-treated HEK293 cells.

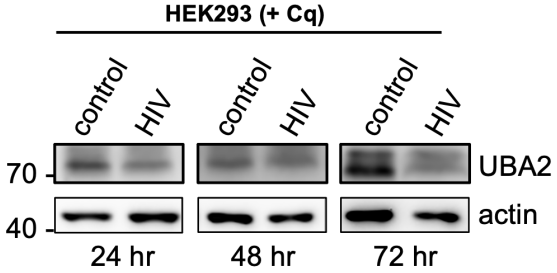

Figure 4.13. UBA2 protein levels of Cq-treated HEK293 cells.

These data, so far, acknowledge that HIV-1 abates UBA2, causing impairment in SUMO conjugation, thus, a decay in cellular sumoylation in vitro. Collectively, these results acquaint that the SUMO-conjugation pathway is tarnished via unknown machinery by HIV-1 genome expression. HIV-1 endorses various insults to the host cells, leading to numerous defects throughout the immune system. HIV-1 hijacks various cellular transcription factors, including NF-κB (Nabel & Baltimore, 1987) whose activation is regulated by sumoylation (Desterro et al., 1998). Although this transcription factor is a regulator of antiviral response, HIV-1 seizes control of the NF-κB-induced transcriptional regulation to enhance its own replication. Conceding the critical functions of SUMOs in immunity, including the versatile behavior of sumoylation in immune signaling (Adorisio et al., 2017) and their interferon (IFN)-induced antiviral activity (Sahin et al., 2014), dysfunction in cellular sumoylation in HIV-1 infection accentu-

ates a novel strategy to incapacitate immunity. Here, by the discovery of sumoylation deficiency, we acknowledge another layer to the sophisticated machinery of HIV-1 infection.

5. DISCUSSION

Human Immunodeficiency Virus is a retrovirus interrupting numerous cellular processes, including cytoskeleton topology (Paoletti et al., 2019), cellular trafficking machineries (Tavares et al., 2021), and epigenetic landscape (Reeder et al., 2015). The manifestation of HIV infection is dependent on the equilibrium of viral manipulation and antiviral defense mechanisms. The cellular immune responses are tightly controlled via post-translational modifications, including sumoylation. For example, sumoylation finetunes NF-κB and interferon signaling (Adorisio et al., 2017). Additionally, interferon stimulates SUMO proteins, restricting the viral infection (Sahin et al., 2014). Considering the role of sumoylation in the immune system, it is not surprising yet very impressing that pathogens has prospered strategies to overwhelm the innate immune responses via reconstructing the cellular sumoylation patterns (Everett et al., 2013; Lowrey et al., 2017; Ribet & Cossart, 2010; Wimmer et al., 2012). HSV-1 impairs the cellular sumoylation by ubiquitylation of sumoylated proteins by the viral factor ICP0 acting as an E3 ubiquitin ligase (Boutell et al., 2002, 2011; Sloan et al., 2015), whereas Listeria monocytogenes proteolytically degrades UBC9, the universal E2 SUMO-conjugating enzyme, through its factor LLO, resulting in the abrogation of cellular sumoylation. Likewise, Klebsiella pneumonia is known to downregulate SUMO expression and trigger desumoylation (Sá-Pessoa et al., 2020). The impairment in the cellular sumoylation system favors the infection, enabling the pathogens to evade the immune responses (Everett et al., 2013; Lowrey et al., 2017; Ribet & Cossart, 2010; Wimmer et al., 2012). Despite the juxtaposition of a collection of HIV proteins, including viral integrase that is sumoylated (Zamborlini et al., 2011), cellular sumoylation in the host-pathogen interaction axis of HIV infection was not documented. Hereby, we illustrated that HIV-1 abolishes sumoylation by targeting UBA2, a subunit of E1 SUMO-activating enzyme *in vitro*.

