The effect of host mRNA decay proteins on HIV-1 genomic RNA metabolism and viral gene expression

by

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July 2018

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

Abstract

mRNA surveillance pathways are host quality control mechanisms that degrade aberrant mRNA to prevent the accumulation of potentially toxic truncated or misfolded proteins. Substrates for mRNA surveillance include transcripts that have pre-termination codons, long 3' untranslated regions (UTRs), retained introns or upstream open reading frames. The human immunodeficiency virus type 1 (HIV-1) genomic RNA (vRNA) also contains long 3' UTRs. However, not only is the vRNA able to evade mRNA surveillance, HIV-1 has also been demonstrated to recruit the mRNA decay proteins to promote vRNA stability and ensure viral gene expression. UPF1 is an integral protein of the nonsense-mediated mRNA decay pathway and Staufen1 is also involved in the post-transcriptional events of mRNA decay, mRNA trafficking and translation. In this work, we further elucidated the roles of these host mRNA decay proteins on the post-transcriptional regulation of HIV-1 vRNA metabolism. The ability of HIV-1 to form a stable viral reservoir is the major obstacle to an HIV-1 cure. In our studies investigating HIV-1 latency, we characterised the roles of mRNA decay proteins on the maintenance of viral latency in a latently-infected model T cell line using fluorescence *in situ* hybridisation - Flow Cytometry (FISH-flow). We observed that UPF1 enhances vRNA stability, thus promoting viral gene expression at a post-transcriptional level. We also demonstrated that two other proteins involved in nonsense-mediated mRNA decay, UPF2 and SMG6, are negative regulators of proviral reactivation and reduce viral gene expression in the same model T cell line. In primary HIV-1-infected CD4+ T cells, UPF1 also enhanced vRNA stability and viral gene expression. UPF2 and SMG6 were also found to restrict HIV-1 gene expression in primary monocyte-derived macrophages, another viral reservoir of HIV-1 infection. In related studies investigating the host response to viral infection, we characterised a novel role for Staufen1 in the rescue of cellular mRNA translation and viral gene expression during HIV-1 nucleocapsid (NC)-induced stress granule assembly. Altogether, the host mRNA decay proteins UPF1, UPF2, SMG6 and Staufen1 affect various stages of vRNA metabolism and HIV-1 replication. These findings can be applied towards an HIV-1 cure using two strategies: the 'kick and kill' strategy to bolster the reactivation of the HIV-1 provirus and effectively decrease the size of the viral reservoir, or a 'block and lock' strategy to permanently silence the HIV-1 provirus.

Résumé

Les mécanismes de surveillance de l'ARN messager (ARNm) sont des voies cellulaires de control de qualité permettant de dégrader les ARNm défectueux afin d'éviter l'accumulation de protéines tronquées ou mal repliées qui seraient potentiellement toxiques pour la cellule. Les processus de surveillance de l'ARNm ciblent les transcrits contenant des codons pré-terminaux, de longues régions non traduites (*unstranslated regions ;* UTR), des introns retenus, ou des cadres de lectures ouverts situés en amont de la séquence codante. L'ARN génomique viral (ARNv) du virus de l'immunodéficience humaine (VIH) contient de longues régions 3'-UTRs. Cependant, non seulement l'ARNv est capable d'échapper à la surveillance cellulaire de l'ARNm, mais le VIH détourne également les protéines de dégradation de l'ARNm afin d'assurer la stabilité de son propre ARNv et l'expression de ses gènes. UPF1 est une protéine essentielle du mécanisme de dégradation de l'ARNm non-sens ; Staufen1 est également impliquée dans les évènements post-transcriptionnels de dégradation de l'ARNm, de circulation de l'ARNm et de translation. Cette étude clarifie les rôles de ces protéines cellulaires de dégradation de l'ARNm dans la régulation post-transcriptionnelle du métabolisme de l'ARNv du VIH-1. L'aptitude du VIH à former un réservoir viral stable est un obstacle majeur dans le développement d'un remède contre le VIH. Nos travaux ont permis de définir le rôle des protéines de dégradation de l'ARNm dans le maintien de la latence virale grâce à l'étude par hybridation *in situ* en fluorescence – cytométrie en flux (FISH-Flow) d'un modèle de cellules T infectées par la forme latente du virus. Nous avons démontré que UPF1 améliore la stabilité de l'ARNv, favorisant ainsi l'expression des gènes viraux au niveau post-transcriptionnel. Nous avons également prouvé dans ce même modèle cellulaire que deux autres protéines, UPF2 et SMG6, impliquées dans la dégradation de l'ARNm non-sens, régulent négativement la réactivation du provirus et diminuent l'expression des gènes viraux. Dans le cas de lymphocytes T CD4+ primaires infectés par le VIH**,** UPF1 augmente la stabilité de l'ARNm et l'expression des gènes viraux. UPF2 et SMG6 restreignent également l'expression des gènes du VIH dans des macrophages primaires dérivés de monocytes, d'autres cellules réservoirs de l'infection au VIH. Dans une étude connexe sur la réponse cellulaire à une infection virale, nous avons caractérisé un nouveau rôle de Staufen 1 dans le

rétablissement de la traduction de l'ARNm cellulaire et dans l'expression des gènes viraux lors de l'assemblage de granules de stress induit par les protéines nucléocapside (NC) du VIH-1. En conclusion, les protéines cellulaires de dégradation de l'ARNm - UPF1, UPF2, SMG6 et Staufen1 - affectent divers stages du métabolisme de l'ARNv et de la réplication du VIH-1. Ces découvertes peuvent être appliquées au développement d'un remède contre le VIH par le biais de deux stratégies : la stratégie « kick and kill » pour stimuler la réactivation du provirus et diminuer efficacement le réservoir viral ; ou la stratégie « block and lock » pour bloquer de façon permanente la réactivation du virus et le garder sous sa forme latente.

Preface

This thesis follows the "Manuscript-based thesis" format in accordance with McGill University's "Guidelines for preparation of a thesis" and consists of 5 chapters. The first chapter is a comprehensive literature review of the two topics central to this thesis: HIV-1 and the host mRNA decay proteins. This chapter also outlines the objectives of the research presented in this thesis. Chapters 2, 3, and 4 are manuscripts that are either published or submitted to scientific journals. References to these publications as well as the contribution of authors are described in the preface of each applicable chapter. The work presented in this thesis represents a significant contribution to knowledge that is the result of independent scholarship and is summarised and discussed in Chapter 5.

Manuscripts not included in this thesis, for which the candidate made significant contributions during the course of their research, include:

- **Rao, S.**, Sine, S. H., Amorim, R., Temzi, A. & DesGroseillers, L., Mouland, A. J. “HIV-1 has impaired ability to dissociate stress granules in novel Staufen1-gene edited cells” (Manuscript in preparation)
- Rance, E., Chen, A., McCullogh, C., Poirier, M., Alpuche-Lazcano, S. P., **Rao, S**., Niu, M., Ming, L., Mouland, A.J., Bell, B., Cochrane, A., & Gatignol, A. “A GagzipGFP HIV-1 Tat/TAR-independent latency model in cell lines derived from human lymphocytes, monocytes and macrophages”. (Manuscript in preparation)
- Le Sage, V., Cinti, A., McCarthy, S., Amorim, R., **Rao, S.**, Daino, G. L., Tramontano, E., Branch, D. R., Mouland, A. J. “Ebola virus VP35 blocks stress granule assembly”. *Virology.* 502:73-83. (February 2017)
- Ajamian, L., Abel, K., **Rao, S.**, Vyboh, K., García-de-Gracia, F., Soto-Rifo, R., Kulozik, A. E., Gehring, N. H., and Mouland, A. J. "HIV-1 Recruits UPF1 but Excludes UPF2 to Promote Nucleocytoplasmic Export of the Genomic RNA". *Biomolecules.* 5 (4): 2808-2839. (October 2015)

Table of Contents

List of figures and tables

List of abbreviations

ADAR1	adenosine deaminase acting on RNA 1
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
APOBEC3G	apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
ARV	antiretroviral
ARE	AU-rich element
AZT	azidothymidine
BMH	branched multiple hairpin
bNAb	broadly neutralizing antibody
BST2	bone marrow stromal cell antigen 2
CA	capsid
cART	combination antiretroviral therapy
CBP	cap-binding complex
CDC	American Centres for Disease Control
CDK9	cyclin dependent kinase 9
CPSF6	cleavage and polyadenylation factor 6
CRISPR	clustered regularly interspaced palindromic repeats
CRF	circulating recombinant forms
CRM1	chromosome region maintenance 1
dCA	didehydro-Cortistatin A
DDX	dead box proteins
DECID	decay inducing complex
DIS	dimerisation initiation site
DMSO	dimethyl sulfoxide
DNA	deoxyrobinucleic acid
dsRBD	double stranded RNA-binding domains
EBM	exon junction binding domain
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
EJC	exon junction complex
ELISA	enzyme-linked immunosorbent assay
Env	envelope
eRF	eukaryotic release factor
ESCRT	endosomal sorting complexes required for transport
FISH	fluorescence *in situ* hybridization
FISH-Flow	fluorescence *in situ* hybridisation - Flow Cytometry

G3BP1	GTPase activating protein (GAP) SH3 domain-binding protein 1
Gag	group-specific antigen
GCN2	general control non-derepressible-2
GST	glutathione S-transferases
HA	hemagglutinin
HAART	highly active antiretroviral therapy
HDACi	histone deacetylase inhibitors
HIV-1	human immunodeficiency virus type 1
HMT	histone methyltransferase
hnRNP	heterogeneous nuclear ribonucleoprotein
HRI	heme-regulated inhibitor
HSC	hematopoietic stem cell
HTLV	human T-cell lymphotropic virus
IAS	International AIDS Society
IDD	intrinsically disordered domain
IF	immunofluorescence
IFN	interferon
Ig	Immunoglobulin
IN	integrase
INS	instability sequence
INSTIs	integrase strand transfer inhibitors
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES	internal ribosome entry site
kb	kilobase
LAV	lymphadenopathy associated virus
LC	low complexity
LDI	long-distance interaction
LRA	latency-reversing agent
LSCM	laser scanning confocal microscopy
LTR	long terminal repeat
MA	matrix
MDM	monocyte-derived macrophage
MHC	major histocompatibility complex
MIF4G	middle portions of eukaryotic initiation factor 4-gamma
NC	nucleocapsid
Nef	negative factor
NES	nuclear export signal
NGD	no-go decay

NLS	nuclear localization signal
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NMD	nonsense-mediated mRNA decay
NPC	nuclear pore complex
NRTIs	nucleoside reverse transcriptase inhibitors
NSD	non-stop decay
Nup	nuclear proteins
NXF1	nuclear RNA export factor 1
ORF	open reading frame
PABP1	poly-A binding protein complex
PACT	PKR activator
PB	P-body
PBS	primer-binding site
PERK	PKR-like ER kinase
PHA	phytohemagglutinin
PIC	pre-integration complex
PIN	PilT N-terminus
PKC	protein kinase C
PKR	protein kinase R
PLA	proximity ligation assay
PMA	phorbol myristate acetate
Pol	polymerase
PPT	polypurine tract
PR	protease
PreP	pre-exposure prophylaxis
PTB	polypyrimidine tract binding protein
PTC	premature translation termination codon
pTEFB	positive transcription elongation factor
RBP	RNA-binding proteins
RENT1	regulator of nonsense transcripts 1
Rev	replication viral factor
RNA	ribonucleic acid
RNP	ribonucleoprotein complex
RRE	Rev response element
RRM	RNA recognition motif
RSE	Rous sarcoma virus stability element
RSV	Rous sarcoma virus
RT	reverse transcriptase

RTC	reverse transcription complex
SA	splice acceptor
Sam68	src-associated protein in mitosis 68kDa
SAMHD1	SAM domain and HD domain-containing protein 1
SBS	Staufen1-binding site
SD	splice donor
SFV	Semliki forest virus
SG	stress granule
SHRNP	Staufen1-containing HIV-1 RNP
SINV	Sindbis virus
SIV	simian immunodeficiency viruses
SL	stem loops
SMD	Staufen-mediated mRNA decay
SMG	suppressor with morphological effect on genitalia
SP	spacer peptide
SR	splicing regulator
SU	gp120 surface protein
TALENs	transcription activator-like effector nucleases
TAR	*trans*-activating response element
Tat	transactivating regulatory protein
TIAR1	TIA-1-related RNA-binding protein
TLR	toll-like receptor
TM	gp41 transmembrane protein
TNPO3	Transportin 3
TraP	treatment as prevention
TRBP	TAR RNA binding protein
UNAIDS	United Nations programme on HIV/AIDS
UPF	up-frameshift protein
US FDA	United States food and drug administration
UTR	untranslated region
Vif	viral infectivity facto
VLP	virus-like particle
Vpr	viral protein R
Vpu	viral protein U
vRNA	HIV-1 genomic RNA
ZFs	zinc-fingers
ZFNs	zinc-finger nucleases

Acknowledgements

First and foremost, I would like to thank my PhD supervisor Dr. Andrew J. Mouland for his guidance and support of the work presented in this thesis. He has played a very large role in the development of my critical thinking abilities and has really shaped me into being an independent scientist. I will always be grateful for how he encouraged me to just “Go for it!” by giving me the opportunities to attend conferences, present at meetings, write manuscripts and teach lectures. He was always receptive to my ideas during our many meetings and applied his scientific expertise to steer me in the right direction. I am also grateful for how he always lightened tougher times with his characteristic sense of humour and encouraged me to give nothing less than my best. I thank him wholeheartedly for giving me a wonderful PhD experience in his laboratory.

Secondly, I would like to thank past and current members of the Mouland Lab, who made coming to work every day so enjoyable and rewarding. Lucia, for patiently training me when I first started and being so comforting in my first few months in a new country. Valerie, for being the epitome of a power woman and inspiring me by her own example to be exemplary in science as well as in life. Alessandro, for being a wonderful lab older brother and navigating the world of NC-stress granules (and polysome profiling) with me. Raquel, for being so patient for the million times she heard me say “Raquel…”, usually followed by a question. I deeply value her support, guidance and companionship during my graduate studies and I cherish the memories of how I ‘forced’ my friendship upon her, our many post-meeting lunches and our numerous discussions about science (How did the first cell divide?) and about life. Abdel and his radiant energy, for being the fabulous person that he is and for just knowing when to lend a shoulder, give a hug or crack a joke. Fernando, Marwan, Sergio, Laure, Meijuan, Kristin, Saina and other members of the Mouland lab, the Lady Davis Institute and the department of Microbiology and Immunology for being excellent peers and for their friendship. I also want to thank Laure, Valerie, Raquel, and Abdel for helping me proofread my thesis and Laure for translating my abstract into French.

