UNDERSTANDING THE ROLE OF NUCLEOPORINS DURING HIV-1 INFECTION

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BY

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DECLARATION

I, Kumaraswami Chintala, hereby declare that this thesis entitled "**UNDERSTANDING THE ROLE OF NUCLEOPORINS DURING HIV-1 INFECTION**" submitted by me under the guidance and supervision of Prof. Sharmistha Banerjee, is an original and independent research work. I also declare that this research work has not been submitted previously in part or full to this university or any other university or institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "**UNDERSTANDING THE ROLE OF NUCLEOPORINS DURING HIV-1 INFECTION**" submitted by **Kumaraswami Chintala, bearing Reg. No 15LBPH15** in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Department of Biochemistry, School of Life Sciences, University of Hyderabad is a bona fide research work carried out by him under my supervision and guidance. This work is original and has not been submitted previously in part or full to this university or any other university or institution for the award of any degree or diploma.

A part of this work has been

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Dedication

To my daughter, Lana Sri

To my Father, Anjaiah

To my mother, Narsamma

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List of Abbreviations

AIDS	acquired immunodeficiency syndrome
APOBEC3	apolipoprotein β-mRNA editing catalytic polypeptide-3
AZT	azidothymidine
cART	combination antiretroviral therapy
CA	capsid
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
CTD	carboxyl-terminal domain
CXCR4	C-X-C chemokine receptor type 4
CYPA	cyclophilin A
DAPI	4’,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
Env	envelope
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gag	group specific antigen
GFP	green fluorescent protein
GLFG	glycine-leucine-phenyl alanine-glycine
GTP	guanosine-5'-triphosphate
HDAC	histone deacetylase
HEXIM1	hexamethylene bis-acetamide inducible 1
HIV-1	human immunodeficiency virus type 1
IN	integrase
kDa	kilodalton

LTR	long terminal repeat
MA	matrix
mRNA	messenger RNA
MX2	myxovirus resistance-2
NC	nucleocapsid
Nef	negative regulatory factor
NE	nuclear envelope
NF-κB	nuclear factor kappa B
NPC	nuclear pore complex
NRE	negative regulatory element
NTD	amino-terminal domain
NUP	nucleoporin
PBS	phosphate-buffered saline
P-TEFb	positive transcription elongation factor b
PIC	preintegration complex
Pol	polymerase
PR	protease
qPCR	quantitative real-time polymerase chain reaction
Rev	regulator of virion expression
RT	reverse transcriptase
RNA	ribonucleic acid
TAR	trans-activating response element
TBS	tris-buffered saline
TBST	tris-buffered saline tween 20
Tat	trans-activator of transcription
Vpr	viral protein R
Vpu	viral protein U

VSV-G	vesicular stomatitis virus envelope glycoprotein G

List of Publications

1. **Kumaraswami Chintala**, Sriram Yandrapally, Warisha Faiz, Chhaya Rani Kispotta, Satarupa Sarkar, Krishnaveni Mishra and Sharmistha Banerjee: Nuclear pore protein NUP98 impedes LTR-driven basal gene expression of HIV-1, viral propagation and infectivity; Frontiers in Immunology (2024). DOI 10.3389/fimmu.2024.1330738

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3. Vengala Rao Yenuganti, Sumbul Afroz, Rafiq Ahmad Khan, Chandrima Bharadwaj, Deepti Kailash Nabariya, Nagaraj Nayak, Madhuri Subbiah, **Kumaraswami Chintala**, Sharmistha Banerjee, Pallu Reddanna, Nooruddin Khan: Milk exosomes elicit a potent anti-viral activity against dengue virus; Journal of Nanobiotechnology (2022). doi.org/10.1186/s12951-022-01496-5

4. Satarupa Sarkar, Kannan Balakrishnan, **Kumaraswami Chintala**, Krishnaveni Mohareer, Tom Luedde , Ananda Ayyappan Jaguva Vasudevan , Carsten Münk, Sharmistha Banerjee: Tough Way In, Tough Way Out: The Complex Interplay of Host and Viral Factors in Nucleocytoplasmic Trafficking during HIV-1 Infection; Viruses MDPI (2022). doi.org/10.3390/v14112503

5. Rajkishor Nishad , Dhanunjay Mukhi , Ashish Kumar Singh , Manga Motrapu , **Kumaraswami Chintala** , Prasad Tammineni , Anil K Pasupulati: Growth hormone induces mitotic catastrophe of glomerular podocytes and contributes to proteinuria; Cell Death Disease (2021). doi.org/10.1038/s41419-021-03643-6

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Chapter 1

Introduction

Chapter 1: Introduction

1.1 HIV-1: The etiological agent of AIDS

Human immunodeficiency virus type-1 (HIV-1) causes acquired immunodeficiency syndrome (AIDS) in humans and is responsible for 40.4 million deaths worldwide since its discovery in 1981. In 2022, there were 39 million people living with HIV infection across the globe, which makes a total of 85.6 million infections since the start of epidemic (1). An individual may acquire HIV-1 via three routes; 1) sexual intercourse; 2) blood transfusion; 3) mother to child, e.g., through breast feeding. Mother to child transmission may also be possible during parturition if there is a blood to body fluid contact. It was estimated that about 85% of HIV-1 infections were transmitted sexually, while the remaining 15% cases were transmitted through contaminated injection needles, blood transfusions from infected individuals to healthy individuals or from mother to child (2). HIV-1 infects and depletes one of the major adaptive immune cells, CD4+ T cells, and progresses through three different phases of course of infection in an infected individual, i.e. acute, chronic and AIDS, followed by eventual death if left untreated (3–5). In acute phase, soon after infection, HIV-1 replicates, disseminates and establishes latency reservoirs, as a result of which there is a decrease in the CD4+ cell count in the blood and this effect is massive in lymphoid tissues such as gut associated lymphoid tissue (GALT) (6–8). Following acute phase, which lasts for a few weeks, HIV-1 enters a chronic phase which is characterised by a decline in the viral RNA copies in the plasma and an increase in the CD4+ cell count from its lowest point. However, in the absence of therapeutic treatment, as this phase progresses, the recovery of CD4+ cell count starts to decrease and viral load begins to stabilise. Although this phase, which lasts for about 10 years, is sometimes referred to as clinical latency because an infected individual does not show any pathological symptoms, there is an actual progressive viral replication that leads to the condition of AIDS. AIDS phase is characterised by a drastic decrease in CD4+ cells, a rapid and continuous viral replication and the occurrence of opportunistic infections such as *Pneumocystis carinii* pneumonia,

Kaposi Sarcoma, mucosal candidiasis, etc. that leads to the death of an infected individual (4,5,9–12).

1.2 Anti HIV-1 strategies: The quest for cure

Antiretroviral therapy: The quality of life of people living with HIV-1 has tremendously improved since the introduction of antiretroviral therapy (ART). The first drug, azidothymidine (AZT) also known as Zidovudine, which is a nucleoside analogue and an inhibitor of viral reverse transcriptase (RT), was introduced in 1987 and has been able to reduce viral RNA copies in the blood (13). However, the first class of ART that targeted RT including AZT had high toxicity and viral resistance (14). This prompted the development of the second class of ART that targeted viral protease with the first, Saquinavir, being introduced in 1995. After extensive testing, clinicians introduced more than one drug in a combination, marking the era of highly active antiretroviral therapy (HAART)/combination antiretroviral therapy (cART) (15,16). The HAART was able to drastically reduce the viral load in the bloodstream and mitigate the incidence of opportunistic infections in infected individuals. These drugs were successful not only in terms of their efficacy in reducing the viral load but also because they have less toxicity and their dosages were minimized. In recent years, several drugs targeting different essential viral steps have been combined into a single pill and given once daily. This allows simpler adherence to the regime of treatment, marking the conversion of threatening AIDS to a less severe chronic disease. Nevertheless, viral resistance for almost every drug reported has been one of the causes of failure of ART against HIV-1 (17). This viral resistance is majorly due to mutations in the viral proteins that the drugs act against, owing to error-prone genome replication mediated by RT. In addition, cessation of ART results in the rebound of the virus from latency, pointing out that an infected individual must take a regime life-long, which makes AIDS incurable.

Latency reversal agents: During latency, cells infected with HIV-1 have integrated viral genome but do not allow viral gene transcription to occur from HIV-1 promoter, long terminal repeat (LTR). This viral latency results from a complex interplay between host cell and virus-mediated molecular mechanisms including epigenetic modifications, transcription interference, and regulation in the availability of the

transcription factors that affect the transcriptional status of HIV-1 promoter, LTR (18)]. Notably, these latent reservoirs are varying in terms of cell types and tissue distribution. While resting memory CD4+ T cells are thought to be the main latent reservoir for HIV-1, macrophages, monocytes, microglia, and astrocytes also act as sanctuaries for the establishment of latency (19–22). Further, these reservoirs are found in a wide variety of tissues such as lymph nodes, the central nervous system, and the intestine (23,24). Therefore, targeting the latent reservoirs that are widely distributed is an extremely daunting task and requires an explicit understanding of the molecular mechanisms that establish and maintain viral latency for the cure of HIV-1. Moreover, the latent form of HIV-1 escapes immunesurveillance as there are no active viral proteins to be recognized by the components of the immune system. This indicates that the strategies by which viral latency is targeted must also meet the requirements that specifically enhance the detection of virus-producing cells by the components of the immune system, e.g. cytotoxic T cell-mediated killing of infected cells, in which provirus is induced to be reversed from the latency. Nevertheless, significant efforts have been put forward to target the underlying mechanisms that establish and maintain viral latency. The initial promising agents that were tested to reverse HIV-1 latency were the inhibitors of epigenetic modifiers, such as the inhibitors of histone deacetylase (HDACi), and histone and DNA methyltransferase (HMTi and DMTi). HDACis such as vorinostat, panobinostat, givinostat, and romidepsin were successful in increasing cell-associated unspliced viral RNA (CA-US RNA) and viral titers in latently infected resting CD4+ T cells, but they failed to reduce the frequency of latently infected cells (25–29). Consequently, the efficacy of histone and DNA methyltransferase inhibitors such as 5-aza-2′-deoxycytidine, epigallocatechin-3-gallate, chaetocin, and BIX-01294 were tested to reverse latency. These inhibitors were shown to restore viral gene expression in resting CD4+ T cells isolated from people living with HIV-1 (PLWH) (30–32). Yet, the reversal effects of these inhibitors were modest, and the clinical trials with these drugs are yet to be performed in PLWH. Several other studies focused on testing the host transcriptional activators and immunomodulatory compounds as the latency reversal agents. Importantly, the agonists of non-canonical NF-κB pathway such as second mitochondria-derived activator of caspases (SMAC) mimetics were shown to increase viral gene expression in vitro latency models, resting CD4+ T cells from

people who are on ART as well as in humanized mouse model of HIV infection (33–35). Nevertheless, these agonists were not successful in reducing the frequency of latent reservoirs. The ex vivo treatment of splenocytes and thymocytes isolated from HIV-infected humanized mice under ART with interleukin 7 (IL-7) increased p24 expression (36). However, the clinical trial on assessing the ART efficacy followed by treatment with IL-7 showed a moderate increase in the CA-US RNA and no profound decrease in the latent reservoir (37).

Vaccines: Despite the fact that the development of ART to prevent HIV-1 infections was an important milestone in scientific history, there is a need to develop an effective vaccine against HIV-1. Importantly, considering factors such as the inaccessibility to ART by the PLWH, the life-long restriction to regimen intake, side effects associated with drugs and the emergence of drug-resistant viral strains, an effective vaccine remains a necessity to end this epidemic. Until 2021, three major approaches were employed in the development of HIV-1 vaccine. While the first two approaches were nearly concluded with poor vaccine efficacy, the third approach based upon the heterologous prime boost produced moderate positive results. Using this heterologous prime-boost approach, the phase 3 clinical trial (RV144) conducted in Thailand, utilized a recombinant canarypox vector as a prime (ALVAC-HIV). This prime was composed of Env (clade E), group-specific antigen (gag) (clade B), and protease (pro) (clade B). Following prime immunization, the participants were given with protein booster (AIDSVAX) consisting of gp120 of different combinations from clade B, MN, and A244 strains. The vaccine efficacy of the RV144 trial was about 60% at 1 year and 31% at 3.5 years. Surprisingly, the trial results indicated that the protection from HIV-1 infection was majorly contributed by non-neutralizing antibodies as opposed to either neutralizing antibodies or CD8+ T cell response (1,38–40). These unexpected results renew the interest in the role of antibodies outside their canonical role as molecules of neutralization and thus focus on antibody-dependent cell-mediated cytotoxicity (ADCC). The currently ongoing trials are based on the hypothesis that non-neutralizing antibodies protect against HIV (41). Since the mRNA-based vaccines have a low risk of infection, ease of manufacturing and flexibility in choosing immunogens, and several mRNA vaccines against Zika, influenza virus and COVID-19 have been successful in treating the

diseases, researchers have focused on developing mRNA-based vaccines for HIV-1. The mRNA-based vaccine, eOD-GT8 60mer, administered to participants in two doses in phase 1 clinical trial (IAVI G001) was an engineered domain of HIV-1 gp120 that binds to CD4+ (41,42). The results from this clinical trial presented at the HIV Research and Prevention virtual conference indicate that 97% of participants administered with eOD-GT8 60mer produced VRC01-class IgG germline B cells, which are considered to be the precursors to the VRC01-class of broadly neutralizing antibodies (bnAbs). However, it is worth noting that these results are yet to show that in these individuals mutations must occur in the genes that encode VRC01-class of bnAbs as the VRC01-class germline precursor cells mature into antibody-producing B cells. Nevertheless, further research and clinical trials are required for the development of an effective and safe HIV-1 vaccine.

1.3 HIV-1 genome and protein organization

HIV-1 genome is a single stranded positive sense RNA and has a size about 9.7 kb in length. The genome consists of 9 genes, some of which overlap, and encodes 15 proteins that are needed for the replication, assembly and release of de novo virions from the infected cell. On either side of the genome flanked are long terminal repeats (LTRs). It is the LTR at 5' end that acts as a viral promoter from which all viral gene expression occurs. The LTR is further subdivided into regulatory elements such as U3 (unique), R (repeat), and U5 (unique). These regulatory elements contain the information that regulates important steps of HIV-1 replication including reverse transcription, integration and gene expression. The cis-acting U3 regulatory element of the LTR promoter is functionally categorized into three domains 1) the modulatory region (−455 nucleotide (nt) to −104 nt, where the nt +1 is defined as the first nucleotide from transcription start site (TSS)); 2) the enhancer (nt −109 to nt −79); 3) the core promoter (nt −78 to nt −1). The regulatory elements R and U5 together form the leader region (nt +1 to nt +188) (43–45). While the modulatory and enhancer regions of U3 regulatory element contain binding sites for inducible transcription factors such as activator protein 1 (AP-1), nuclear factor of activated T-cells (NFAT) and nuclear factor-kappa B (NF-κB), respectively, the core promoter harbors the binding sites for constitutive transcription factors such as specificity protein 1 (Sp1) and TATA-box binding protein (TBP) (46–48). The TSS of LTR exists at the junction

between R and U5 elements. The R element harbors the most important trans-acting responsive region (TAR), to which the viral regulatory protein Tat (transactivator of transcription) binds and helps promote the transcription elongation of viral RNA (49). In addition to the regulation by the virally encoded Tat and host transcription factors, the HIV-1 LTR promoter is also regulated by chromatin remodeling via repositioning of nucleosomes, which are deposited on either side of the TSS upon integration into the host genome. The nucleosomes, Nuc-0, Nuc-1, Nuc-2, on HIV-1 LTR are remodeled in the context-dependent manner and their rearrangements play an important role in the establishment and maintenance of HIV-1 latency. The Nuc-0 is located in the modulatory region of U3 element at ~400bp upstream from the TSS, while Nuc-2 is positioned towards the end of the U5 element, about at ~280bp downstream from the TSS. Similar to cellular genes, Nuc-1 is positioned immediately downstream from the TSS (50). Upon transcription from the viral LTR, three forms of viral RNA are produced: 1) unspliced (9.5 kb) 2) partially spliced (4.5 kb) and 3) completely spliced (2 kb) RNA. While the completely spliced viral RNA encodes three early proteins Tat, Rev (regulator of virion expression), and Nef (negative regulating factor), the partially spliced viral RNA produces Env poly protein (envelope), Vif (viral infectivity factor), Vpr (virus protein r) and Vpu (virus protein unique) proteins. The unspliced viral RNA, which also acts as genomic RNA for releasing virions, encodes two different polyproteins, Gag and Pol. Upon proteolytic processing by HIV-1 protease, Gag (p55) produces structural components of the virus such as MA (matrix) (p17), CA (capsid) (p24) and NC (nucleocapsid) (p7), Pol (p160) produces viral enzymes such as RT (p51), IN (integrase) (p32) and PR (protease) (p12), and Env poly protein (gp160) generates gp120 (surface protein, SU) and gp41 (transmembrane protein, TM). During viral assembly, viral genomic RNA, Gag, Pol, Vpr and host factors are recruited to the plasma membrane harboring gp160 and then viral particles containing these elements are released as immature virions. After release, immature virions undergo maturation by the viral protease to produce mature virions, which are capable of infecting the neighboring cell or the same cell from which virions were released (51,52).

1.4 HIV-1 life cycle

HIV-1 is an enveloped virus that houses a conical core made up of viral capsid protein (CA), which encloses and protects two copies of positive-sense single-stranded RNA molecules and other viral components such as RT, IN, PR and Vpr. The genomic viral RNA is tightly bound by the nucleocapsid protein (p7). The HIV-1 life cycle can be broadly divided into two stages, early and late events. Principally the steps such as the entry of virus, reverse transcription of viral RNA, import of viral capsid into the nucleus and integration of viral DNA into host genome are considered to be early events. On the other hand, the post-integration steps such as transcription of viral genes, translation of viral proteins and finally, release of viral particles are categorized as the late events of HIV-1 replication. Membrane fusion is the key mechanism utilized by HIV-1 to enter into a host cell and mediated by viral membrane proteins gp120 and gp41, which make contact with the target host cell surface receptor CD4+ and the co-receptors, CCR5 or CXCR4 (53). These interactions are accompanied by large structural rearrangements in viral Env protein, chiefly gp41, that induces viral and host membrane fusion allowing viral entry. After membrane fusion, the viral capsid is released into the host cell cytoplasm (53,54).

1.4.1 The journey of HIV-1 across the cytoplasm towards the nucleus: the beginning of interplay between HIV-1 and host protein machinery

The enveloped viruses including HIV-1, upon release into the cytosol, must traffic to the regions of replication within the infected cell. Among these, HIV-1 has long been known to take advantage of microtubule network, which consists of the microtubules (MTs), motor proteins and their adaptor proteins, to move towards the nucleus from the cell periphery (retrograde trafficking), the organelle where HIV-1 integrates its genome for the replication to complete (55–58). To its effective trafficking towards the nucleus, HIV-1 can both stabilize the MTs and hijack the motors and their adaptor proteins. Although most MTs are dynamic in search of cargo for transport, some portions of MTs become highly stable. Indeed, it was shown that HIV-1 stabilizes MTs by recruiting end-binding protein 1 (EB1) to the growing end (also called +end) of the MTs (56). Further, this MT stabilization by HIV-1 was shown to depend on the interaction of viral matrix protein (MA) with EB1 binding protein Kif4,

which stabilizes MTs (56). However, the detailed mechanism of this interaction in the stabilization of the MTs remains elusive. In finding out the components of the cytoskeleton as putative interactors of HIV-1, Fernandez et al. identified MAP1A and MAP1S to play an important role in the stabilization of MTs and the association of incoming HIV-1 cores with microtubules that led to retrograde movement of HIV-1 cores to the nucleus (58). The depletion of these proteins reduced the infectivity of HIV-1 as a result of a defect in the retrograde transport of HIV-1, characterized by the accumulation of cores away from the nucleus (58). A study using Vpr-GFP labeled viral particles showed that HIV-1 traffics in the cytoplasm and accumulates at the perinuclear region as microtubule-associated structures, and that this trafficking depends on the protein dynein, a minus end directed motor protein, implying the critical role of the microtubule network in the retrograde movement of HIV-1 (59). Further, it was also observed that the dynein adaptor protein bicaudal D2 (BICD2) interacts with the incoming HIV-1 viral cores for their movement towards the nucleus, revealing the underlying mechanism of HIV-1 retrograde transport mediated by the dynein and its adaptor protein BICD2 (55). HIV-1, like other viruses, also uses plus end directed motor protein (kinesin) and their adaptor protein FEZ1 (Fasiculation and Elongation Factor zeta 1), which involve in the anterograde transport of molecules, for its net inward movement towards the nucleus (57). The authors of this study proposed a model wherein the opposing motors both contribute to the bidirectional movement of HIV-1 to accomplish the net retrograde movement of HIV-1.

Upon delivery to the sites of nuclear pore complexes (NPCs), the nucleoporins (NUPs), the principal components of NPCs, have been shown to interact with HIV-1 capsid for its import into the nucleus. Among the NUPs that are shown to interact with HIV-1 capsid (discussed below), NUP358 and NUP153 have been shown to participate directly in the import of HIV-1 into the nucleus. The first-ever evidence of interaction between HIV-1 capsid protein (CA) and NUP358 (also known as RanBP2) has been shown by Schaller et al (60). Though the cyclophilin domain of NUP358 has been demonstrated to bind to the capsid (61,62), the relevance of this interaction in the import of capsid is debated because the addition of cyclosporin that abolishes cyclophilin domain-dependent interaction did not prevent NUP358 from binding to capsid (60). However, the following study provides the evidence that

cyclophilin domain of NUP358 loses its association with the CA mutants (P90A in cyclophilin A binding loop and N74D in the hydrophobic binding pocket of capsid) following infection and contributes to the import of HIV-1, suggesting that cyclophilin domain of NUP358 interacts with multiple residues on the HIV-1 capsid and contributes to its import (63). The dependency of HIV-1 on NUP153 has been mapped to the interaction between the C-terminal domain of NUP153, which is rich in FG motifs, and the viral elements CA and IN (64–66). The knockdown of NUP153 moderately decreased 2-LTR circles, a surrogate system to study the import of HIV-1, in the nucleus of infected cells suggesting its positive role in the nuclear import of HIV-1 (64). However, the mechanistic details underlying the involvement of NUP153 in the import of HIV-1 remain elusive and require further studies. NUP62, which is localized in the central channel of NPC, plays a significant role in the nucleocytoplasmic transport of various cargoes by associating with transport receptors. Recently, it was observed that during the initial stages of HIV-1 infection, NUP62 was relocated to cytoplasm from the NPC and colocalized with the incoming viral core (67). However, the mechanism of this relocalization of NUP62 onto the HIV-1 cores and the significance of virus induced displacement of NUP62 from the NPC in the import or any other processes need further studies to be explored. Although the early events occurring between the entry into the host cell and integration into the host genome during HIV-1 infection are not completely clear, the transportation of viral capsid to the cytoplasmic side of the nuclear envelope marks the beginning of the interplay between HIV-1 and nuclear transport machinery, i.e., nuclear pore complexes (NPC).

1.4.2 HIV-1 capsid core entry into the nucleus

The recent experimental evidence in the field supports that capsid core may enter the nucleus in multiple ways; while a few may enter intact, others are partially broken up in the cytoplasm or near the NPCs at the cytoplasmic side of the nuclear envelope (NE) before ingressing into the nucleus (68–70). In the contrary, early investigations were suggestive of the capsid disassembly (uncoating) in the cytoplasm (71–73) or at the nuclear envelope (74,75) and is followed by the import of viral DNA into the nucleus in the form of a subviral complex, called the pre-integration complex (PIC). The most prominent basis for latter postulation was due to

the relatively larger size of the conical capsid core, which has about 60 nm length at the broad end, which surpasses the diameter of the NPC channel (previously described as 40 nm) for it to be transported. While the model of cytoplasmic uncoating was made based majorly on both fluorescence microscopy and a biochemical assay that leverages the TRIM-Cyp mediated restriction of HIV-1 infection (cyclosporine A (CsA)-washout assay), the reports that proposed the model of nuclear membrane-associated uncoating of the capsid largely relied only on the fluorescence microscopy data. In microscopy studies, fluorescence tags were introduced into the assembling virions to determine the status of the capsid during the early steps of HIV-1 replication. These tags were genetically fused directly to the viral components such as Gag as a fluid phase marker, Integrase (IN), and Vpr, or indirectly to the capsid-associated host factors such as cyclophilin A (CYPA) to track the virion particles after the infection (71,76). The loss of capsid-associated fluorescent signal upon cellular entry could therefore be marked as the uncoating of the incoming capsid. The analysis of fluorescent-labeled single viral particles post-entry indicated that the majority of the capsid cores lost the fluorescent signal immediately after viral membrane fusion, while a few retained the signal for some time, losing it eventually in the cytosol, although productive infection was observed. These imaging experiments were taken as indirect clues of the capsid uncoating to be a cytosolic event (71,76). The observation that the concomitant loss of both CYPA and CA signals in the cytosol as well as at the nuclear membrane suggested that an uncoating event might be occurring in the cytosol and at the nuclear membrane (76). In agreement, the studies utilizing biochemical CsA-washout assay, which is based on the ablation of restriction to the HIV-1 infection imposed by TRIM-Cyp by the addition of CsA, and the determination of the percentage of HIV-1 infected cells that become insensitive to TRIM-Cyp (relative half-life of uncoating), also support the model that CA is gradually lost from the capsid core with a half-life of less than 1 h in the cytosol. While these assays laid a critical foundation for investigating capsid uncoating, they also opened up multiple interpretations such as, the CsA might interfere with interactions between capsid and CYPA-domain containing host factors including CYPA that otherwise might maintain the integrity of the capsid (72,73,77). It was, however, shown later that the subviral complexes formed upon uncoating in the cytosol were subjected to proteasomal degradation

and could not generate productive infection, indicating that cytosolic uncoating may not be favorable for HIV-1 (75). In addition, later studies showed that the capsid cores could dock at NPC and undergo disassembly before entering into the nucleus as subviral complexes (74,75). Nevertheless, to support the model of nuclear uncoating, recent culminating evidence from the studies of fluorescence microscopy, wherein CA was directly fused to green fluorescence protein, suggests that the intact or nearly intact capsids enter and disassemble in the nucleus for successful integration and productive infection (68–70). Studies also further indicate that the capsid, while maintaining its integrity, is imported through the NPC, translocated to the sites of integration, and uncoats before integration into the genome (68,69). In line with this evidence, Zila et al., by using combined correlative light and electron microscopy, showed that the diameter of the NPC is sufficiently dilated for the import of intact cone-shaped capsid into the nucleus of T cells (70). While this experimental evidence clearly pointed to the possibilities of various modes of capsid uncoating, many of them employed virus preparation wherein, labeled viral proteins were trans-complemented with wild-type proteins. This may lead to differential labeling of individual virus particles, confounding the microscopy data interpretation, necessitating the use of less invasive labeling strategies to study the early stages of HIV-1 infection, including uncoating. Recently, Schifferdecker et al. established a method, wherein CA is directly labeled with fluorescent dye through genetic code expansion and click chemistry as a less invasive strategy and showed that largely intact capsids enter the nucleus indicating uncoating is a nuclear event that precedes integration (78). Apart from the general conception that the size of the capsid limits access to the nucleus, the differences in the methods, the timing of viral tracking, and the number of particles analyzed in the studies might explain the discrepancy in their conclusion from the new paradigm of nuclear uncoating. Based on these pieces of evidence, one may infer that the models of uncoating described above may not be mutually exclusive and vary depending on the cell type and conditions used in the infection. These models of uncoating are depicted as three schemes (1–3) in Figure 1. It is, therefore, possible to conceive of the following events during the capsid journey toward the nucleus via cytoplasm.

i) *Movement towards the Nucleus*

After entry into the cytosol, some capsid cores may undergo disassembly as they move through the cytosol (Figure 1, Scheme 1), and some, while they are intact, are transported to the cytoplasmic side of the NE via CA-microtubule interactions (57,59,79). Dynein-associated adaptor protein, bicaudal D2 (BICD2), facilitates CA association with microtubules, resulting in the movement toward the nucleus (55,57,58). Complementing the same, the ultrastructural studies by Zila et al. showed that the majority of the capsids proximal to NPC were associated with microtubules and that the average distance of capsids from microtubules was 19-12 nm, which is in agreement with the distance between microtubule and dynein/kinesin-1 as reported earlier (80–82). It has been observed that during HIV-1 infection, the Kinesin-1 motor, KIF5B, induces the relocalization of nucleoporin 358 (NUP358) into the cytoplasm (83). The relocalization allows the interaction between NUP358 and incoming capsid cores for the import of HIV-1. Further, the critical residues (N74 and P90) on the CA are shown to be required for the interaction between NUP358 and CA, and therefore the mutation of these residues (N74D and P90A) induced the accumulation of capsid cores near the cytoplasmic face of the NE indicating that NUP358 promotes capsid import into the nucleus (83). HIV-1, like other viruses, also uses plus end-directed motor protein (kinesin) and its adaptor protein FEZ1 (fasciculation and elongation factor zeta 1), which are involved in the anterograde transport, for net-inward movement of capsid core toward the nucleus (57). The authors of this study proposed a model wherein the opposing motors contribute to the bidirectional movement of HIV-1 to accomplish the net retrograde movement towards the nucleus.

ii) Ingress into the Nucleus through NPC

Two persuasive events can be envisaged for capsid upon nearing the nuclear membrane: a) the docking of capsid cores onto the NPC promotes the accelerated loss of capsid monomers (CA) from the intact capsid, and these partially disassembled subviral complexes (RTC (reverse transcription complex)/PIC) enter into the nucleus (Figure 1, Scheme 2) (74,75). (b) physically compatible diameter of the central channel of NPC allows the intact capsid to penetrate the nucleus without hampering the typical cone shape of HIV-1 capsids (Figure 1, Scheme 3) (although

morphological alterations of capsid core were observed once inside the nucleus) (70). An interesting observation by Zila et al. was that, unlike what was conventionally reported, the central channel of human NPC (in SupT1 cells) was sufficiently dilated with an average diameter of about 64 nm, which is well above the dimension of the broad end of the conical shaped capsid core by about 4 to 9 nm, therefore allowing geometrically feasible entry into the nucleus (70). Intriguingly, this property of dilation of NPC was observed in both infected and uninfected SupT1 cells. The moving capsid cores encounter a high concentration of NUP FG repeats in the central channel.

iii) Release from NPC into the Nucleoplasm

NUP153 and CPSF6 (cleavage and polyadenylation specificity factor 6) are encountered by the capsids when they leave the NPC central channel toward the nuclear side. Upon traversing to the nuclear basket present on the nuclear face of NPC, the sub-viral complexes interact with NUP153 through a conserved hydrophobic pocket on capsid hexamers to complete its translocation and release in the nucleoplasm (66,77,84). NUP153 is a nuclear basket protein that, although positioned to the nuclear face of NPC, has a 200 nm long C-terminal carrying an unstructured FG domain, can reach toward the cytoplasmic face of NPC, where it interacts with PIC-associated CA, IN, and Vpr (85–88). The nuclear protein CPSF6, which is an RNA processing protein, binds to the same site on the capsid as NUP153. It is presumed that CPSF6 replaces NUP153 at the common binding site on capsid, which subsequently releases the capsid containing PIC into the nucleus (66,70). However, a mechanistic model for the movement of partially opened or intact capsids and their eventual release into the nucleus is yet to be determined. One may wonder if capsid binding factors, such as CPSF6, from the inside of the nucleus, generate an inward force, or if there is a gradient of (FG) binding sites to the capsid core inside the channel of NPC, or it could be a result of the variable CA concentrations in cone-shaped capsid core at opposite poles of NPCs.

1.4.3 Cellular factors involved in the capsid uncoating

Though the capsid core protects the genome from being detected by the cellular innate sensors (89–91), the same becomes the target for the cellular restriction

factors including TRIM5 (tripartite motif 5) and MX2 (myxovirus resistance 2) that bind and inactivate the capsid (92,93). Yet, a recent study by Yoh et al. showed that intact capsid is coated by an adaptor protein PQBP1 (polyglutamine binding protein1) which initiates cGAS-dependent innate immune response (94). The details regarding the sensing of HIV-1 viral components as a whole by the host cell and the associated evading strategies by HIV-1 are described in recent reviews (89,95). The list of host factors involved in the capsid uncoating is incredibly growing. Some of these factors include CYPA, transportin 1 (TRN1), TNPO3, and NUP358. CYPA, a cytosolic peptidyl-prolyl cis-trans isomerase, is known to bind to the proline-rich region, namely the CYPA binding loop, in the N-terminal domain of HIV-1 CA (96). In this binding loop, the amino acids, glycine 89 and proline 90 were shown to be crucial for the CA interaction and their changes (G89V and P90A) led to decreased HIV-1 infection (97–100). Consequently, CYPA binding to CA was shown to either stabilize or destabilize the HIV-1 capsid in a cell type-dependent manner, indicating its role in the modulation of HIV-1 capsid disassembly (97). The interaction of nuclear transport receptor transportin 1 (TRN-1) with capsid has been shown to promote both the uncoating and import of the HIV-1 genome. This interaction has been mapped to the NLS located in the CYPA binding loop of capsid (101). Another nuclear transport receptor, transportin SR2 (TRN-SR2)/transportin 3 (TNPO3), has been shown to promote the uncoating of HIV-1 cores (102). However, the detailed mechanism of TNPO3 action during HIV-1 infection remains to be established, given the fact that its depletion has indirect consequences (discussed below). NUP358 contains a cyclophilin domain by which it binds to the capsid at the same site where CYPA binds (103). Though the experimental evidence pointed to the peptidyl-propyl isomerase activity of NUP358, it is still unclear if this enzyme activity leads to the structural changes in the capsid, possibly causing uncoating (61,104). While it may be speculated that opposing stabilizing and destabilizing forces possibly decide the fate of HIV-1 core uncoating, conclusive experiments to validate the factors involved in uncoating remain to be performed. A step-by-step narration of the uncoating of HIV-1 core that discusses all cellular factors involved is elaborated in some recent reviews (105,106).