Our studies showed that the expression of the HIV-1 genome in both HEK293 and Jurkat cell lines declines the cellular sumoylation by both SUMO1 and SUMO2/3 in a time-dependent manner. An accumulation of unconjugated SUMO1 protein levels was

manifested in HIV-1 genome-expressing cells in line with the reduction in sumoylation. The investigation of possible mechanisms revealed that the inhibition of proteasome did not rescue the HIV-1-induced sumoylation loss, whereas the abundance of UBA2 protein is abrogated, indicating an incompetent SUMO conjugation behavior. We investigated the decline in UBA2 protein levels by examining possible scenarios. However, our results demonstrated that HIV-1 does neither transcriptionally downregulate UBA2 expression nor degrade the protein in a proteasome- or autophagy-dependent fashion. Altogether, these entail that HIV-1 engages an insult to the cellular sumoylation machinery by repealing the UBA2 protein.

Although we observed a decline in both SUMO1- and SUMO2/3-conjugated proteins, the patterns of decline differed. The abrogation in cellular sumoylation by SUMO1 was less than by SUMO2/3 in HEK293 cells, but almost the same in Jurkat cells, implying that HIV-1 mostly favors antagonizing SUMO2/3 conjugation. Those SUMO paralogs are competent for conjugation. Nevertheless, the consequence of sumoylation by either paralog may vary (Celen & Sahin, 2020; Sahin et al., 2022; Saitoh & Hinchey, 2000). The cellular stresses, including oxidative stress, trigger the conjugation of SUMO2/3 (Celen & Sahin, 2020; Golebiowski et al., 2009; Saitoh & Hinchey, 2000); however, SUMO1 is heavily conjugated to its substrates under the physiological conditions (Celen & Sahin, 2020). HIV-1 infection is known to accumulate reactive oxygen species, assaulting the cells with oxidative stress (Couret & Chang, 2016; Ivanov et al., 2016; Pace & Leaf, 1995). Considering the role of SUMO2/3 in the control of oxidative stress, the impairment in sumoylation by SUMO2/3 disrupts not only the antiviral responses but defense strategies against HIV-induced oxidative stress as well. Therefore, SUMO2/3 might be the primary target of HIV-1. HSV-1-induced sumoylation loss is also predominantly in SUMO2 (Boutell et al., 2011; Sloan et al., 2015), indicating that viral infections might primarily antagonize SUMO2/3. However, Jurkat cell lines displayed a similar deviation from physiological sumoylation when the HIV-1 genome was expressed. Hence, SUMO1 and SUMO2/3 might equally contribute to the antiviral defense against HIV-1. Of note, the cellular events might alter in primary and cultured cells. Additionally, we detected another distinct pattern in SUMO1 and SUMO2/3. SUMO1, upon the expression of the HIV-1 genome, started

to be concentrated in parallel with the decline in SUMO1-conjugated protein levels in a time-dependent manner. However, unconjugated SUMO2/3 protein levels did not vary significantly. As described, SUMO1 is commonly attached to its substrates under physiological conditions (Celen & Sahin, 2020; Saitoh & Hinchey, 2000), implying that unconjugated SUMO1 availability is lower than unconjugated SUMO2/3. Consequently, the increase in unconjugated SUMO2/3 levels might not be apparent.