I am deeply grateful for CanCURE, the Canadian Initiative for HIV Cure Research created through a partnership between the Canadian Institutes of Health Research (CIHR), the Canadian Foundation for AIDS Research (CANFAR) and International AIDS Society (IAS), and its team grant

to my supervisor that has funded my studies and research. I also extend my gratitude to the Department of Microbiology and Immunology and the Faculty of Medicine at McGill University for supporting my studies with financial awards and for their administrative support. I am also thankful to the members of my PhD advisory committee Dr. Andrés Finzi and Dr. Selena Sagan for their constructive feedback and encouragement of my research. I also thank the Lady Davis Institute (LDI) and the late Dr. Mark Wainberg, Ceasar and Maureen for the access and the maintenance of the BCL3 facility. I would also like to thank the LDI core facilities, especially the ever-helpful and highly competent Christian Young, for the training and services they provided to conduct my scientific research. I am also thankful to our numerous collaborators for assay development, reagents or scientific discussions: Dr. Jens Lykke-Andersen, Dr. Laurent Chatel-Chaix, Dr. Alan Cochrane, Dr. Luc DesGroseillers, Dr. Marc Fabian, Dr. Anne Gatignol, Dr. Niels Gehring, Dr. Rob Gorelick, Ilinca Ibanescu, Dr. Daniel Kaufmann, Dr. Nancy Kedersha, Dr. Andreas Kulozik, Dr. Guy Lemay, Dr. Oliver Muhlemann, Dr. Tamiko Nishimura, Dr. Jean-Pierre Routy, Dr. Michel Tremblay and Dr. Ji Chang You.

I am also very fortunate the have the support of my wonderful friends, family and loved ones to fill me with warmth and happiness, especially during the cold Canadian days. Jonathan, for being with me through every step of the way, for teaching me to break big goals down into smaller pieces, for applying his academic writing skills to give me feedback on my thesis and for taking a stand to only bring out the best in me. Anne-Sophie and Adeline, for being my family away from home and for all the love and laughter. Siddharth, for having my back no matter what and teaching me about the deep value about making meaningful connections. My grandparents and extended family, for the ‘Good morning’ flowers and unconditional love. And most importantly, my wonderful parents Girish and Shobha. Words can barely come close to describe how grateful I am for the love, support, inspiration and wisdom they share with me every single day. They have always believed in me and have taught me to grow wings and fly, but to never lose my roots. To make them proud of me is one of the best feelings in the world and, therefore, I dedicate this thesis to my parents.

Chapter 1

General introduction and literature review

1.1. Preface

This chapter provides a literature review of the topics relevant to the research presented in this thesis. Section 1.2 reviews the history, current prevalence and pathogenesis of HIV infection. The organisation of the HIV-1 genome and the structure of HIV-1 are described in Section 1.3., followed by a detailed review of the viral replication cycle in section 1.4. In section 1.5., the current HIV therapies, the development of an HIV-1 vaccine and the strategies towards an HIV-1 cure are discussed. The mechanisms employed by HIV-1 to evade host cell responses will be described in section 1.6., with an extensive review of the host mRNA decay pathways and its modulation by HIV-1 in section 1.7. Finally, the main objectives of the research presented in this thesis are outlined in section 1.8. All of the material presented in this chapter is a result of independent scholarship.

1.2. HIV and AIDS

Human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS), is a pernicious pathogen that has claimed the life of more than 35 million people worldwide, according to UNAIDS. This section will describe the history and origins of the HIV pandemic, its current prevalence and the pathogenesis of HIV infection.

1.2.1. History and origins

In the late 1970s and early 1980s, the first cases of an emerging syndrome characterised by immune dysfunction, generalised lymphadenopathy and opportunistic infections were reported [1]. Since this condition was observed mainly in the homosexual community, it was first termed 'gay-related immune deficiency syndrome' [2]. In September 1982, the American Centers for Disease Control (CDC) first used the term "acquired immune deficiency syndrome" to describe this condition [3]. In early 1983, a new human retrovirus coined lymphadenopathy associated virus (LAV) was isolated from a patient with generalised lymphadenopathy [4]. This was followed by reports of a similar virus that was preliminarily called human T-cell leukemia virus - III (HTLV-III) being isolated from people with AIDS [5, 6]. In 1986, LAV/HTLV-III was officially termed HIV (subsequently called HIV-1) by the International Committee on the Taxonomy of Viruses [7]. In 1986, a related, but immunologically distinct virus was observed in West Africa and is now called HIV-2 [8]. In 2008, Luc Montagnier and Françoise Barré-Sinoussi were awarded the Nobel Prize in Physiology or Medicine for the identification of HIV as the causative agent of AIDS.

HIV belongs to the lentivirus subgroup of the *Retroviridae* family. The prefix 'lenti' is derived from Latin *lentus* for slow, an indication of the chronic and persistent HIV infection mechanisms that result in immune function deregulation. Both HIV-1 and HIV-2 are zoonotic infections and their cross-species transmission originate from the Simian Immunodeficiency virus (SIV) from different nonhuman primates: chimpanzees and sooty mangabeys, respectively. Phylogenetic studies indicate that HIV-1 was transmitted to the human population as early as 1890 [9] and the first recorded HIV-positive sample dates back to 1959 from a frozen plasma sample from Kinshasa, the Democratic Republic of Congo [10].

1.2.2. Epidemiology and classification

According to UNAIDS, there were 36.7 million people living with HIV in 2016, 19.4 million of them from Eastern and Southern Africa. Table 1.1. depicts the worldwide prevalence of HIV-1. Combination antiretroviral therapy (cART) is currently used to treat HIV infection and it is the standard of care for HIV-1 infection (see section 1.5.1.). The advent of cART has dramatically improved the lives of HIV-infected individuals, with people on cART having near-normal life expectancy [11]. As of June 2017, 20.9 million people have access to cART, which is an outstanding achievement since in 2010, only 7.7 million people were on cART. However, it is important to note that 47% of HIV-infected individuals currently do not have access to cART and 1.8 million people became newly infected with HIV in 2016. Furthermore, 24% of HIV-infected woman do not have access to cART and are unable to prevent mother-to-child transmission of the infection. Therefore, there is a need to identify novel therapeutic or curative strategies for HIV (discussed in section 1.5.).

HIV-1 is categorised into the following groups: M (main), O (outlier), N (non-M, non-O or new) and P (pending classification/identification) [12-16]. The M group contributes to 98% of global HIV-1 isolates and it comprises of 9 subtypes (A-D, F-H and J-K) and at least 89 circulating recombinant forms (CRFs) [17]. Nearly 50% of all people living with HIV-1 have subtype C [17]. Most of the current HIV-1 research is conducted using viruses from subtype B that are mostly prevalent in the Americas, Western Europe and Australasia, despite the fact that this subtype represents only 12% of global HIV infections. Interestingly, B and non-B viruses display differences in their pathogenesis and the development of drug resistance [17-19]. Thus, it is important for further research to validate the cross-reactivity of newer therapies between B and non-B HIV-1 subtypes. The worldwide distribution of HIV-1 group M subtypes is depicted in Figure 1.1.

Region	People living with HIV	New HIV infections in 2016	Total number with access to cART as of July 2017
Worldwide	36.7 million	1.8 million	20.9 million
Eastern and southern Africa	19.4 million	790,000	12.5 million
Asia and the Pacific	5.1 million	270,000	2.5 million
Western and central Africa	6.1 million	370,000	2.3 million
Latin America	1.8 million	97,000	1.1 million
The Caribbean	310,000	18,000	170,000
Middle East and North Africa	230,000	18,000	58,400
Eastern Europe and central Asia	1.6 million	190,000	474,000
Western and central Europe and North America	2.1 million	73,000	1.7 million

Table 1.1.: Worldwide HIV prevalence

Total number of people living with HIV in 2016, the incidence of new infections in 2016 and numbers with access to cART as of June 2017 are described. All data from www.unaids.org.

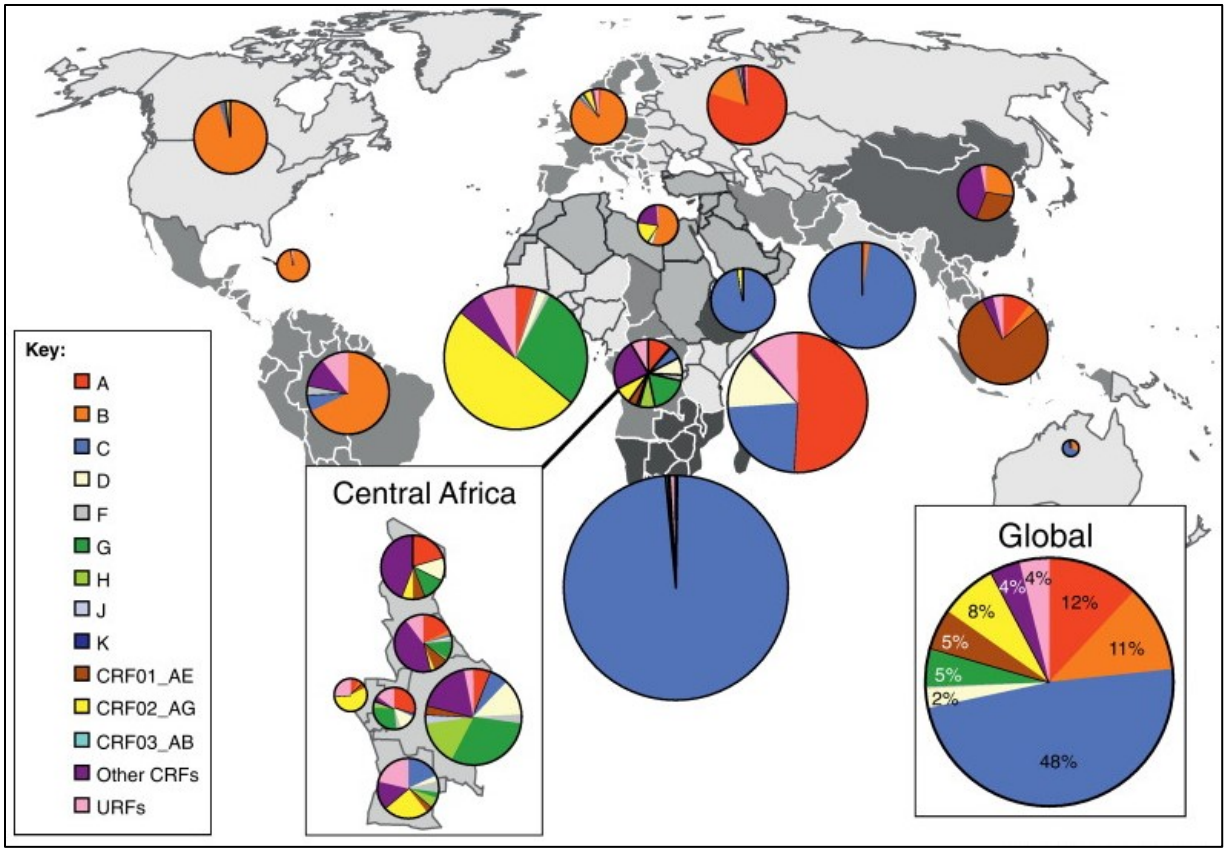

Figure 1.1.: Distribution of HIV-1 Group M subtypes and circulating recombinant forms

In the main figure, the distribution of HIV-1 subtypes by global regions is shown with the relative percentages of infections illustrated by the size of the pie charts based on data obtained from 2004-2007 [17]. In the inset pictures, the HIV-1 distributions found around the world and within Central African countries are represented. Reproduced and adapted with permission from [17].

1.2.3. Transmission and pathogenesis

HIV is mainly transmitted through sexual contact. However, it can also be transmitted non-sexually through blood transfusions, sharing of contaminated needles and from an infected mother to child during birth. The risks of transmitting the virus, but not the pathology of infection, are different based on the route of transmission [20, 21]. The three main stages of an HIV infection are the acute phase, the chronic phase (asymptomatic) and the progression to AIDS (symptomatic) [22]. A graph describing the natural progression of HIV-1 infection is depicted in Figure 1.2. HIV-1 and HIV-2 infections are similar in transmission and presentation of symptoms, but they differ in disease progression and prevalence. HIV-1 is the more predominant and contagious form with a morbidity 40 times higher than the general population [23]. HIV-2 infection, on the other hand, presents slower disease progression, a morbidity only 2 to 5 fold higher than the general population and is mainly observed in western Africa [24-26].

The acute phase is the earliest stage of HIV-1 infection, and generally develops within 2 to 4 weeks post infection. Some infected individuals present flu-like symptoms, such as fever, headaches, and rash [27]. During this period, the virus is actively multiplying and the level of HIV-1 in the blood is very high, which greatly increases the risk of HIV-1 transmission. This is accompanied by a depletion in CD4+ T cell count, a host immune response and an establishment of a viral latent reservoir [28-30]. During the chronic phase of the disease, also referred to clinical latency, the infected individual is usually asymptomatic. The virus multiplies at low levels and there is a gradual, steady depletion of CD4+ T cells. This period lasts for an average of 10 years, but this period is highly variable in length [31]. A drop in CD4+ T cells count to less than 200 cells/µL or an appearance of AIDS-defining opportunistic infections or cancers characterises having progressed to AIDS. This is also accompanied by increased viral loads. The time of progression from acute infection to AIDS varies greatly from one person to another and ranges between as low as 6 months [32] and more than 25 years [33]. The reason for this wide range is yet to be elucidated and may involve both viral and host factors. Without any antiretroviral treatment, people with AIDS usually survive about 3 years.

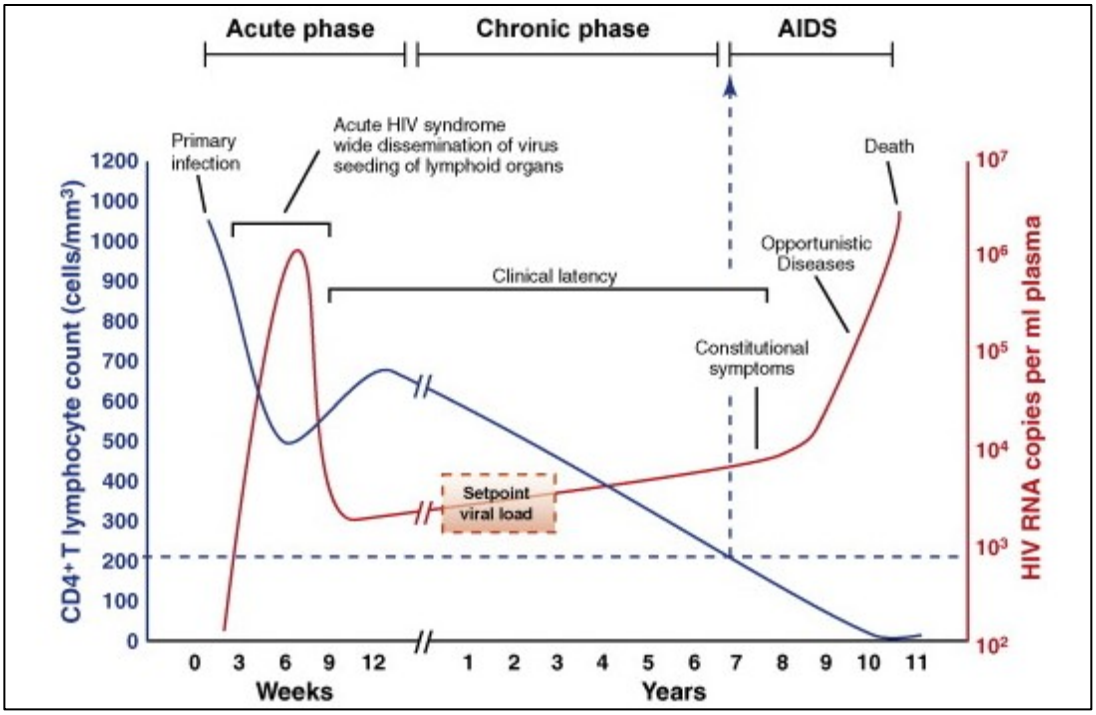

Figure 1.2.: HIV-1 disease progression

The typical three phases of an HIV-1 infection are shown with their characteristic peripheral blood CD4+ T lymphocyte counts and plasma RNA levels (viral load). The acute phase is accompanied by flu-like symptoms, peak virus load and drop in CD4+ T cells. The chronic asymptomatic phase lasts on average 7–10 years and viral replication reaches a steady level known as the 'set point'. AIDS onset is associated with increasing virus replication and declining CD4+ cell counts to <200/mm3. Adapted from [34], reprinted with permission from [35].