1.4.4 Reverse-Transcription

For productive infection by HIV-1, the (+) strand RNA genome must be reverse transcribed into double-stranded DNA by the reverse transcriptase (RT) enzyme and integrated into the host genome by the integrase. Briefly, the RT needs the 3’ OH of a primer to start polymerization, similar to all other DNA polymerases. A cellular tRNA already present within retroviral particles serves as the primer for (+) strand synthesis and anneals to the primer binding site in the 5’ region of the genome to initiate reverse transcription. The process of reverse transcription of viral RNA has been extensively reviewed and may be referred to in (107,108) and references therein. Though the cellular location of the beginning of reverse transcription remains to be elucidated, the mounting evidence suggests the completion of reverse transcription in the nucleus (68,109). Further, several reports demonstrate a coordinated relationship between the uncoating and reverse transcription (73,110–114). Rankovic et al. using time-lapse atomic force microscopy showed that the early stage of reverse transcription increases the pressure inside the capsid core that triggers the capsid disassembly (115). In agreement with this data, it was shown that the first strand transfer synthesis, the initial stage of reverse transcription, is required for the capsid to disassemble but not the late products of the reverse transcription (116). In parallel, Francis et al. utilizing fluorescence microscopy for single viral particle tracking observed the gradual loss of CYPA-DsRed from capsid upon initiation of reverse transcription, loss of this fluorescence indicating the uncoating (76). While these studies strengthen the paradigm that reverse transcription promotes the uncoating, experimental evidence shows that the alterations in capsid stability, by the introduction of point mutations in CA, impair the process of reverse transcription, substantiating a coordinated relationship between these processes (114,117). Further, it is proposed that the viral capsid can act as a container to maintain an effective concentration of RT molecules and possibly other components required for the reverse transcription, and given the fact that RT frequently dissociates from the template and reverse transcription requires two template switching events, intact capsid may therefore ensure to hold the RT molecules near the sites of reverse transcription (110). To the best of our knowledge, nuclear transport proteins including NUPs have not been directly linked to the reverse transcription of the viral genome. However, host factors, some of which are either

positive or negative regulators, have a significant regulatory role in the process of viral genome reverse transcription (107).

1.4.5 Integration

The linear double-stranded viral DNA (vDNA), the product of reverse transcription, complexes with integrase and host factors to form an ill-defined sub-viral complex called the preintegration complex (PIC), and the vDNA associated with this complex must be integrated into the chromatin of the infected cells by viral IN in a two-step process (3' processing and strand transfer). The viral genetic material becomes a part of the cellular genome once it has been transformed into DNA and integrated, rendering the cell permanently infected. Therefore, as long as the infected cell continues to divide, the provirus is copied and faithfully inherited (118). Though several host factors associated with PIC have been identified, their functional role in HIV-1 integration remains elusive (119). Many of the transport receptors including KPNA2/KPNB1, KPNA4, TRN-1, and TNPO3 (120), and NUPs including NUP62 (121) and NUP153 (87) have been shown to interact with IN, while their molecular function in the integration is yet to be elucidated. The depletion of NUP62 has been shown to reduce its abundance with host chromatin and cause a decrease in the viral integrations, suggesting its direct role in the integration that depends on the IN interaction (121). Though NUP153 can interact with both IN and CA, the relevance of these interactions either in the import or integration needs to be functionally elucidated (85,87,122). In addition, another NPC-associated NUP98 is also shown to be involved in the integration of viral DNA but the mechanism behind this function is still unknown (85). Given the fact that certain NUPs including NUP98 and NUP153 are mobile and move on and off the NPC (123,124), it is plausible that their dynamic nature of interactions with viral determinants might perform required functions at required places. For instance, NUP153 can promote both PIC import by interacting with CA at NPC and its integration into the genome in the nucleoplasm by interacting with IN. Another important aspect of productive HIV-1 infection is the trafficking of PIC to the integration sites and, thus, its selection in the genome. Accumulating evidence indicates that HIV-1 favors gene-dense regions that are transcriptionally active, particularly genomic regions that are close to the speckle-associated domains (SPADs), while disfavoring the heterochromatin regions such as lamina-associated

domains (LADs) for its integration (125–128). Several host factors have been shown to play an important role in selecting HIV-1 integration sites in the genome. Among these, LEDGF/p75 (lens epithelium-derived growth factor) and CPSF6 have been extensively studied for their role in targeting the viral genome in gene-rich regions (125,126,129,130). Though these proteins, through their differential interacting viral determinants (LEDGF/p75 with IN; CPSF6 with CA), help viral integrations into relatively gene-dense regions, the zonal locations of integration in the nucleus were shown to vary for each of these proteins. While LEDGF/p75 promotes integration in the nuclear periphery, CPSF6 helps integration into the nuclear interior regions that are proximal to SPADs (125,128,131). Further studies are required to clarify these differential interactions and their effects on the integration site selection. Interestingly, it has been recently observed that though initially the transcriptionally active proviruses were seen in the nuclear periphery (near the nuclear envelope), after a few cell divisions, these proviruses were observed throughout the nucleus, indicating that the nuclear distribution of transcriptionally active proviruses was dynamic, and the distance of the location of HIV-1 proviruses from nuclear envelope does not correlate with its transcriptional activity (132). In parallel, studies have shown that NUPs, namely NUP358 and NUP153, and TNPO3 are involved in the integration of viral DNA into gene-dense regions (133,134). Depletion of NUP358 and TNPO3 caused alterations in the integration site selection (from gene-rich regions to gene-sparse regions), and this effect was mapped to depend on the HIV-1 Gag (133). It is suggested that the reduction of HIV-1 infection upon TNOP3 depletion seemed to be an indirect consequence of the cytoplasmic accumulation of CPSF6, which is restrictive for infection, and therefore the role of TNPO3 in integration site selection remains to be answered (135). Like NUP358, the nuclear basket protein NUP153 was shown to interact, although at a different region, with CA to promote integration into gene-rich regions (85,134). Integration site analysis of HIV-1 with N57 mutations that disrupt the interaction between capsid and NUP153 showed reduced integration into transcriptionally active genes similar to HIV-1 in the cells devoid of CPSF6, indicating that the integration site selection depends on several factors in the infected cells (122). Although other NUPs, including NUP214, NUP98, and NUP62 were shown to interact with CA, their participation in the integration site selection remains to be elucidated (121,136).

1.4.6 Transcription

As mentioned in section 1.3, viral gene transcription occurs from 5'LTR promoter and is regulated by viral regulatory protein Tat and a plethora of host transcription factors. Although anti-sense transcript is produced from 3'LTR, its production is less prevalent in comparison to sense transcript, which is generated from 5'LTR at up to ~2500-fold. It is suggested that anti-sense transcript may be involved in the viral latency, and however, the underlying molecular mechanism needs to be explored. Nearly fifty transcription factors are either predicted or shown to bind HIV-1 5'LTR (50). How these factors regulate viral gene expression and whether they act in a concerted manner are still open questions to investigate. HIV-1 transcription is driven by the host RNA polymerase II (RNA Pol II) and broadly subdivided into initiation, promoter clearance, elongation and termination stages (137). It was initially thought that RNA Pol II-mediated transcription of cellular genes is mainly regulated at the initiation stage, and that, in contrast, the elongation stage plays a less prominent role, merely adding ribonucleotides to the growing RNA chain (138–140). However, the first study that changed this view of a less prominent role of elongation in the transcription came from HIV-1, where the elongation phase of transcription was shown to be a rate-limiting factor for the HIV-1 gene expression (141). It was subsequently identified that the pausing of RNA Pol II soon after initiation was due to the concerted actions of negative regulators of transcription such as DRB sensitivity inducing factor (DSIF) and negative elongation factor (NELF) (142,143). HIV-1 transcription from integrated provirus can be Tat-dependent and –independent transcription. In the absence of Tat, RNA Pol II together with general transcription factors such as TBP, TFIIA, TFIIB, TFIIF, TFIIE and TFIIH binds to the LTR promoter and begins to transcribe RNA using integrated viral DNA as a template. This transcription activity is also enhanced by inducible transcription factors such as NF-kB and Sp1 (50,144). Nevertheless, because of the stem-loop structure formed by TAR RNA, this initiation of transcription only results in stalling of RNA Pol II at the promoter proximal region and the major production of short abortive transcripts of about less than 50nt (50,141). When Tat is available and binds to the TAR structure, the RNA Pol II is freed from promoter-proximal pausing and synthesizes full-length viral RNA transcripts (145). Yet, the follow-up studies showed that the efficient production of full length viral RNA not only depends on Tat-TAR element interaction

but also on the interaction of Tat and other cellular factors. The Tat was shown to interact and recruit the ubiquitously expressed cellular cofactor, positive transcription elongation factor b (P-TEFb), to the HIV-1 LTR (146–148). The positioning of P-TEFb, which is a heterodimer composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1), next to the paused RNA Pol II allows CDK9 to phosphorylate the larger subunit of RNA Pol II, promoting the elongation of transcription (149,150). Besides, P-TEFb is also known to phosphorylate DSIF and NELF, and this phosphorylation frees RNA Pol II from pausing at promoter-proximal region, resulting in the synthesis of full length viral transcripts (151,152). As a regulatory mechanism, nearly half of the nuclear P-TEFb is kept inactive in the complexes enriched with 7SK small nuclear RNA (7SK snRNA). These inactive complexes lack the ability to phosphorylate their substrates including RNA Pol II, DSIF and NELF (153,154). Further, it was also identified that the presence of nuclear hexamethylene bis-acetamide inducible 1 (HEXIM1) in these P-TEFb/7SK snRNA complexes is indispensable for negative regulation of P-TEFb (155,156). The novel positive (bromodomain-containing protein 4 (Brd4)) (157,158) and negative (Lupus antigen (La)-related protein 7 (LARP7)) (159) regulators of P-TEFb were identified, and the studies to understand their mechanism of action in HIV-1 gene transcription are under investigation. Although both Tat and HEXIM1 bind to the same domain in the CycT1 of P-TEFb, it is unclear how Tat outcompetes with HEXIM1 for the extraction P-TEFb from the 7SK snRNA complex (160,161). It is also interesting to note that several other cellular factors including ELL1/2, AFF1/4, ENL, and AF9 have been identified to be part of Tat- P-TEFb complex, now called as super elongation complex (SEC), and shown to promote the Tat mediated transactivation of transcription (162–164). It is important to note that many of the NUPs including NUP153, NUP98, NUP62 and TPR were shown to bind the HIV-1 LTR and that among these, NUP153 and TPR enhanced the HIV-1 gene expression, but the underlying mechanism is yet to be explored (165). Although the authors of this study specifically confirmed the association of NUP153, NUP98, NUP62 and TPR with HIV-1 LTR, the use of mab414 (monoclonal antibody 414), which recognizes those NUPs that share the phenylalanine–glycine (FG)-repeat motifs such as NUP358, NUP214, NUP153 and NUP62 to immunoprecipitate HIV-1 LTR made the interpretation ambiguous that whether the NUP358 and NUP214 specifically

associated with the HIV-1 LTR. As described earlier, an important aspect of metazoan gene transcription regulation is the recruitment of Nuc-1 to the promoter, and HIV-1 gene expression regulation is no exception to this. With the Nuc-1 positioned downstream to the TSS, RNA Pol II does not have the access to chromatin to synthesize full length mRNAs and only produces short abortive transcripts. As with the cellular genes, Nuc-1 positioning on HIV-1 LTR is regulated by chromatin remodeling complexes, Brg-associated factor (BAF) and polybromo-associated BAF (PBAF), which are also known by switch/sucrose non-fermentor (SWI/SNF) complexes and share several subunits but have opposite functions in metazoan gene regulation. While BAF helps position Nuc-1 to downstream of TSS inhibiting transcription elongation (166), PBAF remodels Nuc-1, facilitated by HATs such as p300/CBP and hGCN5, and thereby removes transcription elongation block (167). The explicit understanding of the mechanisms that regulate the recruitment of transcription factors and chromatin remodeling at HIV-1 LTR might provide new therapeutic targets for the functional cure of HIV-1.

1.4.7 Export of viral RNA and virion release

As mentioned above, the completely spliced (2kb) viral RNA encodes three early proteins Tat, Rev, and Nef (168). Nef, an important early factor that helps in establishing HIV-1 infection, once synthesized in the cytoplasm, is not known to enter into the nucleus. As of now, the role of Nef in the export of viral RNA directly or indirectly is not shown. However, Nef has been shown to downregulate cell-surface proteins, such as MHC I and CD4+, facilitating evasion of elimination of infected cell by cytotoxic T lymphocytes (CTLs) and preventing superinfection respectively (169,170). It also counteracts the antiviral SERINC proteins and downregulates many other cell-surface proteins (171,172). The two other early regulatory proteins, Tat and Rev, enter the nucleus after they are synthesized in the cytosol. As discussed above, Tat acts as a trans-activator and enhances transcription elongation by binding to the TAR motif of newly transcribing viral RNA. On the other hand, Rev shuttles across the nuclear membrane to carry the cargo of partially spliced and unspliced viral RNAs by binding to the rev response elements (RRE) present in these viral RNAs. The accessory and poly proteins (Gag, Env, Pol, Vpr, Vpu and Vif) are translated from the partially spliced (4.5kb) and unspliced viral RNA (9.5kb) as

they arrive to the cytoplasm. Viral genomic RNA, Gag, Pol, Vpr along with host factors are recruited by host endosomal sorting machinery to the plasma membrane harboring gp160 and then viral particles containing these elements are bud-off from the plasma membrane as immature virions. After release, immature virions undergo maturation by the viral protease to produce mature virions, which are capable of infecting the neighboring cell or the same cell from which virions were released (51,52).

1.5 Restriction factors that block HIV-1 at different steps of its life cycle

Interferons (IFNs), induced by viral infection, are the major players in innate defense pathways against any virus. IFN signaling culminates to initiate the transcription of interferon-stimulated genes (ISGs), many of which function as host restriction factors (RFs), which hinder almost every step of the viral life cycle. Thus, IFN signaling is the core of the innate defense mechanism against viruses in general. The RFs directly interact with viral components, precluding viral replication, and creating an antiviral state in the infected host cell. On the other hand, the evolutionary success of HIV-1 pathogenesis owes to its intricate strategies that block host RFs using its encoded components, such as Vpr (173), Vpu (174), Vif (175), and Nef (176). HIV-1 also targets components of IFN signaling, limiting the expression levels of RFs. RFs induced by IFNs include IFITM proteins, TRIM5α (Tripartite motif-containing protein-5), APOBEC3 proteins, SAMHD1 (SAM and HD domain containing protein 1), MX2, ISG15, SERINC (serine incorporator)-3/5, Schlafen11, ERManI (endoplasmic reticulum α1,2-mannosidase-I), TSPO (Tryptophan-Rich Sensory Protein), ZAP (Zinc-finger antiviral protein), GBP5 (Guanylate binding protein-5) and BST2 (Bone marrow stromal cell antigen-2)/Tetherin (177–179). While the mode of antiviral action of several RFs such as IFITMs, MX2, Schlafen11, ZAP, ERManI, GBP5 and ISG15 for HIV-1 is known, the associated counter mechanisms by HIV-1 are still not clear.

Restriction during entry: IFITMs-mediated antiviral activity ranges from inhibition of viral entry to inhibition of viral protein synthesis, suggesting their broad spectrum of antiviral action (180–182)). Specifically, IFITM2 and IFITM3 avert viral entry, while IFITM1 prevents Gag production (70). Further, the IFITMδ20 isoform also causes selective restriction of the tropic variant, X4-virus, which is abundant during the late phase of HIV-1 infection (183).

***Restriction during reverse transcription*:** APOBEC3 family proteins, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H, show potent restriction of Vif deficient HIV-1 (184–186). APOBEC3G requires both deaminase-dependent (causing mutations in viral cDNA) and independent mechanisms (attenuating reverse transcriptase activity) to exert its antiviral activity against HIV-1 (187–190). Though APOBEC3F is induced along with APOBEC3G among other ISGs, its antiviral activity does not appear to depend on deaminase catalytic activity to the extent that is required by APOBEC3G (191) and is packaged into virions (192).

***Restriction during nuclear entry*:** MX2 or MXB, another IFNα-induced ISG, reduces nuclear accumulation of viral DNA in the nucleus of infected host cells without affecting the reverse transcription indicating its antiviral role at early stages of HIV-1 infection (193,194). MX2 also prevents the uncoating of the viral capsid, leading to the abrogation of HIV-1 infection (195). HIV-1 CA mutations that allow alternative entry into the nucleus or abrogating MX2- capsid interactions make the virus resistant to MX2, suggesting MX2 exerts its antiviral role through interaction with CA (194,195).

Restriction during viral budding and release: ISG15 is a ubiquitin-like protein and the highest expressed ISG (196). ISG15 prevents both the assembly and the release of HIV-1 virions in producer cells (197,198). Though the ISGylation (conjugation to targets) of HIV-1 viral components is not known, ISG15 blocks ubiquitination of viral Gag and host tumor susceptibility gene Tsg101 (a component of ESCRT-I), affecting virion assembly and release (198). It also disrupts the interaction between VPS4 and LIP5, which are a part of the endosomal sorting complex required for membrane budding and release of HIV-1 virions (197).

1.6 The HIV-1-accessory proteins counteract the restriction factors

HIV-1 uses its accessory proteins such as Vif, Vpr, Vpu, and Nef to overcome the restriction imposed by host RFs. HIV-1 Vif-mediated counter mechanism against APOBEC3G has been the subject of study for the last two decades. It was shown that Vif prevents the antiviral activity of APOBEC3G by promoting its degradation via 26s proteasome (199). In addition, later studies suggested that Vif also reduces the levels of APOBEC3G by inhibiting the translation of mRNA encoding this protein and transcription of the gene, APOBEC3G, probably by competing with host transcription

cofactor CBFb, which otherwise binds and activates this gene (200,201). Vif also affects the incorporation and deaminase activity of APOBEC3G, suggesting the multiple ways by which it interferes with RF activities (202–204). In addition, Vpr promotes the degradation of APOBEC3G with the help of its binding protein, VprBP, suggesting a common antiviral pathway affected by two HIV-1 proteins (205). Initial reports regarding the antagonism between HIV-1 Vpu and RF tetherin showed that Vpu binds and promotes the internalization of membrane-associated tetherin through their transmembrane interactions, which leads to beta transducing repeat-containing protein (TrCP2) dependent degradation (174). Furthermore, Vpu, through its cytoplasmic domain, is also suggested to displace tetherin from the sites of viral assembly leading to counteracting tetherin-mediated antiviral effect (206). The anti-tetherin role of Vpu has also been associated with IFN resistance by HIV-1 (207). Serine Incorporator protein 5 (SERINC5) is another potent RF, but not induced by IFN that gets packaged into virions in producer cells and prevents viral fusion with the target cell (172,208,209). HIV-1 Nef counteracts this inhibition by promoting SERINC5 trafficking into the endosomal compartment for degradation, thus reducing intracellular SERINC5 in producer cells (172,208).

1.7 The scope of the thesis

As discussed in the previous sections, many members of the NUP family were shown to positively regulate several steps of HIV-1 replication, including import of capsid, integration of viral DNA and its site selection, transcription and export of viral RNA. It is interesting to note that, apart from their well-known canonical function at the NPC in the transport of viral components such as capsid/viral subcomplexes and RNA, NUPs also non-canonically promote some of essential steps of HIV-1 replication. The increasing evidence also hints that NUPs may play additional roles in HIV-1 replication and that more NUPs that are yet to be identified may take part in the replication of HIV-1. Besides, some of the NUPs were also shown to aid the host restriction factor MX2 to inhibit the replication of HIV-1, indicating both pro- and anti-viral roles for NUPs towards HIV-1 replication. Here, I examined the expression levels and localization of NUPs such as NUP98, NUP62, NUP155, NUP133, NUP107 and NUP85 during HIV-1 infection in different cell lines that represent both immune and non-immune origin. Further, my research work addresses the possible

non-canonical function of NUP98 in the regulation of HIV-1 gene transcription. My investigation provides new insights into the HIV-1 gene transcription regulation by NUP98, and further understanding of the molecular mechanism underlying NUP98 mediated regulation of HIV-1 gene transcription is required, which may be therapeutically targeted for the functional cure of HIV-1.

Chapter 2

Materials and methods

Chapter 2: Materials and methods

All experiments were performed as per the guidelines of institutional biosafety committee.

2.1 Cell lines and reagents

SupT1 cells (gifted by Dr. Shahid Jameel, International Centre for Genetic Engineering and Biotechnology, New Delhi, India) were grown in Roswell Park Memorial Institute's medium (RPMI-1640, HiMedia, Cat. # AL162A) with 10% fetal bovine serum (FBS, Cat. #10270106, Invitrogen, USA), 100 µg/ml streptomycin and 100U/ml penicillin (Cat. # A001A, HiMedia, India). THP-1 monocyte leukemia cell line (Cat#TIB-202, ATCC) were grown in RPMI-1640 with 10% FBS, 100 µg/ml streptomycin and 100U/ml penicillin. HEK293T cells (gifted by Dr. Reddy's Institute of Life Sciences, Hyderabad, India), 1321N1 (gifted by Dr. Anand Kumar Kondapi, University of Hyderabad, India) and TZM-bl cells (gifted by Prof. Ranga Udaykumar, Jawarlal Nehru Centre for Advanced Scientific Research, Bengaluru, India) were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, Cat. # AL066A, HiMedia, India) with 10% FBS, 100 µg/ml streptomycin and 100U/ml penicillin as recommended.

SupT1 cell line is a CD4+ T cell type and physiologically represents the target cell type for HIV-1. 1321N1 cell line is a cell type of astrocyte, which is one of the neural cell types that can get infected by HIV-1 and contributes to neuroinflammation and HIV-associated neurocognitive disorder (HAND). TZM-bl cell line is genetically engineered from HeLa cell line and stably expresses receptor (CD4+) and co-receptor (CCR5). This cell line also has separate integrated copies of reporter genes such as luciferase and β-galactosidase and their expression is under the control of HIV-1 promoter. Due to their extensive use as cell models in HIV-1 research, we included both HEK293T and TZM-bl in our study. We considered SupT1 as a permissive cell line and 1321N1, TZM-bl and HEK293T as non-permissive cell lines for HIV-1 infection, depending on the entry pathway HIV-1 uses to enter into the cells.

Protein A/G agarose beads (Cat. # sc-2003) were purchased from Santa Cruz Biotechnology, USA. Antibodies against NUP155 (Cat. # ab199528), NUP133 (Cat. # ab155990), NUP107 (Cat. # ab73290) and HIV-1 p24 (Cat. # ab9071) were purchased from Abcam, USA. Antibody against NUP85 was purchased from Invitrogen, USA (Cat. # PA5-84522). Antibodies against NUP98 (Cat. # PAB196Hu01) and NUP62 (Cat. # PAC257Hu01) were purchased from Cloud-Clone Corp., USA. Antibodies against GAPDH (Cat. # sc-47724), GFP (Cat. # sc-9996) and HEXIM1 (Cat. # sc-390059) were purchased from Santa Cruz Biotechnology, USA. Antibody against β-tubulin (Cat. # AC008) was purchased from ABclonal, USA. Anti-NF-κB p65 antibody was purchased from Cell Signaling technology, USA (Cat. # 4764T). Anti-HDAC1 antibody used in the study was a kind gift from Prof. Arunasree, University of Hyderabd, India (Cat. # BML-SA401-0100). Anti-rabbit HRP (Cat. # sc-2357) and anti-mouse HRP (Cat. # sc-516102) were purchased from Santa Cruz Biotechnology, USA. Anti-rabbit IgG Alexa Fluor 594 (Cat. # A-11037) was purchased from Invitrogen, USA. Anti-rabbit IgG Alexa Fluor® 488 (Cat. # ab150077) was purchased from Abcam, USA. Lipofectamine 2000 (Cat. # 11668-019) was purchased from Invitrogen, USA.

2.2 Plasmids

The plasmids including the HIV-1 molecular clone pNL4.3 and pHEF VSV-G were a kind gift from Dr. Udaykumar Ranga (Jawaharlal Nehru Centre for Advanced Scientific and Research, Bangalore, India) (210). The plasmids pLTR-luc and pIndie-C1 were a kind gift from Dr. Debashis Mitra (National Centre for Cell Science, Pune, India) (211–215). The plasmids pEGFPC1, full length GFP-NUP98 and GFP-NUP62 were a kind gift from Dr. Radha Chauhan (National Centre for Cell Science, Pune, India). The plasmids expressing different domains of NUP98 cloned in pEGFPC1 and full length Myc-NUP98 cloned in pcDNA were a kind gift from Dr. Maureen Powers (Emory University, Atlanta, USA) (216). pSIV$_{AGM}$-Luc-R^{-}E^{-}Δvif was a kind gift from Carsten Munk (Heinrich-Heine-University, Düsseldorf, Germany) (217). pNLC4.3GFP was a kind gift from Prof. Barbara Muller (University of Heidelberg, Germany) (218). The plasmid HIV-1 LTR-GFP was obtained from addgene (Cat. #115809). The pLKO plasmids expressing shRNA targeting NUP98 (TRCN0000291177) and scrambled shRNA were obtained from ShRNA Resource

Centre (Indian Institute of Science, Bangalore, India). HIV-1 Tat cloned in pcDNA3.1 was described previously (219). The deletion mutant constructs such as ΔNRE LTR, ΔNF-κB LTR, and ΔSP1 LTR were generated by site-directed mutagenesis using respective primers listed in the Table A2 (annexures) and pLTR-Luc construct as a template.

2.3 Transfections, virus preparations and infections

In all the experiments unless otherwise stated, HEK293T cells were seeded overnight before transfection at 4*10^5 cells per well in a 6-well plate in 2 ml of complete DMEM medium (10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin). Next day morning, medium was replaced with 1.8 ml of fresh complete DMEM medium 30 min before transfection. Cells were then transfected or co-transfected with indicated plasmids for 6 hours (6 h) at 37ºC with 5% CO_2 using Lipofectamine 2000 according to manufacturer's protocol (Cat. # 11668-019). After 6 h, cells were fed with 2 ml of fresh complete DMEM medium and further incubated for 48 h at 37ºC with 5% CO_2. For VSV-G pseudotyped HIV-1 NL4.3 virus preparation, HEK293T cells were seeded at 80% confluence per well in a 6-well plate before transfection, and transfection was performed by calcium phosphate method as previously described (220,221). Briefly, transfection mixtures were made with the plasmids pNL4.3 and pHEF VSV-G at 3:1 (total 3 µg per well) via calcium phosphate–DNA co-precipitation in the following manner. The required amounts of plasmid DNA (total 3 µg per well) and 10 µl of 2.5 M $CaCl_2$ were diluted in a sterile H_2O to a final volume of 100 µl. This Ca/DNA solution was then quickly mixed with equal volumes (100 µl) of 2x HBS (HEPES buffered saline: 140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.05). This calcium phosphate–DNA co-precipitates containing transfection mix was immediately sprinkled onto the cells fed with 1.8 ml of complete DMEM. Cells were then incubated for 6 h at 37ºC with 5% CO_2. Transfection medium was removed and fresh 2 ml complete DMEM was added to the cells and cells were incubated at 37ºC with 5% CO_2 for the formation of HIV-1 NL4.3 virus. Forty eight hours post-transfection (48 hpt), culture supernatant containing virus was collected, centrifuged at 500g for 10 min for removal of cell debris and filtered through 0.45 µm syringe driven filter. Virus was then precipitated with 50% polyethylene glycol (PEG) 6000

(final concentration 8.5%) and 4 M NaCl (final concentration 0.3 M) at 4ºC overnight. Virus was pelleted at 7000g for 10 min at 4ºC in a fixed rotar, resuspended in a required volume of incomplete DMEM and aliquots were stored at -80ºC (220). Concentration of virus (p24 equivalents) was estimated by p24 ELISA according to manufacturer's protocol (ABL, Cat. # 5447).

SupT1 cells were infected with HIV-1 NL4.3 virus as previously described (222). Briefly, cells were added with required amounts of HIV-1 NL4.3 virus (2 ng p24 equivalents/1*10^5 cells) in 1 ml of complete RPMI medium with diethylaminoethanol (DEAE) dextran (10 µg/ml) per well in a 6-well plate, subjected to centrifugation at 350g for 40 min at 15ºC (spinoculation) and incubated for 2 h at 37ºC, 5% CO2 for viral entry. Then cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) twice before they were incubated in 2 ml of complete RPMI medium at 37ºC with 5% CO_2 for 96 h. HEK293T cells were incubated with HIV-1 NL4.3 virus (2 ng p24 equivalents/1*10^5 cells) in 1 ml of complete DMEM medium with DEAE dextran (10 µg/ml) per well in a 6-well plate for 4 h at 37ºC, 5% CO2 for viral entry. Then cells were washed with PBS twice before they were incubated in 2 ml of complete DMEM medium at 37ºC with 5% CO2 for 96 h.

2.4 Lentivirus production and shRNA Knockdown in HEK293T and SupT1 cells

For knockdown of NUP98 in HEK293T cells, cells were seeded overnight before transfection at 4*10^5 cells per well in a 6-well plate and then transfected with shRNA expressing plasmids for 6 h at 37ºC with 5% CO_2 using Lipofectamine 2000. Cells were then fed with fresh complete DMEM and further incubated for 48 h at 37ºC with 5% CO_2. Forty eight hours post-transfection, cells were washed once with PBS and lysed in NP40 buffer (50 mM Tris pH 8.0,150 mM NaCl, 1.0% NP-40, 1x protease inhibitor cocktail); 100 µl of NP40 buffer was used to lyse the cells harvested from one well of a 6-well plate. The efficiency of knockdown of NUP98 by shRNA was determined by western blotting using anti-NUP98 antibody. Lentivirus used for the knockdown of NUP98 in SupT1 cells, was prepared by transfecting HEK293T cells with packaging and transfer vectors as described above. Briefly, HEK293T cells, per well in a 6-well plate, were co-transfected with VSV-G encoding

pMD2.G (250ng), Gag-Pol encoding psPAX2 (1000ng) and pLKO.1-Puro (1000ng) harbouring NUP98 specific shRNA sequences by calcium phosphate method. Forty eight hours post-transfection, virus was collected, centrifuged at 500g for 10 min for removal of cell debris and filtered through 0.45 µm syringe driven filter and was stored at -80°C. SupT1 cells were transduced with lentivirus harbouring either Sc shRNA or NUP98 specific shRNA sequences for 24 h. Seventy two hours post-transduction, cells were harvested, lysed in NP40 buffer and lysates were analysed for the knockdown of NUP98 by western blotting using anti-NUP98 antibody.

2.5 Western blotting

For basal expression of NUPs, adherent cells such as 1321N1, HEK293T and TZM-bl were maintained in 100 mm dishes in complete DMEM media until they reached 80% confluency. The suspension cells, SupT1 (CD4+), were maintained in T75 flasks in complete RPMI medium until they reached 80% confluency. On the other hand suspension cells, THP1 were treated with phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml for 24 h in complete RPMI medium in 100 mm dishes (at 80% confluency), and further incubated for 24 h in PMA-free complete RPMI for differentiation into macrophages. Once they reached 80% confluency, the cell lines such as SupT1, 1321N1, HEK293T, TZM-bl and THP1 were harvested, washed with PBS and resuspended in 300 µl of NP40 buffer. Then, cells were vortexed for 30 min at 4ºC, followed by centrifugation at 12000 rpm for 20 min at 4ºC. The cell lysates were resuspended at one-fifth volume in 5x SDS loading dye (250 mM Tris-Cl pH 6.8, 10% SDS (sodium dodecyl sulfate), 5% β-mercaptoethanol, 0.02% bromophenol blue, 30% glycerol) and boiled at 95ºC for 5 min. The protein lysates were then resolved on 10% SDS-PAGE for 1.5 h at 100 V and transferred onto the nitrocellulose membrane for 1.5 h to 2 h at 60 V under wet conditions. Membrane blots were blocked with 5% skim milk in TBST (tris-buffered saline with tween 20, 0.1%) at room temperature for 1 h on the shaker. Blots were then probed with primary antibodies diluted in TBST with 1% BSA at 4ºC overnight on the shaker. All the primary antibodies used in the study were diluted at 1:2000. After three washes with TBST, blots were incubated with secondary antibodies conjugated to HRP (horseradish peroxidase) at room temperature for 1 h on the shaker. The secondary antibodies such as anti-rabbit HRP and anti-mouse HRP were diluted at 1:10000 in

TBST with 1% BSA. After three washes with TBST, blots were developed using chemiluminiscence detection kit (Cat.# K-12045-D10, advansta, USA). Similarly, at indicated time points, cells (transfected/infected) were harvested, washed twice with PBS, resuspended in NP40 lysis buffer and processed for western blotting as described above. Using ImageJ-win64 software, the protein bands of interest were quantified and the values were normalized to that of the corresponding loading controls (GAPDH or tubulin) for each blot individually.