We wanted to explain how HIV-1 abolishes cellular sumoylation. Therefore, we acknowledged possible schemes. HIV-1, like HSV-1, might utilize a viral factor as a STUbL (Boutell et al., 2002, 2011; Sloan et al., 2015) or hijack a cellular E3 ubiquitin ligase to tag sumoylated proteins with ubiquitin, equipping them for proteasomal degradation. We also investigated whether HIV-1 endangers the SUMO conjugation cascade as proteosome-independent degradation of UBC9 in Listeria monocytogenes infection (Ribet et al., 2010). Our studies displayed that UBA2 protein levels were reduced by approximately 50% at 72 hours post-transfection in both HEK293 and Jurkat cells. Nonetheless, SAE1, the other subunit of E1 SUMO-activating enzyme, and UBC9 levels did not deviate from the cells transfected with a control vector. The impairment in the SUMO conjugation pathway could simply resolve the decrease in cellular sumoylation in HIV-1-expressing cells. However, the mechanistic facet of this UBA2 decrease remains unclear. Since HIV factor the tat is known to have transactivation activity (Karn & Stoltzfus, 2012) and to reconstruct the epigenetic topology of the host (Reeder et al., 2015), HIV is able to reprogram the cellular transcriptome during infection. In order to explore the mechanism, we investigated the transcriptional regulation of UBA2 expression, yet UBA2 mRNA levels remained the same in HIV-1-expressing cells. We showed that HIV-1 does not utilize viral or cellular STUbLs to ubiquitylate sumoylated proteins directly. However, viral factors vif, vpx (in HIV-2), and vpu recruit the E3 ubiquitin ligases to overcome the restriction factors (Seissler et al., 2017), indicating that HIV-1 might ubiquitylate UBA2 specifically to eliminate cellular sumoylation. Therefore, we also inhibit proteosomes to understand whether UBA2 is degraded in a proteosome-dependent manner. Nevertheless, the UBA2 protein was not rescued through proteasome blockage, implying that HIV-1 does not ubiquitylate, thus, does not address UBA2 to the proteasome. HIV-1 can finetune autophagy

versatilely in its hosts (Killian, 2012; Santerre et al., 2021; Wang et al., 2012). Therefore, we investigated the cellular sumoylation and UBA2 levels by blocking autophagy. Inhibition of autophagy rescued neither cellular sumoylation nor UBA2 levels, indicating HIV-1 does not harness autophagy for UBA2 clearance. Overall, these suggest that a reduction in UBA2 levels results in deficiencies in SUMO conjugation. However, the mechanism underlying this UBA2 decay is not clearly understood. These suggest that HIV-1 might degrade UBA2 in a proteosome-independent manner through sequestering UBA2 by cellular proteases considering that HIV utilizes Furin and Furin-like proteases to process viral env and tat proteins (Gu et al., 1995; Hallenberger et al., 1992; Tikhonov et al., 2004). HIV also interacts with cellular translation machinery to express its own genome. Moreover, HIV protein rev is an RNA-binding protein that translocates viral RNAs from the nucleus to the cytoplasm (Karn & Stoltzfus, 2012). Contemplating the regulation of RNAs and their translation in HIV infection, this virus might be able to interact with cellular RNAs, including UBA2 mRNA. This interaction might lead to the spatial downregulation of UBA2 expression by translational repression as well. Thereby, the translational efficiency of UBA2 mRNA and the contribution of cellular proteases should be investigated.

Interferon signaling act through SUMO proteins to produce an antiviral response to restrict viral infection (Sahin et al., 2014). The interplay between interferon signaling and HIV infection is not very well understood (Utay Douek, 2016). Additionally, interferon's anti-HIV activity is low compared to anti-HSV-1 activity when ICP0 is deleted (Sahin et al., 2014). Considering that ICP0 antagonizes cellular sumoylation by sequestering sumoylated proteins into the proteasome for degradation (Boutell et al., 2002, 2011; Sloan et al., 2015) and our findings explaining HIV-1 also diminishing cellular sumoylation, the limited anti-HIV-1 activity of interferons might be explained. This can be further investigated via the identification of HIV-1 factors dampening UBA2 and by the examination of interferon activity on HIV-1 restriction when those viral factors are deleted.

Overall, our study elucidates that HIV-1 assaults cellular sumoylation, possibly via invading SUMO conjugation cascade by targeting UBA2. The HIV-1-induced

sumoylation loss might have high-degree outcomes, including the impairment in immune signaling in accordance with the roles of SUMO proteins in immune signaling. Thus, this study decodes a novel aspect of the manifestation of sophisticated HIV-1 infection mechanism to ravage its host, invading the immune system to escape from immune responses.