1.3. HIV-1 genome organisation and structure

This section will describe the organisation of the HIV-1 genomic RNA and the structure of mature HIV-1.

1.3.1. HIV-1 genome

The HIV-1 genome consists of two copies of a 9-kb positive-sense RNA that have nine open reading frames (ORFs) enclosed within the core of a mature virus particle. The genomic RNA, hereby called vRNA, is capped and polyadenylated, similar to other cellular mRNAs [36]. The vRNA is flanked by non-coding regions called the 5' and 3' untranslated regions (UTRs) that contain numerous regulatory sequences that mediate transcription, splicing, vRNA dimerization, vRNA packaging, and reverse transcription. The trans-activating response region (TAR) in the 5'UTR is a binding site for the viral protein Tat and is required for viral transcription [37]. The primer-binding site (PBS) follows the TAR region and this is where tRNAlys3 binds, thus initiating the process of reverse transcription (see section 1.4.1.2.) [38, 39]. The packaging of the vRNA into progeny virus particles is primarily dependent on the interaction of the nucleocapsid (NC) domain of the main HIV-1 structural protein Gag with the four RNA stem-loops (SL1, SL2, SL3, and SL4) that compose the core encapsidation signal, psi (ψ) [40]. The dimerisation initiation site (DIS) and the major splice donor (SD) sites are present on SL1 and SL2 respectively and contribute to viral encapsidation and vRNA splicing [40]. The various cis-acting RNA elements on the vRNA are depicted in Figure 1.3.

The HIV-1 proviral DNA is generated by the reverse transcription of the vRNA into DNA and integration of the double-stranded HIV-1 DNA into the human genome. The HIV-1 proviral DNA is flanked at both ends by long terminal repeat (LTR) sequences and the 5' LTR region codes for the promotor for transcription of the viral genes.

The three main HIV-1 translation products of the HIV-1 provirus are the structural Group-specific antigen (Gag), the Envelope (Env) and the Polymerase (Pol). They are initially synthesized as polyprotein precursors that are subsequently processed by viral or cellular proteases into mature, particle-associated proteins. The 55-kDa Gag precursor, $Pr55^{Gag}$, is the major HIV-1 structural protein. It is cleaved by the viral protease (PR) into the matrix (MA), capsid (CA), nucleocapsid (NC), p6, and the two spacer peptides SP1 and SP2, during or after the release of progeny virions. The 160-kDa GagPol polyprotein, $Pr160^{GagPol}$, is autocatalysed to generate the viral enzymes: protease (PR), the heterodimeric reverse transcriptase (RT), and integrase (IN) proteins. Proteolytic digestion by a cellular protease converts the glycosylated 160-kD Env precursor, gp160, into the gp120 surface (SU) and gp41 transmembrane (TM) proteins [41]. The other six ORFs encode the regulatory viral proteins: the transactivating regulatory protein (Tat), the Replication viral factor (Rev), the Negative factor (Nef), the Viral Infectivity Factor (Vif), the Viral Protein R (Vpr), and the Viral Protein U (Vpu) [36]. The HIV-1 genome organisation and its protein products are illustrated in Figure 1.4.

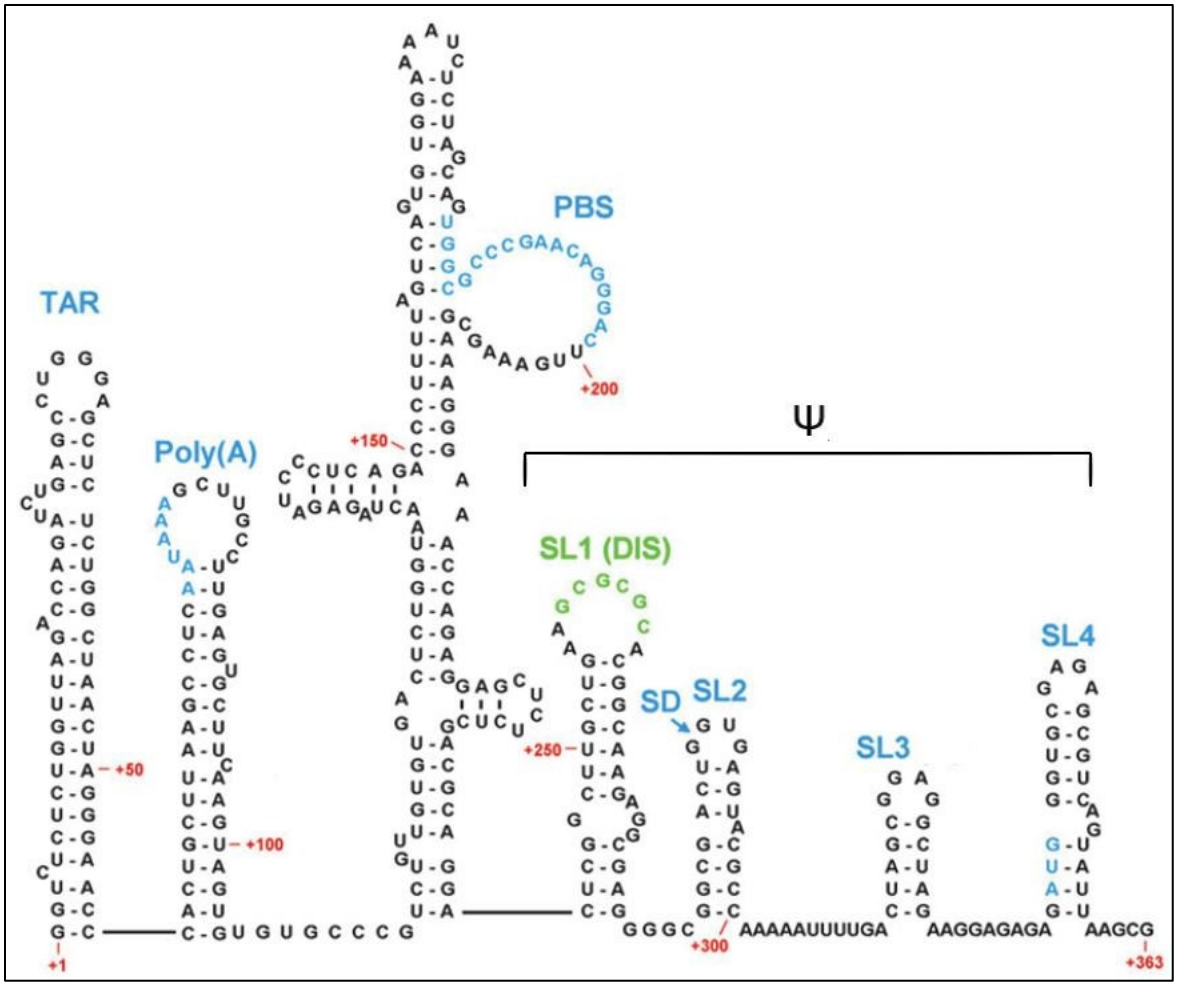

Figure 1.3. Cis-acting RNA elements in the 5'UTR of the vRNA

Illustration of the various cis-acting RNA structures in the HIV-1 5' UTR important for virus replication. These are the TAR element, the poly (A) hairpin, the PBS and the packaging signal Ψ stem-loops 1–4 containing the DIS, the major splice donor SD, and the Gag start codon, respectively. Nucleotides and numbering correspond to the HIV-1 HXB2 sequence. Adapted from [42-44].

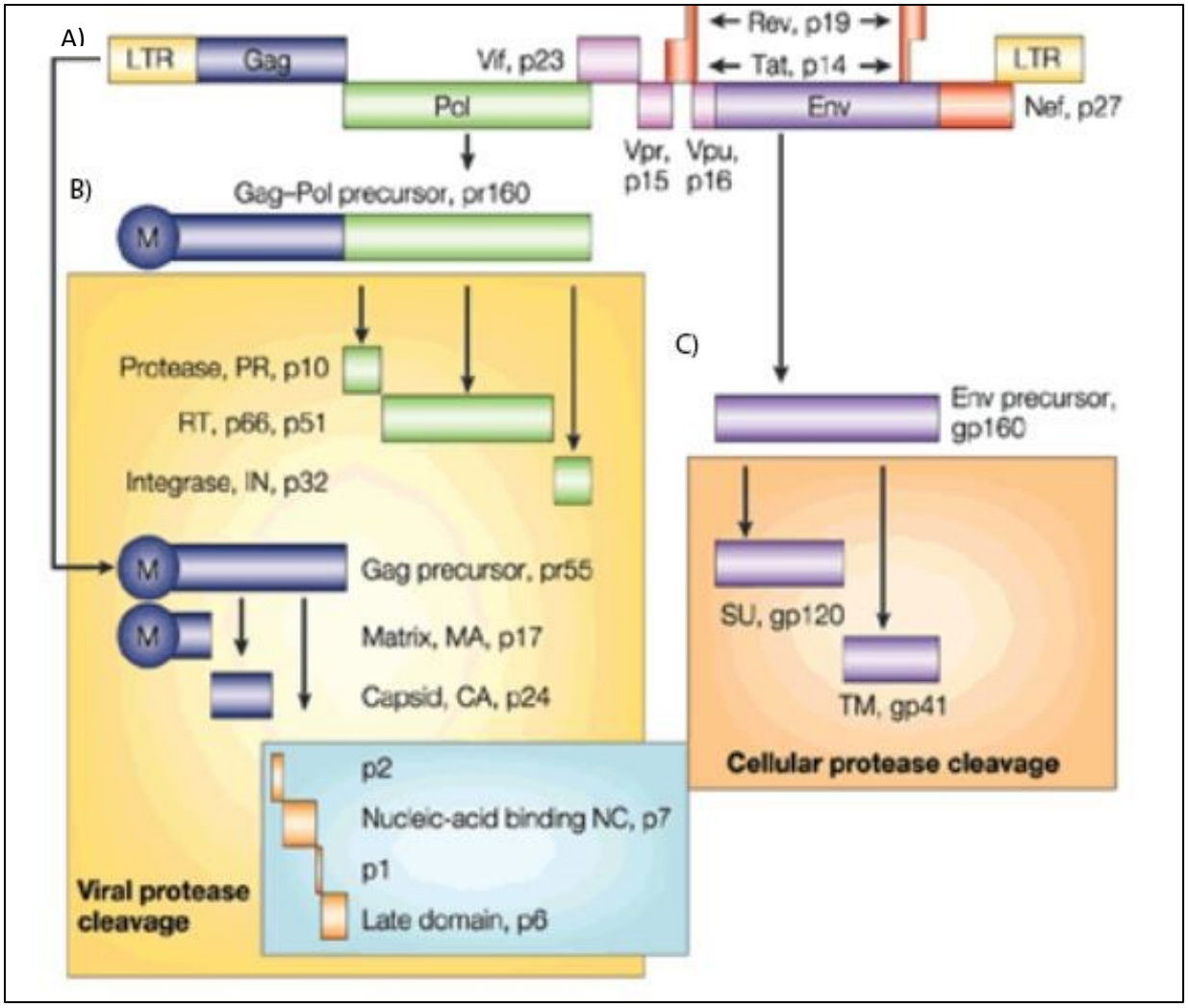

Figure 1.4.: HIV-1 genomic organisation and protein products

A) The HIV-1 genome organisation and the various open reading frames that code for the viral proteins and polyproteins are depicted in different colours. The protein products following proteolytic processing of B) Gag/GagPol and C) Env are also depicted. Adapted and reprinted with permission from [45].

1.3.2. HIV-1 structure

The mature HIV-1 particle is a sphere of approximately 120 nm diameter and is enveloped by a lipid bilayer acquired during the process of budding from the host cell [46]. HIV-1 envelope trimeric gp120–gp41 complexes, composed of the transmembrane glycoprotein gp41 and the external envelope glycoprotein gp120, are embedded in the membrane. The matrix protein (MA) lies below the lipid envelope and surrounds a cone-shaped core comprised of capsid (CA). This viral core contains the two positive-strand vRNAs to which the nucleocapsid (NC) proteins are bound. Numerous host and viral proteins are packaged into the virus and these include protease, the reverse transcriptase, Vpr, Vif and Nef [47]. The mature HIV-1 particle is illustrated in Figure 1.5.

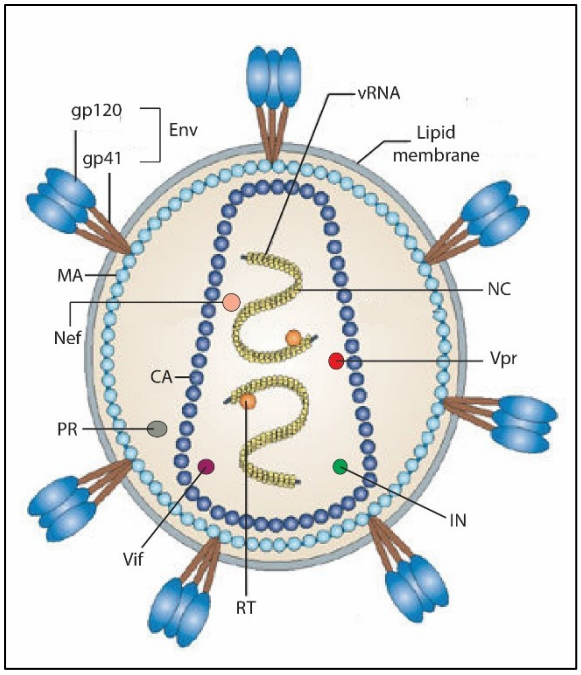

Figure 1.5.: Mature HIV-1 structure

The HIV-1 Env composed of trimeric gp120–gp41 complexes are embedded in a lipid membrane. The cytoplasmic tail of gp41 interacts with the MA. The CA composes the conical core that contains two vRNAs surrounded by the NC protein. PR, RT, Vpr, Vif and Nef are also present in the virus. Adapted and reprinted with permission from [48].