2.6 RT-qPCR

For basal expression of NUP mRNA, adherent cells such as 1321N1, HEK293T and TZM-bl were maintained in 100 mm dishes in complete DMEM media until they reached 80% confluency. The suspension cells, SupT1 (CD4+), were maintained in T75 flasks in complete RPMI medium until they reached 80% confluency. On the other hand suspension cells, THP1 were treated with phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml for 24 h in complete RPMI medium in 100 mm dishes (at 80% confluency), and further incubated for 24 h in PMA-free complete RPMI for differentiation into macrophages. Once they reached 80% confluency, the cell lines such as SupT1, 1321N1, HEK293T, TZM-bl and THP1 were harvested, washed with PBS and resuspended in 1 ml of TRIzol. Similarly, at indicated time points, cells (transfected/infected) were harvested, washed twice with PBS and resuspended in 1 ml of TRIzol. Total RNA was isolated as previously described (223) and treated with Dnase I to remove the contaminating genomic DNA. 1 µg RNA was used to obtain cDNA using iScript cDNA synthesis kit (Cat. # 1708891, Biorad, USA), which was then used as template for amplification of NUP mRNA or HIV-1 Env mRNA by iTaq Universal SYBR Green Supermix (Cat. # 172-5121, Biorad, USA) using primers listed in Table A1 (annexures). Expression of NUP mRNA or HIV-1 Env mRNA was normalized to GAPDH mRNA as an internal control.

2.7 Cell viability by trypan blue dye exclusion assay

Viability of the cells was determined by trypan blue dye exclusion assay as previously described (54). Briefly, cells were harvested at indicated time points and resuspended in PBS. Then, cells were mixed with equal volumes of trypan blue (0.4%), and both viable and non-viable cells were counted based on the dye

exclusion in the haemocytometer. The percentage of viable cells was determined by dividing viable cells with total number of cells.

2.8 Luciferase activity assay in HEK293T cells

HEK293T cells were seeded overnight before transfection at 1*10^5 cells per well in 24-well plate in 500 µl of complete DMEM and then transfected with reporter plasmids along with expression plasmids or molecular clone pNL4.3 for 6 h at 37ºC with 5% CO2 using Lipofectamine 2000 (Invitrogen, USA). Cells then were fed with fresh complete DMEM and further incubated for 48 h at 37ºC with 5% CO2. Forty eight hours post-transfection, cells were washed once with PBS and 120 µl of reporter lysis buffer (Cat.# E397A) was added to each well, followed by 2 freeze-thaw cycles (each cycle: freezing at -80ºC for 1 h and thawing at room temperature for 15 min) for cell lysis. Cell lysates were obtained by centrifugation at 12000 rpm for 10 min at 4ºC in microcentrifuge tubes. Luciferase activity (relative light unit (RLU)) in the cell lysates was measured using luciferase assay reagent (LAR: Cat.# E1483) using luminometer (Turner Biosystems); 5 µl of cell lysate was mixed with 50 µl of LAR in a transparent microcentrifuge tube and immediately RLUs were obtained using luminometer. For each sample, RLUs were normalized to the protein quantified by BCA method according to manufacturer's protocol (Cat. #786-570).

2.9 TZM-bl reporter assay

β-galactosidase activity: Overnight before infection with HIV-1 NL4.3, TZM-bl cells were seeded in 96-well plate at 1*10^4 cells per well in 100 µl of complete DMEM medium. Next day morning, cells were allowed for binding with HIV-1 NL4.3 at different dilutions or p24 equivalents (ng) for 3 h at 37º C with 5% CO_2. Forty eight hours post-infection, cells were washed once with PBS, and 100 µl of lysis buffer (0.1 M Tris-pH 7.8 and 0.5% triton X-100) was added to each well and incubated the plate at 37º C for 15 min for cell lysis. Then, plate was centrifuged at 3000 rpm for 10 min at room temperature and 50 µl of lysate was mixed with 50 µl ONPG substrate solution. The reaction mix was incubated at 37º C until the yellow colour appeared (Note: incubation time depends on the infectivity of the virus; it can be up to 4 h). The reaction was stopped with 1 M Na_2CO_3 (100 µl) and the absorbance was taken at 420 nm.

ONPG substrate solution (6 ml)	
100X $MgCl_2$	60 µl
10X ONPG (15 mg/ml)	600 µl
0.1 M sodium phosphate buffer pH 7.5	Up to 6 ml

100X $MgCl_2$ (100 µl)	
0.3 M $MgCl_2$	50 µl
β-mercapto ethanol (14.7 M)	45 µl
H_2O	5 µl

Luciferase activity: Overnight before infection with HIV-1 NL4.3, TZM-bl cells were seeded in 24-well plate at 1*10^5 cells per well in 500 µl of complete DMEM medium. Next day morning, cells were allowed for binding with HIV-1 NL4.3 (5 ng p24/1*10^5 cells) for 3 h at 37ºC with 5% CO_2. Forty eight hours post-infection, cells were washed once with PBS and 120 µl of reporter lysis buffer was added to each well and followed by 2 freeze-thaw cycles as described above. Luciferase activity in the cell lysates was measured as mentioned above.

2.10 Chromatin immunoprecipitation

HEK293T cells, 48 h post-transfection with Myc-NUP98 and HIV-1 LTR constructs, were harvested in microcentrifuge tube from one well of a 6-well plate, washed with PBS and subjected to crosslinking as previously described (224). Briefly, cells were added with 1 ml of 1% formaldehyde in PBS for 15 min at 37ºC. Crosslinking was followed by quenching with 125 mM glycine in PBS for 5 min at 37ºC and cells were collected by centrifugation at 2,000 g for 5 min at 4°C. After washing with PBS twice, cells were resuspended in 200 µl NP40 buffer and incubated on ice for 15 min and subjected to sonication; six cycles of pulses 20 seconds on and 30 seconds off on ice with 30% power. The supernatant was separated from lysed cell debris by centrifugation at 12000 rpm at 4ºC for 20 min. The supernatants containing the sheared chromatin fragments of about 400-800 bp were incubated with protein A/G agarose beads pre-conjugated to anti-myc antibody at 4ºC overnight under shaking conditions. For pre-conjugation, 2 µg of anti-myc antibody was mixed with 20 µl of pre-washed protein A/G agarose beads for each sample. The chromatin-immunoprecipitates with protein A/G agarose beads were pelleted-down by centrifugation at 2000 rpm for 2 min at room temperature and washed with TBST

three times. The protein bound DNA was eluted by adding 120 µl of elution buffer (1% SDS, 100 mM $NaHCO_3$) to the protein A/G beads and vortexing at 30ºC for 15 min. The beads were removed by centrifugation 2000 rpm for 2 min at room temperature and supernatant was collected. To this supernatant, 2 µl of RNase A (10 mg/ml) and 4.8 µl of 5 M NaCl were added and incubated at 65°C overnight under shaking conditions. Next day morning, 2 µl of proteinase K (20 mg/ml) was added and further incubated for 1 h at 60ºC under shaking conditions. The DNA was purified by Qiagen PCR purification kit. The column purified DNA quantified by spectrometry was used as template for qPCR using primers (Table A3 (annexures)) designed in the HIV-1 LTR promoter.

2.11 Co-immunoprecipitation

HEK293T cells were co-transfected with pNL4.3 and either pcDNA or Myc-NUP98. Forty eight hours post-transfection, cells were washed with PBS and lysed in NP-40 buffer. Similarly, HEK293T cells infected with HIV-1 NL4.3 were transfected with either pcDNA or Myc-NUP98. Forty eight hours post-transfection, cells were washed with PBS and lysed in NP-40 buffer. Protein A/G agarose beads were washed with TBST buffer and incubated with cell lysate for 4 h at 4°C to remove proteins that may non-specifically bind to the protein A/G agarose beads (pre-clearing step). Then the beads were removed by centrifugation at 2000 rpm for 2 min and the supernatant was added to the fresh protein A/G agarose beads pre-conjugated with 2 µg anti-myc antibody and incubated for overnight at 4ºC on a rocker. After overnight of incubation, beads were washed three times by centrifugation at 2000 rpm for 2 min with TBST buffer. The sample with the beads were then dissolved in SDS loading buffer and processed for western blot analysis.

2.12 Immunostaining of the cells for the confocal imaging

For the localization of NUPs at basal state, adherent cells such as 1321N1, HEK293T and TZM-bl cells were seeded on coverslips in a 12-well plate at 80% confluency overnight before the start of the immunostaining. THP cells were differentiated with PMA on coverslips in a 12-well plate at 80% confluency as described above. On the following day cells were washed with PBS and fixed in 3.7% formaldehyde solution in PBS for 20 min at room temperature. In case of

SupT1, cells when they reached 80% confluency were washed with PBS, fixed in 3.7% formaldehyde solution for 20 min at room temperature and allowed to bind to poly-L-lysine coated slides. Then, the fixed cells were permeabilized with 0.5% triton X-100 in PBS for 10 min at room temperature and washed with PBS three times. The permeabilized cells were blocked for 1 h at room temperature with 3% BSA in PBS. Cells were then allowed for incubation with primary antibodies diluted in 3% BSA in PBS for 1 h at room temperature. All the primary antibodies used in the study were diluted at 1: 400. After three times wash with PBS, cells were incubated with diluted secondary antibodies such as Anti-rabbit IgG Alexa Fluor 594, Anti-rabbit IgG Alexa Fluor® 488 or Anti-mouse IgG Alexa Fluor® 647 under dark conditions for 1 h at room temperature. All the secondary antibodies were diluted at 1:1000 in 3% BSA in PBS. After three washes with PBS, the coverslips were mounted onto the slides with mounting medium containing DAPI (Abcam, USA). Cells were imaged under Leica TCS SP8 microscopy using HC PL APO CS2 63X/1.40 OIL objective. Similarly, at indicated time points, cells (transfected/infected) were harvested, washed twice with PBS and processed for immunostaining as described above.

2.13 Data and statistical analysis

All the experiments were performed at least three times. The represented values were the mean with standard deviation. For statistical analysis, Student's paired t-test was conducted for all the experiments except otherwise mentioned, for which one-way ANOVA with Tukey's multiple comparisons test was conducted using GraphPad Prism 5. $P<0.05$, $P<0.01$ and $P<0.001$ were considered to be statistically significant and represented as *, ** and **, respectively.

Chapter 3

Differential regulation of NUPs by HIV-1

Chapter 3: Differential regulation of NUPs by HIV-1

3.1 Introduction

Nuclear pore complexes (NPC), which are conduits embedded in the nuclear membrane/envelope (NE), define the structural and functional properties of the nucleus. These megadalton complexes (125 MDa) allow the transport of molecules larger than 40 kDa from cytoplasm to nucleus and vice versa in a manner that requires energy in the form of GTP. Although the constituents of NPC, nucleoporins (NUPs) which arrange in multiple copies and occupy specific position as subcomplexes in the NPC, are traditionally seen as static components, the growing body of research suggests that majority of the NUPs are dynamic and shown to have off-NPC function, i.e., the regulation of gene expression, division of the cell and maintenance of transcriptional memory of interferon inducible genes (225–228). In light of this, research findings show that the interaction of NUPs with chromatin regulates genome organization and gene expression (227). In agreement with this, several microscopy studies show the nucleoplasmic localization of NUPs such as NUP62, NUP214, NUP153 and NUP98, suggesting that these NUPs are not only part of the static structures but also dynamically move on and off-NPCs, and play off-NPC roles under certain conditions (123,124,216,229).

During the disease progression to AIDS, HIV-1 infects several cell types in the human body that include CD4+ T cells, macrophages, dendritic cells, astrocytes, endothelial and epithelial cells, which have differential regulation on HIV-1 life cycle, from highly replicative to abortive. As discussed in the chapter 1, HIV-1 utilizes NUPs at several steps during its replication. These steps include docking of capsid (CA) onto NPC (NUP358), import of viral DNA (NUP153) and export of viral RNA (NUP98, NUP214, NUP62) (65,83,121,122,230–233). Further, the evidence shows that NUPs such as NUP62, NUP98, NUP358 and NUP153 are required for the HIV-1 DNA integration and its integration site selection in the host genome (104,232,234). With the increasing evidence suggesting non-canonical role of NUPs in HIV-1 lifecycle, a systematic study in different cell types is required to expand our understanding on the role of NUPs in the context of HIV-1 infection. In this study, we set out to understand the regulation of NUPs such as NUP98, NUP62, NUP155, NUP133, NUP107 and NUP62 during HIV-1 infection in different cell types.

3.2 Results

3.2.1 Basal expression of Nucleoporins varies among the cell types

Previously, it was shown that the expression of nucleoporins vary at protein levels among different tissues and cell types (235). Here we studied the basal expression of NUPs both at protein and RNA levels in different cell types known to be infected with HIV-1 such as SupT1, 1321N1, TZM-bl, HEK293T and THP1. In addition, we also examined the localization of these nucleoporins by immunostaining followed by confocal microscopy. From the data obtained from western blotting and quantitative real time PCR (RT-qPCR), we infer that the expression of NUPs at protein and RNA levels varied within a cell type and among the cell types used in the study (Figure 3-1A, B, C). Strikingly, for a few NUPs such as NUP98 and NUP62, the expression at RNA level remained nearly the same in all the cell types (Figure 3-1C). However, the expression of these NUPs at protein level was low in 1321N1 and THP1, respectively, in comparison to other cell types (Figure 3-1A, B). This observation suggests that turn-over of these NUPs may be differentially regulated between the cell types. It should be noted that the expression of NUP98, NUP133 and NUP85 at protein level in THP1 cells was below detectable level (Figure 3-1A). It is noteworthy that the protein levels of NUP107 and NUP85 seemed to reflect at RNA levels in all the cell types (Figure 3-1B, C). The gene for NUP98 expresses two alternatively spliced forms of mRNA; one encodes shorter NUP98-6kDa protein and other encodes longer NUP98-NUP96 precursor protein. While NUP98-6kDa, upon autoproteolytic cleavage at its C-terminal end, produces full length functional NUP98 (98kDa) and a 6kDa shorter peptide, NUP98-NUP96 precursor upon autoproteolytic cleavage, gives rise to both full length functional NUP98 (98kDa) and NUP96 (96kDa) proteins. In our study, antibody that was raised against N- terminal end of the NUP98 detected both longer form of the precursor protein (194kDa) and shorter form, NUP98 (98kDa) itself (Figure 3-1A). It is interesting to note that the longer form of the precursor protein was hardly detectable in SupT1 cells, where as its expression was readily detectable in other cell types such as 1321N1, TZM-bl and HEK293T (Figure 3-1A). On the other hand, the level of shorter form of NUP98 (98kDa) in SupT1 was as high as that was observed in TZM-bl and HEK293T cells (Figure 3-1A). This observation leads to interpret that there may be two possible ways through which the homeostasis of precursor form and short form of NUP98 is

maintained in SupT1 cells; 1) the processivity of the longer precursor form in SupT1 cells is so efficient that at physiological state the shorter form of NUP98 (98kDa), could only be existed but not the other longer precursor form; 2) most of the splicing events only results in mRNA that encodes shorter form of NUP98 (98kDa), thereby at physiological state the less of precursor form and more of shorter form of NUP98 would be observed. Further, though the level of precursor protein was highly present in 1321N1, its processed form such as NUP98 was low in this cell type in comparison to all other cell types. This indicates that the turn-over of NUP98 is differentially regulated in 1321N1 cell type in comparison to other cell types. Taken together, nucleoporin expression both at protein and RNA levels varies between the cell types.

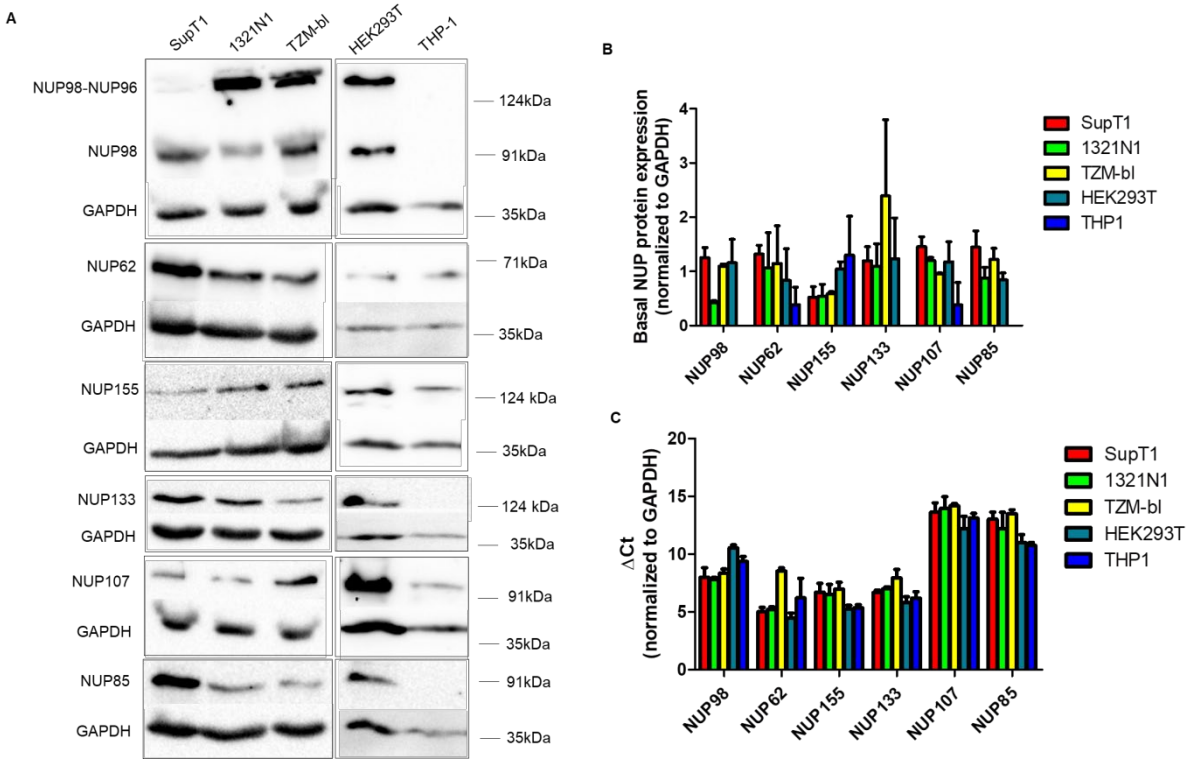

Figure 3-1. Basal expression of nucleoporins across different cell types. **(A-C)** Actively growing cells (80% confluency) except THP1, which were differentiated with PMA, were harvested and subjected either for protein extraction or RNA isolation. Protein extraction was used for western blotting. **(A)** Blots were probed with anti- NUP98, NUP62, NUP155, NUP133, NUP107, NUP85 and GAPDH antibodies. NUP protein expression was normalized to the loading control GAPDH using image J software. **(B)** Bar graphs represent the mean NUP's expression from three independent experiments. RNA was subsequently reverse transcribed and quantified by RT-qPCR. NUP RNA expression was normalised with internal

control GAPDH RNA (ΔCt). **(C)** Bar graph represents the mean NUP Ct values from three independent experiments.

3.2.2 The localization of Nucleoporins varies among the cell types

It is reasonable to presume that, given that the main function of NUPs is to transport molecules between the nuclear and cytosolic compartments, the localization of all the NUPs is expected to be mainly at the nuclear envelope (NE). But, however, previous reports show that few NUPs including NUP62, NUP214 and NUP98 were not only observed at the NE but also both in cytoplasm and nucleoplasm (124,236). We believed that it is important to test the localization of NUPs in the cell types that we used to show the basal expression of NUPs such as NUP98, NUP62, NUP155, NUP133, NUP107 and NUP85 both at protein level and RNA level. However, the antibodies against NUP107 and NUP85 worked poorly for the imaging despite trying several protocols, and hence we eliminated the localization studies for these two NUPs and continued for the localization of other NUPs, which are, NUP98, NUP62, NUP155 and NUP133. After fixation and immunostaining, using the oil-lens objective of confocal microscopy the cell was optically sliced into 3D stacks so that each slice of the stack, which also included the nuclear compartment, was analysed for the localization of concerned NUP. To identify and distinguish nucleus from the cytosolic compartment, the counterstain DAPI was used. We further focused and analysed the middle slice of the stacks so as to gain insights into the localization of NUPs both in cytosolic and nuclear compartments. As a representative image for the reader, we showed the middle slice of the stack that reflects both cytosolic and nuclear compartments for each NUP for each cell type (Figure 3-2). It is to be noted that for the cell types with thin rim of cytoplasm, such as SupT1 cells, it was difficult to clearly distinguish nuclear boundary by the NE from the cytosolic compartment through immunostaining followed by confocal microscopy. In fact, for SupT1 cells, which are circular in shape and have minuscule cytosol, it was not elementary for us to discriminate the thick staining of NE by the NUPs from the cytosolic compartment in comparison to other cell types used in our study that have distinguishable cytosolic and nuclear compartments. From the 3D sectioning of the nucleus, it was further noticed by the DAPI staining that the nucleus of SupT1 cells seemed multilobed, which is in agreement with the previous reports (70). Moreover, in SupT1

cells, these multilobed nuclear structures were also distinctly visualised with NE staining by all the NUPs except NUP62.

The imaging analysis of NUPs denoted many remarkable observations, which we highlighted in the Table 3-1. All the NUPs except NUP62 were observed at NE in all the cell types. For NUP62, the localization was mainly noticed as a diffusing signal in the nucleoplasm in all the cell types. The lack of NE staining by NUP62 suggests that the antibody may not have access to the epitopic region of the NUP62 that is differentially oriented in the NPC but has access to the nucleoplasmic NUP62. We also noticed that the nucleoplasmic NUP62 was completely absent in nucleolus in all the cell types tested. Although the presence of NUP62 in the cytoplasm of 1321N1, HEK293T, and THP1 cells was readily observed as a diffuse signal or foci, its localization in the cytoplasm of TZM-bl cells was completely absent. Interestingly, in the cytoplasm of HEK293T cells the foci of NUP62 were bigger and more distinct than that were observed in the cytoplasm of 1321N1 and THP1 cells. For NUP98, the localization was observed in the cytosol, NE, and nucleoplasm. However, its localization differed from one cell type to the other. While NE staining and intranucleoplasmic diffuse signal from NUP98 were observed in all the cell types, distinct puncta/bodies were seen in the nucleoplasm of both TZM-bl and HEK293T cells. Nevertheless, not all the HEK293T cells in an imaging field contained these bodies. Further, the number of these bodies per nucleus and the size of each body varied between TZM-bl and HEK293T cells. As compared to HEK293T cells, TZM-bl cells had more and smaller bodies. Additionally, the overlapping of DAPI with NUP98 staining showed that the intranuclear bodies in HEK293T cells were colocalized with nucleolus, as opposed to TZM-bl cells. As discussed more in detail in Chapter 4, these bodies were known to be called GLFG bodies, and shown to be devoid of any of the known markers of nuclear bodies. The studies are now beginning to understand and characterise the composition of these bodies, and it remains to be determined how these bodies function both structurally and functionally. It would be curious to speculate if there are cell-specific and/or cell-stage-specific GLFG bodies and if they are involved in specific functions. The NUP133, the component of Y-complex (also known as scaffold complex), was found to localize to the cytoplasm, NE, and nucleoplasm in each cell type tested. Like for NUP98 in TZM-bl and HEK293T cells, NUP133 was found to form structurally similar nuclear bodies in

these two cell types. While the size of the nuclear body seemed to be similar in both the cell types there were more bodies found in the TZM-bl cells than that were in HEK293T cells. Further, as far as the cell-specific occurrence of the bodies formed by NUP133 was concerned, all TZM-bl cells were shown to have these bodies, whereas only a minority of HEK293T cells possessed them in their nucleoplasm. Interestingly, 1321N1 and THP1 cells also exhibited bodies in their nucleoplasms, but these were less distinct than those found in HEK293T and TZM-bl cells. As part of the Y-complex the NUP133 was manifested to be recruited to GLFG bodies (237). This previous observation suggests that the nuclear bodies that were shown in our study by immunostaining of NUP133 might be the GLFG bodies. Further studies are needed to understand if there is a common mechanism through which the GLFG bodies are formed in the cell types where we observed these bodies.

In all cell types, NUP155 was found to localize to the cytoplasm, nucleoplasm, and nuclear envelope. Surprisingly, in THP1 cells NUP155 localized as a single large body within the nucleoplasm, which was absent in all other cell types. This cell-specific nuclear body containing NUP155 was found to be exclusively colocalized with nucleolus. Like other NUPs, the localization of NUP155 in the cytosol of HEK293T appeared as distinct puncta. Unlike in HEK293T cells, most of NUP155 signals in the cytosol of TZM-bl were near NE.

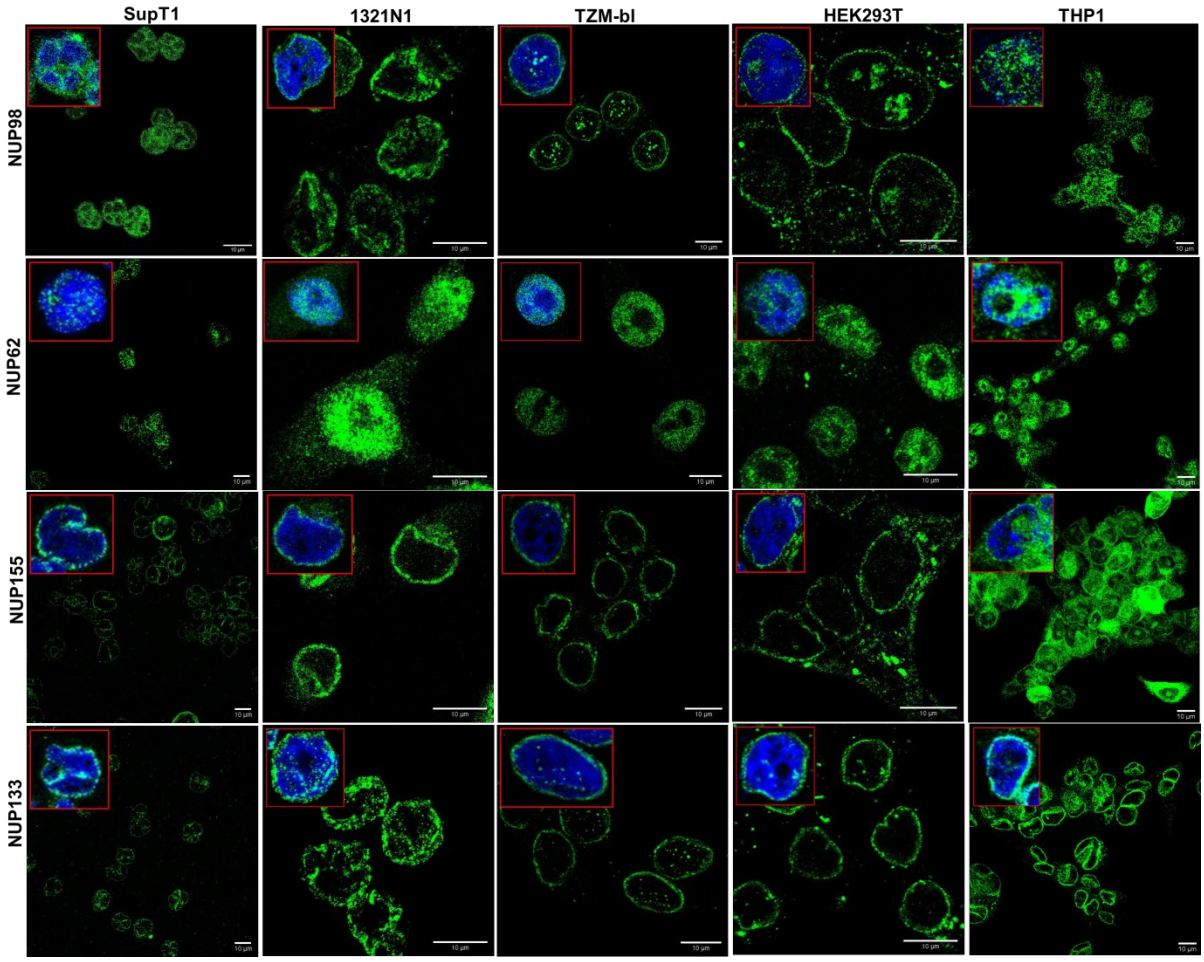

Figure 3-2. Localization of NUPs across different cell types. Actively growing cells (80% confluency) except THP1, which were differentiated with PMA, were fixed and subjected to intracellular immunostaining using anti- NUP98, NUP62, NUP155 and NUP133 antibodies. After incubation with Anti-rabbit 488 antibody and staining with DAPI, cells were observed under confocal microscopy using green and blue exciting lasers. Inlet shows the merge image of a selected cell in a field of view. Scale bar is 10 μm.

Table 3-1 Localization of NUPs across different cell types

NUP	SupT1	1321N1	TZM-bl	HEK293T	THP1
NUP98	Nuclear envelope Intranuclear diffuse	Nuclear envelope Intranuclear diffuse Cytoplasmic diffuse Small nuclear bodies	Nuclear envelope Intranuclear diffuse Small nuclear bodies Cytoplasmic diffuse and puncta	Nuclear envelope Intranuclear diffuse Large nuclear bodies Cytoplasmic diffuse and puncta	Nuclear envelope Intranuclear diffuse Cytoplasmic diffuse and puncta Small nuclear bodies

NUP62	Intranuclear diffuse	Intranuclear diffuse	Intranuclear diffuse	Intranuclear diffuse Cytoplasmic puncta	Intranuclear diffuse
NUP155	Nuclear envelope	Nuclear envelope Cytoplasmic diffuse	Nuclear envelope Cytoplasmic diffuse and puncta	Nuclear envelope Cytoplasmic diffuse and puncta	Nuclear envelope Cytoplasmic diffuse Large nuclear body
NUP133	Nuclear envelope	Nuclear envelope Small nuclear bodies	Nuclear envelope Intranuclear diffuse Small nuclear bodies Cytoplasmic diffuse and puncta	Nuclear envelope Intranuclear diffuse Small nuclear bodies Cytoplasmic diffuse and puncta	Nuclear envelope Cytoplasmic diffuse

3.2.3 Differential regulation of NUPs by HIV-1 infection

As mentioned in Chapter 2 (Methodology), for our studies, we considered the permissive cells as those cells that permit the entry of HIV-1 through the classical pathway, which requires the interaction of HIV-1 envelope proteins such as gp120 and gp41 with the host cell receptor (CD4+) and co-receptor (CCR5/CXCR4). On the other hand, the cells that do not allow the entry of HIV-1 via the classical pathway are considered as non-permissive cells as they lack receptor and co-receptor. In our HIV-1 infection studies, we included SupT1 as a permissive cell line and 1321N1, TZM-bl and HEK293T as non-permissive cell lines. SupT1 cell line is a CD4+ T cell type and physiologically represents the target cell type for HIV-1. 1321N1 cell line is a cell type of astrocyte, which is one of the neural cell types that can get infected by HIV-1 and contributes to neuroinflammation and HIV-associated neurocognitive disorder (HAND). TZM-bl cell line is genetically engineered from HeLa cell line and stably expresses receptor (CD4+) and co-receptor (CCR5). This cell line also has separate integrated copies of reporter genes such as luciferase and β-galactosidase and their expression is under the control of HIV-1 promoter. Due to their extensive use as cell models in HIV-1 research, we included both HEK293T and TZM-bl in our study. After the preparation and quantification of HIV-1 by p24 ELISA (Figure 3-3A), we assessed the infectivity of the virus using TZM-bl cell line as a reporter system. We infected the TZM-bl cells with increasing concentrations of HIV-1 in terms of its

p24 equivalents. After 48 hpi, the reporter assay was performed using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate for β-galactosidase. We noticed that increasing concentrations of virus incrementally enhanced the activity of β-galactosidase (Figure 3-3B). This implies that the HIV-1 produced under experimental conditions we supplied retained the infectivity. We used this virus capable of causing infection in subsequent experiments.