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APPENDIX A: PLASMID MAP

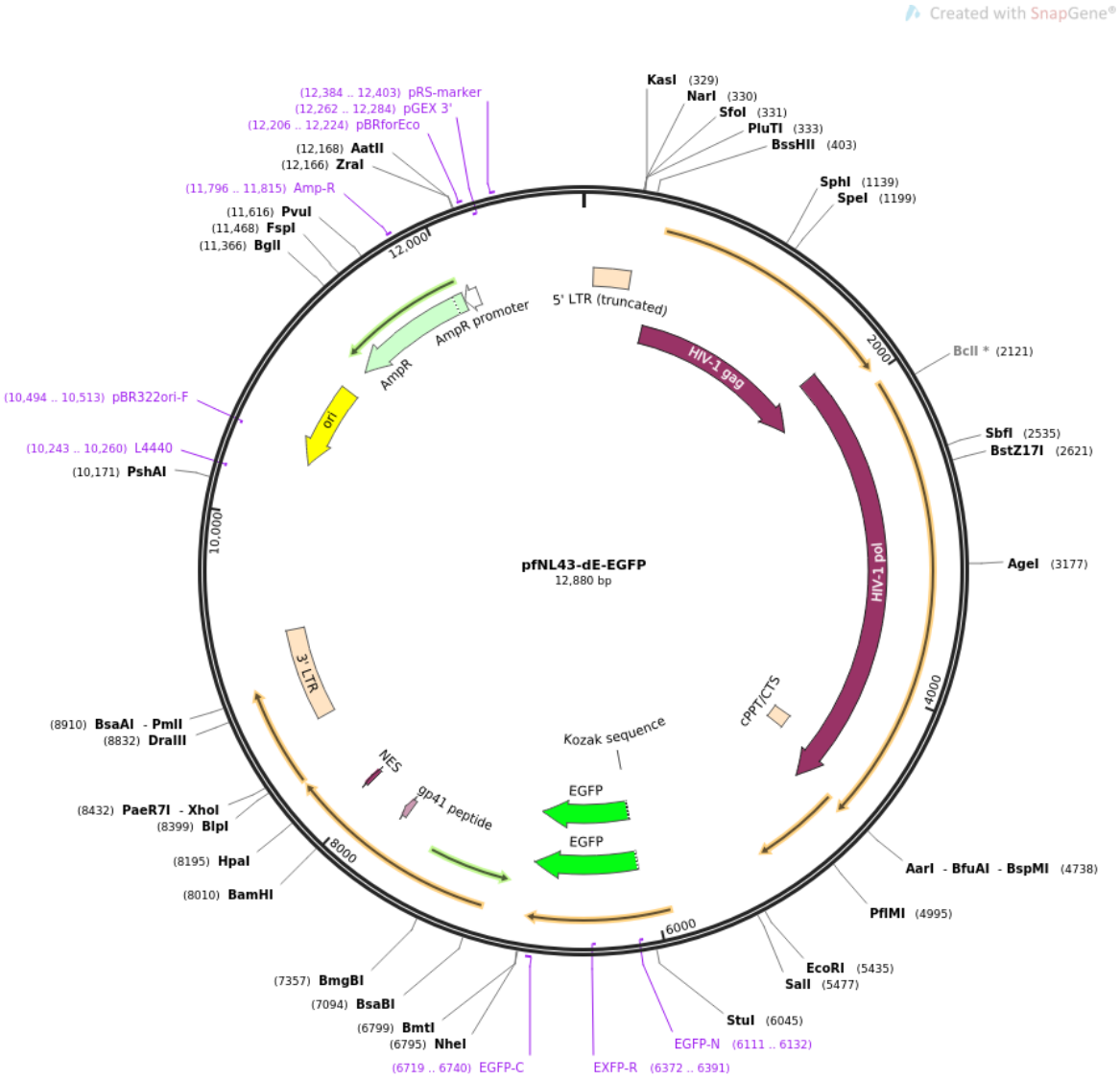

Figure A.1. The map of pfNL-43-dE-EGFP.

APPENDIX B: PERMISSION FOR QUOTED FIGURES AND TABLES

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