1.4. The HIV-1 replication cycle

The HIV-1 replication cycle can be divided into early and late stages, based on the process of integration. This section will first describe the various steps involved in of the HIV-1 life cycle (depicted in figure 1.6.). The concept of HIV-1 latency and the various factors that contribute to its maintenance will then be discussed in section 1.4.3.

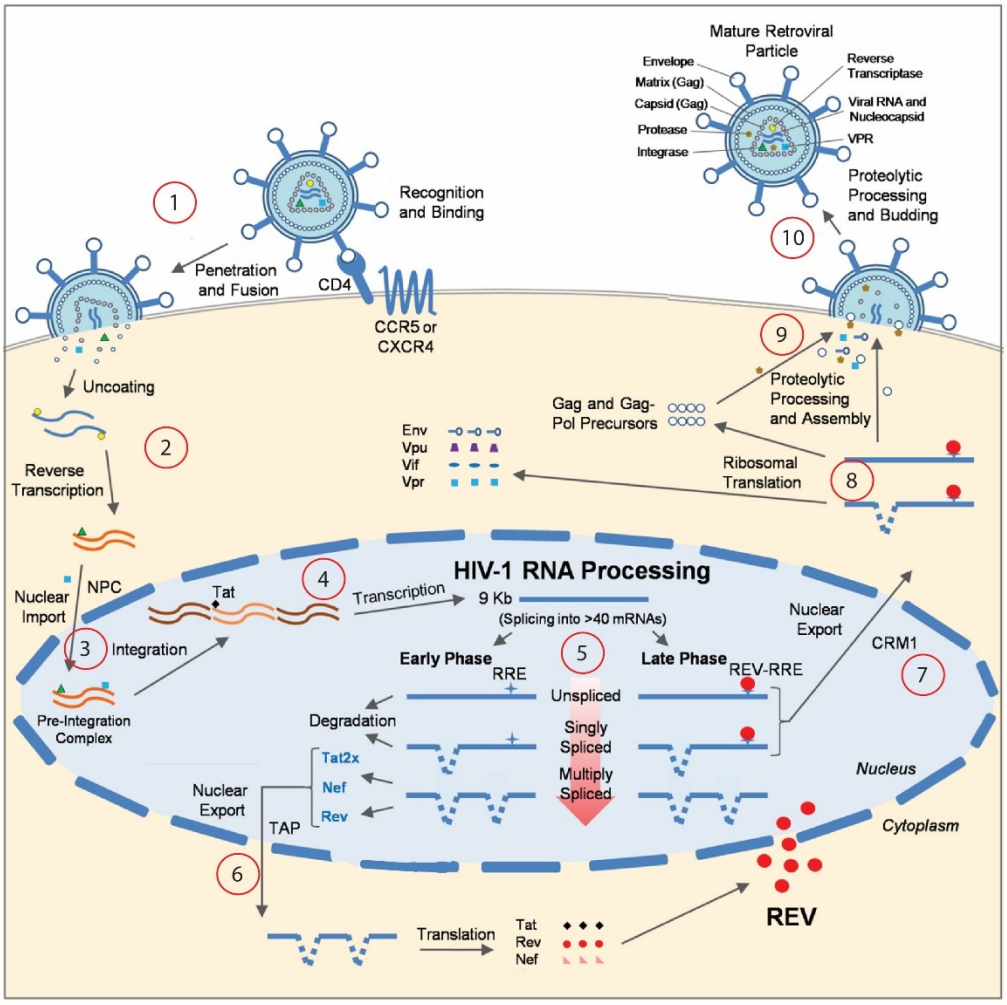

Figure 1.6.: Overview of the HIV-1 life cycle

The early stages of the HIV-1 life cycle is comprised of 1) attachment, fusion and entry, 2) uncoating and reverse transcription, and 3) nuclear import and integration. The steps in the late stages are 4) transcription, 5) vRNA splicing, nuclear export of 6) multiply spliced and 7) unspliced or singly spliced transcripts, 8) translation and vRNA encapsidation, 9) viral assembly, budding and release and 10) maturation. Adapted from [49].

1.4.1. Early HIV-1 replication stages:

1.4.1.1. Attachment, fusion and entry:

The first step of the viral replication cycle involves the binding of the gp120 component of the viral envelope trimer with the CD4 receptor of the host cell [50]. The discovery that CD4 is the main entry receptor for HIV-1 was aided by the observation that HIV-1 infection specifically targets and depletes CD4+ T cells [51]. CD4 is a member of the immunoglobulin (Ig) superfamily and plays a role in the stabilization of the interaction between the T cell receptor and the class II major histocompatibility complex (MHC-II) molecules on antigen-presenting cell [52]. Apart from CD4+ T cells, the CD4 receptor is expressed on monocytes, macrophages and dendritic cells. Despite the expression of CD4 receptor, the efficiency of infection varied between different cell types. This observation paved the way for the discovery of the requirement of either the CXCR4 or CCR5 co-receptor for HIV-1 attachment [53-55]. Differential co-receptor usage explains the tropism of viruses, since T-tropic viruses that preferentially infect T cells use the CXCR4 co-receptor that is primarily present on T cells; whereas the M-tropic viruses that usually infect macrophages use the CCR5 receptor that is expressed on macrophages [56, 57]. The binding of CD4 to gp120 induces a conformational changes that promotes gp120 binding to its co-receptors. Following binding of the coreceptors CXCR4 or CCR5, a sub sequential series of conformational changes take place resulting in the formation of a prebundle and the insertion of the hydrophobic region of gp41 into the target cellular membrane. This insertion brings the viral and cellular membranes to close proximity, thereby allowing fusion of both membranes and release of the viral core into the target cell [58, 59]. The different steps in typical HIV-1 entry into host cells are presented in Figure 1.7. It is important to note that even though receptor/co-receptor binding is the main mode of entry of HIV-1 into its target cells, the virus can also enter the host cell by other mechanisms. HIV-1 has been reported to enter the host cells via endocytosis [60], however viral entry is pH-independent [61]. Cell-to-cell transmission aided by the host cell receptors LFA-1 and ICAM can also occur in HIV-1 virological synapses [62]. Other host receptors such as DC-SIGN and Galectin1 have also been reported to promote cell-to-cell transmission of the virus [63, 64].

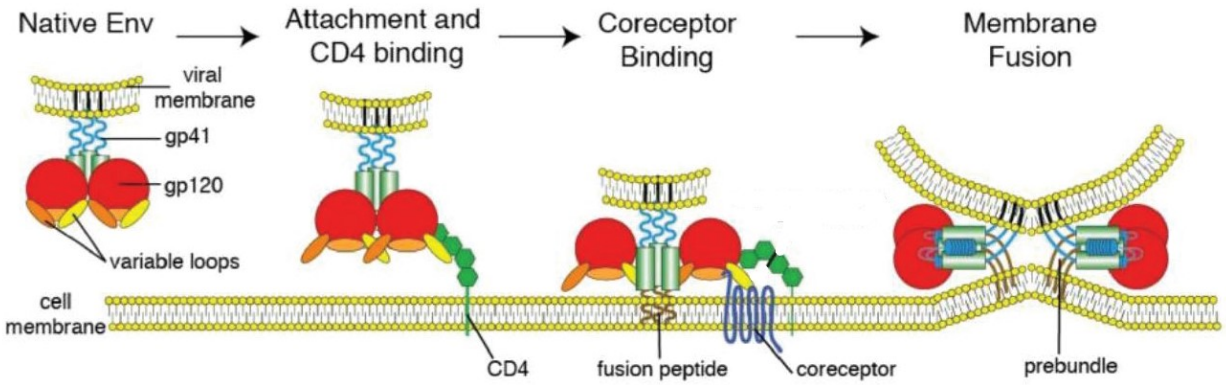

Figure 1.7.: The steps involved in HIV-1 entry and fusion

The main steps of HIV-1 entry are the binding of gp120 (red) to its primary cellular receptor CD4 (green). CD4 binding results in conformational changes that allow binding of gp120 to the coreceptor-either CCR5 or CXCR4. Coreceptor binding results in triggering of the fusion machinery and formation of the six-helix bundle required to drive fusion of the viral and host cell membranes. Adapted from [65].

1.4.1.2. Uncoating and Reverse Transcription:

Upon fusion with the host cell membrane, the viral core composed of the conical CA lattice enclosing the NC-bound viral RNA genome and the viral proteins Vpr, Vif, Nef, RT, and IN is released into the cytosol of the host cell [66]. The CA lattice then undergoes a process of progressive disassembly thereby giving rise to the reverse transcription complex (RTC). This multiprotein complex is composed of the vRNA, RT, NC, IN, Vpr and some remaining CA [67]. Recent findings demonstrate that in the presence of a reverse transcription inhibitor, uncoating is significantly delayed, suggesting that reverse transcription and uncoating are linked [68]. RTCs are primed to initiate the process of reverse transcription which uses the vRNA as a template for the synthesis of complementary DNA (cDNA), a step characteristic of retroviruses [69]. The enzyme that catalyzes this reaction is the viral RT that has two enzymatic functions: DNA/RNA-dependent polymerase activity and RNaseH activity [70]. The NC protein also plays an important role during reverse transcription due to its molecular chaperone and nucleic-acid aggregating activities [71]. The first step of reverse transcription is the binding of tRNAlys3 to the PBS site on the vRNA and tRNAlys3 serves as a template for the RT enzyme generating the (-)DNA strand [39]. This is followed by the degradation of the (+)strand RNA via the RNAseH activity of the RT enzyme [72]. The vRNA contains two specific purine-rich sequences, known as the polypurine tracts (PPTs), that are resistant to the RNase H cleavage and are necessary for the process of reverse transcription. Finally, the (-)DNA strand is then used as a template for the (+)DNA strand synthesis and the double-stranded proviral cDNA is synthesized [69]. The various steps involved in the process of reverse transcription are described in Figure 1.8.

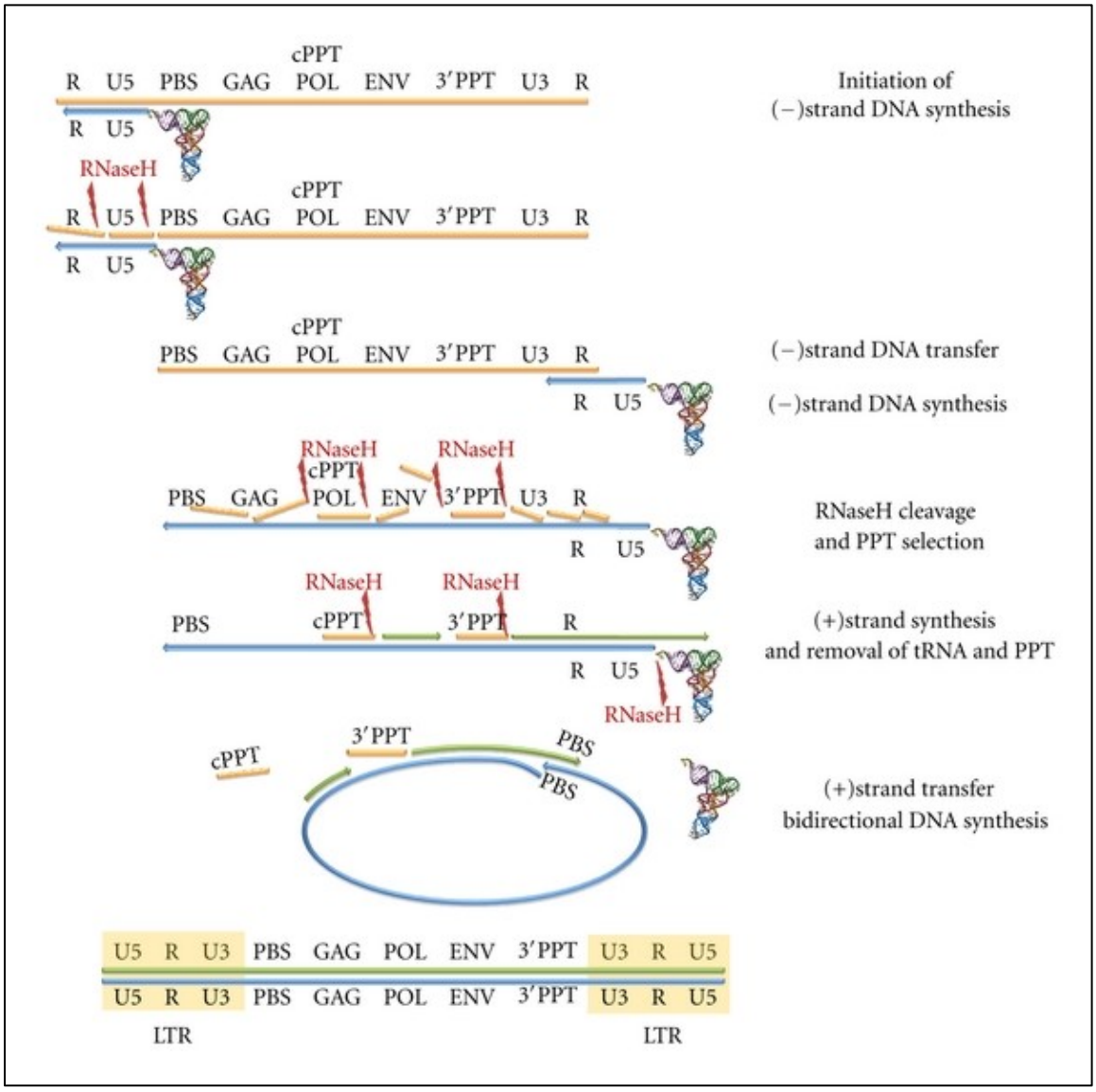

Figure 1.8.: The different steps involved in HIV-1 reverse transcription

tRNALys3 binds to the PBS near the 5′-end of the (+)strand RNA genome (orange) and behaves as a primer for (−)strand DNA (blue) synthesis. Strand transfer of the (−)strand DNA and its further elongation takes place. DNA synthesis proceeds, and the RNase H function cleaves the RNA strand of the RNA:DNA at numerous points leaving intact two PPTs. (−)strand DNA synthesis (green) initiation using PPTs as primers takes place, concluding in the formation of a linear dsDNA product. Reprinted with permission from [73].

1.4.1.3. Nuclear Import and Integration:

Upon completion of reverse transcription, the newly synthesized viral cDNA assembles into a new complex called the pre-integration complex (PIC) that is comprised of both viral and host proteins essential for the step of nuclear import [74]. Since the size of the PIC is greater than the passive diffusion limit of nuclear pores, it has long been assumed that the PIC must harbor determinants that promote active nuclear import [75]. The viral proteins implicated to play a role in nuclear import are CA, MA, Vpr and IN [76-79]. It has also been proposed that a cis-acting, triple-stranded DNA structure known as the central DNA flap, which is a product of lentiviral reverse transcription, promotes nuclear import of the PIC [80]. Host proteins also play a role in nuclear import such as Transportin 3 TNPO3, the nuclear proteins Nup153 and Nup98, Importin 7 and cleavage and polyadenylation factor 6 CPSF6 [81-85]. Through a series of interactions between the PIC, the nuclear pore complex (NPC), and host factors, the PIC complex enters the nucleus [86].