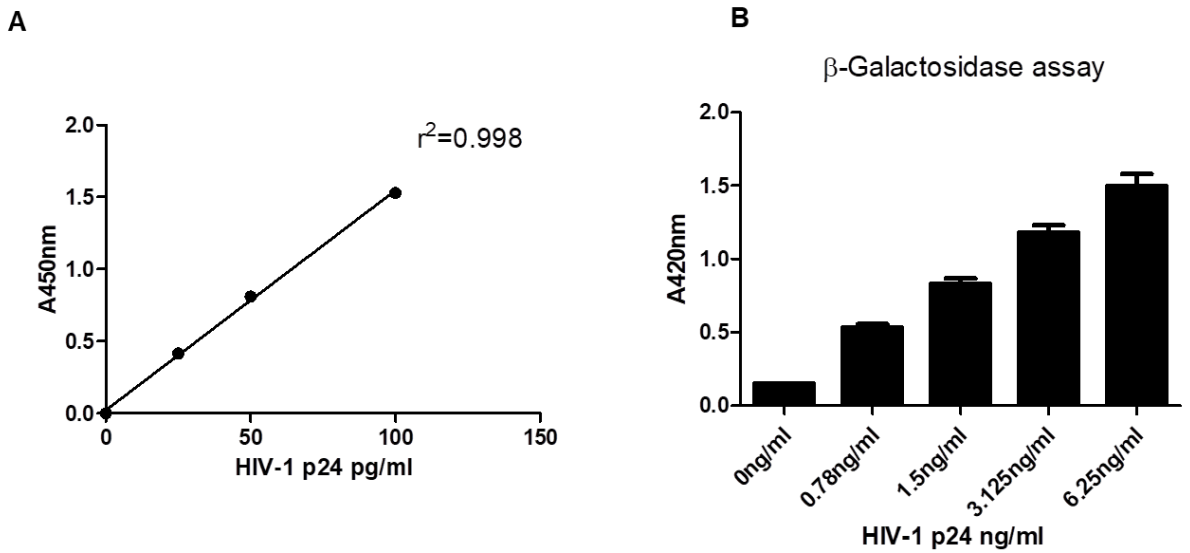

Figure 3-3. HIV-1 NL4.3 infectivity in reporter cell line TZM-bl. **(A)** Culture supernatant containing virus (VSV-G pseudotyped HIV-1 NL4.3) was collected from HEK293T cells and estimated in terms of p24 equivalents by p24 ELISA using the standard. **(B)** TZM-bl cells were infected with HIV-1 NL4.3, and 48 hpi, cell lysate was used for β-galactosidase assay.

To understand the impact of HIV-1 on the NPC components, we first checked the expression of some of the NUPs such as NUP98, NUP62, NUP155, NUP133, NUP107 and NUP85 in permissive cell line such as SupT1. For this, we had chosen several time points representing both the early and late stages of HIV-1 replication. SupT1 cells were infected with VSV-G pseudotyped HIV-1 virus (hereafter referred to as HIV-1 NL4.3). Initially, at post-infection early time points such as 0hr, 3hrs, 6hrs, 9hrs, and 12hrs, which represent early stages of HIV-1 replication, cells were harvested and analysed for the expression of NUPs by western blotting. Though we observed the dynamic change in the expression of NUPs during these post-infection

time points, the data was statistically non-significant and indicated that the early stages of HIV-1 replication did not influence the levels of NUPs (Figure 3-4).

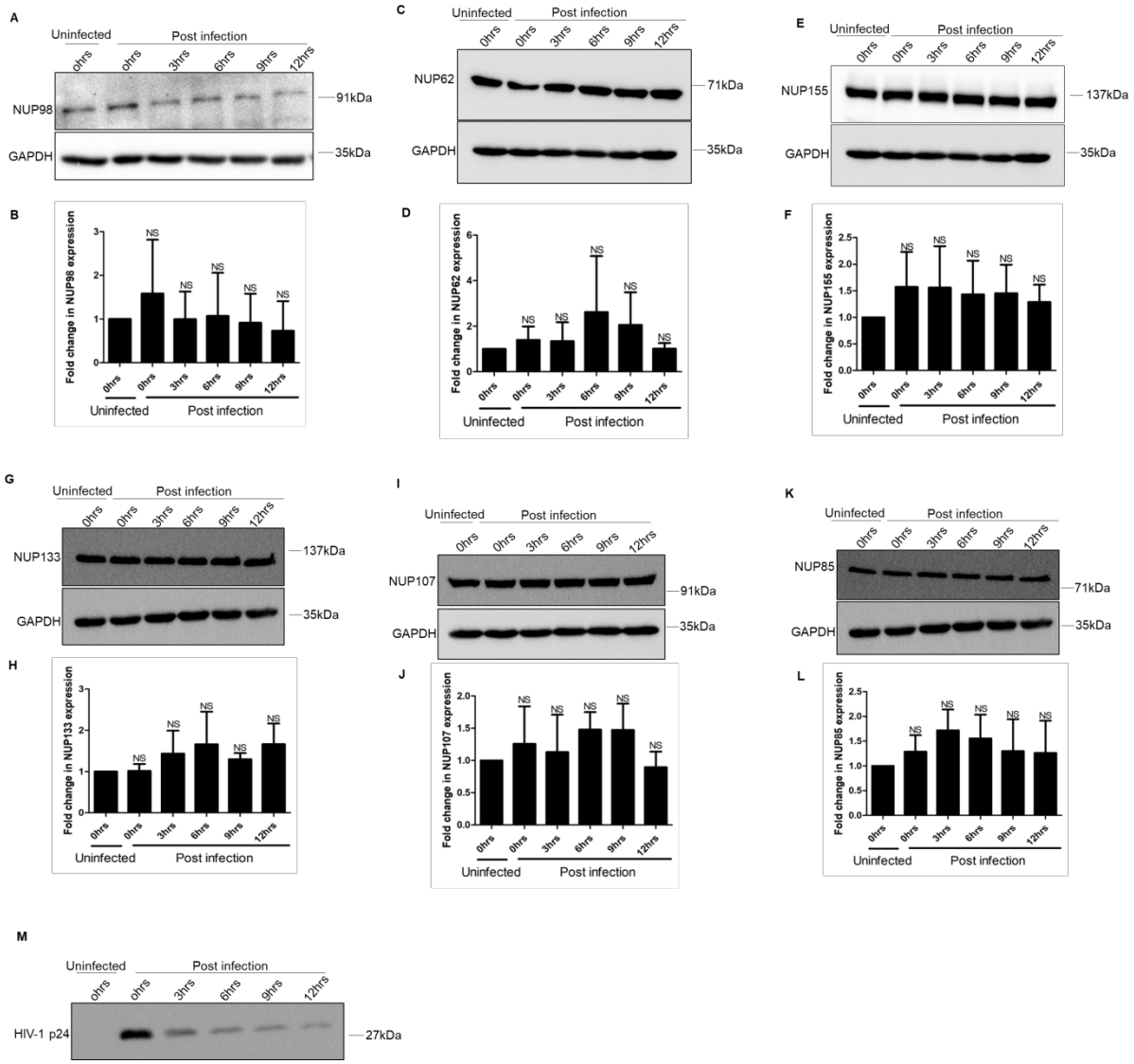

Figure 3-4. Expression of NUPs during early stage of HIV-1 replication in SupT1 cells. **(A-M)** SupT1 cells were infected with HIV-1 NL4.3 virus. At indicated time points post infection, cells were lysed and lysate was used for western blotting. Blots were probed with anti-NUP98 **(A)**, NUP62 **(C)**, NUP155 **(E)**, NUP133 **(G)**, NUP107 **(I)**, NUP85 **(K)** and HIV-1 p24 **(M)** antibodies. NUP's expression was normalized to the loading control GAPDH using image J software. **(B, D, F, H, J, L)** Bar graphs represent the mean fold change of NUP's expression from three independent experiments relative to the control cells (uninfected), which was taken as one. NS, P>0.05

Having observed no effect of HIV-1 infection on the levels of NUPs during early time points, we further tested two later post-infection time points (48 and 96 hpi) in

SupT1, which represent late stages of HIV-1 replication that include active transcription and translation of viral proteins, assembly of viral proteins and release of viral progeny, on the levels of NUPs. At 48 hpi, western blotting results revealed that there were no statistically significant changes in the levels of any NUP (Figure 3-5). At last, at 96 hpi, while the levels of NUP62, NUP155, NUP133, NUP107 and NUP85 remained unchanged, the level of NUP98 was significantly decreased by 4.7-fold (Figure 3-6).

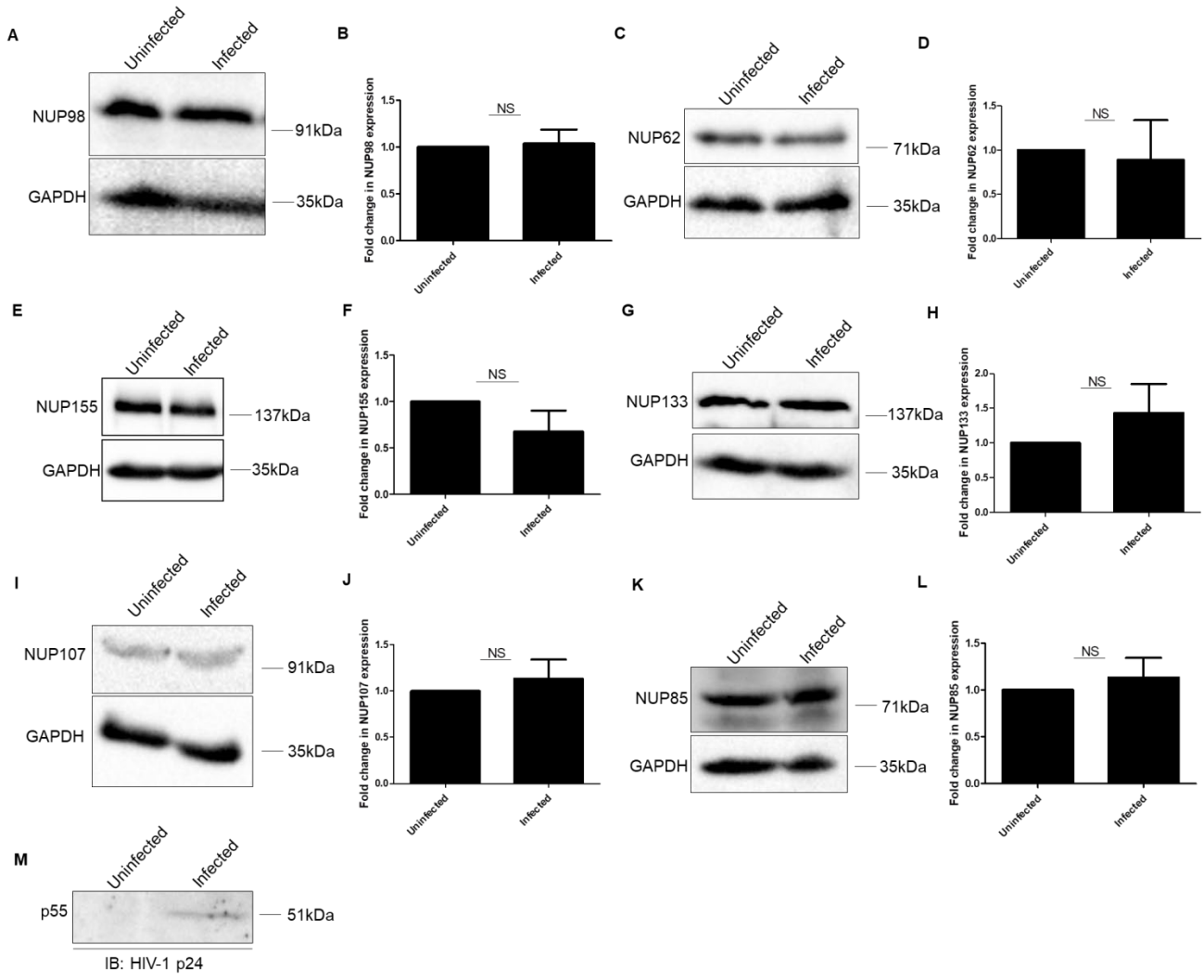

Figure 3-5. Expression of NUPs during late stage of HIV-1 replication in SupT1 cells (48 hpi). **(A-M)** SupT1 cells were infected with HIV-1 NL4.3 virus. Two days after infection (48 hpi), cells were lysed and lysate was used for western blotting. Blots were probed with anti-NUP98 **(A)**, NUP62 **(C)**, NUP155 **(E)**, NUP133 **(G)**, NUP107 **(I)**, NUP85 **(K)** and HIV-1 p24 **(M)** antibodies. NUP's expression was normalized to the loading control GAPDH using image J software. **(B, D, F, H, J, L)** Bar graphs represent the mean fold change of NUP's

expression from three independent experiments relative to the control cells (uninfected), which was taken as one. NS, P>0.05.

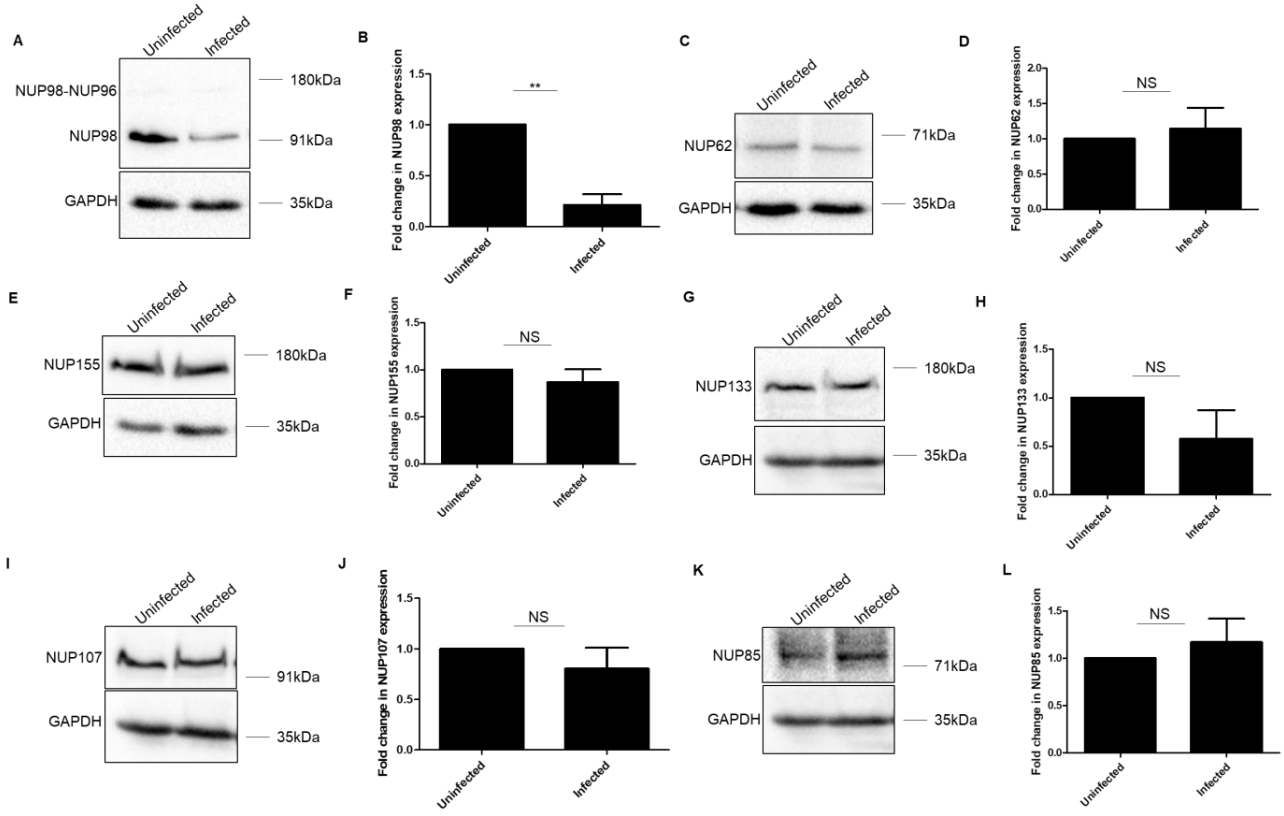

Figure 3-6. Expression of NUPs during late stage of HIV-1 replication in SupT1 cells (96 hpi). **(A-M)** SupT1 cells were infected with HIV-1 NL4.3 virus. Four days after infection (96 hpi), cells were lysed and lysate was used for western blotting. Blots were probed with anti-NUP98 **(A)**, NUP62 **(C)**, NUP155 **(E)**, NUP133 **(G)**, NUP107 **(I)**, NUP85 **(K)** and HIV-1 p24 **(M)** antibodies. NUP's expression was normalized to the loading control GAPDH using image J software. **(B, D, F, H, J, L)** Bar graphs represent the mean fold change of NUP's expression from three independent experiments relative to the control cells (uninfected), which was taken as one. NS, P>0.05; **, P<0.01.

Having tested the levels of NUPs at different time points in SupT1 and found that the NUP98 level was decreased at 96 hpi, we further determined the levels of NUPs in non-permissive cells such as 1321N1, TZM-bl, and HEK293T at 96 hpi. The expression of all the tested NUPs remained unchanged in 1321N1 cells at this time point (Figure 3-7). Like in SupT1, the expression of NUP98 was significantly decreased in HEK293T cells (Figure 3-7A, B). Particularly, in TZM-bl cells, as opposed to all other cell types, the expression of NUPs such as NUP62 and NUP133 was significantly reduced (Figure 3-7C, D, G, H). The decrease in the levels of

NUP62 and NUP133 in TZM-bl is in consistent with the previous reports (238). The differential regulation of NUPs by HIV-1 at a late time point, i.e. 96 hpi, indicates that only the late stage of HIV-1 infection affected the levels of NUPs both in permissive (SupT1) and non-permissive cell types (TZM-bl and HEK293T). With the western blotting of lysates from all the cell types with anti-p24 antibody, I further normalized the intracellular p55 levels to the respective loading control GAPDH for each cell type and observed that the percentage infectivity of HIV-1 was slightly less in HEK293T cells in comparison to SupT1 cells (Figure 3-8A, B, C).

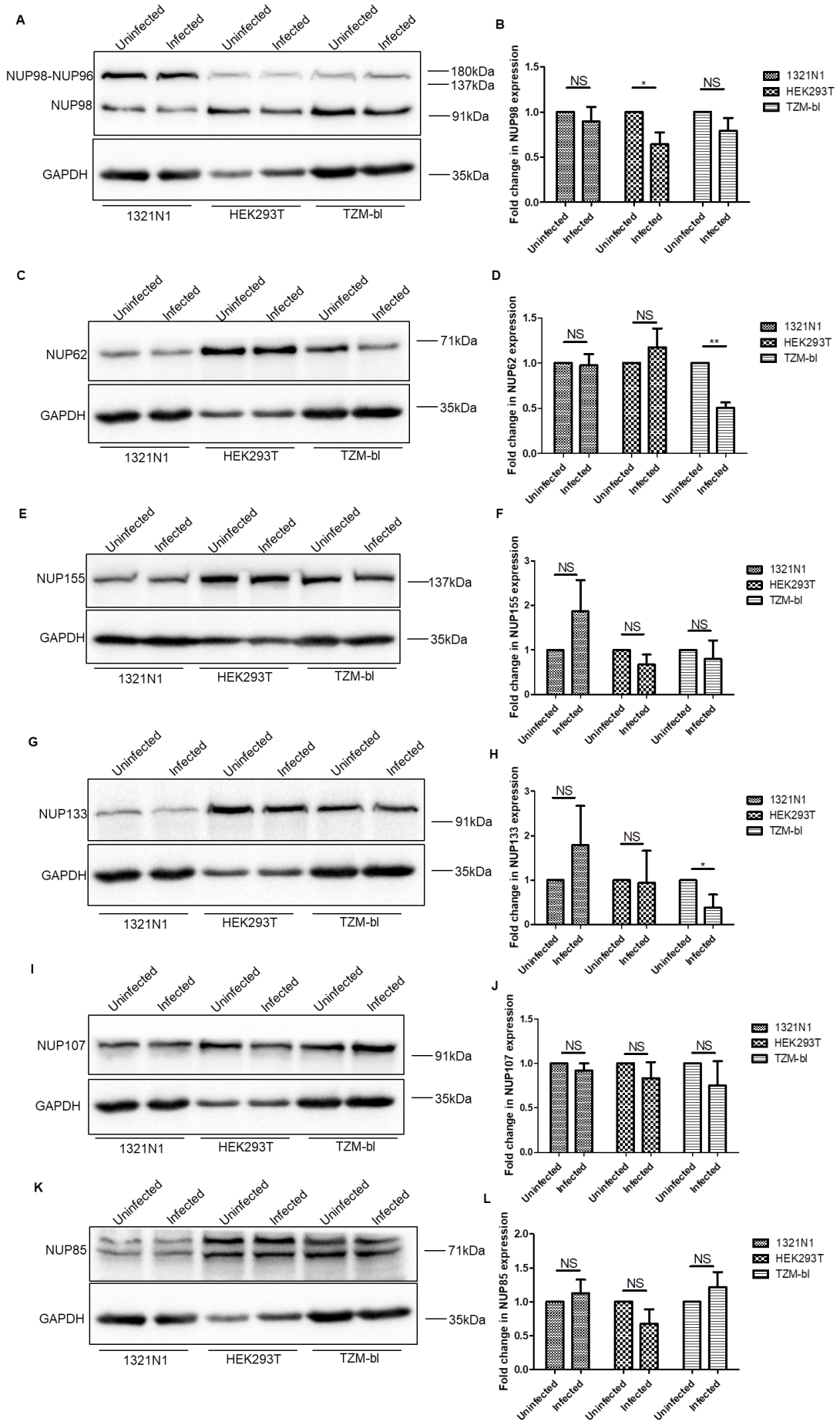
A
Uninfected
Infected
NUP98-NUP96
NUP98
GAPDH
180kDa
137kDa
91kDa
35kDa
1321N1
HEK293T
TZM-bl
B
Fold change in NUP98 expression
NS
*
1321N1
HEK293T
TZM-bl
C
NUP62
71kDa
D
Fold change in NUP62 expression
**
E
NUP155
137kDa
F
Fold change in NUP155 expression
G
NUP133
91kDa
H
Fold change in NUP133 expression
I
NUP107
J
Fold change in NUP107 expression
K
NUP85
71kDa
L
Fold change in NUP85 expression

Figure 3-7. Expression of NUPs during late stage of HIV-1 replication in 1321N1, HEK293T and TZM-bl cells. **(A-L)** Cells were infected with HIV-1 NL4.3 virus. Four days after infection (96 hpi), cells were lysed and lysate was used for western blotting. **(A, C, E, G, I, K)** Blots were probed with anti-NUP and anti-GAPDH antibodies. NUP expression was normalized to the loading control GAPDH using image J software. **(B, D, F, H, J, L)** Bar graphs represent the mean fold change of NUP expression relative to the control cells (uninfected), which was taken as one. Experiments were performed at the least three times; NS, P>0.05; *, P<0.05.

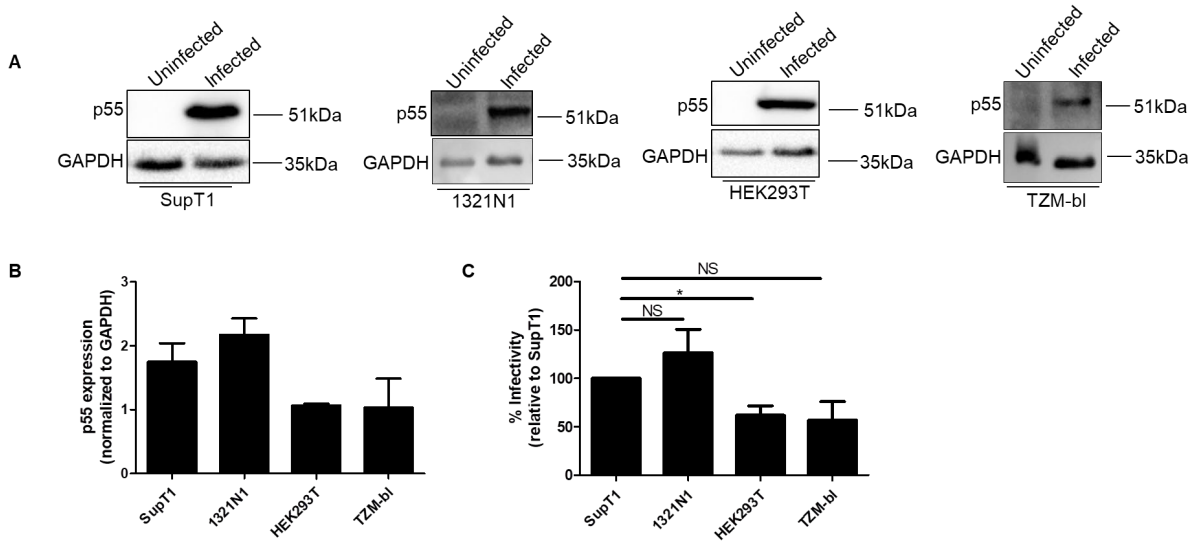

Figure 3-8. Intracellular HIV-1 p55 levels in SupT1, 1321N1, HEK293T and TZM-bl cells at 96 hpi. **(A-C)** Cells were infected with HIV-1 NL4.3 virus. Four days after infection (96 hpi), cells were lysed and lysate was used for western blotting. **(A)** Blots were probed with anti-HIV-1 p24 and anti-GAPDH antibodies. **(B)** Bar graphs represent the mean intracellular p55 levels normalized to the loading control GAPDH using image J software. **(C)** Bars represent the mean infectivity percentage relative to the SupT1 cells, which was taken as one. Experiments were performed at the least three times; NS, P>0.05; *, P<0.05.

To further explore if only the late stage of HIV-1 infection is enough to modulate the expression of NUPs, we took advantage of the proviral plasmid construct pNL4.3, which allows the synthesis of viral genomic RNA and proteins and release of virions upon transfection, mimicking the late stage of HIV-1 infection. We examined the expression of NUPs in HEK293T cells transfected with pNL4.3 by western blotting. Similar to infection condition, we found that NUP98 was significantly downregulated by 2.1-fold in the cells transfected with pNL4.3 (Figure 3-9 A, B). However, unlike

during the infection, the levels of NUP62 and NUP85 were also downregulated by 1.2- and 2.1-fold, respectively, whereas NUP133 was upregulated by 1.5-fold (Figure 3-9C, D, G, H, K, L). But the expression of other nucleoporins such as NUP155 and NUP107 remained unchanged under transfection conditions (Figure 3-9E, F, I, J). The differential expression of NUPs during HIV-1 infection in different cell types was listed in Table 3-2.

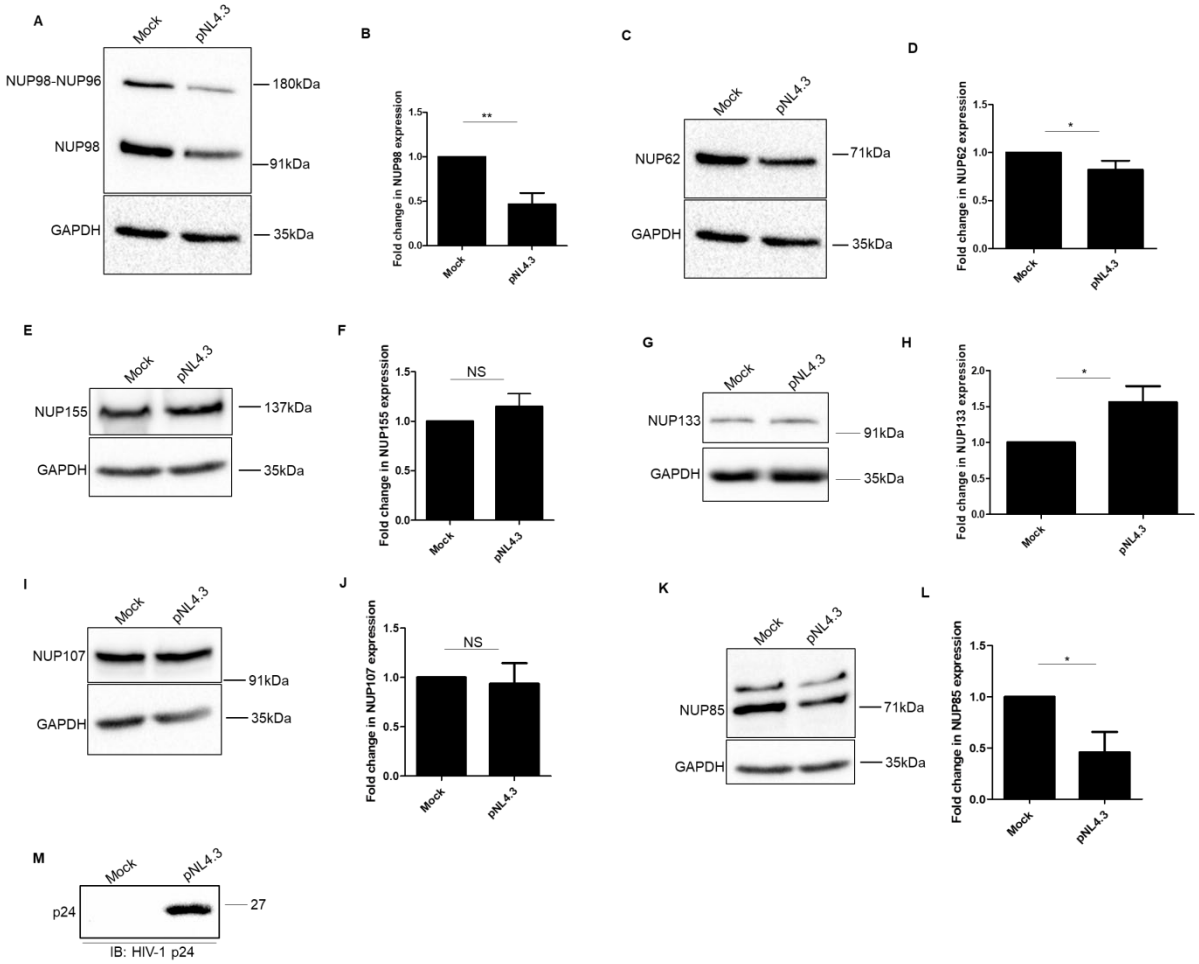

Figure 3-9. Differential expression of NUPs in HEK293T cells transfected with pNL4.3. **(A-M)** HEK293T cells were transfected with proviral plasmid pNL4.3. Two days after transfection, cells were lysed and lysate was used for western blotting. Blots were probed with anti-NUP98 **(A)**, NUP62 **(C)**, NUP155 **(E)**, NUP133 **(G)**, NUP107 **(I)**, NUP85 **(K)** and HIV-1 p24 **(M)** antibodies. NUP's expression was normalized to the loading control GAPDH using image J software. **(B, D, F, H, J, L)** Bar graphs represent the mean fold change of

NUP's expression from three independent experiments relative to the control cells (mock), which was taken as one. *, P<0.05; **, P<0.01; NS, P>0.05.

Table 3-2 Differential expression of NUPs upon HIV-1 infection in different cell types

NUP	SupT1 (96 hpi)	1321N1 (96 hpi)	TZM-bl (96 hpi)	HEK293T	
				96 hpi	48 hpt
NUP98	Downregulated	Unchanged	Unchanged	Downregulated	Downregulated
NUP62	Unchanged	Unchanged	Downregulated	Unchanged	Downregulated
NUP155	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged
NUP133	Unchanged	Unchanged	Downregulated	Unchanged	Upregulated
NUP107	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged
NUP85	Unchanged	Unchanged	Unchanged	Unchanged	Downregulated

3.2.4 HIV-1 infection does not affect mRNA levels of NUPs

To further understand the regulation of NUPs by HIV-1, I checked the mRNA levels of NUPs under study in all the cell types, for which NUPs protein expression was analysed. Four days post-infection (96 hpi) with HIV-1 NL4.3, cells were harvested; RNA was isolated for cDNA synthesis and quantified by qPCR as mentioned in the chapter 2. The mRNA expression analysis of NUPs was based on the $2^{-\Delta\Delta Ct}$ method. Briefly, the mRNA expression for each NUP was normalised to internal control GAPDH mRNA (ΔCt) under both infection and non-infection conditions for each cell type. Then, the levels of each NUP mRNA in infected cells was normalised that of uninfected cells ($-\Delta\Delta Ct$) and fold change in NUP mRNA expression was measured ($2^{-\Delta\Delta Ct}$). The mRNA quantification analysis showed that HIV-1 did not seem to regulate the expression of any NUP, if not NUP155, under study at mRNA level in all the cell types (Figure 3-10A-D). In TZM-bl cells, the expression of NUP155 mRNA was downregulated by HIV-1 infection (P<0.01) (Figure 3-10D).

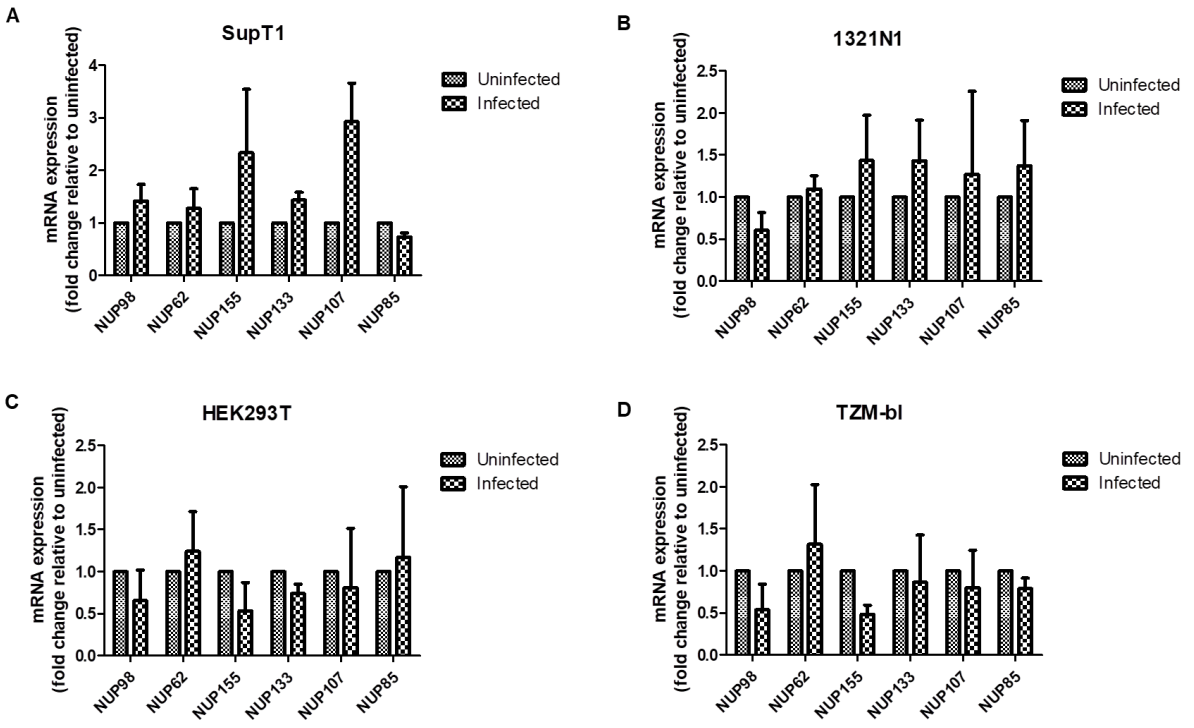

Figure 3-10. NUP mRNA expression during late stage of HIV-1 replication in SupT1, 1321N1, HEK293T and TZM-bl cells. **(A-D)** Cells were infected with HIV-1 NL4.3 virus. Four days after infection (96 hpi), cells were harvested and RNA was isolated. NUP mRNA expression was normalized to the internal control GAPDH. Bar graphs represent the mean fold change of NUP expression in infected cells relative to the uninfected cells, which was taken as one. Experiments were performed at least three times.