The last step of the early stages of the HIV-1 life cycle is the integration of the viral DNA into the host cell genome. The viral enzyme integrase IN plays a crucial role in this process along with multiple host proteins such as LEDGF/p75 and INI1 [87]. Integration takes place in three steps: 3' end processing, strand transfer and gap repair. 3' end processing takes place before the PIC is translocated into the nucleus, where the IN enzyme cleaves and removes two nucleotides (usually GT) at both extremities of the LTR generating a 5' overhang and a free 3'OH group. In the nucleus, IN catalyzes a staggered cleavage in the cellular target DNA. Strand transfer takes place where and the 3' recessed ends of the viral DNA are joined to the 5' overhanging termini of the cleaved cellular DNA. Finally, cellular repair enzymes close the gap and the viral DNA is successfully integrated into the host cell genome [41].

1.4.2. Late HIV-1 replication stages:

1.4.2.1. Transcription:

The transcription of the integrated HIV-1 provirus is driven by the LTR promoter employing the cellular RNA polymerase-II [88]. At first, this transcription generates low levels of a full length, poly-cistronic mRNA that is multiply spliced to generate a 2-kB mRNA. This multiply spliced RNA can be exported into the cytoplasm using the nuclear RNA export factor 1 (NXF1) pathway and codes for the viral proteins Tat, Rev and Nef [89]. Tat then shuttles back into the nucleus and plays a pivotal, essential role in vRNA transcription. Tat binds to the positive transcription elongation factor pTEFb, composed of cyclin T1 and the cyclin dependent kinase 9 (CDK9), and together this complex then binds to the 5' transactivation response RNA (TAR) on initiated transcripts where CDK9 phosphorylates RNA polymerase II [90]. Phosphorylation results in a burst in proviral transcription and, following alternative splicing events, generates multiple variants of 9-kb unspliced RNAs, 4-kb singly-spliced RNAs and the multiply-spliced 2-kb RNAs [89].

1.4.2.2. Viral RNA splicing:

HIV-1 alternative splicing is a controlled process generating more than 40 different mRNAs that are broadly categorised into 9-kb unspliced RNAs, 4-kb singly-spliced RNAs and the multiply-spliced 2-kb RNAs [90, 91]. These mRNAs are generated as a consequence of alternative selection of the four splice donors (SD) and the eight splice acceptors (SA) embedded in the viral genome and the presence of adjacent cis-acting RNA elements [89, 92]. These cis-acting elements can either be enhancing or silencing sequences. They modulate alternative splicing of the HIV-1 primary RNA transcript by binding to the splicing regulators (SR) proteins and heterogeneous ribonucleoprotein particles (hnRNPs) [93]. The unspliced RNA (vRNA) codes for Gag and GagPol; singly-spliced RNA species code for Vif, Vpr, Tat and Env/Vpu; and Tat, Rev and Nef are generated from multiply-spliced transcripts [89]. The different HIV-1 RNA species are illustrated in Figure 1.9.

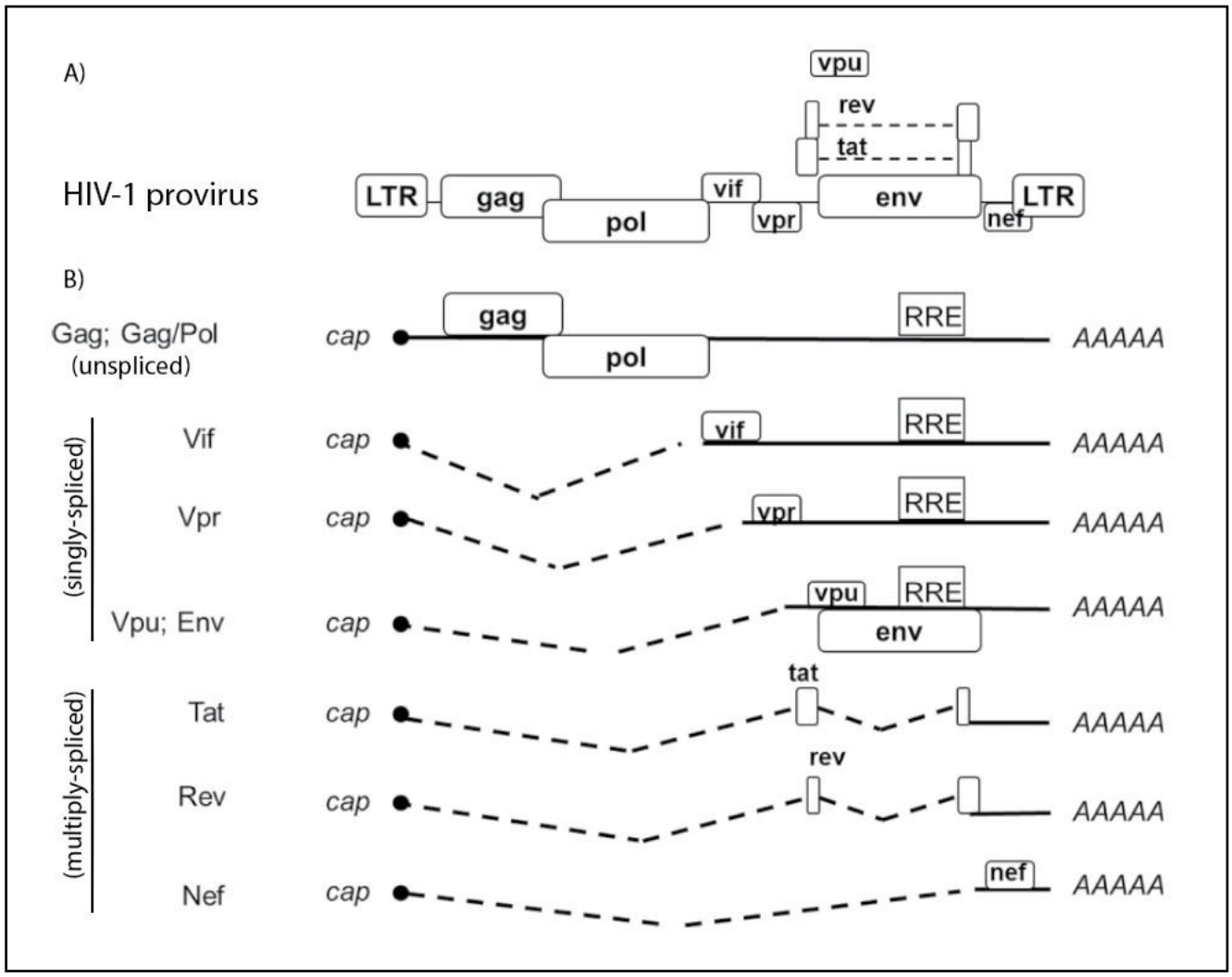

Figure 1.9.: HIV-1 RNA splicing products

A) The LTR promoter from the HIV-1 provirus drives HIV-1 transcription. B) The unspliced, genomic RNA (vRNA) and various transcripts generated by alternative splicing, with the corresponding proteins they code for on the left, are depicted. The dashed lines connect the major splice donor sites to the appropriate splice acceptor. Adapted and reprinted with permission from [40].

1.4.2.3. Viral RNA export and stability:

Multiply-spliced viral RNAs can be exported from the nucleus into the cytoplasm via the NXF1 pathway [89]. However, singly-spliced and unspliced HIV-1 RNAs contain introns that block their NXF1-mediated nuclear export. HIV-1 has overcome this hurdle through the action of the viral protein Rev [94]. Rev, translated from multiply-spliced viral RNA in the cytoplasm, is imported to the nucleus through an interaction between its nuclear localisation signal (NLS) and the nuclear import receptor importin β. Rev then binds to and multimerises on the cis-acting RNA element called the Rev Responsive Element (RRE) present on both the singly spliced and unspliced viral RNAs via its NLS region [95, 96]. Rev also interacts with RanGTP and the export protein CRM1 (chromosome region maintenance 1) via its nuclear export signal (NES) to form a nuclear Rev-viral RNA RNP that can be exported via the nuclear pore [97]. Once in the cytoplasm, the complex is disassembled through the hydrolysis of RanGTP to RanGDP. Therefore, HIV-1 ensures the nucleocytoplasmic export of the singly spliced and unspliced RRE-containing transcripts via the action of Rev. Numerous host proteins have been reported to synergize Rev-mediated export pathway such as UPF1, hnRNP A1, DDX3, DDX1 and Sam68 [98-102]. The unspliced vRNA contains numerous cis-acting RNA sequences that impair mRNA stability, nucleocytoplasmic export and translation initiation [103, 104]. These instability sequences (INS) have high AU contents and are present on Gag, Pol and Env genes [103, 105-107]. In the absence of Rev, the unspliced and singly spliced HIV-1 transcripts that are retained in the nucleus are either downregulated by further splicing or are degraded [108-110]. The inhibitory of effects of the INS on viral gene expression are counteracted by Rev-RRE interaction [103]. Therefore, the viral protein Rev functions to promote both the nucleocytoplasmic export and stability of the vRNA.

1.4.2.4. Viral RNA translation and encapsidation:

Following its nucleocytoplasmic export, the vRNA plays a critical role in the formation of new infectious virions. The vRNA has two main fates: to serve as the template for the major HIV-1 structural and enzymatic proteins Gag and GagPol, or to be packaged into virions to serve as the genomic vRNA for budding viruses. The majority of vRNA is not captured for encapsidation but serves other roles in generating viral proteins or as a cofactor for assembly [111-114]. The regulation of the vRNA's fate, that is, whether it is translated or encapsidated, is reported to be via a putative model involving a conformational switch in the secondary structure of the vRNA [115]. The vRNA long-distance interaction (LDI) secondary structure is used in translation, while the branched multiple hairpin (BMH) secondary structure is used for genome encapsidation [115]. The binding of the NC protein to the vRNA is said to cause a rearrangement from the LDI to the BMH conformation [116]. The different conformations of the vRNA are illustrated in Figure 1.10.

HIV-1 transcripts are predominantly translated by cellular cap-dependent mechanisms; however, translation from two internal ribosome entry sites (IRES) can also occur [117-119]. Although co-translation of the vRNA seems to enhance its packaging [114], translation of the vRNA is not required to generate infectious virus particles [111]. It has also been proposed that viruses assemble distinct RNPs to serve as a scaffold for encapsidation [120, 121]. The host cell protein Staufen1 has been reported to facilitate viral encapsidation via an interaction with the NC domain of the Gag polyprotein [120, 122, 123]. It has been demonstrated that during HIV-1 infection, there is assembly of Staufen1-containing HIV-1 RNPs (SHRNPs) that is involved in assembly, localization and encapsidation of vRNA [120]. The selectivity of HIV-1 genomic RNA in virus particles is thought to be mediated by the encapsidation signal (Psi, ψ). Psi is a cis-acting RNA sequence of no less than 100 nucleotides in length and binds to the NC region of Gag. The binding of NC to Psi mediates the incorporation of two single-stranded RNA molecules linked together by the dimer initiation site (DIS) into the budding virion. [40, 124].

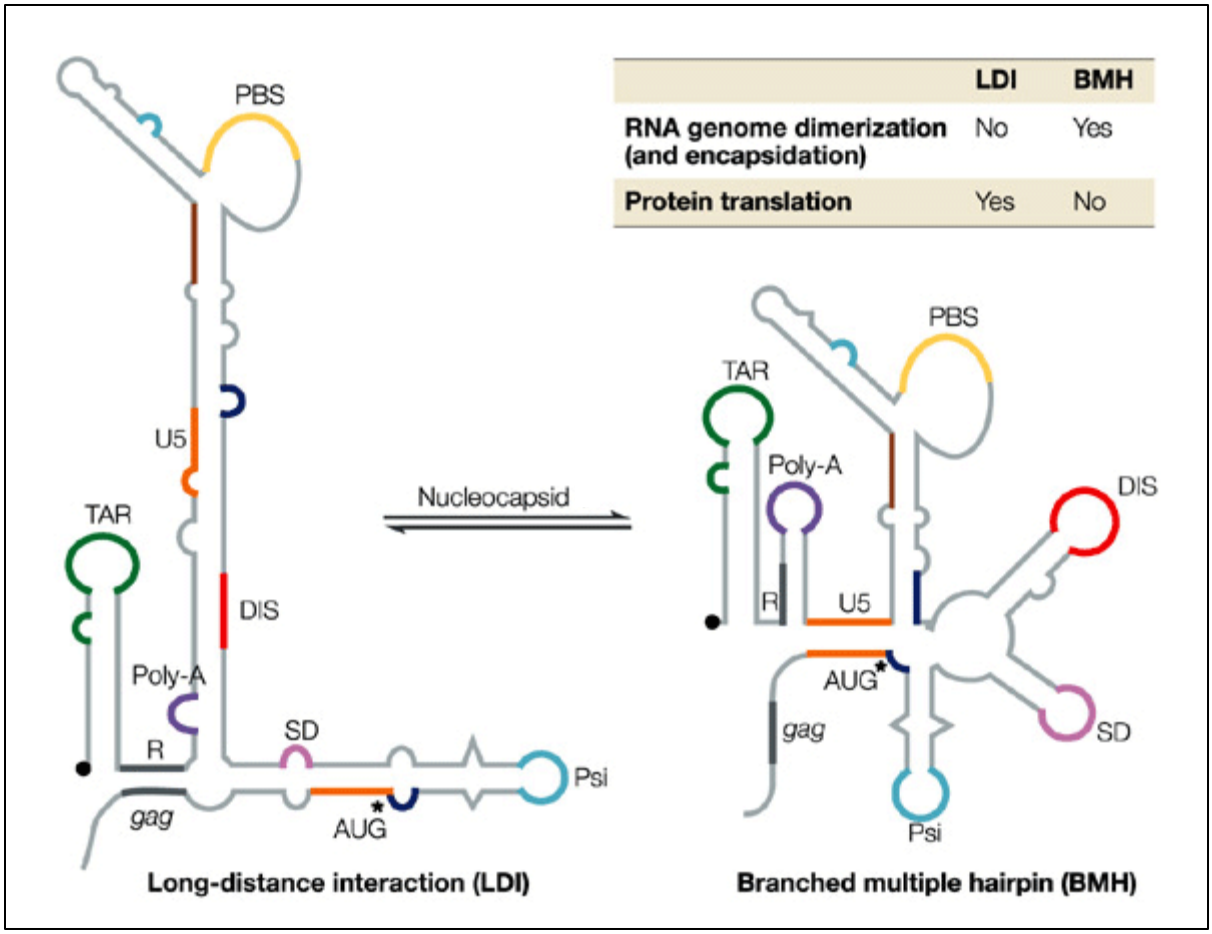

Figure 1.10.: Secondary structure of the HIV-1 vRNA

The long-distance interaction (LDI) secondary structure is proposed to promote vRNA translation. The binding of HIV-1 NC is said to induce a conformational change to the branched multiple hairpin (BMH) secondary structure, thus allowing the genome to be encapsidated through exposure of the dimerization initiation site (DIS). Reprinted with permission from [125].