It is generally accepted that cellular processes are confined to specific compartments within a cell and require proteins that carry these processes to be localised in those compartments. Many viruses have evolved strategies to sequester proteins into particular compartments so that certain phases of viral replication are accomplished in those compartments. Keeping this in view, we surveyed whether the localization of NUPs both in permissive cells (SupT1) and non-permissive cells (HEK293T) were changed during the late stage of HIV-1 replication by immunostaining and followed by microscopy. However, Microscopy studies showed that the cellular localization of NUPs remained unaltered during HIV-1 NL4-3 infection (96 hpi) in SupT1 cells (Figure 3-11) or transfection with pNLC4.3 GFP in HEK293T cells (Figure 3-12). Taken together, we conclude that HIV-1 regulates the cell-specific expression of NUPs and does not influence the localization pattern of these proteins.

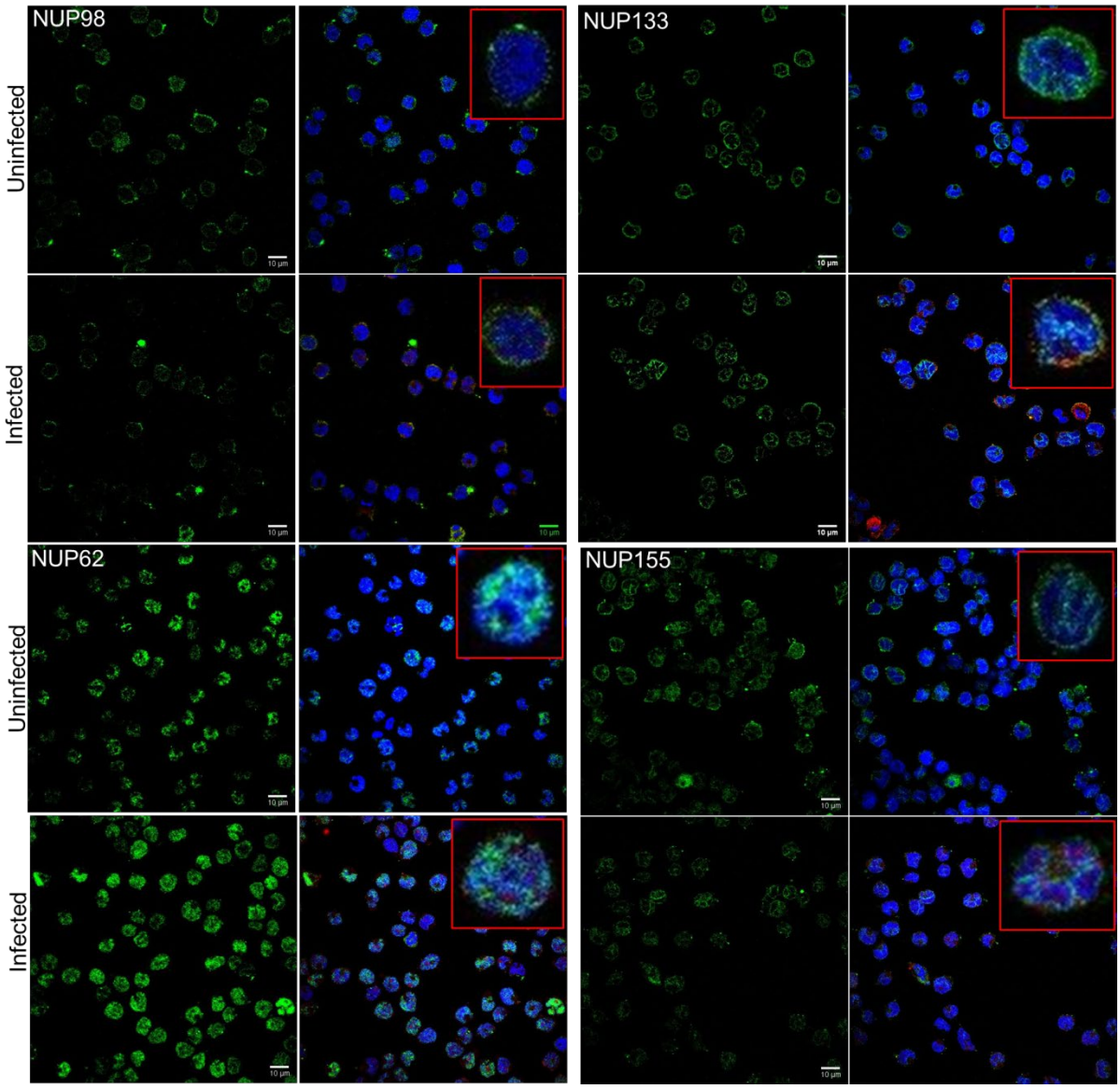

Figure 3-11. Localization of NUPs during late stage of HIV-1 replication in SupT1 cells (96 hpi). Cells were infected with HIV-1 NL4.3 virus. Four days after infection (96 hpi), cells were fixed and subjected to intracellular immunostaining using anti- NUP98, NUP62, NUP155, NUP133 and HIV-1 p24 antibodies. After incubation with Anti-rabbit 488, anti-mouse 647 antibodies and staining with DAPI, cells were observed under confocal microscopy using green, far red and blue exciting lasers. Inlet shows the merge image of a selected cell in a field of view. Scale bar is 10 µm

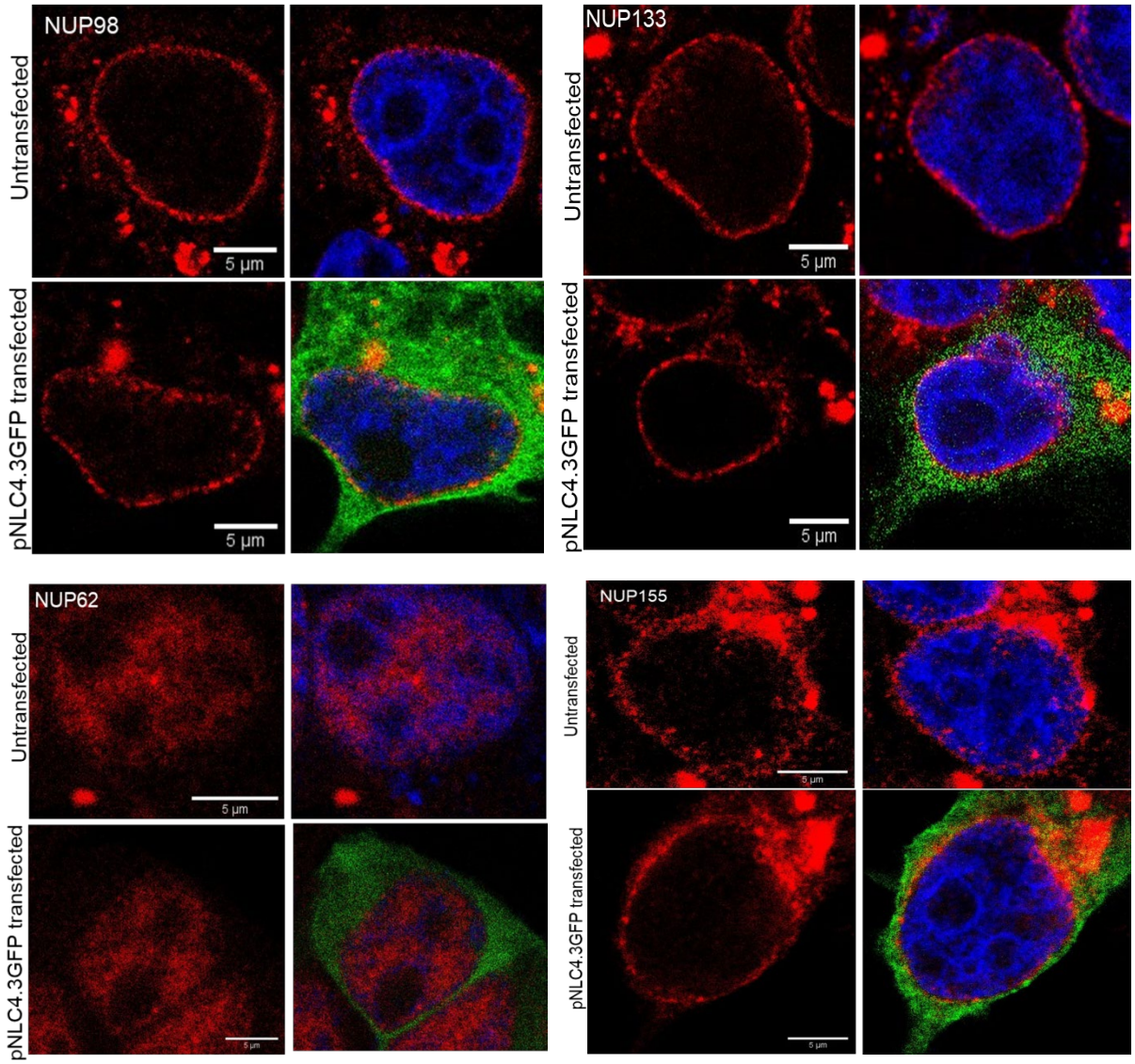

Figure 3-12. Localization of NUPs during late stage of HIV-1 replication in HEK293T cells. Cells were transfected with pNLC4.3 GFP. Forty eight hours post-transfection, cells were fixed and subjected to intracellular immunostaining using anti- NUP98, NUP62, NUP155 and NUP133 antibodies. After incubation with Anti-rabbit 546 antibody and staining with DAPI, cells were observed under confocal microscopy using green, red and blue exciting lasers. Scale bar is 5 µm.

3.3 Discussion

In this study, I assessed the basal expression of NUPs both at protein and RNA levels in different cell types known to be infected by HIV-1 such as SupT1, THP1, 1321N1, HEK293T and TZM-bl cells. After normalization with internal control such as GAPDH for each cell type, I observed that the expression of NUPs, both at the protein and RNA levels varied within a cell type as well as among the cell types. For

the first time to the best of my knowledge, I show differential expression of these NUPs (both at protein and RNA levels) among aforementioned cell types. I further evaluated the localization of the NUPs in these cell types by immunostaining followed by confocal microscopy. The differences in the localization of the NUPs within a cell type and among cell types are listed in the Table 3-1. Remarkably, in the cytoplasm of HEK293T cells, all the tested NUPs were found to be localized as distinct foci in addition to their presence at NE. However, NUP62 was absent at NE, although it formed cytoplasmic foci. It will be interesting to investigate if the same cytosolic foci consist of all the NUPs tested and if there is a common mechanism underlying the formation of these foci in HEK293T cells. As expected, the intranuclear bodies (GLFG bodies) in TZM-bl cells were observed with antibodies against NUP98 and NUP133. The intranuclear bodies in HEK293T cells that were visualised with anti-NUP98 and NUP133 antibodies differ in terms of number, size and the location in the nucleoplasm. While NUP98 localized mainly as a single large intranuclear body and colocalized with nucleolus, the pattern of NUP133 localization in HEK293T cells was similar to its localization in TZM-bl. This means that both in TZM-bl and HEK293T cells, the intranuclear bodies that contained NUP133 may be chemically similar in composition and have a common pathway that form these bodies and carryout similar functions. In HeLa cells, from which TZM-bl cells were genetically engineered, it was described that both NUP98 and NUP133 were found to be part of the same intranuclear bodies and shared same functions (237). However, in HEK293T cells, it is unlikely that the intranuclear bodies that are positive for either NUP98 or NUP133 are chemically similar in composition and carryout similar functions as these bodies were differentially colocalized with nucleoli and had different sizes. Interestingly, previous reports showed that the intranuclear GLFG bodies were devoid of any known markers of other nuclear bodies in HeLa cells, indicating that these specialized intranuclear structures may have different nuclear functions to carryout (124). Future investigation is, indeed, needed to test whether the intranuclear bodies differentially stained by NUP98 and NUP133 in HEK293T cells have a similar underlying mechanisms for the formation of these structures and have the same set of functions to carry out. Interestingly, in THP1 cells the intranuclear localization of NUP155 was very distinct from its localization in all other cell types used in the study. In THP1 cells, NUP155 was localized as a single large nuclear body and confined exclusively to the nucleolus, which was not observed in

all other cell types, in addition to its appearance at NE and in the cytosol as a diffuse signal. Previous reports describe that NUP155 was localized to both the cytoplasmic and nucleoplasmic faces of the NPC and that NUP155 was required for the formation of both NE and NPC during cell division in *Caenorhabditis elegans* embryos and in *Xenopus laevis* egg extracts (239). It is likely that NUP155 may also be involved in the nucleolar function in a cell type-dependent manner as its localization was observed in the nucleolus of THP1 cells. It should be noted that the immunostaining of intracellular proteins could be affected by the epitopic accessibility of the antigen to the antibody used in the study. For example, an antibody raised against a particular region of NUP can detect it at one cellular location but not in other cellular locations because of masking of epitopic region in protein complexes, whose composition may differ depending on the cellular location and cell type they are expressed in. Thus, one should be cautious in concluding the cellular localization of any protein of interest.

I further explored the expression levels of NUPs in SupT1 cells during HIV-1 NL4.3 infection. Although the initial stages of HIV-1 infection did not affect the levels of NUPs, late time point, i.e., 96 hours post-infection (96 hpi), downregulated the NUP98 but not the other tested NUPs. Other cell types such as 1321N1, HEK293T, and TZM-bl were also infected with HIV-1 NL4.3 to see its effect on the levels of NUPs. Like in SupT1, in HEK293T cells NUP98 was significantly downregulated but to a lesser extent than what was observed in SupT1. Nevertheless, the levels of other NUPs were unaffected by HIV-1 NL4.3 in HEK293T cells. Interestingly, the levels of all the NUPs were unaffected in 1321N1 by HIV-1 NL4.3 at this time point (96 hpi). In TZM-bl cells, while the levels of NUP98, NUP155, NUP107, and NUP85 were not affected, the expression of NUP62 and NUP133 was decreased. To understand the specificity of the effect of late stage of HIV-1 infection on the levels of NUPs, the late-stage condition of HIV-1 infection in HEK293T cells was mimicked by transfecting the cells with the molecular clone pNL4.3, essentially skipping the initial steps of HIV-1 replication such as entry, reverse transcription and integration. Under this condition, a few disparities were observed in the levels of NUPs from what was observed during HIV-1 NL4.3 infection in HEK293T cells. While NUP98's expression was downregulated, the expression of NUP62, NUP133, and NUP85 was also modulated in contrast to the condition of infection with HIV-1 NL4.3. Taken together,

the regulation of expression of the NUPs by HIV-1 infection varies from one cell type to the other. I also tested the effect of HIV-1 infection on the localization of the NUPs, whose protein levels were tested, in both SupT1 and HEK293T cells. However, I did not observe the discernible changes in the localization pattern of these NUPs during the late stage of HIV-1 infection. Previously, it was observed that the expression (Jurkat T cells and HeLa cells) and localization (HeLa cells) of NUP62 were modulated in cells transfected with pNL4.3 and that the regulation of NUP62 was associated with increased HIV-1 replication (238,240). In line with this evidence, in our study, the expression of NUP62 was downregulated in HEK293T cells transfected with pNL4.3. However, its localization in HEK293T cells remained unchanged under similar conditions. It should be noted that while our antibody detected NUP62 exclusively in the nucleoplasm, in the previous observations it was found to be localized majorly at the NE (238,240).

Chapter 4

Nucleoporin NUP98 suppresses the basal HIV-1 gene expression through its N-terminal domain and reduces HIV-1 infectivity

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The nuclear pore protein NUP98 impedes LTR-driven basal gene expression of HIV-1, viral propagation, and infectivity

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Chapter 4: Nucleoporin NUP98 suppresses the basal HIV-1 gene expression through its N-terminal domain and reduces HIV-1 infectivity

4.1 Introduction

NPCs and their constituents (NUPs) play fundamental roles in the completion of viral life cycle for several viruses including HIV-1. Given their essential participation during HIV-1 replication, several proteomic studies have focused and identified many NUPs to be dysregulated during HIV-1 infection (230,241,242). However, the functional association of many of the NUPs identified in these studies with respect to HIV-1 infection remains uncharacterized. Since, in our study, NUP98 expression was downmodulated during both transfection and infection conditions, we focused our investigation on NUP98. While NUP98 was previously shown to promote the export of HIV-1 RNA via Rev-hCRM1 mediated transport from the nucleus to cytosol across the nuclear membrane barrier (233), recent evidence also suggested that NUP98 was required for MX2 mediated HIV-1 restriction (243), pointing out multiple and conflicting roles for NUP98 as pro- or anti- HIV-1 factor. Here, we aimed to further understand the multifaceted function of NUP98 in HIV-1 infection and decipher the underlying mechanism driven by NUP98 that regulate HIV-1 propagation.

4.2 Results

4.2.1 The viral regulatory proteins HIV-1 Tat and Rev do not affect the endogenous levels of NUP98 protein

The downregulation of NUP98 at the late time point (i.e., 96 hpi) by HIV-1 in both SupT1 and HEK293T and during pNL4.3 transfection in HEK293T pointed that the modulation of NUP98 occurred during the late-stage of HIV-1 replication. Using its components, HIV-1 is known to regulate many of the host factors to survive and efficiently produce viral progeny from an infected cell. Among the virally encoded factors, HIV-1 regulatory proteins such as HIV-1 Tat and Rev were previously shown to affect the homeostasis of many host-cellular proteins (244,245). I therefore investigated the effect of these viral factors on the endogenous levels of NUP98. To test this, HEK293T cells were transfected with pcTat and pcRev independently. As a control, cells were also transfected with pcDNA. The over-expression of either HIV-1 Tat or Rev alone in HEK293T cells did not affect the endogenous levels of NUP98,

suggesting these viral factors may not have a direct role in the regulation of NUP98 expression (Figure 4-1).

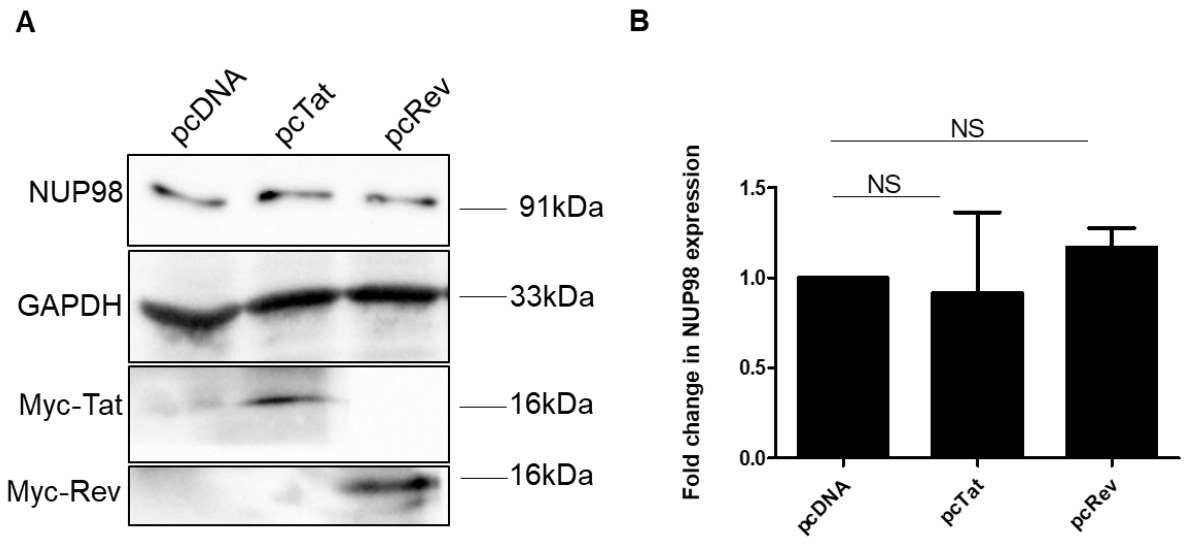


Figure 4-1. HIV-1 Tat and Rev do not affect the expression of NUP98 in HEK293T cells. HEK293T cells were transfected with pcDNA, pcTat or pcRev. Two days after transfection, cells were lysed and lysate was used for western blotting. **(A)** Blots were probed with anti-NUP98, GAPDH and myc antibodies. NUP98 expression was normalized to the loading control GAPDH using image J software. **(B)** Bar graphs represent the mean fold change of NUP98 expression from three independent experiments relative to the control cells (pcDNA). NS, P>0.05.

4.2.2 Overexpression of NUP98 reduces HIV-1 viral transcript, protein levels and viral titers

Continuing with our observation that HIV-1 reduced the intracellular pool of NUP98 in both SupT1 and HEK293T cells, we sought to understand the effect of transiently overexpressed NUP98 on the late stages of HIV-1 infection by measuring the intracellular levels of HIV-1 protein p55 (Gag) in HEK293T cells. To achieve this, HEK293T cells were co-transfected with NUP98 over-expressing plasmid (GFP-NUP98) and pNL4.3 and western blotting was performed to estimate the intracellular p55 levels. Western blot analysis showed that the intracellular levels of p55 were significantly decreased upon over expression of GFP-NUP98 in comparison to control cells by 1.7-fold (Figure 4-2A, B). The expression of GFP-NUP98 was verified in the cell lysates by western blotting (Figure 4-2C). We next estimated the quantity of viral antigens (p24 equivalents/viral titers) released in the culture supernatant of

HEK293T cells co-transfected with GFP-NUP98 and pNL4.3 by p24 ELISA. In agreement with cellular viral protein p55 levels, viral antigens in the culture supernatants of HEK293T cells transfected with GFP-NUP98 were attenuated by 3-fold compared to the control cells (Figure 4-2D). The decrease in both intracellular p55 levels and viral antigen-release led us to speculate if NUP98 actually reduced total viral RNA. To this end, HEK293T cells were co-transfected with GFP-NUP98 and pNL4.3 as mentioned above. After 48 h of transfection, cells were harvested and total viral RNA was quantified by RT-qPCR using the primers listed in Table A1 (annexures). Indeed, we observed that the overexpression of NUP98 reduced total viral transcript (Env mRNA) levels (Figure 4-2E).

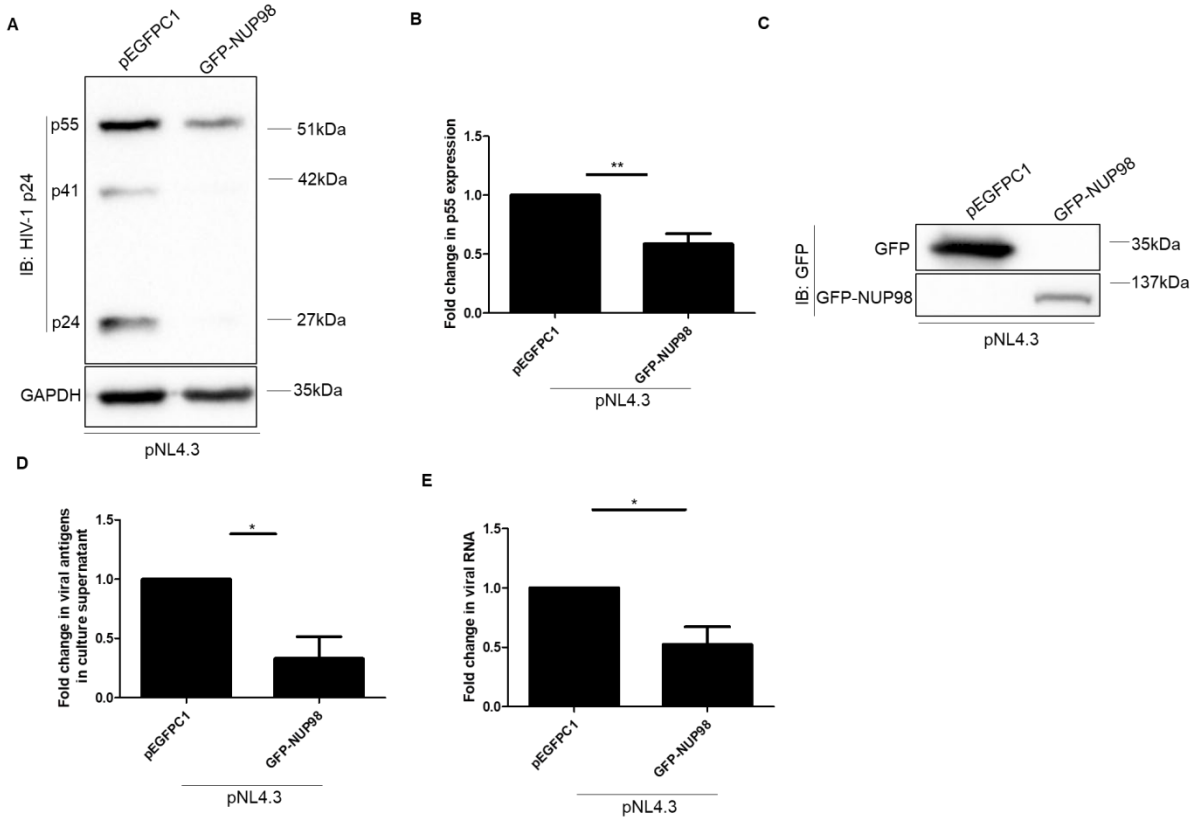


Figure 4-2. Overexpression of NUP98 reduces HIV-1 p55 protein, viral titers and viral RNA levels upon pNL4.3 transfection in HEK293T cells. **(A-E)** HEK293T cells were co-transfected with pNL4.3 and either pEGFPC1 or GFP-NUP98. Forty eight hours post-transfection (48 hpt), cells were harvested and culture supernatant was collected. **(A)** Blots were probed with anti-HIV-1 p24 and GAPDH antibodies. p55 expression was normalized to the loading control GAPDH. **(B)** Bars represent the mean fold change of p55 expression relative to the vector control. **(C)** The GFP-NUP98 expression was verified by western blotting using anti-

GFP antibody. **(D)** Bars represent the mean fold change of viral antigens in culture supernatant relative to the vector control. **(E)** Bars represent the mean fold change of intracellular viral RNA relative to the vector control. The experiments were performed at the least three times. *, P<0.05; **, P<0.01.

To test if NUP98 mediated reduction of viral p55, RNA levels and viral antigen-release was also true for cells infected with HIV-1 NL4.3, HEK293T cells were first transfected with GFP-NUP98 for 24 hrs and then challenged with HIV-1 NL4.3 for 48 h. As expected, even under these infection conditions, intracellular p55 levels were reduced by GFP-NUP98 (Figures 4-3A, B). The expression of GFP-NUP98 was verified in the cells transfected with GFP-NUP98 plasmid construct by western blotting using anti-GFP antibody (Figure 4-3C). In addition, viral RNA levels and viral antigen-release were also reduced upon NUP98 transient overexpression (Figures 4-3D, E). The reduced total viral RNA upon NUP98 overexpression, thus, explains the decrease in the intracellular p55 levels and viral antigen-release. The trypan blue dye exclusion assay showed that transient overexpression of NUP98 had negligible effect on the cell viability (Figure 4-3F).

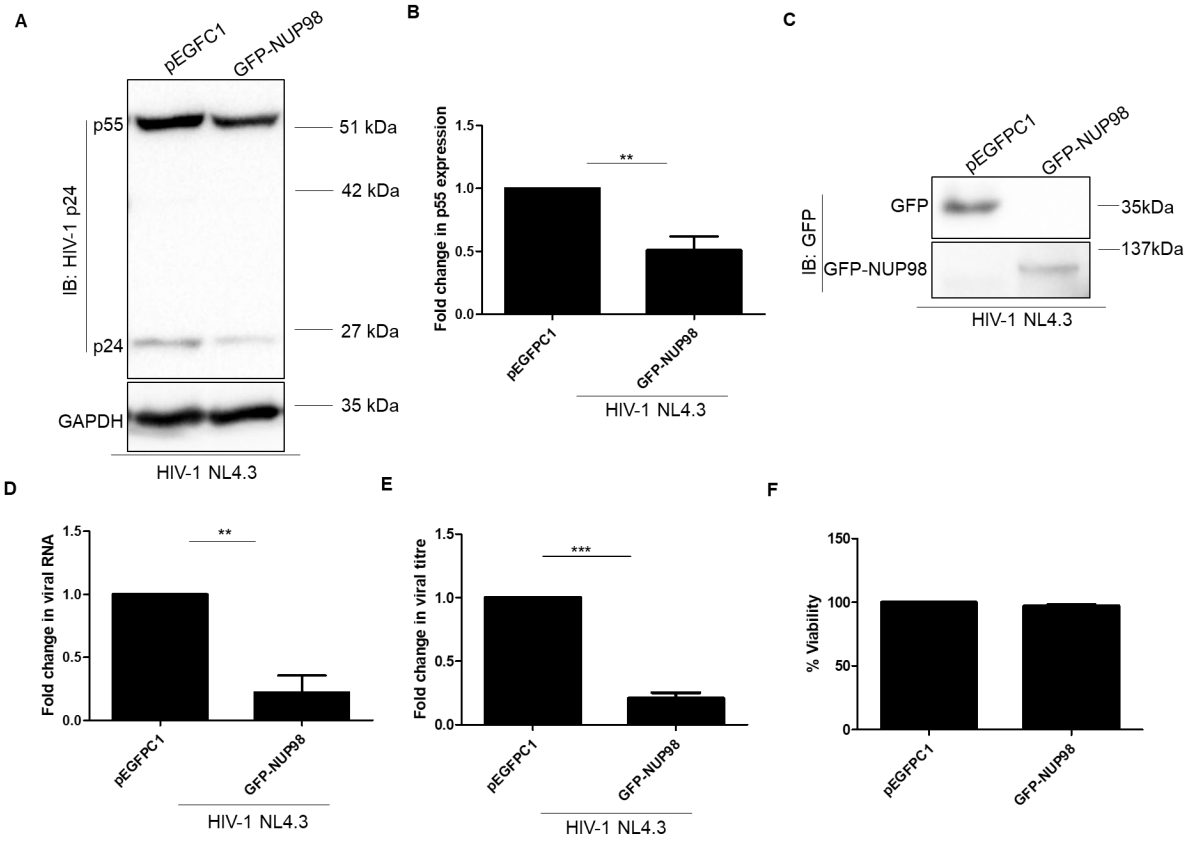


Figure 4-3. Overexpression of NUP98 reduces HIV-1 p55, RNA levels and viral titers during HIV-1 NL4.3 infection in HEK293T cells. **(A-E)** HEK293T cells were transfected with either pEGFPC1 or GFP-NUP98. 24 hrs post-transfection, cells were infected with HIV-1 NL4.3, and 48 hpi cells were harvested either for lysate preparation or RNA isolation, and culture supernatant was collected for p24 ELISA. **(A)** Blots were probed with anti- HIV-1 p24 and anti-GAPDH antibodies. p55 expression was normalized to the loading control GAPDH. **(B)** Bars represent the mean fold change of p55 expression relative to the vector control. **(C)** The GFP-NUP98 expression was verified by western blotting using anti-GFP antibody. **(D)** Bars represent the mean fold change of viral RNA expression relative to the vector control. **(E)** Bars represent the mean fold change of viral antigens relative to the vector control. **(F)** HEK293T cells were transfected with either pEGFPC1 or GFP-NUP98. Forty eight hours post-transfection, cells were harvested, stained with trypan blue and counted using haemocytometer. Bars represent the percentage of viability of cells transfected with GFP-NUP98 relative to vector control (100%).The experiments were performed at the least three times. **, P<0.01; ***,P<0.001.

We then checked if any of the other NUPs under this study could also affect the HIV-1 virion production in general. Therefore, as another FG rich NUP, the role of NUP62 on intracellular p55 protein expression and viral antigen release in the culture supernatant was evaluated. Upon co-transfection with pNL4.3 and GFP-NUP62, we observed no significant effect on either intracellular p55 protein expression or viral antigen release by transiently expressed GFP-NUP62 (Figures 4-4A, B). The expression of GFP-NUP62 was verified in the cells transfected with GFP-NUP62 plasmid construct by western blotting using anti-GFP antibody (Figures 4-4C). Thus, this data suggests that the inhibition of HIV-1 protein expression was NUP98 specific in our conditions with respect to the selected NUPs that we were studying.