1.4.2.5. Viral assembly, budding and maturation:

The Gag polyprotein is a central player in viral assembly and can alone assemble into virus-like particles. After its synthesis, Gag associates to plasma membrane sites rich in PI(4,5)P2 and cholesterol, such as lipid rafts and tetraspanin-enriched microdomains [126]. Gag is targeted to these sites by virtue of the myristoylation of its MA domain where it accumulates and multimerizes through several Gag-Gag interactions [127]. The Env glycoprotein complex of gp41 and gp120 traffics to cell membranes from the endoplasmic reticulum and is recruited to an assembling virus. Encapsidation of the vRNA takes place as described in the previous section. Budding of the virus as the plasma membrane is facilitated by the interactions of the C-terminal p6 domain of Gag and the members of the endosomal sorting complexes required for transport (ESCRT) machinery, TSG101 and ALIX [128, 129]. Maturation begins during the process of budding and is carried out by the viral enzyme protease. The PR cleaves GagPol into free PR, IN and RT as well as the Gag polyproteins into MA, CA and NC proteins [130]. This process triggers major morphological changes to the structure of the virus resulting in the generation of a mature, infectious viral particle (Figure 1.11.) and in the successful completion of the viral replication cycle. The overall processes of viral assembly, budding and maturation are illustrated in Figure 1.12.

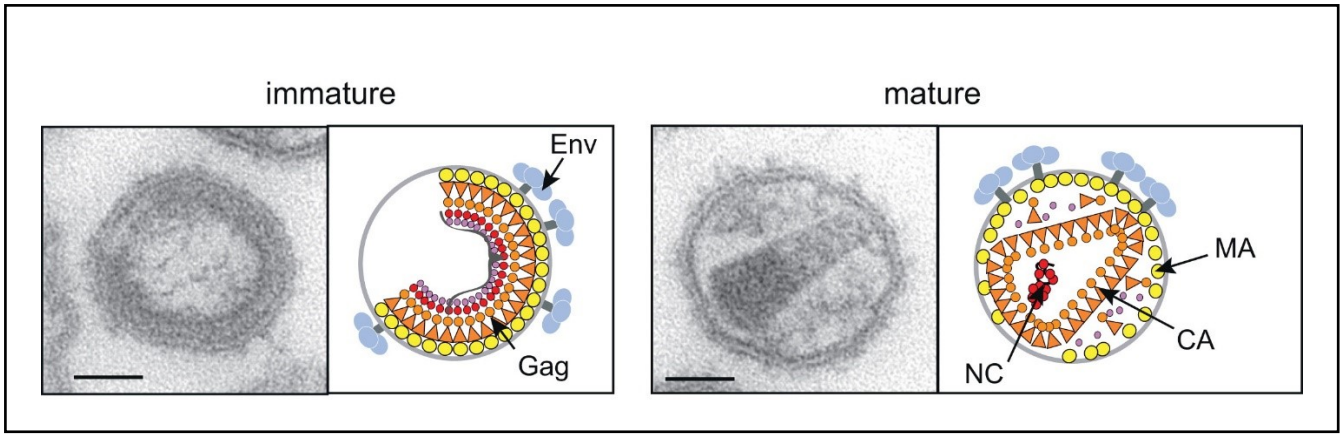

Figure 1.11.: Morphological changes induced by HIV-1 maturation

Thin-section electron micrographs of immature and mature HIV-1 and sketches indicating the position of Gag domains and their rearrangement after HIV-1 maturation are depicted. Reproduced with permission from [131].

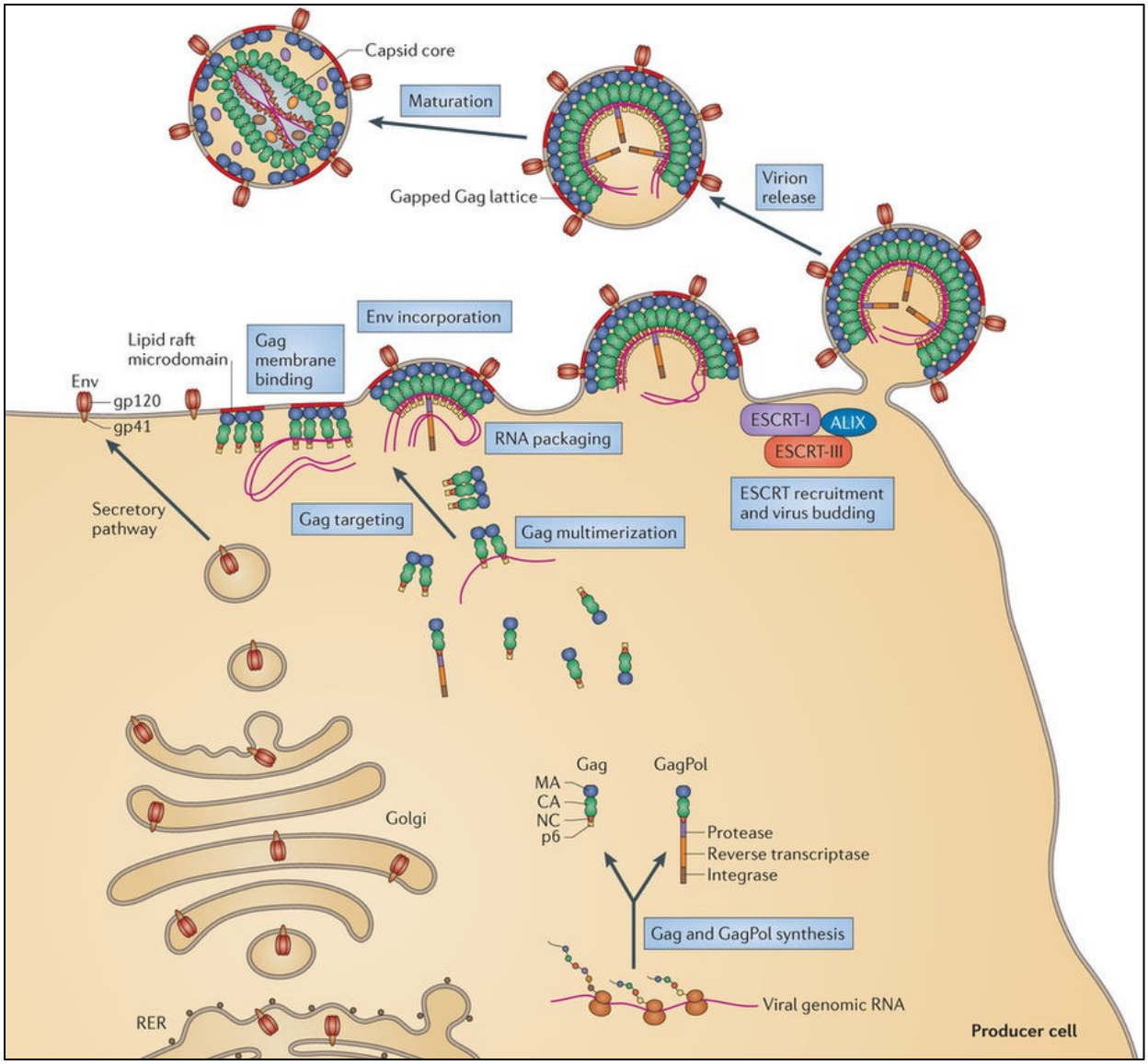

Figure 1.12.: HIV-1 assembly, budding and maturation

The steps of Gag and Env trafficking to the plasma membrane, Gag multimerisation, vRNA encapsidation, ESCRT-mediated viral release and protease-mediated viral maturation are illustrated. Reprinted with permission from [132].

1.4.3. HIV-1 Latency

Latency in HIV-1 infection is defined as a reversibly non-productive state of infection which is characterised by the presence of infected cells that do not actively produce viral particles, but retain the ability to do so [133]. It was first discovered in 1997 when integrated, replication-competent HIV-1 was recovered from resting memory CD4+ T cells [134, 135]. The viral reservoir persists in the presence of cART and is the major obstacle towards an HIV-1 cure [136]. The reservoir is mainly comprised of resting, memory CD4+ T cells, along with other cell types such as monocytes, macrophages, dendritic cells and astrocytes in peripheral blood and other anatomical compartments such as the gut, lymph nodes and central nervous system [137, 138]. This section describes the molecular mechanisms underlying HIV-1 latency. For the implications of HIV-1 latency in HIV cure research, see section 1.5.3.

Following integration into the host cell, there are many molecular mechanisms that govern the maintenance of HIV-1 latency. The site of integration itself contributes the maintenance of latency and HIV-1 is believed to preferentially integrate into actively transcribing genes and transcriptional interference can contribute to HIV-1 latency [139-141]. Since the HIV-1 provirus is embedded into the host chromatic, epigenetic regulation of chromatic structure has also been demonstrated to influence HIV-1 latency [142-144]. The HIV-1 LTR is organised into nucleosomes near the transcription start site which promotes the maintenance of latency [145-147]. The CpG-methylation of the HIV-1 LTR also contributes to latency by preventing the binding of transcription factors such as NF-κB and Sp1 [148]. Histone acetylation and methylation also influence viral gene expression with the histones of latent HIV-1 proviruses being deacetylated and having more methylation marks [149]. The lack of the transcription factors NF-κB, NFAT and Sp1 in resting T cells also promotes latency [150]. The viral proteins Tat and Vpr also have been reported to influence HIV-1 latency. Defects in Tat promote HIV-1 latency by inhibiting transcription [151, 152] and Vpr promotes LTR transactivation and viral reactivation [153, 154].

Most of the investigations on the molecular mechanisms that control HIV-1 latency have focussed on impediments at the level of viral integration and proviral transcription [149]. However, following transcription, just like any other cellular mRNA, the vRNA needs to be effectively spliced, exported and translated to express viral proteins, indicating that post-transcriptional

events can also affect viral latency. For example, a study that showed that latent, resting CD4+ cells contain not only integrated proviral DNA as expected, but also cell-associated unspliced HIV-1 RNA [155]. In a similar vein, another report showed that latent resting CD4+ T cells contain both genomic vRNA as well as multiply spliced HIV-1 RNA sequestered within the nucleus, and that those could be efficiently rescued through the overexpression of the host protein polypyrimidine tract binding protein (PTB) [156]. Two characterised primary T cell models of latency have also demonstrated a post transcriptional block to HIV-1 reactivation [157, 158]. In a model where latency is established by infecting resting cells after chemokine treatment [159], high levels of multiple spliced RNA and low levels of vRNA are sequestered in the nucleus with the absence of viral proteins [157], highlighting a block at the level of nuclear export. In a different resting cell latency model [160], latently infected cells had unspliced transcripts ~100-fold more abundant than singly spliced transcripts and ~10,000-fold more abundant than multiply spliced transcripts [158], thereby highlighting the roles of splicing in the maintenance of HIV-1 latency. In addition, numerous microRNAs have been implicated in the maintenance of HIV-1 latency (reviewed in [161]), providing another example of how post-transcriptional events can affect proviral reactivation.

1.5. HIV-1 therapies

Combination antiretroviral therapy (cART) is currently used to treat HIV-1 and is comprised of an arsenal of drugs that act at different stages of the viral life cycle. In the following section, the advent of this therapy, its advantages and its limitations will be first described. The recent advances in the field of HIV-1 vaccine research will then be discussed. Finally, this section will focus on the strategies employed towards the development of an HIV-1 cure.

1.5.1. HIV-1 drugs:

In the early days of the AIDS epidemic, HIV-1 infection had a very high mortality rate. However, the landscape of the disease has changed dramatically since the discovery of antiretroviral drugs (ARV). In 1987, azidothymidine (AZT), a nucleoside analogue targeting the HIV-1 RT enzyme, was the first ARV to be discovered [162]. However, the use of AZT monotherapy led to the emergence of resistance mutations. An understanding of HIV-1 biology has led to the discovery of 27 other ARVs approved by the United States Food and Drug Administration (US FDA) (Table 1.2.). In 1996, combination antiretroviral therapy (cART) (then called highly active antiretroviral therapy or HAART) was first implemented [163, 164]. cART is now the standard of care for HIV-1 infection and includes a combination of three or more ARVs from at least two different classes. Based on the stage of the viral replication cycle that the drugs act upon, the current ARVs are classified into the following 6 classes: CCR5 inhibitors, fusion inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) block, integrase strand transfer inhibitors (INSTIs), and protease inhibitors [164]. The stage of the life cycle that these ARVs target are depicted in Figure 1.13. The current US-FDA approved ARVs are listed in Table 1.2.

The implementation of cART has been extremely beneficial and has resulted in HIV-1 infection being likened to a chronic condition rather than an acutely life-threatening disease, with patients on successful cART having near normal life expectancy [11, 165]. Furthermore, cART implementation has begun to reduce the spread of new cases of HIV-1 infection [166]. cART is also used as Pre-exposure Prophylaxis (PreP) in individuals with high risk of exposure, highlighting another important function of cART in HIV-1 transmission prevention [167].

Despite these advantages, cART is not without drawbacks. One of the biggest disadvantages of cART is that it is not curative. To effectively supress viremia, the infected individual must be on therapy for the entire duration of their lifetime. This is due to the ability of the virus to form a stable latently infected reservoir (see section 1.4.3. on viral latency) [28]. This implies that despite cART treatment, replication-competent HIV-1 persists even after prolonged suppression of viremia [135]. It is estimated that it would take around 70 years of cART treatment to clear HIV-1 from the viral reservoir [134]. Upon interruption of treatment, there is a rapid rebound of plasma viral loads [168]. This necessity for lifelong HIV-1 treatment has many drawbacks such as adverse side effects that prevent drug adherence, the development of drug resistance and economic repercussions [169-171]. Furthermore, of the 36.7 million people currently infected with HIV-1, 15.8 million people are not on cART (UNAIDS). Therefore, there still remains a need for novel longer-lasting ARVs, an HIV-1 vaccine or curative HIV-1 therapies.

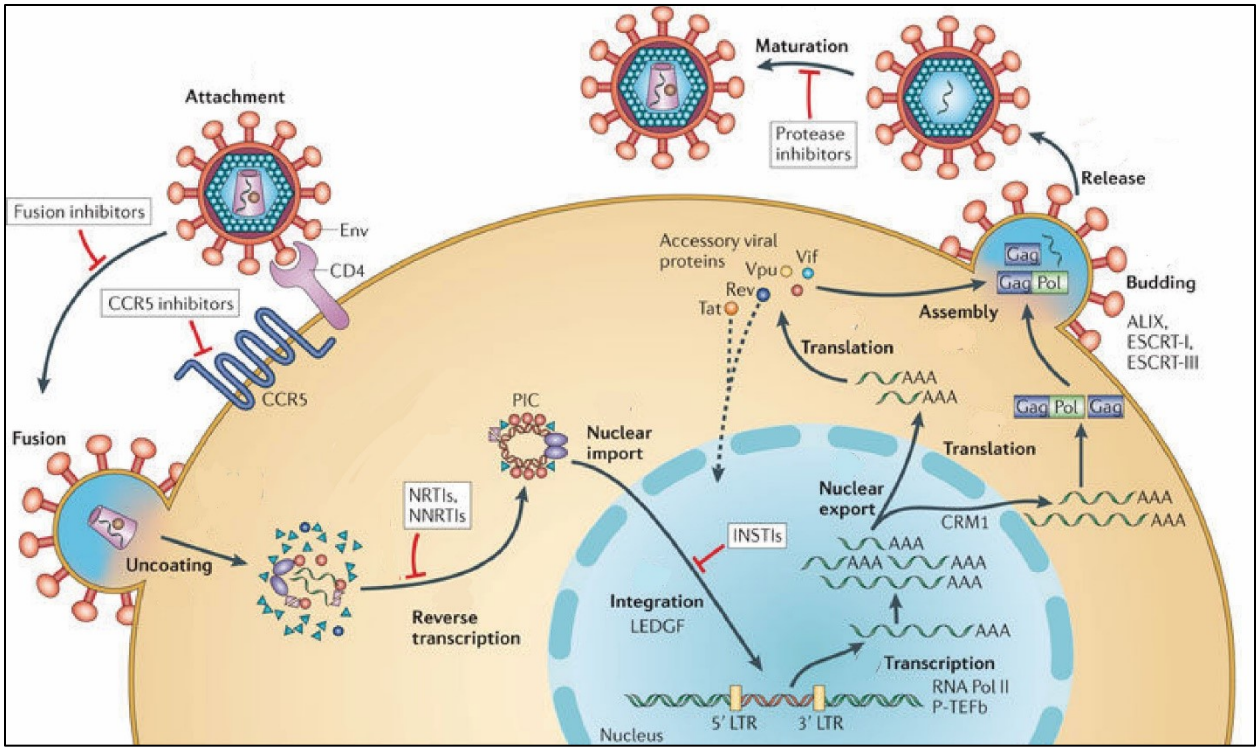

Figure 1.13.: ARV intervention at stages of the HIV-1 replication cycle

The steps at which CCR5 inhibitors, fusion inhibitors, NRTIs, NNRTIs, INSTIs and protease inhibitors inhibit HIV-1 replication are highlighted. Adapted and reprinted with permission from [172].

Abbreviation	ARV	Year of FDA approval
CCR5 Inhibitors		
MVC	Maraviroc	2007
Fusion Inhibitors		
T-20	Enfuvirtide	2003
Nucleoside Reverse Transcriptase Inhibitors		
AZT/ZDV	Zidovudine	1987
ddI	Didanosine	1991
ddC	Zalcitabine	1992
d4T	Stavudine	1994
3TC	Lamivudine	1995
ABC	Abacavir	1998
TDF	Tenofovir	2001
FTC	Emtricitabine	2003
Non-Nucleoside Reverse Transcriptase Inhibitors		
NVP	Nevirapine	1996
DLV	Delavirdine	1997
EFV	Efavirenz	1998
ETR	Etravirine	2008
RPV	Rilpivirine	2011
Integrase Inhibitors		
RAL	Raltegravir	2007
DTG	Dolutegravir	2013
EVG	Elvitegravir	2014
Protease Inhibitors		
SQV	Saquinavir	1995
RTV	Ritonavir	1996
IDV	Indinavir	1996
NFV	Nelfinavir	1997
APV	Amprenavir	1999
LPV/RTV	Lopinavir+Ritonavir	2000
ATV	Atazanavir	2003
FOS-APV	Fosamprenavir	2003
TPV	Tipranavir	2005
DRV	Darunavir	2006

Table 1.2.: Current list of US-FDA approved ARVs

Table adapted from: http://www.avert.org/antiretroviral-drugs.htm

1.5.2. HIV-1 vaccines:

Over the last few years, many strategies have been successfully implemented to reduce the transmission of HIV-1, such as the use of cART treatment as prevention (TraP) [166], ARVs as PreP [173] and the use of vaginal microbicides [174]. Despite these interventions, there were still 1.8 million people who acquired the disease in 2016. A study that modeled the impact of current and novel approaches on the control of the HIV-1 pandemic found that the development of an HIV-1 vaccine could serve as the single most effective way to prevent the spread of HIV-1 [175]. A successful HIV-1 preventative vaccine remains elusive to this date and according to International AIDS Vaccine Initiative Clinical Trials Database, of the over 200 HIV-1 vaccine candidates / regimens have been clinically tested since 1986, only six of them have completed efficacy trials. RV144 remains the only efficacy trial that demonstrated a modest vaccine efficacy of 31.2% [176].

Two main points challenge the development of a successful HIV-1 vaccine. Firstly, the immune-mediated correlates of protection for HIV-1 have not been conclusively identified. Secondly, the virus is capable of profound and durable variability, particularly within the viral envelope gene that encodes the proteins most readily accessible by the immune system [177]. Most effective vaccines prevent infection by eliciting the production of neutralising antibodies. However, the efficacy in the RV144 trial was associated with non-neutralizing IgG antibodies to Env that could mediate viral clearance through antibody-dependent cell-mediated cytotoxicity (ADCC) or by the stimulation of CD8+ T cells [178].

Currently, there are multiple clinical trials running to build on the RV144 findings, as well as using strategies to generating or delivering broadly neutralizing antibodies (bNAbs) or generating effector memory T cell responses with the use of live, replicating viral vectors (reviewed in [179]. Most recently at the 9th International AIDS Society conference (IAS 2017), exciting development results from an early stage Phase 1/2a APPROACH study using a mosaic HIV-1 vaccine were announced and was shown to reduce the pre-exposure risk of infection by 94%. The use of this vaccine also resulted in complete protection in 66% of participants after six exposures. Another important avenue of HIV-1 vaccine research is the development of therapeutic vaccines that

could help HIV-1 infected individuals control the infection without cART, prevent progression to AIDS and potentially even reduce the size of the viral reservoir (reviewed in [180]). The development of a therapeutic vaccine could pave the way for a functional cure for HIV-1 (see section 1.5.3.1.).

1.5.3. HIV-1 Cure:

There are two main avenues to pursue when working towards an HIV-1 cure. The first is a sterilising cure in which every cell from the viral reservoir containing a replication-competent provirus is eliminated. The second is a functional cure by which the HIV-1 infected individual is able to control viral replication and prevent progression to AIDS in the absence of cART. These two strategies and the various methods to achieve them are discussed in the following sections.

1.5.3.1. Functional Cure:

A functional cure implies the control of HIV-1 replication in the absence of cART, without necessarily eliminating the virus from reservoirs. This phenotype is seen in a small group of patients (less than 1%) who are naturally able to control their viral loads to below detection level for years [181]. The molecular mechanisms underlying suppression of viremia in elite controllers have been investigated [182] and one of the factors that could contribute to viral control is the smaller size of the viral reservoir of the elite controllers [183]. Another group of people with a similar phenotype are the post-treatment controllers who demonstrate the control of the plasma viremia following cessation of cART [184]. Early initiation of cART has been linked to the reduced size of the viral reservoir and longer durations of ART-free remissions such as that seen in the Mississippi baby, patients of the ANRS VISCONTI (Viro-Immunologic Sustained Control after Treatment Interruption) cohort and in a young woman from the French Agence Nationale de Recherche sur le Sida (ANRS) EPFCO10 pediatric cohort [185-188]. Thus, one strategy to achieve a functional cure is the early initiation of cART. However, according to studies in simian models, the latent reservoir is seeded as early as 3 days after infection [189]. Although early treatment is extremely beneficial, it might not be enough to prevent viral control in most patients where the infection is diagnosed later that 3 days post infection.

An alternative strategy suggested for a functional cure is called the “block and lock” strategy in which HIV-1 transcriptional inhibitors such as didehydro-Cortistatin A (dCA) could block ongoing viremia during cART, thereby locking the HIV-1 promoter in persistent latency [190, 191]. In the quest for an HIV-1 cure, it would also be interesting to evaluate if post-transcriptional inhibitors of viral gene expression could be used to elicit a state of deep latency in cells. Therapeutic vaccines could also serve as a functional cure by stimulating the host immune system to clear out virus producing cells following reactivation from latency, thus controlling viremia [192].

Gene therapy using zinc-finger nucleases (ZFNs), TALENs (transcription activator-like effector nucleases), RNA interference or the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system is another approach to achieve an HIV-1 cure [193, 194]. One gene therapy strategy is to create host cells that have a mutation in the CCR5 gene so that they will become resistant to R5 tropic viruses [195, 196]. Another method is to use gene-editing techniques to excise the HIV-1 provirus from the host genome. With the advent of CRISPR/Cas9 technology, several groups have been able to successfully eliminate the HIV-1 provirus from primary T cells, pro-monocytes Jurkat cells and microglial cells in vitro [197-199]. This technique has an important pitfalls being that although Cas9/sgRNA can inhibit HIV-1 replication initially, HIV-1 escape variants are soon produced due to the non-homologous end-joining repair, and contained mutations around the Cas9 cleavage sites [200]. Gene-editing strategies should therefore be designed with precision and consider the implications of viral escape.

1.5.3.2. Sterilising Cure:

Since the major obstacle to an HIV-1 cure is the establishment of a latent viral reservoir, one strategy towards an HIV-1 cure is to eliminate the latent reservoir using the "kick and kill" strategy [201]. This strategy entails the activation of virus production using latency-reversing agents (LRAs) (the "kick") followed by the triggering their cell death through virus-induced cytolysis or immune-mediated clearance (the "kill"). LRAs serve to increase viral gene expression from latency, and include histone deacetylase inhibitors (HDACis), histone methyltransferase (HMT) inhibitors, DNA methyltransferase inhibitors, bromodomain inhibitors, protein kinase C (PKC) agonists, PI3K/Akt pathway inhibitors and agonists for the innate immune receptors TLR7 or TLR9 [202-204]. To date, clinical trials with LRAs have demonstrated that activation of viral gene expression is possible *in vivo*, but there is limited to no reduction in the size of the viral reservoir [133, 205]. Most latency-reversing agents act at the level of transcription to promote viral gene expression. However, post-transcriptional events also affect viral gene expression and reactivation from latency [155, 157, 158, 160, 161, 206]. This is highlighted in a recent study in which CD4+ T cells from HIV-1 infected patients were reactivated with the LRAs romidepsin or PMA/ionomycin and only 2 to 10% of cells that expressed vRNA produced viral proteins [207]. Viral gene expression needs to be efficiently achieved for the reactivation 'kick', through the use of transcriptional activators in combination with drugs that affect the post-transcriptional processes controlling viral gene expression. The 'kill' part of this strategy involves boosting both the cell-mediated and humoral immune responses to eliminate virus-producing cells. This could involve the passive transfer of broadly neutralising antibodies to control the virus [208]; the use of therapeutic vaccines to stimulate CD4+ T cell responses or non-neutralising antibody production to promote cell clearance via ADCC [180]; or the use of compounds that induce the apoptosis of HIV-1 infected cells [205].

So far, only one person has attained what appears to be a sterilising cure through combination of irradiation of blood cells followed by an HIV-1 resistant cell transplant [209]. Timothy Brown, also known as the Berlin patient, received an allogeneic hematopoietic stem cell (HSC) transplant to treat acute myeloid leukemia. He received the transplant from an HLA-matched donor who also had the homozygous CCR5Δ32/Δ32 deletion, a genotype that renders cells resistant to infection by HIV-1 R5 tropic viruses [209]. Since his transplant in 2007, Timothy Brown has maintained undetectable viral loads in the absence of cART. He remains the only individual with an established infection who has been cured of HIV-1.

1.6. Viral evasion of host responses

One of the main reasons why HIV-1 is such a debilitating illness is that it targets and depletes the CD4+ T cells, which are the main players of the adaptive immune response. Upon infection, the host cells mount intrinsic defence mechanisms to inhibit viral replication. These include the presence of host proteins that could restrict specific steps of the viral replication cycle (restriction factors) and the expression of interferon stimulated genes such as PKR. HIV-1 has evolved strategies to counteract these host defences, as described in this section.

1.6.1. PKR and Tat:

The interferon (IFN)-inducible PKR is a dsRNA sensor and a key player in the innate antiviral immune response [210, 211]. PKR activation inhibits both viral and cellular translation by phosphorylating the alpha subunit of the translation elongation initiation factor eIF2α, thus leading the assembly of translationally silent RNPs known as stress granules (SGs) [212]. HIV-1 uses several different mechanisms to overcome this inhibition, including inactivation of PKR by the action of the viral protein Tat [213], the recruitment of PKR inhibitors such as the TAR RNA binding protein (TRBP) [214] and adenosine deaminase acting on RNA 1 (ADAR1) [215], and by the modification of the function of the PKR activator (PACT) into a PKR inhibitor [216].

1.6.2. APOBEC3G and Vif:

The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) is a cytidine deaminase that catalyzes the conversion of cytidine to uridine. APOBEC3G is packaged into assembling virions. Upon infection of target cells, it causes potentially lethal dC to dU mutations on the newly reverse-transcribed minus-strand of viral DNA [217]. To evade restriction by the ABOBEC3 proteins, the viral protein Vif targets APOBEC3F/G/C for degradation via the host ubiquitin-proteasome pathway [218].

1.6.3. BST2/Tetherin and Vpu:

Bone marrow stromal cell antigen 2 (BST2, also named tetherin) inhibits the release of nascent HIV-1 particles by retaining the budding virions at the cell surface [219, 220]. Tetherin is

downregulated by the HIV-1 protein Vpu by two possible mechanisms: the ubiquitination and lysosomal degradation of BST2, or the mistrafficking of BST2 in the clathrin-dependent membrane trafficking pathways [221]. Apart from Tetherin, Vpu also downregulates the CD4 receptor [222].

1.6.4. SERINC5 and Nef:

SERINC5 is a member of the serine incorporator protein family that normally facilitates the incorporation of serine in the biosynthesis of sphingolipids and phosphatidylserine. Recent reports have shown that SERINC5 acts as an HIV-1 restriction factor by impairing the fusogenecity of HIV-1 particles when incorporated into the virion [223, 224]. Nef antagonises SERINC5 activity by triggering its redistribution into an endocytic compartment, thus excluding SERINC5 incorporation into virions [223, 224]. Nef also has highly characterised functions in downregulating CD4 and MHC-II [225].

1.7. Host mRNA decay pathways and HIV-1

Eukaryotic cells employ numerous regulatory mechanisms to ensure proper mRNA metabolism and gene expression at each step of mRNA biogenesis. mRNA surveillance and decay pathways are examples of such host quality-control mechanisms that mediate the degradation of aberrant mRNAs and can prevent the accumulation of potentially toxic mistranslated proteins in the host cell [226, 227]. Aberrant mRNAs include those that arise from defective template genes or a defect in a step of mRNA biogenesis. Nuclear mRNA surveillance mechanisms degrade RNAs in response to defects in 5'-capping, transcription elongation, splicing, 3'-end formation and nuclear export [228-237].