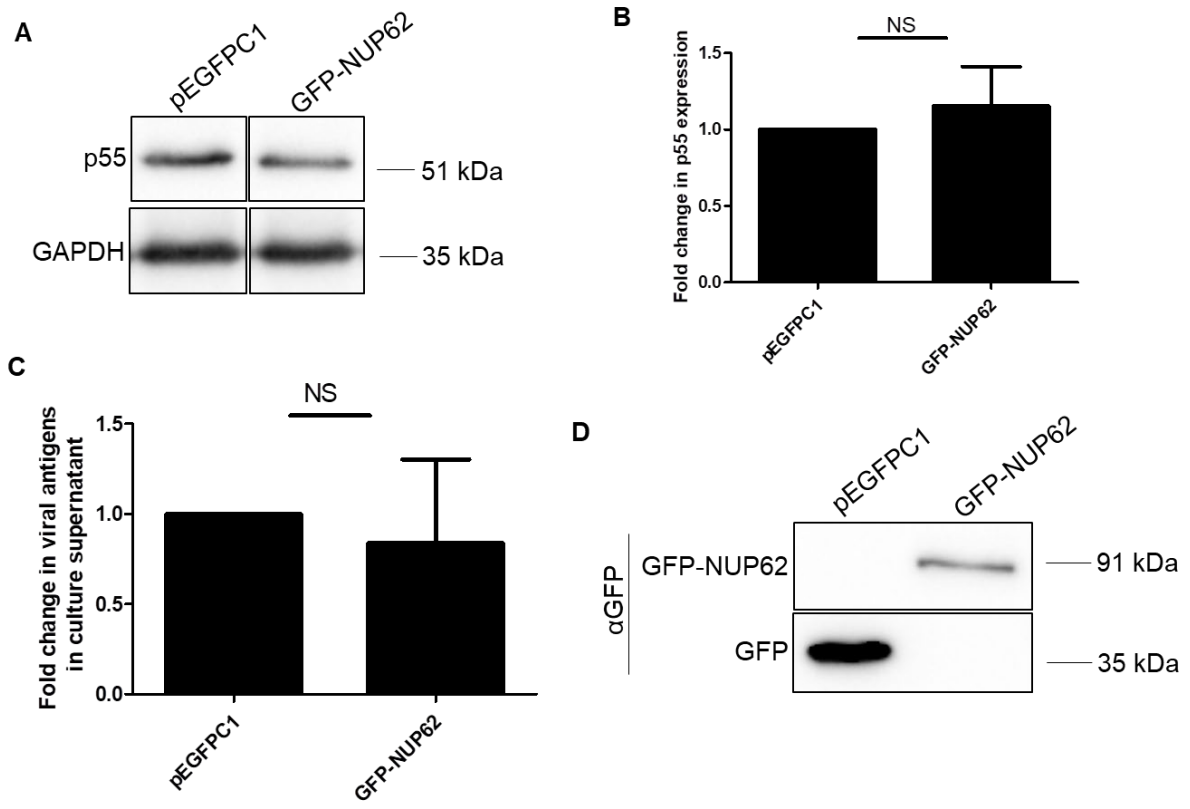


Figure 4-4. Overexpression of NUP62 does not affect HIV-1 p55 protein levels and viral titers upon pNL4.3 transfection in HEK293T cells. **(A-D)** HEK293T cells were co-transfected with pNL4.3 and either pEGFPC1 or GFP-NUP62. Forty eight hours post-transfection, the cells were harvested and culture supernatant was collected. Cells were lysed and the lysates were used for western blotting. **(A)** Blots were probed with anti-HIV-1 p24 and GAPDH antibodies. **(B)** p55 expression was normalized to the loading control, GAPDH and the bars represent the mean fold change in expression of p55 relative to the vector control. **(C)** The viral antigens from the culture supernatant was estimated by p24 ELISA and the bars represent the mean fold change of viral antigens relative to the vector control. **(D)** The GFP-NUP62 expression was verified by western blotting using anti-GFP antibody. The experiments were performed at least three times. NS, P>0.05.

We also extended our study to determine whether transiently expressed NUP98 could also affect intracellular p55 levels produced from another proviral construct, pIndie-C1 (subtype C). pIndie-C1 is an infectious molecular clone isolated from the HIV-1 subtype C strain 93IN101 of pandemic potential, which is prevalent in India (48). In line with the data obtained from pNL4.3 (subtype B), p55 levels expressed from pIndie-C1 were significantly reduced upon transient expression of NUP98

(Figure 4-5A, B). This implies that NUP98 broadly affects the replication of different subtypes of HIV-1.

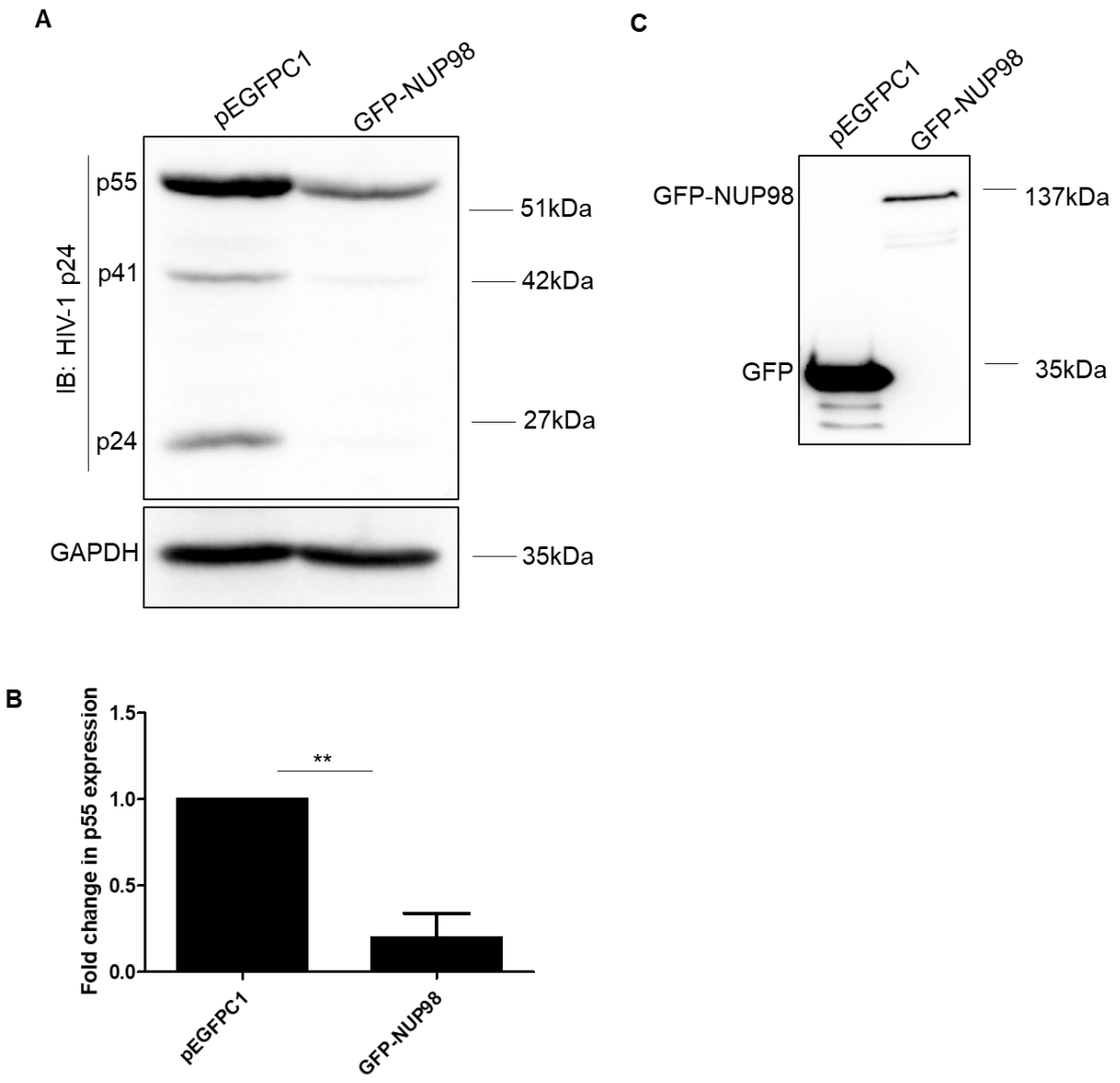


Figure 4-5. Overexpression of NUP98 reduces HIV-1 p55 protein levels upon pIndie-C1 transfection in HEK293T cells. **(A-C)** HEK293T cells were co-transfected with pIndie-C1 and either pEGFPC1 or GFP-NUP98. Forty eight hours post-transfection, cells were harvested and subjected to lysate preparation for western blotting. **(A)** Blots were probed with anti-HIV-1 p24 and anti-GAPDH antibodies. **(B)** p55 expression was normalized to the loading control GAPDH using image J software. Bars represent the mean fold change of p55 expression relative to the pEGFPC1 transfected cells. **(C)** The GFP-NUP98 expression was verified by western blotting using anti-GFP antibody. The experiments were performed at least three times. **, P<0.01.

4.2.3 Knockdown of NUP98 enhances HIV-1 viral transcript, protein levels and viral titers

To corroborate the effect of NUP98 on HIV-1 replication, the endogenous NUP98 in SupT1 cells was depleted using shRNA. Previously it was shown that NUP98 promotes the integration of HIV-1 and that the depletion of NUP98 led to decreased infectivity (231). To surpass the effect of NUP98 depletion on the integration of HIV-1 DNA, SupT1 cells were first infected with HIV-1 NL4.3 for 24 hrs and then transduced with lentivirus containing shRNA that targeted NUP98. Seventy two hours post-transduction, cells were harvested to analyse the intracellular p55 protein and viral RNA levels by western blotting and RT-qPCR, respectively and culture supernatants were collected to measure virus associated p24 by ELISA. The analysis of western blotting showed that the endogenous NUP98 was significantly downregulated in comparison to control cells transduced with Scrambled (Sc) shRNA (Figures 4-6A, B). The depletion of endogenous NUP98 enhanced the intracellular p55 levels as well as virus associated p24 in the culture supernatant (Figures 4-6C, D, E). Moreover, viral RNA transcripts were also increased upon depletion of NUP98, suggesting that NUP98 plays an important anti-viral role in the HIV-1 gene expression (Figure 4-6F). The viability of cells that were depleted of NUP98 was assessed by trypan blue dye exclusion assay and we observed that depletion of NUP98 had negligible effect on the cell viability in comparison to control cells (Figure 4-6G). Taken together, we conclude that NUP98 reduced the intracellular viral RNA, viral p55 levels and released virus titers.

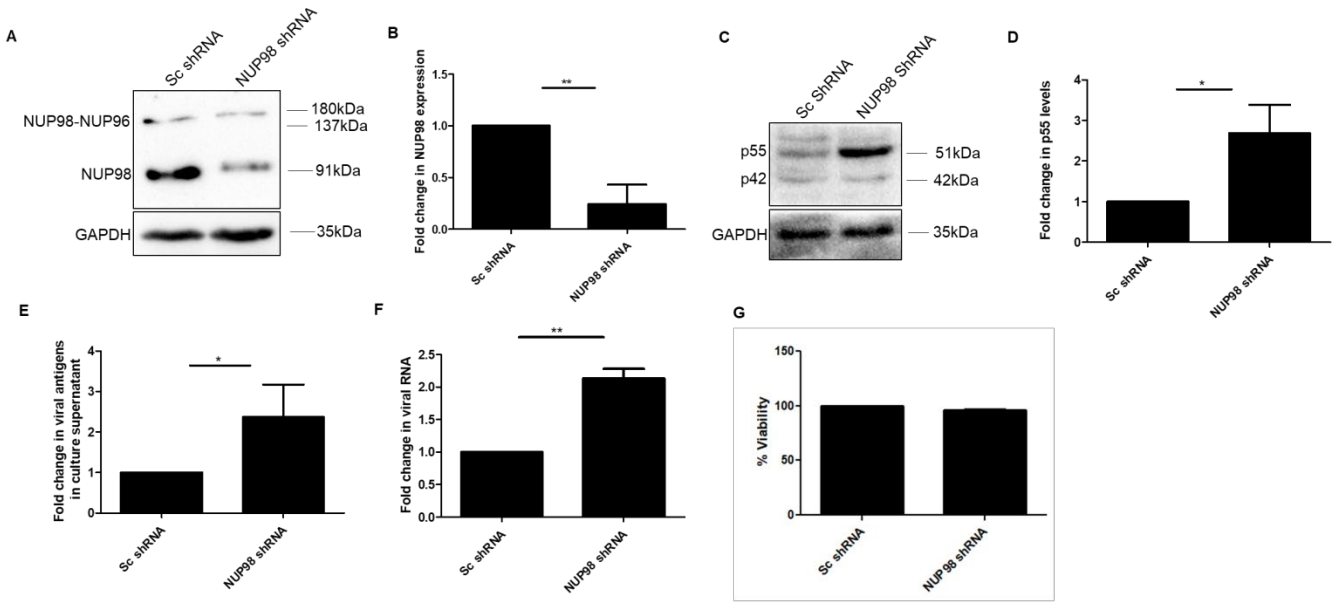


Figure 4-6. Knockdown of NUP98 in SupT1 cells ameliorates HIV-1 RNA, protein levels and viral titers. **(A-F)** SupT1 cells were infected with HIV-1 NL4.3 for 24 hrs and then transduced with lentivirus containing either control Sc shRNA or NUP98 shRNA. Seventy two hours post-transduction cells were harvested either for lysate preparation or RNA isolation, and culture supernatants were collected for p24 ELISA. **(A)** Blots were probed with anti-NUP98 and anti-GAPDH antibodies. NUP98 expression was normalized to the corresponding loading control GAPDH. **(B)** Bars represent the mean fold change of NUP98 expression relative to Sc shRNA. **(C)** Using the same lysates as in (A), blots were probed with anti-p24 and anti-GAPDH antibodies. p55 expression was normalized to the loading control GAPDH. **(D)** Bars represent the mean fold change of p55 expression relative to Sc shRNA. **(E)** Bars represent the mean fold change of viral antigens relative to Sc shRNA. **(F)** Bars represent the mean fold change of viral RNA expression relative to Sc shRNA. **(G)** Seventy two hours post-transduction, SupT1 cells were harvested, stained with trypan blue and counted using haemocytometer. The bars represent the mean percentage viability of cells transduced with NUP98 shRNA relative to Sc shRNA control (100%). The experiments were performed at least three times. *, P<0.05; **, P<0.01.

4.2.4 NUP98 reduces the infectivity of HIV-1

It is well established that the components of host cellular defense system affect viral infection by reducing the infectivity of the virus released from the producer cells. As we observed that NUP98 attenuated both the intracellular p55 and viral titers from the producer cell, we reasoned whether NUP98 might also affect the infectivity of the

HIV-1 particles released from the producer cell. Towards this, HIV-1 was prepared from HEK293T cells by co-transfecting with the plasmids pNL4.3 and GFP-NUP98. As a control, cells were also co-transfected with pNL4.3 and pEGFPC1. The equal amounts of virus particles (5ng/ml p24 equivalents) produced from either control cells or NUP98 overexpressing cells were then used to infect the target reporter TZM-bl cells. Forty eight hours post-infection (48 hpi), luciferase activity driven from HIV-1 LTR promoter was evaluated. To our surprise, the infectivity of the virus prepared from producer cells expressing NUP98 was decreased by 2-fold in comparison to control cells (Figure 4-7A). Further, to examine the infectivity of the virus emerged from the SupT1 cells lacking endogenous NUP98, HIV-1 viral particles were prepared from SupT1 cells transduced with either Sc shRNA or NUP98 shRNA containing lentivirus. TZM-bl cells were infected with equal amounts of these viral particles (5ng/ml p24 equivalents) as previously described. We observed that the infectivity of the virus produced from NUP98 depleted cells was enhanced in relative to the virus produced from control cells (Sc shRNA) (Figure 4-7B). Thus, our both overexpression and knockdown of NUP98 studies suggest that NUP98 abrogates the infectivity of HIV-1 in a target cell during new rounds infections.

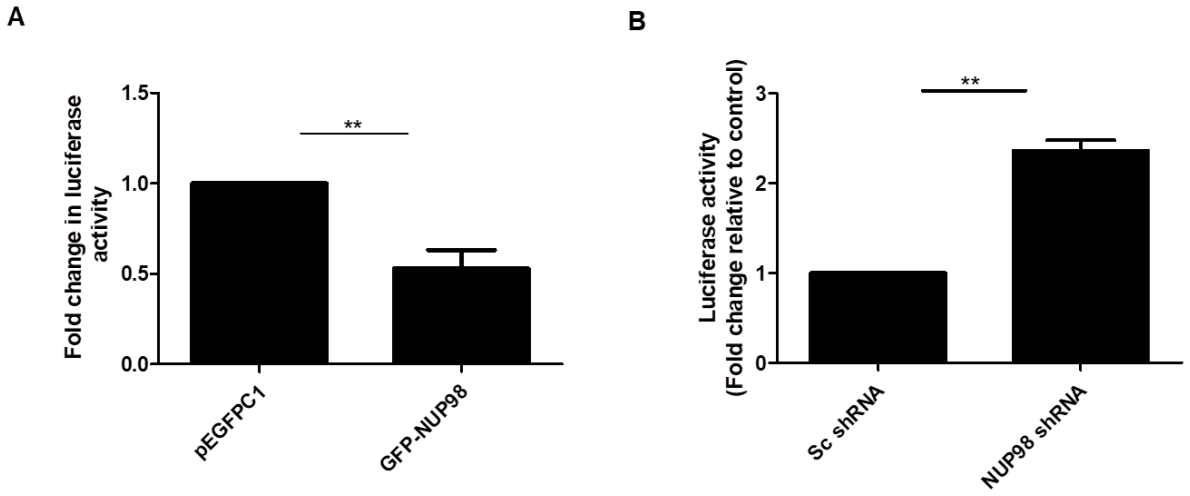


Figure 4-7. NUP98 reduces the infectivity of released HIV-1 particles. **(A)** HEK293T cells were co-transfected with pNL4.3 and either pEGFPC1 or GFP-NUP98. Forty eight hours post-transfection, culture supernatant containing viral particles was collected and used to infect TZM-bl cells. Forty eight hours post-infection (48 hpi), cells were harvested and cell lysate was used for luciferase assay. Bars represent the mean fold change in luciferase activity relative to the virus collected from pEGFPC1 transfected cells. **(B)** The equal

amounts of virus (p24 equivalents) collected from shRNA transduced cells (Sc shRNA and NUP98 shRNA) were used to infect TZM-bl cells. Forty eight hours post-infection, cells were harvested for luciferase assay. Bars represent the mean fold change of luciferase activity relative to the Sc shRNA control. The experiments were performed at the least three times. **, P<0.01.

Since we observed that NUP98 reduced the infectivity of released viral particles, we checked if NUP98 is packaged into the emerging virions from the producer cells (SupT1 and HEK293T). We first probed the presence of endogenous NUP98 by western blotting in the virion particles released from the producer cells such as SupT1 and HEK293T. We found that the endogenous NUP98 was not packaged into the released virion particles produced either from SupT1 or HEK293T cells (Figures 4-8A, B). We further examined if the transiently overexpressed NUP98 could be packaged into the virion particles. To achieve this, HEK293T cells were co-transfected with pNLC4.3 GFP and GFP-NUP98 or pEGFPC1. Forty eight hours post-transfection, the transiently expressed NUP98 was probed in the viral lysates by western blotting with anti-GFP antibody. However, owing to suppressive effect of transiently expressed NUP98 on intracellular p55 levels, the viral particles released were very low to be detected by western blotting. Thus, it is difficult to conclude whether transiently expressed NUP98 could be packaged into the released virions under these conditions (Figure 4-8C).

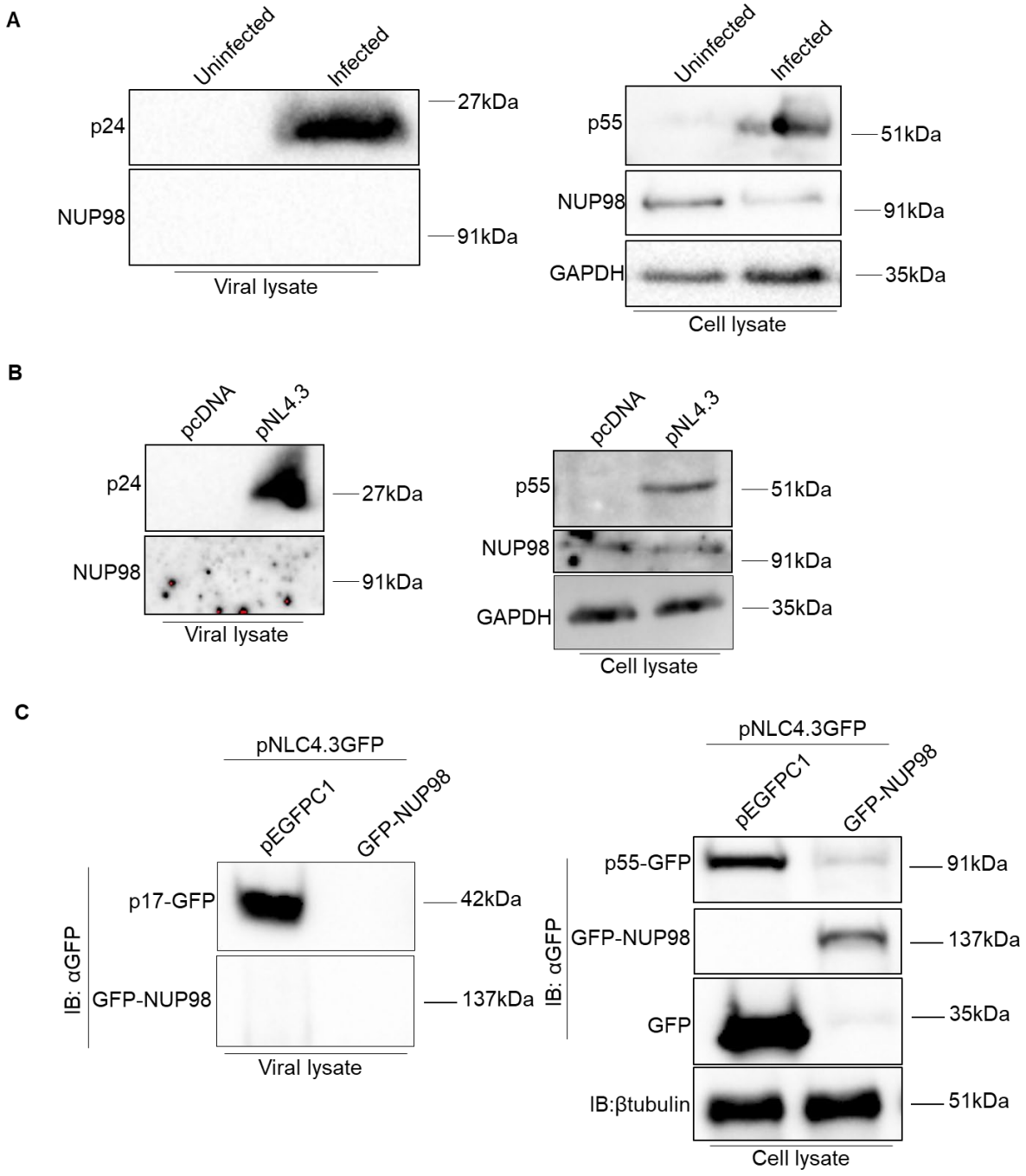


Figure 4-8. NUP98 is not co-packaged into the released viral particles. **(A)** SupT1 cells were infected with HIV-1 NL4.3, and four days post-infection, cells were harvested and the culture supernatant was collected. Culture supernatant containing the viral particles was subjected to PEG precipitation as described in methods and resuspended in NP40 lysis buffer. The resulting viral lysates and cell lysates were probed with antibodies against HIV-1 p24, GAPDH and NUP98. **(B)** HEK293T cells were transfected with either pcDNA or pNL4.3. Forty eight hours post-transfection, cells were harvested and the culture supernatant was collected. Culture supernatant containing the viral particles was subjected to PEG

precipitation and the resulting viral pellet was resuspended in NP40 lysis buffer. The viral and cell lysates were probed with anti-HIV-1 p24, anti-GAPDH and anti-NUP98 antibodies. **(C)** HEK293T cells were co-transfected with pNLC4.3GFP and either pEGFPC1 or GFP-NUP98 and viral lysates were processed as described in **(B)**. The viral and the cell lysates were probed with anti-GFP and anti-tubulin antibodies.

4.2.5 NUP98 associates with HIV-1 LTR and decreases HIV-1 LTR-driven transcription

As overexpression and knockdown studies indicated that NUP98 reduced both viral protein and RNA levels, we investigated if NUP98 affected the HIV-1 LTR-driven transcription by associating with HIV-1 LTR. To check this hypothesis, HEK293T cells were co-transfected with Myc-NUP98 and plasmid construct containing full length HIV-1 LTR promoter (pLTR-Luc), and 48 h later ChIP-qPCR was performed using anti-myc antibody. For the qPCR analysis, the primers were designed to amplify the region towards the end of HIV-1 5'LTR, i.e. +68nt to +168nt, where +1nt indicates the transcription start site in the LTR promoter (Table A3 (annexures)). We found that transiently expressed NUP98 was enriched at HIV-1 LTR by 6-fold in comparison to vector control (Figure 4-9A). Having confirmed that NUP98 associates with HIV-1 LTR, we next investigated its impact on HIV-1 LTR-driven transcription. Towards this, we used pLTR-Luc construct that expresses luciferase gene from the HIV-1 LTR as a reporter system. HEK293T cells were co-transfected with pLTR-Luc, and pEGFPC1 or GFP-NUP98 in absence or presence of pNL4.3. All HIV-1 viral proteins were provided in the form of molecular clone pNL4.3 to mimic the condition wherein the regulation of LTR activity by viral proteins including HIV-1 Tat, which is the main viral transcription regulator of LTR promoter, would be ensured. We indeed observed that the transiently expressed GFP-NUP98 decreased the expression of luciferase gene from HIV-1 LTR in comparison to vector control, irrespective of whether pNL4.3 was provided or not (Figure 4-9B). These results indicate that NUP98 prevents basal viral gene expression from HIV-1 LTR promoter. To further understand if the inhibition of basal transcription of HIV-1 LTR by NUP98 could be rescued by HIV-1 Tat, HEK293T cells were co-transfected with pLTR-Luc and GFP-NUP98 or pEGFPC1 in presence or absence of pcDNA Tat. We observed that NUP98 reduced the luciferase activity by 3.9-fold even in the presence of Tat, indicating that Tat could not rescue the NUP98-mediated downregulation of HIV-1

LTR activity (Figure 4-9C). Further, we substantiated these observations using pLTR-GFP construct which retains LTRs, genes for early viral regulatory proteins, Tat, and Rev, but lacks genes for Env, Gag, Gag-Pol, Nef, Vif, and Vpr. In place of Gag, Pol, Vif, and Vpr, GFP was inserted such that its expression is directly under the control of HIV-1 LTR. The expression of GFP from the LTR promoter was then analysed by the western blotting using anti-GFP antibody and normalized to the loading control β-tubulin. Even under these conditions, NUP98 was able to reduce the GFP expression driven by HIV-1 LTR by 10-fold (Figures 4-9D, E). To corroborate the role of NUP98 in the regulation of HIV-1 LTR-driven transcription, the expression of NUP98 was depleted in HEK293T cells by transfecting the cells with target shRNA plasmid. As a control, cells were also transfected with scrambled (Sc) shRNA plasmid, independently. The depletion of NUP98 protein was observed in the cells transfected with shRNA targeting NUP98 but not in the cells transfected with Sc shRNA (Figures 4-9F, G). Forty eight hours post-transfection with shRNAs, cells were re-transfected with pLTR Luc. After 24 hrs incubation, cells were harvested and luciferase activity was performed. The data from luciferase assays showed that the HIV-1 LTR activity was increased by 1.5-fold in the cells depleted of NUP98 in comparison to the cells transfected with Sc shRNA, corroborating that NUP98 decreased the transcription from HIV-1 LTR promoter (Figure 4-9H). These data, thus, provide the evidence that NUP98 specifically suppressed the HIV-1 LTR-driven viral basal gene expression.

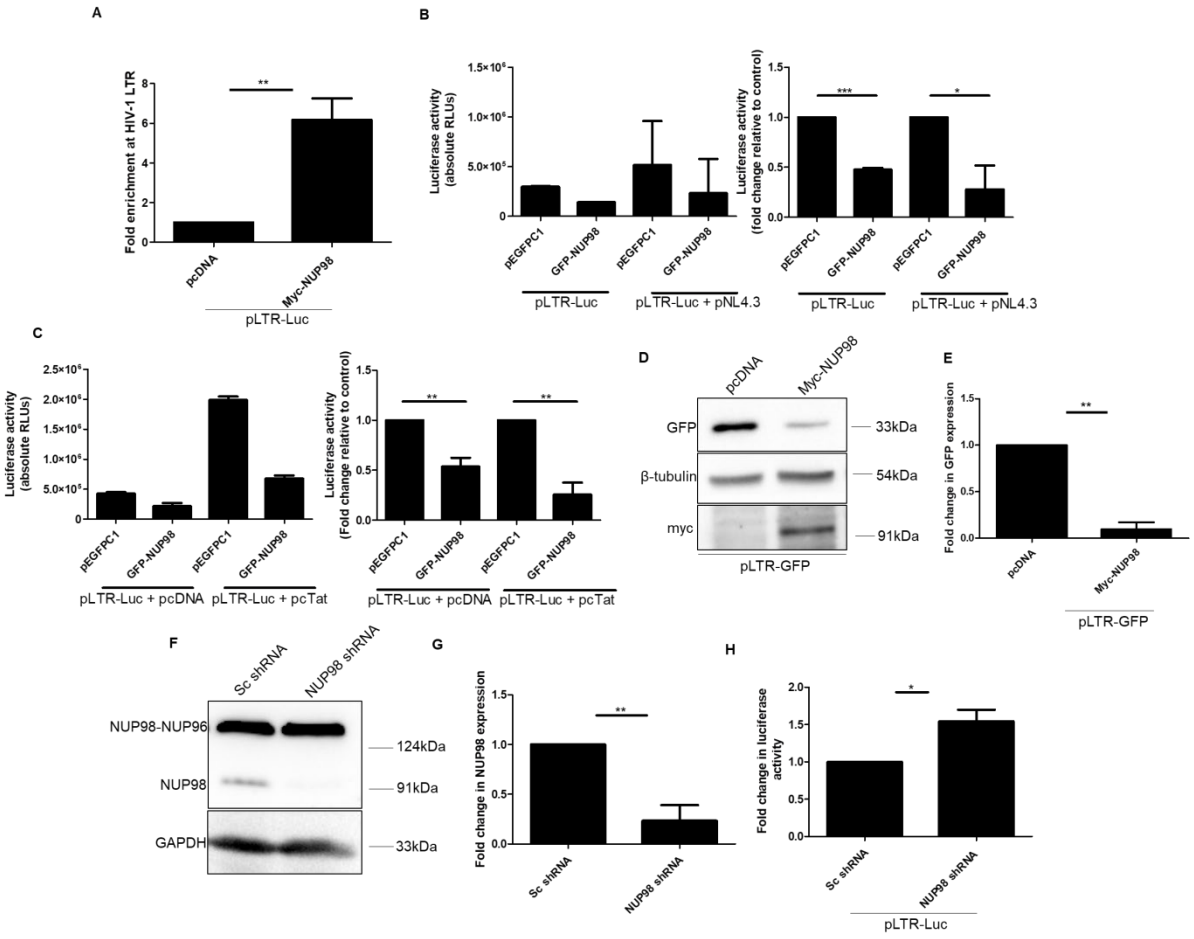


Figure 4-9. NUP98 associates with HIV-1 LTR and diminishes HIV-1 LTR-driven gene expression. **(A)** HEK293T cells were co-transfected with pLTR-Luc and either pcDNA or Myc-NUP98. Forty eight hours post-transfection, ChIP-qPCR assay was performed as mentioned in the methodology (chapter 2). Bars represent the mean fold enrichment of Myc-NUP98 at HIV-1 LTR relative to vector control. **(B)** HEK293T cells were co-transfected with pLTR-Luc and either pEGFPC1 or GFP-NUP98 in presence or absence of pNL4.3. Forty eight hours post-transfection, cells were lysed in reporter lysis buffer and lysate was used for luciferase activity. Bars represent the mean basal luciferase activities (B, left panel) and the mean fold change of luciferase activities in relative vector control (B, right panel). **(C)** HEK293T cells were co-transfected with pLTR-Luc, pcDNA or pcTat and either pEGFPC1 or GFP-NUP98. Forty eight hours post-transfection, cells were lysed in reporter lysis buffer and lysate was used for luciferase activity. Bars represent the mean basal luciferase activities (C, left panel) and the mean fold change of luciferase activity relative to vector control (C, right panel). **(D-E)** HEK293T cells were co-transfected with pLTR-GFP and either pcDNA or Myc-NUP98. Forty eight hours post-transfection, cells were lysed and lysate was used for western blotting. **(D)** Blots were probed with antibodies against GFP, β-tubulin and myc. GFP expression was normalized to the loading control β-tubulin. **(E)** Bars represent the

mean fold change of GFP expression relative to vector control. **(F-G)** HEK293T cells were transfected with either NUP98 targeting shRNA or scrambled (Sc) non-specific shRNA. **(F)** Blots were probed with anti-NUP98 and anti-GAPDH antibodies. The expression of NUP98 was normalized to the loading control GAPDH. **(G)** Bars represent the mean fold change of NUP98 expression relative to Sc shRNA control. **(H)** Forty eight hours post-transfection with shRNAs, cells were again transfected with pLTR-Luc and incubated further for 24 hrs. Cells were lysed in reporter lysis buffer and lysate was used for luciferase activity. Bars represent the mean fold change of luciferase activity in relative Sc shRNA control. The experiments were performed at least three times. *, P<0.05; **, P<0.01.

To further validate if the negative effect of NUP98 was indeed HIV-1 LTR specific, I tested the effect of NUP98 on two other viral promoters such as cytomegalovirus (CMV) and simian immunodeficiency virus (SIV) promoters. HEK293T cells were co-transfected with pEGFPC1 and Myc-NUP98 or pcDNA. Here, I used pEGFPC1 plasmid, representing a CMV promoter under which GFP is constitutively expressed. The expression of GFP from the CMV promoter was then analysed by the western blotting using anti-GFP antibody and normalized to the loading control β-tubulin. However, the overexpression of NUP98 did not change the levels of GFP expressed from the CMV promoter (Figures 4-10A, B). It should be noted that since CMV promoter in pEGFPC1 construct does not contain all the elements of the CMV, we cannot exclude the possible inhibitory effect of NUP98 on CMV-driven gene expression in the context of CMV infection. Similarly, we also tested if NUP98 could affect SIV promoter by co-transfecting HEK293T cells with pSIV$_{AGM}$-Luc-R^{-}E^{-}Δvif and GFP-NUP98 and measuring SIV promoter driven luciferase activity. We found that NUP98 had no influence on SIV LTR promoter (Figure 4-10C), suggesting that NUP98 specifically suppresses HIV-1 LTR activity.

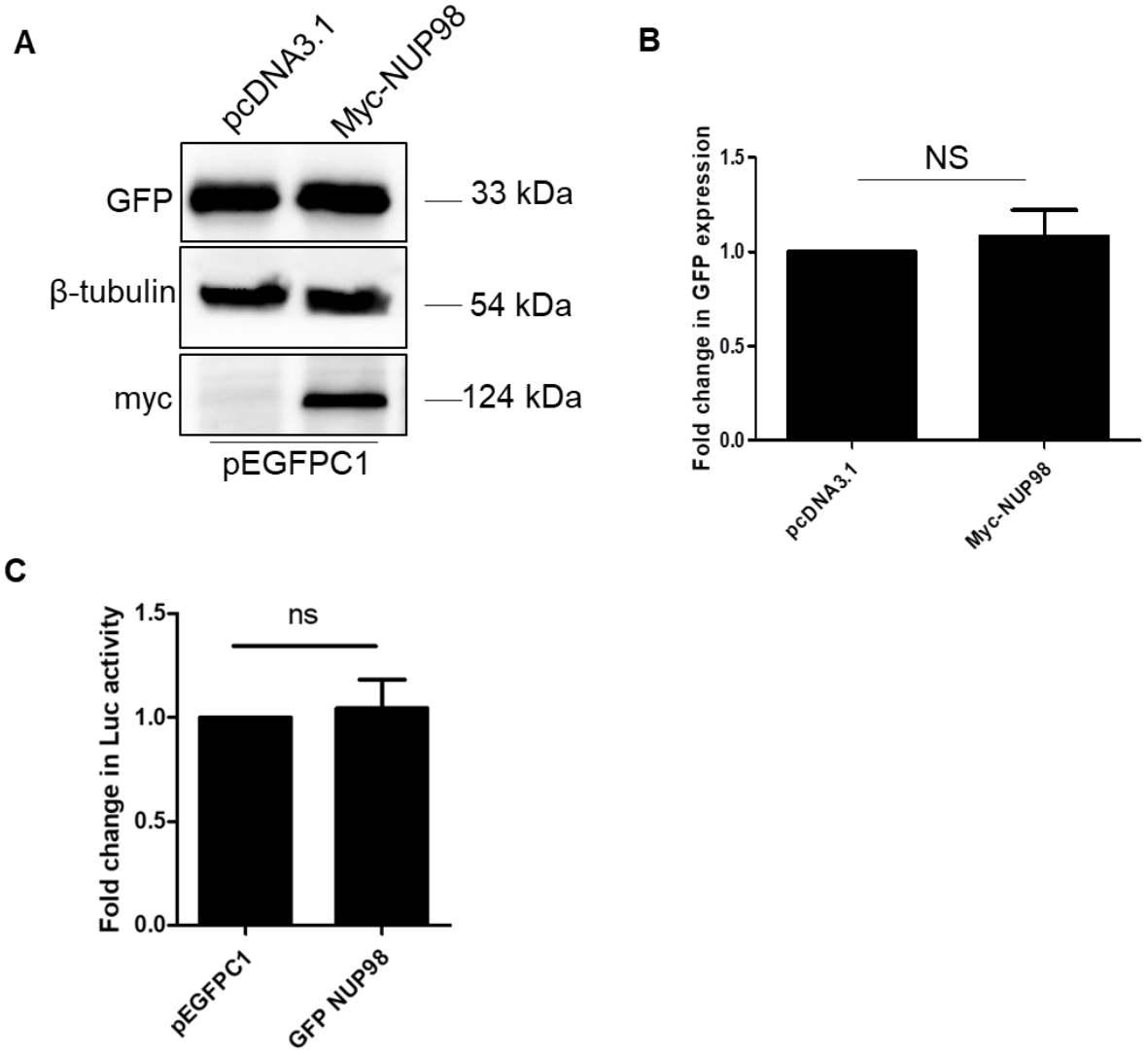


Figure 4-10. NUP98 does not influence CMV and SIV promoter activities. **(A, B)** HEK293T cells were co-transfected with pEGFPC1 and either pcDNA or Myc-NUP98. Two days after transfection, the cells were lysed and lysates were used for western blotting. **(A)** Blots were probed with anti-GFP, anti-β-tubulin and anti-myc antibodies. **(B)** GFP expression was normalized to the loading control β-tubulin and the bars represent the mean fold change in the expression of GFP relative to the vector control. **(C)** HEK293T cells were co-transfected with pEGFPC1 or GFP NUP98 and $pSIV_{AGM}$-Luc-$R^-E^-\Delta$vif. Two days after transfection, the cells were lysed and the lysate was used for luciferase activity. The bars represent the mean fold change in luciferase activity relative to vector control. The experiments were performed at least three times. NS, P>0.05.

4.2.6 NUP98-mediated decrease in viral gene expression is dependent on NRE region of HIV-1 LTR

The HIV-1 LTR promoter is functionally divided into negative regulatory element (NRE), enhancer, core and TAR regions (246). The NRE of LTR was known to downmodulate the LTR-directed HIV-1 gene expression (246). While the enhancer region contains binding sites for transcription factors such as nuclear factor-kappa B (NF-κB), the core region harbors the binding sites for constitutive transcription factors such as specificity protein 1 (Sp1) and TATA-box binding protein (TBP). As above experiments suggested that NUP98 negatively affected the basal viral gene expression, which was independent of Tat, we hypothesized that the elements upstream to TAR region might play a regulatory role in the NUP98-mediated lowering of viral gene expression. To test this hypothesis, we created deletion mutants of HIV-1 LTR using pLTR-Luc construct as a template, lacking NRE region (ΔNRE-LTR), binding sites for NF-κB and Sp1 (ΔNF-κB-LTR and ΔSp1-LTR) (Figure 4-11A) by site-directed mutagenesis (SDM) with the primers listed in Table A2 (annexures). HEK293T cells were co-transfected with these deletion mutants and GFP-NUP98 or pEGFPC1. The comparison of basal luciferase activities of these LTR deletion mutants with that of WT LTR in cells transfected with pEGFPC1 suggested that the deletion mutants ΔNF-κB-LTR and ΔSp1-LTR significantly lost the promoter activity (Figure 4-11B). This observation indicates that the binding sites for NF-κB and Sp1 are important for basal activity of LTR promoter. In contrast, deletion of NRE region (ΔNRE LTR) did not affect the basal transcription activity (Figure 4-11B). We further normalized the luciferase activities for each LTR constructs in presence of GFP-NUP98 with the luciferase activities measured in the background of the vector control (pEGFPC1). In consistent with above experiments, the negative effect of NUP98 on WT LTR activity was evident relative to the vector control (pEGFPC1) (Figure 4-11C). Moreover, the promoter activities of ΔNF-κB-LTR and ΔSp1-LTR constructs were also shown to be suppressed by the NUP98 in relative to the vector control, suggesting that these elements of LTR may not be required for NUP98-mediated suppression of HIV-1 gene expression (Figure 4-11C). Interestingly, the NUP98-mediated suppressing effect on the luciferase activity from the ΔNRE LTR construct was not observed (Figure 4-11C), indicating that this region being involved in NUP98-mediated LTR activity suppression. We next performed

ChIP-qPCR analysis using primer sets (Table A3 (annexures)) that amplify specific regions of HIV-1 LTR (Figure 4-11D) to determine the occupancy of Myc-NUP98. We observed the enrichment of Myc-NUP98 on both the NRE and NF-κB/Sp1 regions (Figure 4-11E). However, considering that the deletion of neither NF-κB nor Sp1 binding sites on the HIV-1 LTR affected the NUP98-mediated suppression of LTR activity, we conclude that NUP98-mediated lowering of viral gene expression is dependent on NRE region of HIV-1 LTR. It should be further noted that the involvement of NF-κB and Sp1 in NUP98 mediated regulation of HIV-1 LTR activity may not be ruled out and future investigation is needed to understand the molecular events underlying NRE-specific suppressive effect of NUP98 on HIV-1 gene expression.

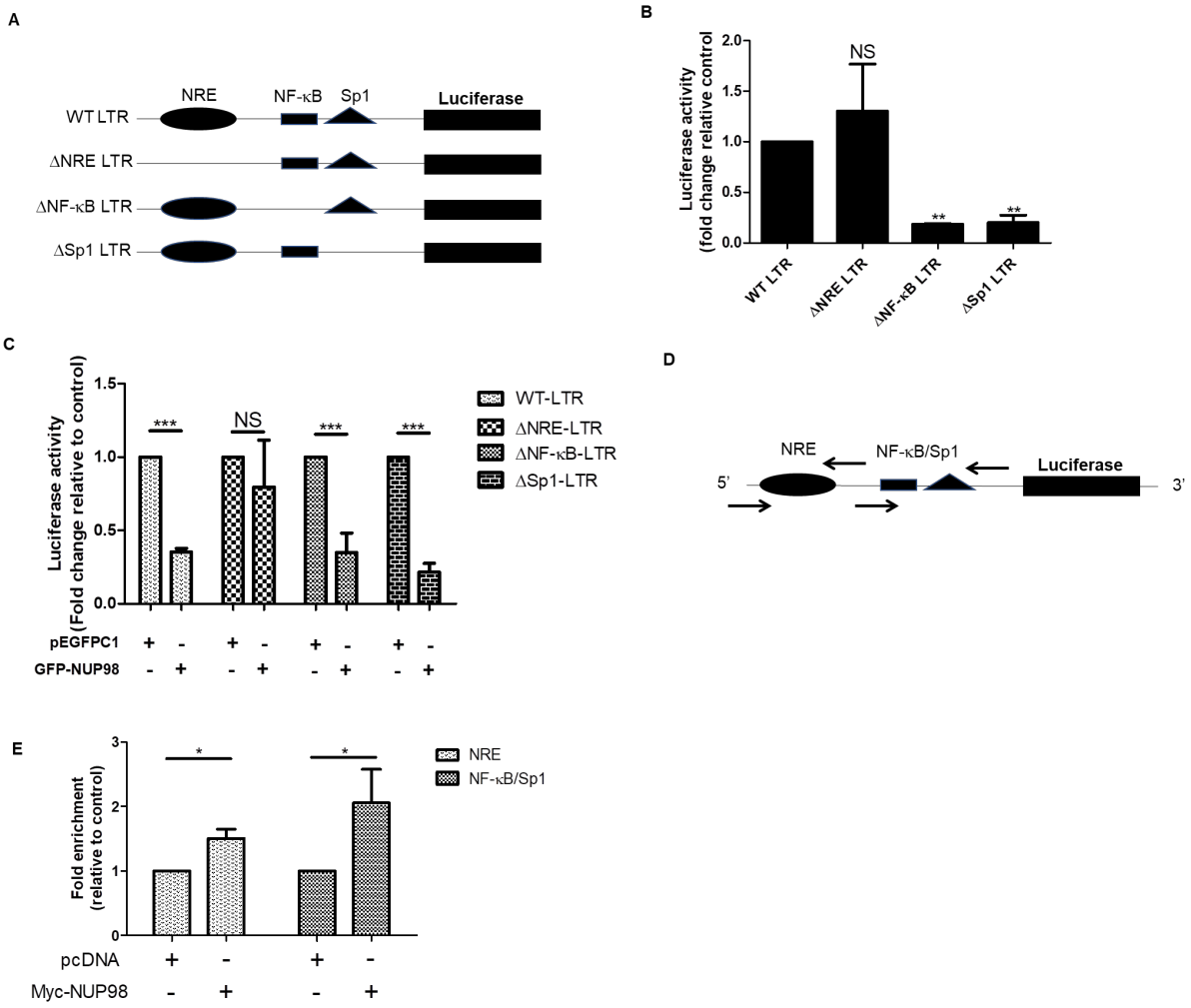


Figure 4-11. NRE of LTR is required for NUP98-mediated perturbation of HIV-1 LTR-directed gene expression. **(A)** The Schematic representation of full-length wild type (WT) LTR and its deletion mutant plasmid constructs. **(B, C)** HEK293T cells were co-transfected

either with pEGFPC1 or GFP-NUP98 and pLTR-Luc or LTR deletion mutant plasmid constructs. Forty eight hours post-transfection, cells were lysed in reporter lysis buffer and lysate was used for luciferase activity. **(B)** Bars represent the mean basal luciferase activities of LTR mutant constructs relative to WT LTR vector. **(C)** Bars represent the mean fold change of luciferase activities of LTR constructs in presence of GFP-NUP98 relative to vector control (pEGFPC1). **(D)** The Schematic representation of primers that amplify NRE and NF-κB/Sp1 binding sites. **(E)** HEK29T cells were co-transfected with either pcDNA or Myc-NUP98 and pLTR-Luc. Forty eight hours post-transfection, cells were harvested for ChIP-qPCR assay. Bars represent the mean fold enrichment of NUP98 at NRE and NF-κB/Sp1 binding sites in relative to vector control. The experiments were performed at the least three times. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis in **(C)** using GraphPad Prism 5. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, $P>0.05$.

We further checked if transiently expressed NUP98 could interact with the some of the well-established host protein regulators of HIV-1 LTR such as NF-κB subunit p65, hexamethylene bis-acetamide-inducible protein 1 (HEXIM1) and histone deacetylase 1 (HDAC1). The western blotting analysis of immunoprecipitated complexes showed that none of these factors interacted with transiently expressed NUP98 (Figure 4-12). Although a previous study showed that fused NUP98 proteins, i.e. NUP98-HOXA9 and NUP98-PMX1, interact with HDAC1 and are involved in the regulation of genes implicated in acute leukemia (247), our experiments with transiently expressed NUP98 indicate that un-fused NUP98 might not interact with HDAC1. Nevertheless, based on Co-IP analysis with transiently expressed NUP98, we cannot rule out the functional interaction between endogenous NUP98 and NF-κB, HEXIM1 or HDAC1. Future investigations will be required to understand the possible physical interaction between NUP98 and members of protein complexes that regulate the HIV-1 gene expression.

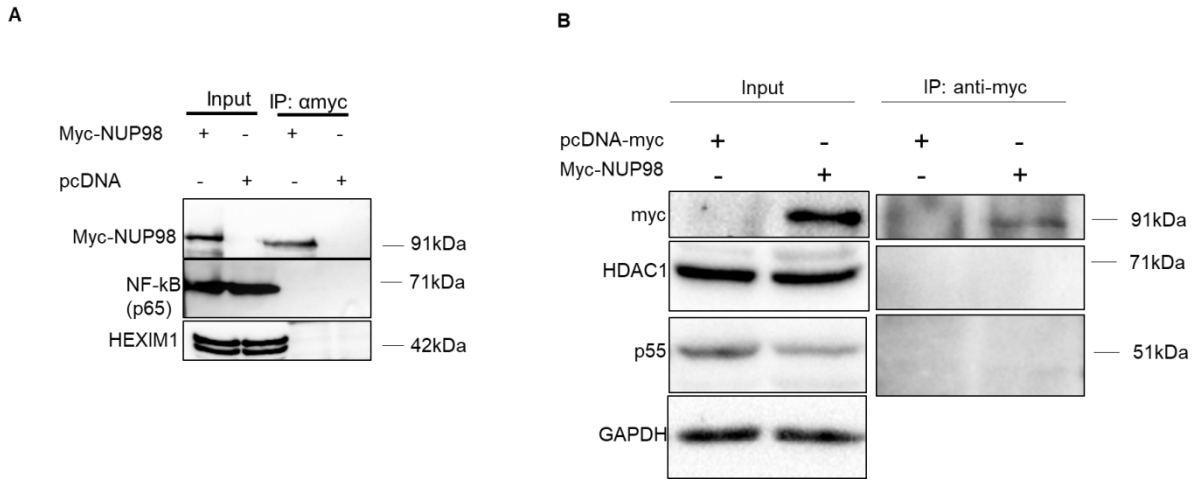


Figure 4-12. NUP98 does not interact with NF-κB subunit p65, HEXIM1 or HDAC1. **(A)** HEK293T cells were co-transfected with pNL4.3 and either pcDNA or Myc-NUP98. Two days after transfection, the cells were lysed and the lysate was used for co-immunoprecipitation by anti-myc antibody and followed by western blotting. The presence of Myc-NUP98, p65 and HEXIM1 in the immunoprecipitates was assessed by probing the blots with anti-Myc, anti-p65 and anti-HEXIM1 antibodies, respectively. **(B)** HEK293T cells infected with HIV-1 NL4.3 were transfected with either pcDNA or Myc-NUP98. Two days after transfection, the cells were lysed and the lysate was used for co-immunoprecipitation by anti-Myc antibody and followed by western blotting. The presence of Myc-NUP98, HDAC1 and p55 in the immunoprecipitates was assessed by probing the blots with anti-Myc, anti-HDAC1 and anti-HIV-1 p24 antibodies, respectively.

4.2.7 N-terminal region of NUP98 (1-504) contributes to NUP98 mediated inhibition of HIV-1 LTR-driven transcription

Next, we deliberated on understanding the contribution of different domains of NUP98 in the regulation of HIV-1 LTR-driven transcription. The constructs that express domains of NUP98 were previously described (124) and schematically shown in Figure 4-13A. HEK293T cells were co-transfected with pLTR Luc, pcDNA Tat and pEGFPC1 or NUP98 GFP or plasmid expressing different domains of NUP98. As observed in previous experiments, the full length NUP98 (1-920) reduced the promoter activity of HIV-1 LTR (Figure 4-13B). A similar effect was also observed for GFP-ΔC (1-863), which has 6kDa region removed from the C-terminal end of NUP98 (Figure 4-13B). The GFP-NTD NUP98 (1-225), which expressed only the amino (N)-terminal domain of NUP98, was also found to reduce the HIV-1 LTR-driven activity by 2-fold (Figure 4-13B). We further found that the GLFG (glycine-

leucine-phenylalanine-glycine) domain of NUP98 (221-504) reduced the LTR activity significantly by 1.6-fold, whereas the CTD (carboxyl-terminal domain) of NUP98 (506-920) did not affect the LTR activity (Figure 4-13B). The suppression of HIV-1 LTR gene expression by both NTD and GLFG domains indicates that the N-terminal region spanning both these domains (1-504) is required for NUP98 to downregulate HIV-1 LTR activity, with the NTD playing the dominant role.

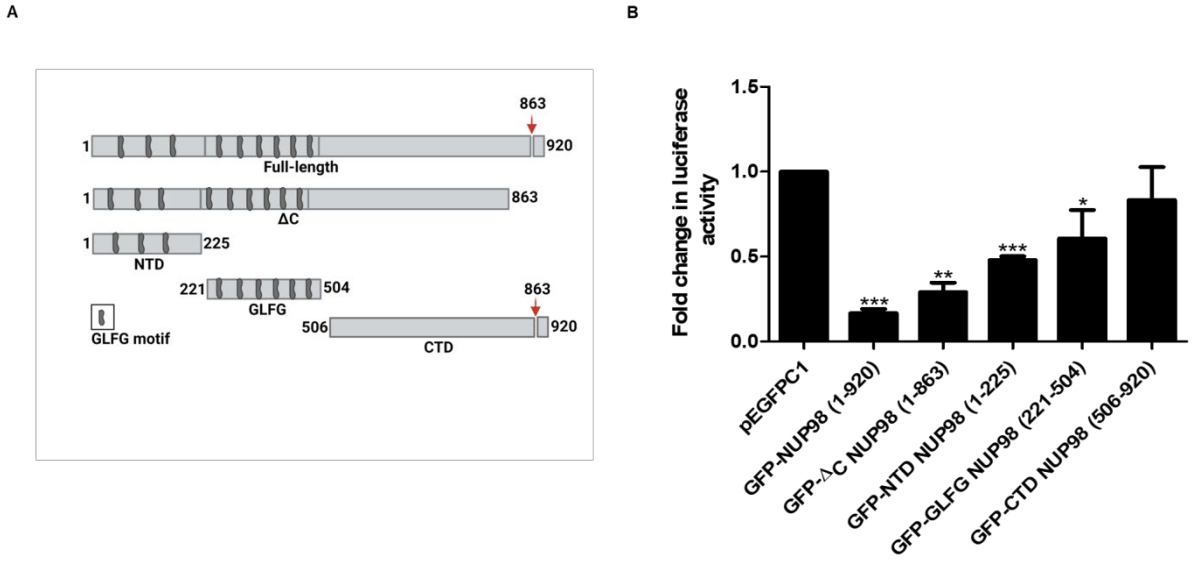


Figure 4-13. N-terminal region of NUP98 inhibits HIV-1 LTR-driven gene expression. **(A)** Schematic representation of full length NUP98 and domains of NUP98; down arrow indicates the autoproteolytic cleavage site. **(B)** HEK293T cells were co-transfected with pLTR-Luc, pcTat and plasmids expressing full length and domains of NUP98. Forty eight hours post-transfection, cells were lysed in reporter lysis buffer and lysate was used for luciferase activity. Bars represent the mean fold change of luciferase activity in relative to the vector control (pEGFPC1). *, P<0.05; **, P<0.01; ***, P<0.001.

4.2.8 Overexpression of NUP98 induces the formation of nuclear bodies and HIV-1 does not influence the formation of these bodies

Previous studies demonstrated that NUP98 localizes to both cytoplasmic and nucleoplasmic sides of the NPC, and in some cell types, to the interior of the nucleus as nuclear GLFG bodies (123,248). In our study, we found that endogenous NUP98 in HEK293T cells to be localized to the cytosol as foci, to the NE as part of NPC and to the nucleolus as bodies (Figure 3-2). We further evaluated the localization of transiently expressed NUP98 in HEK293T cells. Cells were transfected with GFP-NUP98 and imaged with confocal microscopy. In agreement with the localization of

endogenous NUP98, transiently expressed GFP-NUP98 localized in the cytosol as foci, weakly to the NPC in a few cells and in the nucleoplasm as diffuse signal and bodies (Figure 4-14A). However, the nuclear bodies, which are formed upon transient expression of NUP98, are smaller in size and higher in number in comparison to endogenous NUP98 (compare Figure 3-2 and Figure 4-14A) . Some of these bodies were also peripherally localized to the nucleolus (Figure 4-14A). Concordantly, it was previously shown that NUP98 moves-on and -off the NPC into the nuclear bodies in the nucleoplasm and requires GLFG domain for the formation of these bodies (hence, also known as GLFG bodies) (124). However, the functional relevance of these intranuclear bodies in the regulation of genes is not clear as these structures were shown to be devoid of active RNA polymerase II (124). Instead, it was rather thought that GLFG bodies might serve as storages structures from which NUP98 could traffic to the promoters of the genes under different cellular conditions (124,249). We further overexpressed myc tagged version of NUP98 and looked for its localization following immunostaining with anti-myc antibody. Surprisingly, as opposed to GFP-NUP98, like endogenous NUP98, myc tagged NUP98 localized to NPC and formed single large nuclear body. This large nuclear body was exclusively localized to the nucleolus (Figure 4-14B). The difference in the localization pattern between tagged versions of NUP98 (GFP vs. Myc) may be due to the following possible reasons: 1) GFP tag; high molecular weight GFP-NUP98 is not compatible to be localized to the interior of nucleolus but compatible to be localized in the nucleoplasm and in the periphery of nucleolus. 2) Accessibility to the epitope; nucleolar Myc-NUP98 has the accessibility to anti myc antibody but not the nucleoplasmic bodies containing Myc-NUP98. Next, we investigated whether HIV-1 could influence the formation of these nuclear bodies induced by NUP98. For this, cells were transfected with GFP-NUP98 and, then, infected with HIV-1 NL4.3. The imaging analysis suggests that HIV-1 does not influence NUP98 induced nuclear bodies (Figure 4-14C).

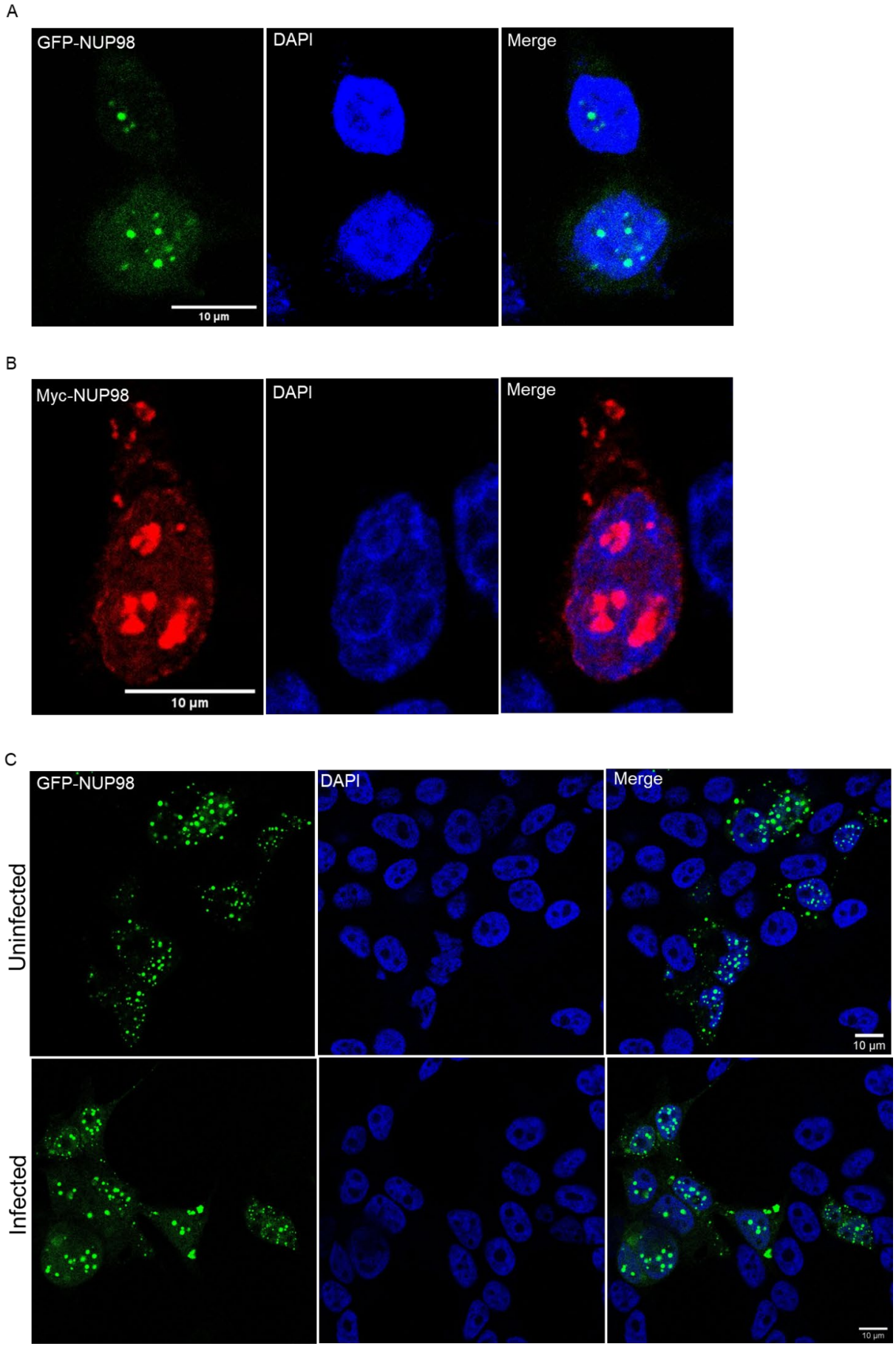
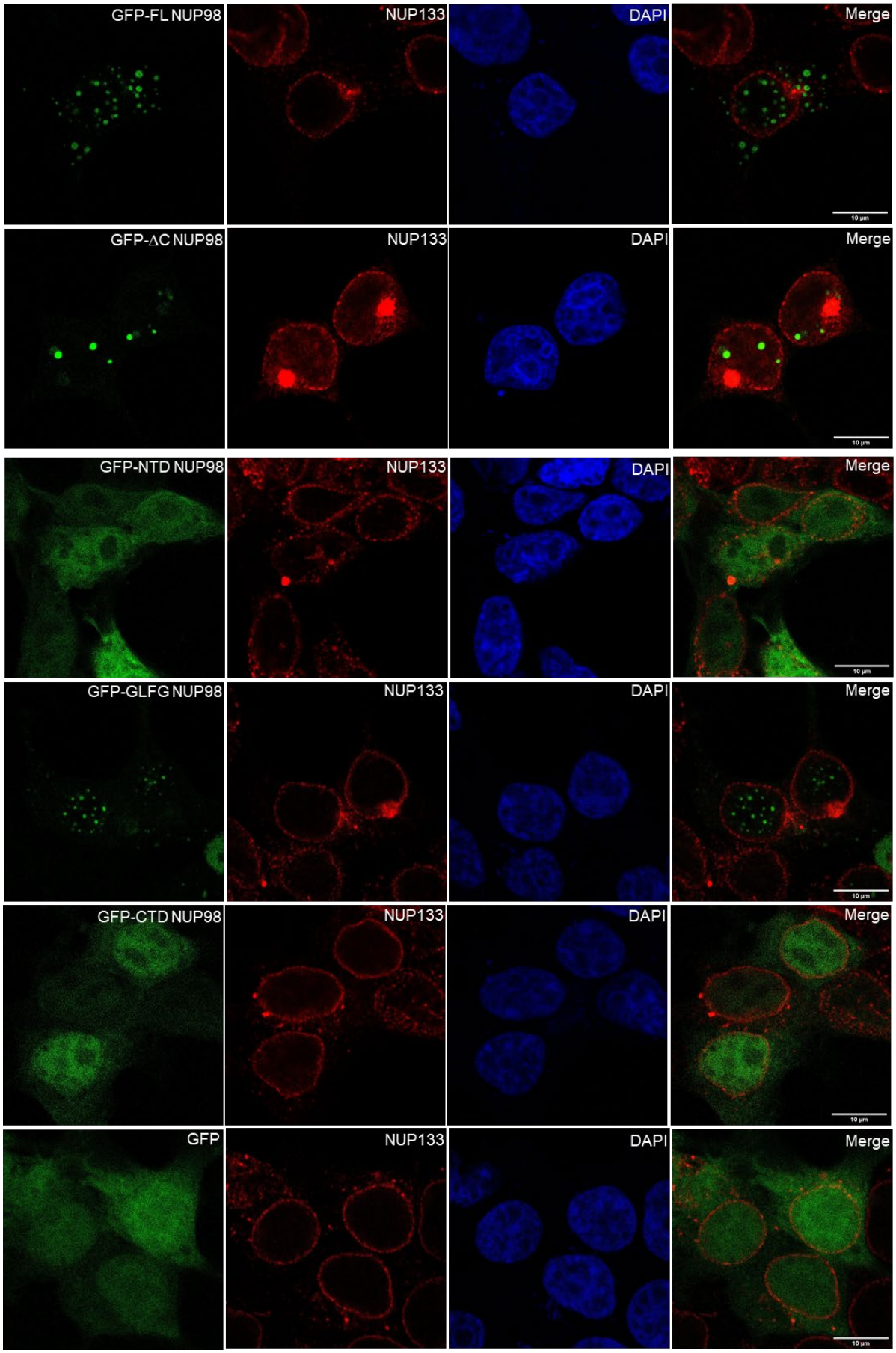

A
GFP-NUP98
DAPI
Merge
10 µm
B
Myc-NUP98
DAPI
Merge
10 µm
C
GFP-NUP98
DAPI
Merge
Uninfected
Infected
10 µm
10 µm

Figure 4-14. NUP98 induces the formation of intranuclear bodies. **(A)** HEK293T cells were transfected with GFP-NUP98. Twenty four hours post-transfection, cells were imaged under confocal microscopy. **(B)** HEK293T cells were transfected with Myc-NUP98. Twenty four hours post-transfection, cells were fixed and immunostained with anti-myc antibody, followed by incubation with anti-mouse 647 secondary body. **(C)** HEK293T cells were transfected with GFP-NUP98. Twenty four hours post-transfection, cells were infected with HIV-1 NL4.3. After 24 hpi, cells were imaged under confocal microscopy. Scale bar is 10 µm.

Previously, it was observed that the entire nuclear pore sub-complex, Y-complex, is recruited and localized to nuclear GLFG bodies in sublines of HeLa. Though several reports went on to show that GLFG bodies were formed in HEK293T cells upon overexpression of NUP98, it is not clear whether these bodies recruit Y-complex or any of the components of Y complex in this cell type. Therefore, we sought to understand if NUP133, as a component of Y-complex, is colocalized to nuclear bodies with transiently expressed NUP98 in HEK293T cells. Our microscopy analysis indicated that NUP133 did not colocalize with the GLFG bodies that were formed upon overexpression of NUP98 in HEK293T (Figure 4-15). We further examined the localization of aforementioned truncated versions of NUP98, and their colocalization with NUP133. While, like full length NUP98, C-terminally truncated NUP98 (1-863) and GLFG domain (221-504) formed intranuclear GLFG bodies, NTD (1-225) and CTD (506-920) of NUP98 appeared as diffuse signal and did not form nuclear bodies (Figure 4-15). Further, NUP133 did not colocalize with any of truncated versions of NUP98 (Figure 4-15). This lack of colocalization of NUP133 with the GLFG bodies indicates that Y-complex components may not be the part of these bodies and not physically involved in the formation of these bodies themselves in HEK293T cells. Further, it should be noted that although NTD of NUP98 could not form the GLFG bodies, it affected the HIV-1 LTR-driven gene expression as efficiently as full length NUP98 did, suggesting that the diffusive NUP98 might play an important role in the regulation of HIV-1 LTR but not the GLFG bodies themselves, which might act as only storage structures (Figure 4-13B).


GFP-FL NUP98
NUP133
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GFP-GLFG NUP98
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Figure 4-15. NUP98 induced intranuclear bodies do not recruit NUP133. HEK293T cells were transfected with plasmids expressing full length and different domains of NUP98. Twenty four hours post-transfection, cells were fixed and immunostaining was performed using anti-NUP133 antibody, followed by incubation with anti-rabbit 546 secondary antibody. Confocal images showing the localization of full length and different domains of NUP98 (green) and endogenous NUP133 (red); cells were also counterstained with DAPI (blue). Scale bar 10 μm.

4.3 Discussion

Since HIV-1 infection downregulated the NUP98 protein levels both in SupT1 and HEK293T cells, I hypothesized that NUP98 might play an important role in HIV-1 replication. I, therefore, investigated the possible role of NUP98 in the late stages of HIV-1 replication such as transcription and translation of viral proteins and release of virions. As surmised, the transient expression of NUP98 in HEK293T cells decreased the HIV-1 RNA and protein levels that arose either from integrated HIV-1 proviral DNA (infection with HIV-1 NL4.3) or unintegrated proviral DNA (transfection with pNL4.3). Concordantly, the viral titers from the culture supernatant were also reduced under similar conditions. However, the effect on viral titers was greater than that was observed on p55 protein levels (HIV-1 NL4.3: 5-fold vs.1.9-fold; pNL4.3: 3-fold vs. 1.7-fold), implying that NUP98 might independently regulate both viral protein levels and virion release. To understand its global effect across the subtypes of HIV-1, I also explored whether NUP98 affected the most prevalent subtype of HIV-1, i.e. subtype C (Indie-C1). Indeed, I observed that NUP98 lowered the p55 levels from proviral DNA pIndie-C1, indicating that NUP98 employs a common mechanism of action in subverting the replication of HIV-1 subtypes that genetically differ from each other.

For the last two decades, several host factors that mitigate the infectivity of HIV-1 released from the producer cells, and their underlying molecular mechanisms were identified (reviewed in (89)). In fact, some of these host factors were also shown to be packaged in the releasing virions and thwart initial steps of viral replication such as entry of the virus and reverse transcription in the target cells (250–263). To check whether the infectivity of the virus generated from producer cells expressing NUP98 was affected, HEK293T cells expressing GFP-NUP98 were transfected with pNL4.3 and the resulting virus was used to infect the reporter cell line, TZM-bl (target cell).

The luciferase activity from the lysates of TZM-bl showed that the virus produced under NUP98 overexpression condition lost the infectivity in comparison to control cells by 2-fold. Thus, NUP98 seems to have a multifaceted anti-HIV-1 role affecting different steps of HIV-1 lifecycle. This notion could be stood out considering that NUP98 is localized to different compartments of the cell such as cytosol, NPC, and nucleoplasm, and that NUP98 is shown to interact with a plethora of cellular factors that are implicated in the transcription, splicing, and export of RNA and translation of host proteins. Nevertheless, it would be of great interest to uncover the underlying mechanism behind NUP98's mediated subversion of infectivity of HIV-1 generated from producer cells.

Given that the levels of p55 were decreased by transient expression of NUP98, I speculated if NUP98 affected viral RNA levels. Indeed, the decreased HIV-1 p55 protein levels correlated with decreased viral RNA levels upon overexpression of NUP98 in HEK293T cells. Further, the direct correlation between the levels of viral protein and viral RNA that were either reduced upon overexpression of NUP98 or increased upon depletion of NUP98 led me to investigate the possible role of NUP98 in the regulation of HIV-1 LTR promoter, which controls the expression of viral genes. In light of HIV-1 gene transcription, latency establishment, and maintenance for the cure from HIV-1, many studies have focused on identifying host transcription factors and their underlying mechanisms that regulate the viral gene expression. While several factors that either promote or prevent HIV-1 gene transcription were clarified, the list of these factors and associated mechanisms is essentially expanding. Though my investigation did not find the interaction between NUP98 and the transcription factors such as NF-κB (p65), HEXIM1 and HDAC1, interestingly, I noticed that NUP98 made the association with HIV-1 LTR, the underlying mechanism of which should be investigated in the future. In agreement with our observation, a previous study in search of whether or not NUPs associated with HIV-1 LTR had identified NUP98 to be associated with this promoter (264). To understand the significance of the association of NUP98 with HIV-1 LTR, I explored whether or not NUP98 is involved in the regulation of LTR-driven gene expression. The analysis of reporter gene expression from HIV-1 LTR indicated that NUP98 reduced the transcription activity of HIV-1 LTR. Further, it was also interesting to find that NUP98-mediated reduction in the LTR activity was independent of viral protein

Tat, indicating that NUP98 basally overturns the HIV-1 gene expression. In addition, reporter gene expression analysis further indicated that NUP98 could not affect the other viral promoters such as CMV and SIV, suggesting that NUP98 specifically affected HIV-1 LTR. Lastly, I found that the N-terminal region of NUP98 and NRE of LTR are required for the NUP98 to inhibit the HIV-1 LTR-driven gene expression.

As mentioned above, previous studies reported that NUP98 move-on and -off the NPC into the GLFG bodies in the nucleoplasm (124). In support of this data, evidence from microscopic studies suggests that only elevated levels of NUP98 could form GLFG bodies in certain sublines of HeLa that naturally express high levels of endogenous NUP98 or in cells that transiently express exogenous NUP98 (237,265,266). In our study, we also observed that both endogenous and transiently expressed NUP98 formed nuclear GLFG bodies. Interestingly, in the context of HIV-1 gene expression, NTD of NUP98 (1-225), which has 3 GLFG motifs mitigated the HIV-1 LTR-driven gene expression and localized as a diffuse signal but did not form GLFG bodies in the nucleus. On the other hand, the GLFG domain (221-504) of NUP98, which has 6 GLFG motifs formed the intranuclear bodies but less significantly inhibited the HIV-1 LTR-driven gene expression. These observations indicate that the diffused NUP98 contributed more to the transcription regulation of HIV-1 LTR than that is present in the GLFG bodies. Previous observations suggest that the integration of HIV-1 DNA occurs in the transcriptionally active genes positioned proximally to the nuclear periphery and that the NUPs associated with NPC make contact with HIV-1 provirus (104,127,264,267–273). Though these observations correlate the interaction of proviral DNA with NUPs as a positive factor for the proviral gene expression, it is also possible that the NUPs whether or not physically associated with NPC may also negatively regulate the expression of proviral genes. To add complexity to our understanding on provirus in the host genome, it has been recently observed that though transcriptionally active provirus was initially detected near the NE, its distribution across the nucleus became random and dynamic after several cell divisions (267). The authors from this study further showed that the transcriptional activity of the provirus doesn’t correlate with the distance between the NE and the provirus in the genome, indicating that the absolute interaction of NPC-associated NUPs with HIV-1 provirus may not be needed for the viral gene expression to occur. The caveat from these studies is that

the observations made were based on the transcriptionally active proviruses and did not have clue about the transcriptionally inactive proviruses, which upon activation drives viral gene expression and thus, gives rise to new viral progeny. Nevertheless, it would be tempting to speculate that the transcriptionally active or inactive HIV-1 proviruses that are far from NE, i.e., towards the interior of the nucleus, could make contact with off-NPC NUPs and therapeutic interventions that disrupt the interactions between HIV-1 genome and NUPs may help patients recover from HIV-1/AIDS.

Chapter 5

Summary and future perspectives

Chapter 5: Summary and future perspectives

5.1 Summary of the thesis

Upon examining the expression of NUPs NUP98, NUP62, NUP155, NUP133, NUP107, NUP85-both at protein and RNA levels-in cell types such as SupT1, THP1, 1321N1, HEK293T and TZM-bl cells under basal conditions, I found that the basal levels of NUPs expression were different among the cell types. I further evaluated the localization of the NUPs in these cell types under basal conditions by immunostaining using confocal microscopy. Interestingly, the localization of the NUPs-NUP98, NUP62, NUP155 and NUP133- varied within a cell type as well as among the cell types under study. Nonetheless, all the NUPs except NUP62, whose localization was nearly excluded from NE, were found to be localized at NE in all the cell types, probably as part of NPCs. Interestingly, in case of HEK293T cells all the tested NUPs were found to be localized as distinct foci in the cytoplasm in addition to their presence at NE. In case of TZM-bl cells, which were genetically engineered from HeLa cells, the intranuclear bodies were readily observed with antibodies against NUP98 and NUP133. Although the intranuclear bodies in HEK293T cells were visualised with anti-NUP98 and NUP133 antibodies, their number, size and the location in the nucleoplasm differed in comparison to those that found in TZM-bl. Future investigation is, indeed, needed to see whether the intranuclear bodies stained by NUP98 and NUP133 in HEK293T and TZM-bl cells have similar underlying mechanism for their formation and same set of functions to carryout. Interestingly, in THP cells the intranuclear localization of NUP155 was very distinct from its localization in all other cell types used in the study. In THP1 cells, NUP155 was localized as a single large nuclear body and confined exclusively to nucleolus, in addition to its appearance at NE and in the cytosol as a diffuse signal.

Having observed varied expression and localization of NUPs in different cell types under basal conditions, I further explored the protein expression levels of NUPs NUP98, NUP62, NUP155, NUP133, NUP107 and NUP85 in all the cell types under study during HIV-1 NL4.3 infection. During late stages of HIV-1 infection, i.e., 96 hpi, the expression of NUP98 was significantly downregulated in both SupT1 and HEK293T cell types, whereas in other cell types such as 1321N1 and TZM-bl, its

expression was unaffected. Specifically, in TZM-bl cells the expression of NUP62 and NUP133 were downregulated, while other NUPs remained unaffected. Interestingly, the expression of any NUP under study was unchanged in the case of 1321N1 cells during HIV-1 NL4.3 infection. To understand the specificity of the effect of late stages of HIV-1 infection on the levels of NUPs, the late stages condition of HIV-1 infection in HEK293T cells was mimicked by transfecting the cells with the molecular clone pNL4.3, essentially skipping the initial steps of HIV-1 replication such as entry, reverse transcription and integration. Under these conditions, a few disparities were observed with the levels of NUPs from what was observed during HIV-1 NL4.3 infection in HEK293T cells. While, like during HIV-1 NL4.3 infection, NUP98's expression was downregulated, the expression of NUP62, NUP133 and NUP85 was also modulated in contrast to the condition of infection with HIV-1 NL4.3. This difference is probably due to the differential strength of viral protein expression that emerges from the viral promoter, which is in turn affected by the chromatin environment (integrated vs. unintegrated). Taken together, the regulation of expression of the NUPs by HIV-1 infection varies from one cell type to the other. I also tested the effect of HIV-1 infection on the localization of the NUPs, whose protein levels were tested, in both SupT1 and HEK293T cells. However, I did not observe the discernible changes in the localization pattern of these NUPs during the late stages of HIV-1 infection.

As I observed the lowered endogenous levels of NUP98 upon HIV-1 infection in both SupT1 and HEK293T cells, I speculated if NUP98 had any antiviral function against HIV-1. To understand this, I undertook both overexpression and knockdown of NUP98 approaches. Both these complementary studies suggested that NUP98 downmodulated the HIV-1 gene expression, which resulted in the decreased intracellular viral RNA, protein levels as well as viral particle release in the culture supernatants. The direct correlation between the levels of viral RNA and viral protein that were reduced upon overexpression of NUP98 led me to investigate the possible role of NUP98 in the regulation of HIV-1 LTR promoter, which controls the expression of viral genes. I first confirmed that NUP98 associated with HIV-1 LTR promoter by performing ChIP-qPCR assay using transiently expressing Myc-NUP98 construct. In line with this evidence, I found that NUP98 downmodulates the LTR promoter activity using a reporter plasmid construct, in which the reporter gene

(luciferase/GFP) expression is directly under the control of HIV-1 LTR promoter. Interestingly, the downmodulation of LTR promoter activity by NUP98 could not be rescued by viral regulatory protein HIV-1 Tat, suggesting that NUP98 diminishes the basal HIV-1 gene expression. To claim the specific activity of NUP98 on HIV-1 LTR promoter, I tested its effect on other viral promoters such as cytomegalovirus (CMV) and simian immunodeficiency virus (SIV). However, NUP98 did not change the reporter gene expression driven by these viral promoters, suggesting that NUP98 specifically inhibited the HIV-1 LTR promoter activity. To further understand the underlying mechanism of LTR directed gene expression inhibition by NUP98, we constructed LTR promoter mutants upstream of the luciferase gene, lacking either negative regulatory element (NRE) or binding sites for NF-kB or Sp1. I observed that LTR lacking NRE lost sensitivity to the inhibition by NUP98, while other LTR mutants did not, suggesting that NRE of the LTR is required for the NUP98 to show its inhibitory activity. In agreement, I also showed that NUP98 preferably occupied NRE of the LTR by ChIP-qPCR assay. Lastly, I identified that N-terminal region of NUP98 is required for its negative impact on LTR activity.

Interestingly, the virion particles released from NUP98 overexpressing producer cells had reduced infectivity, whereas the virion particles released from NUP98 depleted producer cells enhanced their infectivity, indicating that NUP98 reduces the infectivity of released viral particles through yet unknown mechanism(s).

Based on the evidence presented here, I conclude that NUPs were differentially regulated by HIV-1 that depended on the cell type used in the study. I further conclude that NUP98, which is conventionally involved in the transport of molecules as a member of nuclear pore complex, non-canonically functions as an anti-HIV-1 factor through two different mechanisms: 1) limiting the viral gene transcription through interaction with HIV-1 LTR and 2) lowering the infectivity of the virus released from a producer cell. Further, it could be conceived that NUP98 might suppress the HIV-1 promoter by either blocking host transcription factors from binding and activating the HIV-1 promoter or bringing epigenetic markers onto the HIV-1 promoter so that viral genes remain silenced.

The downregulation of this anti-viral factor during infection can be a host-restriction evasion strategy employed by HIV-1. We believe that more insights into the

understanding of molecular events underlying NUP98 mediated HIV-1 gene expression repression will help us understand the complex biology behind the host-HIV-1 conflicts.

5.2 Future directions

This study demonstrates yet another host factor that reduced the HIV-1 gene expression by occupying the LTR promoter. The evidence presented here provides new insights into the regulation of HIV-1 gene expression, which is a prerequisite for productive HIV-1 infection. It will be interesting to see how NUP98 is recruited to HIV-1 LTR promoter and whether it interacts with known/unknown activators or repressors that regulate HIV-1 gene expression. The understanding of the molecular events underlying NUP98 mediated gene expression repression could help us understand the complex biology behind the host-HIV-1 interactions. It will also be interesting to understand how NUP98 reduces the infectivity of the virion particles and molecular mechanisms therein. I believe our baseline information unfolds thought-provoking perspectives such as to investigate if there is a family of NUPs which specialize additionally as anti-viral factors, and if such anti-viral properties of NUPs are cell-specific which may be decisive in HIV-1 propagation.

Annexures

Table A1. List of primers used in the RT-qPCR

Target genes	Primers (5'-3')
NUP98	FP: CCGTGATACCGAAGTTGAAAGC
	RP: AGATGCCTGCAAGACCTCAC
NUP62	FP: GGATCACCTTTGACTGAGCGA
	RP: TGCCACAACCCCAAACTACA
NUP155	FP: CTGAAAACGGAAATCCCCAGC
	RP: GCTCATTTGCCTTCTGAAGTTCT
NUP133	FP: GGCTTGTCTGGTGTGCAAAG
	RP: TCGGCACTCCAGTGGAAATC
NUP107	FP: CGACAGCCTTGTTCTTTCTACC
	RP: TGAAGACTGCGTAAGTCGGG
NUP85	FP: GCCAACAGTCACTAAAAATCAGAGA
	RP: CGTCAATTCTCTGGAGGCCC
GAPDH	FP: TGTTGCCATCAATGACCCCTT
	RP: CTCCACGACGTACTCAGCG
HIV-1 Env	FP: GCAGTGGGAATAGGAGCTTTGTTC
	RP: GAGCTGTTGATCCTTTAGGTATCTTTCC

qPCR conditions

Initial denaturation: 95ºC- 2 min

Denaturation: 95ºC-30 sec
Annealing: 55ºC-30 sec
Extension: 72ºC-40 sec
} 40 cycles

Final extension: 72ºC-10 min

Table A2. List of primers used in the creation of pLTR-Luc mutant constructs by site-directed mutagenesis

Construct name	Primers (5'-3')
ΔNRE LTR-Luc	FP: CTTACAAGGACCCTGAGAGAGAAGTGTTAG
	RP: TCTCAGGGTCCTTGTAAGTCATTGGTCTTA
ΔNF-κB LTR-Luc	FP: TTGTTACAAAGGGAGGCGTGGCCTGGGCGG
	RP: CGCCTCCCTTTGTAACAAGCTCGATGTCAA
ΔSp1 LTR-Luc	FP: ACTTTCCAGGAGCCCTCAGATGCTGCATAT
	RP: TGAGGGCTCCTGGAAAGTCCCCAGCGGAAA

Table A3. List of primers used in the ChIP-qPCR

Target site amplified	Primers (5'-3')
HIV-1 LTR (+68 nt to +168 nt)	FP: GCCTCAATAAAGCTTGCCTTGA
	RP: TCCACACTGACTAAAAGGGTCTGA
HIV-1 LTR (NRE)	FP: ATCTACCACACACAAGGCTACTTCC
	RP: CCACTCTAACACTTCTCTCTCAGGGT
HIV-1 LTR (NF-κB/Sp1)	FP: TTTGACAGCCGCCTAGCATTTC
	RP: CATCTGAGGGCTCGCCACTCC

qPCR conditions

Initial denaturation: 95ºC- 2 min

Denaturation: 95ºC-30 sec
Annealing: 55ºC-30 sec
Extension: 72ºC-40 sec
} 40 cycles

Final extension: 72ºC-10 min

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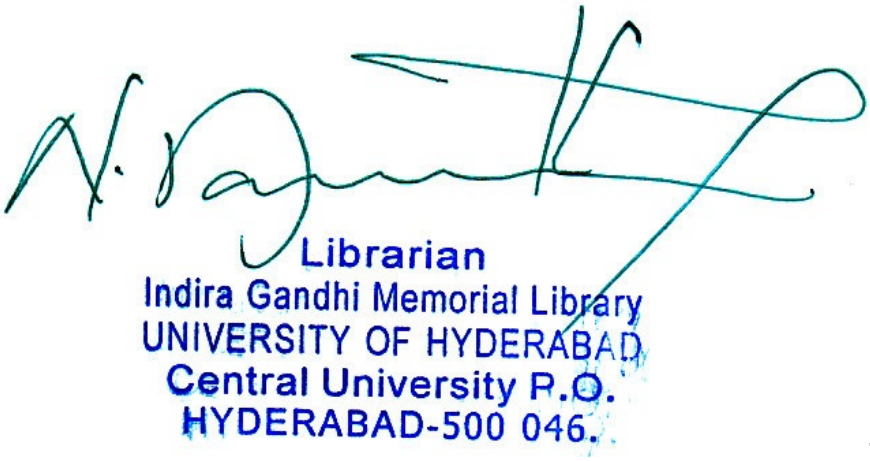

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The nuclear pore protein NUP98 impedes LTR-driven basal gene expression of HIV-1, viral propagation, and infectivity

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Nucleoporins (NUPs) are cellular effectors of human immunodeficiency virus-1 (HIV-1) replication that support nucleocytoplasmic trafficking of viral components. However, these also non-canonically function as positive effectors, promoting proviral DNA integration into the host genome and viral gene transcription, or as negative effectors by associating with HIV-1 restriction factors, such as MX2, inhibiting the replication of HIV-1. Here, we investigated the regulatory role of NUP98 on HIV-1 as we observed a lowering of its endogenous levels upon HIV-1 infection in $CD4^+$ T cells. Using complementary experiments in NUP98 overexpression and knockdown backgrounds, we deciphered that NUP98 negatively affected HIV-1 long terminal repeat (LTR) promoter activity and lowered released virus levels. The negative effect on promoter activity was independent of HIV-1 Tat, suggesting that NUP98 prevents the basal viral gene expression. ChIP-qPCR showed NUP98 to be associated with HIV-1 LTR, with the negative regulatory element (NRE) of HIV-1 LTR playing a dominant role in NUP98-mediated lowering of viral gene transcription. Truncated mutants of NUP98 showed that the attenuation of HIV-1 LTR-driven transcription is primarily contributed by its N-terminal region. Interestingly, the virus generated from the producer cells transiently expressing NUP98 showed lower infectivity, while the virus generated from NUP98 knockdown $CD4^+$ T cells showed higher infectivity as assayed in TZM-bl cells, corroborating the anti-HIV-1 properties of NUP98. Collectively, we show a new non-canonical function of a nucleoporin adding to the list of moonlighting host factors regulating viral infections. Downregulation of NUP98 in a host cell upon HIV-1 infection supports the concept of evolutionary conflicts between viruses and host antiviral factors.

KEYWORDS

nuclear pore complexes, NUP98, HIV-1 LTR, transcription, viral gene expression

Abbreviations: NPCs, nuclear pore complexes; NUP, nucleoporin; HIV-1, human immunodeficiency virus-1; LTR, long terminal repeat; Tat, transactivator of transcription; VSV-G, vesicular stomatitis virus glycoprotein G; HEXIM1, hexamethylene bisacetamide-inducible protein 1; NF-κB, nuclear factor kappa B; P-TEFb, positive transcription elongation factor b; GLFG, glycine leucine phenyl alanine glycine; ChIP, chromatin immunoprecipitation.

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MINI REVIEW
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Dodging the Host Interferon-Stimulated Gene Mediated Innate Immunity by HIV-1: A Brief Update on Intrinsic Mechanisms and Counter-Mechanisms

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Host restriction factors affect different phases of a viral life cycle, contributing to innate immunity as the first line of defense against viruses, including HIV-1. These restriction factors are constitutively expressed, but triggered upon infection by interferons. Both pre-integration and post-integration events of the HIV-1 life cycle appear to play distinct roles in the induction of interferon-stimulated genes (ISGs), many of which encode antiviral restriction factors. However, HIV-1 counteracts the mechanisms mediated by these restriction factors through its encoded components. Here, we review the recent findings of pathways that lead to the induction of ISGs, and the mechanisms employed by the restriction factors such as IFITMs, APOBEC3s, MX2, and ISG15 in preventing HIV-1 replication. We also reflect on the current understanding of the counter-mechanisms employed by HIV-1 to evade innate immune responses and overcome host restriction factors. Overall, this mini-review provides recent insights into the HIV-1-host cross talk bridging the understanding between intracellular immunity and research avenues in the field of therapeutic interventions against HIV-1.

Keywords: HIV-1, restriction factors, PRR, viral counter mechanisms, ISG interferon stimulated genes, PAMP

Abbreviations: HIV-1, Human Immuno deficiency virus 1; RF, Restriction factor; IFN, Interferon; PRR, Pattern recognition receptor; PAMP, Pathogen associated molecular pattern; cGAS, cyclic GMP-AMP Synthase; IFI16, Interferon γ induced protein 16; STING, Stimulator of interferon gene; IKK, Inhibitor of NFκB kinase; TBK, TANK binding kinase; IRF, Interferon regulatory factor; RLR, Retinoic acid inducible gene I (RIG-I) like receptor; TLR, Toll like receptor; MDA5, Melanoma differentiation associated protein 5; MAVS, Mitochondrial associated viral sensing protein; ISG, Interferon stimulated gene; IRE, Interferon response elements; MX2, Myxovirus resistance protein 2; IFITM, Interferon induced transmembrane protein; APOBEC3, Apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3; SERINC5, Serine incorporator protein 5; Vif, Viral infectivity factor; Vpr, Viral protein R; Vpu, Viral protein U; PR, viral protease; CA, Capsid; TrCP2, beta transducing repeat-containing protein; TRBP, TAR-RNA Binding Protein.

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RESEARCH

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Milk exosomes elicit a potent anti-viral activity against dengue virus

Vengala Rao Yenuganti[1*†], Sumbul Afroz[1,2†], Rafiq Ahmad Khan[2], Chandrima Bharadwaj[2], Deepti Kailash Nabariya[1], Nagaraj Nayak[3], Madhuri Subbiah[3], Kumaraswami Chintala[4], Sharmistha Banerjee[4], Pallu Reddanna[1] and Nooruddin Khan[1,2*†]

Abstract

Background: Exosomes are nano-sized vesicles secreted by various cells into the intra and extracellular space and hence is an integral part of biological fluids including milk. In the last few decades, many research groups have proved the potential of milk exosomes as a sustainable, economical and non-immunogenic drug delivery and therapeutic agent against different pathological conditions. However, its anti-viral properties still remain to be unearthed.

Methods: Here, we have been able to isolate, purify and characterize the milk derived exosomes from Cow (CME) and Goat (GME) and further studied its antiviral properties against Dengue virus (DENV), Newcastle Disease Virus strain Komarov **(**NDV-K) and Human Immunodeficiency Virus (HIV-1) using an in-vitro infection system.

Results: TEM, NTA and DLS analysis validated the appropriate size of the isolated cow and goat milk exosomes (30–150 nm). Real-time PCR and immunoblotting results confirmed the presence of several milk exosomal miRNAs and protein markers. Our findings suggest that GME significantly decreased the infectivity of DENV. In addition, we confirmed that GME significantly reduces DENV replication and reduced the secretion of mature virions. Furthermore, heat inactivation of GME did not show any inhibition on DENV infection, replication, and secretion of mature virions. RNase treatment of GME abrogates the anti-viral properties indicating direct role of exosomes in DENV inhibition. In addition GME inhibited the infectivity of NDV-K, but not HIV-1, suggesting that the GME mediated antiviral activity might be virus specific.

Conclusion: This study demonstrates the anti-viral properties of milk exosomes and opens new avenues for the development of exosome-based therapies to treat viral diseases.

Keywords: Goat milk exosomes, Dengue virus, Anti-viral, And NDV-K

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Review

Tough Way In, Tough Way Out: The Complex Interplay of Host and Viral Factors in Nucleocytoplasmic Trafficking during HIV-1 Infection

Satarupa Sarkar [1], Kannan Balakrishnan [1,2,3,†], Kumaraswami Chintala [1,†], Krishnaveni Mohareer [1], Tom Luedde [2], Ananda Ayyappan Jaguva Vasudevan [2,4], Carsten Münk [2] and Sharmistha Banerjee [1,*]

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Abstract: Human immunodeficiency virus-1 (HIV-1) is a retrovirus that integrates its reverse-transcribed genome as proviral DNA into the host genome to establish a successful infection. The viral genome integration requires safeguarding the subviral complexes, reverse transcription complex (RTC) and preintegration complex (PIC), in the cytosol from degradation, presumably effectively secured by the capsid surrounding these complexes. An intact capsid, however, is a large structure, which raises concerns about its translocation from cytoplasm to nucleus crossing the nuclear membrane, guarded by complex nuclear pore structures, which do not allow non-specific transport of large molecules. In addition, the generation of new virions requires the export of incompletely processed viral RNA from the nucleus to the cytoplasm, an event conventionally not permitted through mammalian nuclear membranes. HIV-1 has evolved multiple mechanisms involving redundant host pathways by liaison with the cell's nucleocytoplasmic trafficking system, failure of which would lead to the collapse of the infection cycle. This review aims to assemble the current developments in temporal and spatial events governing nucleocytoplasmic transport of HIV-1 factors. Discoveries are anticipated to serve as the foundation for devising host-directed therapies involving selective abolishment of the critical interactomes between viral proteins and their host equivalents.

Keywords: HIV-1; nucleocytoplasmic trafficking; capsid import; viral mRNA export; HIV-1 Rev; nucleoporins

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1. Introduction

Viruses carry DNA or RNA as their genomic blueprints for propagating inside prokaryotic or eukaryotic hosts. In eukaryotes, most DNA viruses, and a few RNA viruses, have to complete the daunting task of crossing the nuclear membrane twice; first, to enter the nucleus to replicate their genomes, and second to export their RNA and other factors out of the nucleus for translation of viral proteins and their assembly to form new progeny. Viruses have evolved adequate mechanisms and bear factors that aid in hijacking host import–export machinery across the nuclear membrane and completing the productive infection. While there may be overlapping host players and common mechanisms employed in infections caused by different viruses, the strategies employed by a specific virus always involve certain unique steps. DNA viruses, such as baculoviruses, herpes viruses, or adenoviruses, dock at the nuclear pore complex (NPC), but not all viruses pass through NPC

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Growth hormone induces mitotic catastrophe of glomerular podocytes and contributes to proteinuria

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Abstract

Glomerular podocytes are integral members of the glomerular filtration barrier in the kidney and are crucial for glomerular permselectivity. These highly differentiated cells are vulnerable to an array of noxious stimuli that prevail in several glomerular diseases. Elevated circulating growth hormone (GH) levels are associated with podocyte injury and proteinuria in diabetes. However, the precise mechanism(s) by which excess GH elicits podocytopathy remains to be elucidated. Previous studies have shown that podocytes express GH receptor (GHR) and induce Notch signaling when exposed to GH. In the present study, we demonstrated that GH induces TGF-β1 signaling and provokes cell cycle reentry of otherwise quiescent podocytes. Though differentiated podocytes reenter the cell cycle in response to GH and TGF-β1, they cannot accomplish cytokinesis, despite karyokinesis. Owing to this aberrant cell cycle event, GH- or TGF-β1-treated cells remain binucleated and undergo mitotic catastrophe. Importantly, inhibition of JAK2, TGFBR1 (TGF-β receptor 1), or Notch prevented cell cycle reentry of podocytes and protected them from mitotic catastrophe associated with cell death. Inhibition of Notch activation prevents GH-dependent podocyte injury and proteinuria. Similarly, attenuation of GHR expression abated Notch activation in podocytes. Kidney biopsy sections from patients with diabetic nephropathy (DN) show activation of Notch signaling and binucleated podocytes. These data indicate that excess GH induced TGF-β1-dependent Notch1 signaling contributes to the mitotic catastrophe of podocytes. This study highlights the role of aberrant GH signaling in podocytopathy and the potential application of TGF-β1 or Notch inhibitors, as a therapeutic agent for DN.

Introduction

Glomerular complications are the predominant cause of end-stage kidney disease, and clinical conditions, such as diabetes and hypertension are associated with glomerular dysfunction and proteinuria. Glomerular podocytes are highly differentiated specialized visceral cells that account for ~30% of glomerular cells. These cells provide epithelial coverage to the capillaries and together with glomerular basement membrane (GBM) and perforated endothelial cells, constitute a glomerular filtration barrier. The unique cytoplasmic extensions of podocytes are known as foot processes, which attach to the GBM and interdigitate with neighboring foot processes to form the slit diaphragm (SD). The sophisticated architecture of SD contributes to the glomerular permselectivity. The process of progressive podocyte damage characterized by podocyte hypertrophy, detachment of podocytes, and, finally, irreversible loss of podocytes has been observed in human and experimental models of nephropathy and glomerular diseases[1]. Injury and depletion of podocytes, leading to podocyte insufficiency and capillary collapse, have been implicated in glomerulosclerosis and resulting chronic kidney disease.

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