Following export into the cytoplasm, the processes of mRNA decay and translation are highly interconnected [238, 239]. Translation-dependent mechanisms of mRNA decay are nonsense-mediated mRNA decay (NMD), no-go decay (NGD) and non-stop-decay (NSD). NMD induces the degradation of mRNAs that are unspliced or aberrantly spliced; or of mRNAs that contain introns, upstream ORFs, premature termination codons (PTC) or long 3'UTRs [226, 240]. NGD results in the endonucleolytic cleavage of mRNAs that contain strong stalls in translational elongation whereas NSD degrades mRNAs that lack a stop codon [241-243]. Besides these, some other methods of mRNA decay that depend on cis-acting RNA elements are conditionally used to regulate gene expression such as the AU-rich element (ARE) mediated decay, miRNA-mediated decay and Staufen-mediated decay (SMD) [244-246]. The non-translating mRNAs can accumulate in two types of mRNP granules: P-bodies, which contain the mRNA decay machinery; and stress granules, which contain sequestered translation initiation components [238, 247-249]. It is currently hypothesised that cytoplasmic mRNAs can cycle between actively translating polysomes; and non-translating P-bodies, and stress granules [238].

Since viruses utilise the host cell machinery for their own gene expression, they have evolved ways not only to evade the mRNA surveillance mechanisms, but also to modulate RNA granules and hijack mRNA decay proteins to ensure viral gene expression [250-252]. In this section, some host mRNA decay pathways and the mechanisms by which HIV-1 modulate them will be described.

1.7.1. Nonsense-mediated mRNA decay:

1.7.1.1. NMD proteins and functions:

NMD is an important quality-control mechanism in cells that identifies and degrades aberrant mRNAs including those that harbour premature-termination codons (PTCs), thereby preventing the accumulation of potentially toxic truncated proteins [226, 253, 254]. Although NMD was previously implicated only in the degradation of aberrant mRNAs, it is now widely accepted that NMD also targets up to 25% of other physiological mRNAs for degradation in response to cellular needs [226, 255-257]. NMD is controlled by the up-frameshift proteins (UPF) and their associated suppressor with morphological effect on genitalia (SMG) proteins (described in Table 1.3). The key player in NMD is UPF1, which is a member of the SF1 subfamily of RNA helicases. [258].

NMD protein	Molecular weight (kDa)	Interaction with other NMD proteins	Functions in NMD
UPF1	123	UPF2, SMG1, SMG6, SMG5/7	Central player in NMD, direct RNA binding, helicase activity, ATPase activity
UPF2	148	UPF1, SMG1, UPF3A, UPF3B	Regulates UPF1 helicase activity, stimulates SMG1 kinase activity, establishes a physical link between UPF1 and UPF3A/B
UPF3A	55	UPF2, EJC	Establishes a physical link between UPF1-UPF2 and the EJC
UPF3B	56	UPF2, EJC	Establishes a physical link between UPF1-UPF2 and the EJC, functionally dominant over UPF3A
SMG1	410	UPF1, UPF2, SMG8, SMG9	Kinase involved in the phosphorylation of UPF1 at various SQ and TQ motifs
SMG6	160	UPF1, EJC	Executes the endonucleolytic cleavage of the target mRNA
SMG5	114	UPF1, SMG7	Forms a complex with SMG7, recruits PP2A for UPF1 dephosphorylation, recruits DCP2 and DCP1a for target mRNA decapping
SMG7	122	UPF1, SMG5	Forms a complex with SMG5, recruits CCR4-NOT complex for target mRNA deadenylation
SMG8	110	SMG1, SMG9	Regulates of SMG1 kinase activity
SMG9	58	SMG1, SMG8	Regulates of SMG1 kinase activity

Table 1.3.: NMD proteins and their functions

Adapted from [254].

1.7.1.1.1. UPF1

UPF1, also known as the regulator of nonsense transcripts 1 (RENT1) or suppressor with morphogenetic defects in genitalia 2 (SMG2), has RNA-dependent adenosine triphosphatase (ATPase) as well as ATP-dependent RNA helicase activities [259]. UPF1 is 1118 amino acids long and contains a CH domain in its N-terminal region from amino acids 115 to 294 that is rich in cysteines and histidines [260]. The CH domain contains zinc fingers that are known to promote RNA-binding and binds to instability sequences (INS) in RNA [260, 261]. The CH domain also serves as the binding site for the eukaryotic release factor 3 (eRF3), up-frameshift protein 2 (UPF2) and Staufen1 [246, 262-264]. UPF1 contains seven helicase motifs and its ATPase activity spans two helicases motifs (Ia and II) [261]. The SQ domain on UPF1 is located at the C-terminus region of the protein from the amino acids 915 to 1118. It is rich in the amino acids serine and glutamine, and contains critical phosphorylation sites [265]. UPF1 is phosphorylated by SMG1, a phosphatydylinositol 3-kinase related protein, [266-269]. Conversely, UPF1 is dephosphorylated by SMG6 and the SMG5/7 complex via the recruitment of PP2A [270, 271]. The phosphorylation of UPF1 plays an important role in regulating UPF1's cellular activities and the binding of other proteins involved in NMD to UPF1. UPF1 also contains a nuclear export (NES) and a nuclear localization signal (NLS) located between the amino acids 55-416 and 596-697, respectively, and is localised in both the nucleus and cytoplasm [272]. Apart from its role in NMD, UPF1 also has defined roles in DNA repair and replication [273, 274], RNA stability [275-277], telomere metabolism [273] and cell cycle progression [274] (reviewed in [264]). UPF1 is also a component of cytoplasmic P-bodies [278]. The structure of UPF1 and its different domains are illustrated in Figure 1.14.

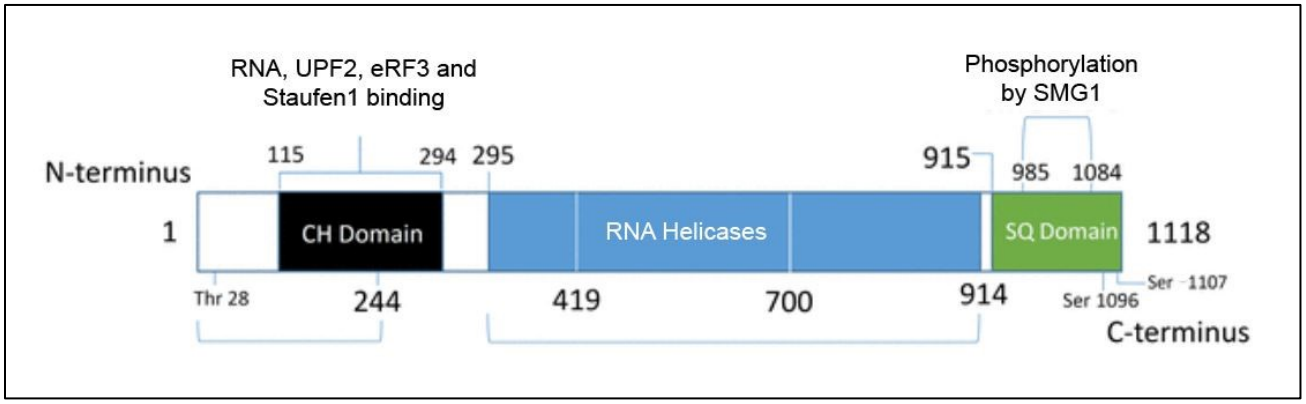

Figure 1.14: UPF1 protein structure and domains

The structure UPF1 with indications of different domains and motifs are depicted. This figure is not to scale. Numbers positioned above and below indicate the amino acid positions. Important serine and threonine phosphorylation sites are indicated. Adapted and reprinted with permission from [279].

1.7.1.1.2. UPF2

The other UPF proteins that play critical roles during NMD are UPF2 and the two isoforms of UPF3: UPF3A and UPF3B. UPF2 is a phosphoprotein of 1272 amino acids containing four core regions: three middle portions of eukaryotic initiation factor 4-gamma (MIF4G) domains and one C-terminal region [279]. The C-terminus of UPF2 is involved in binding to UPF1 [280]. UPF2 interacts with the RNA recognition motif (RRM) domain of UPF3 via its third MIF4G domain [281]. UPF2 acts as a molecular bridge between UPF1 and UPF3 [262]. The binding of UPF2 to UPF1 has been reported to induce a conformational change in UPF1 that stimulates UPF1's RNA helicase activity and dampens its RNA-binding capability [260, 262]. UPF2 is also involved in promoting the phosphorylation of UPF1 [279, 282]. The UPF2 protein has multiple NLSs and one NES but is primarily localised in the cytoplasm and, to a much smaller extent, in the nucleoplasm [283]. UPF2 has characterised functions in fetal liver development, spermatogenesis and testicular development [284-286]. The structure of UPF2 and its different domains are illustrated in Figure 1.15.

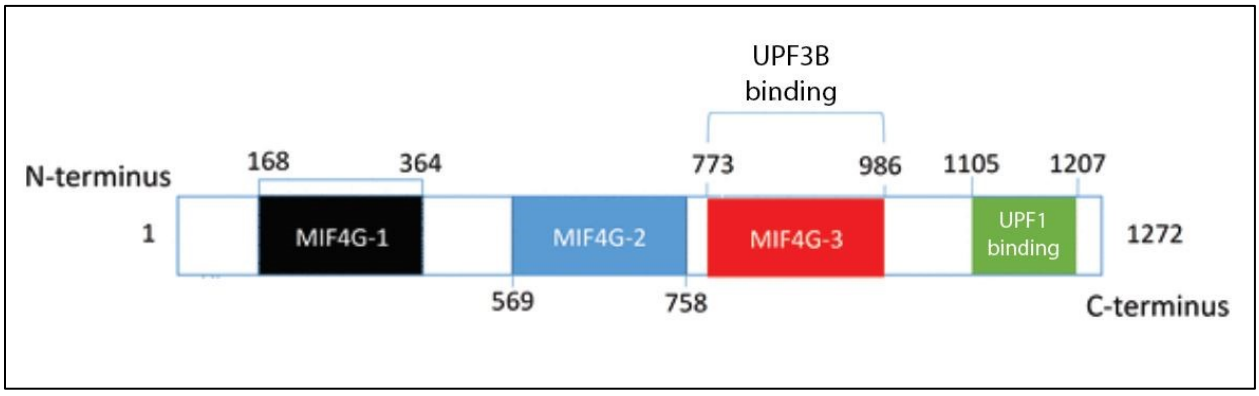

Figure 1.15.: UPF2 protein structure and domains

The structure UPF2 with indications of its different domains and motifs are presented. This figure is not to scale. Numbers positioned above and below indicate the amino acid positions. Adapted and reprinted with permission from [279].

1.7.1.1.3. UPF3

In humans, UPF3 exists in two different isoforms, UPF3A and UPF3B, that contain an RRM at their N-terminus by which they interact with UPF2 [287]. Both isoforms compete for binding to UPF2 and that there is a unidirectional regulation between UPF3B and UPF3A such that the association of UPF3B to UPF2 results in a decrease in UPF3A protein levels [288]. Compared to UPF3B, UPF3A only weakly triggers NMD [289]. UPF3B contains an exon-junction complex (EJC) binding motif (EBM) that is critical for binding to Y14, an essential component of EJCs, and for activating NMD [290]. Both UPF3B and UPF3A undergo alternative splicing which generate different isoforms and result in the formation of different complexes with UPF1 [281]. UPF3AL, the longer isoform, preferentially associates with a complex that contains phosphorylated UPF1 (P-UPF1) and UPF2 which is called a post-phosphorylation complex. The shorter isoform UPF3AS is found in a complex called the pre-dephosphorylation complex that contains PP2A, P-UPF1 and SMG5/7 [291]. UPF3A and UPF3B compete for UPF2 binding and the binding of UPF3B to UPF2 has been demonstrated to have a detrimental effect on UPF3A levels [288]. UPF3A also has been shown to have antagonistic functions on NMD due to its gene duplication with UPF3B [292]. The UPF3 proteins are mainly nuclear and contain NES and NLS signals. The UPF3 proteins also have reported roles in the differentiation of neural stem cells, respiratory function and the plant cell's response to salt stress [293-295].

1.7.1.2. NMD pathways:

The exact mechanism by which the cells elicit NMD is yet to be elucidated, but three models are proposed: the exon–junction complex (EJC) model, the UPF1 3'-UTR sensing and potentiation model, and the faux 3'-UTR model [279].

1.7.1.2.1. EJC-dependent NMD pathway:

The EJC model is the most widely accepted model of NMD. In this model, host mRNA transcripts that harbor a PTC located at least 50-55 nucleotides upstream of the EJC and within the initial ORF are susceptible to NMD. The EJC is about 350kDa and comprises at least 10 different proteins such as UPF3B, Y14, MAGOH, eIF4AIII and Barentz, which get deposited 20-24 nucleotides upstream of exon-exon junctions following splicing. During the first round of translation, the ribosome travels along the mRNA and displace the EJCs until it reaches a stop codon. Once the ribosome reaches the stop codon that is not followed by an EJC, then normal termination of translation occurs. However, in PTC-containing transcripts, the ribosomes encounter the PTC and recruit UPF1, SMG1 complex (containing SMG1, SMG8 and SMG9) and the eukaryotic restriction factors eRF1 and eRF3 to form the SURF (SMG1:UPF1:eRF1:eRF3) complex [296, 297]. UPF1 within the SURF complex interacts with UPF2 that is bound to UPF3B at the EJC, which leads to the formation of the decay inducing complex (DECID). The binding of UPF2 to UPF1 results in a conformational change in UPF1 that promotes the phosphorylation of UPF1 by SMG1 [268]. Phosphorylated UPF1 plays an important role in the recruitment of proteins involved in the later RNA decay events via the endonucleolytic cleavage or deadenylation pathways. The more common pathway of to degrade aberrant mRNA in NMD is via endonucleolytic cleavage and involves the host protein SMG6 [298]. SMG6 contains an exon junction binding domain (EBM) [299], a 14-3-3-like domain that binds to phosphorylated UPF1 [270] and a PilT N-terminus (PIN) domain [300] that possesses the endonuclease activity [300-302]. The binding of SMG6 to P-UPF1 leads to the endonucleolytic cleavage of the aberrant mRNA [303]. This is followed by the degradation of the 5’ and 3’ ends of the mRNA fragment by XRN1 and the exosome, respectively [304]. Another pathways for the decay of aberrant mRNA is via the binding of the SMG5/7 proteins to P-UPF1. SMG5 recruits mRNA-decapping proteins DCP2 and DCP1a, while SMG7 recruits CCR4–NOT deadenylase complex that results in the decapping and subsequent degradation of the aberrant mRNA by XRN1 [305-308]. Figure 1.16. is a schematic depiction of the EJC-dependent NMD pathway.

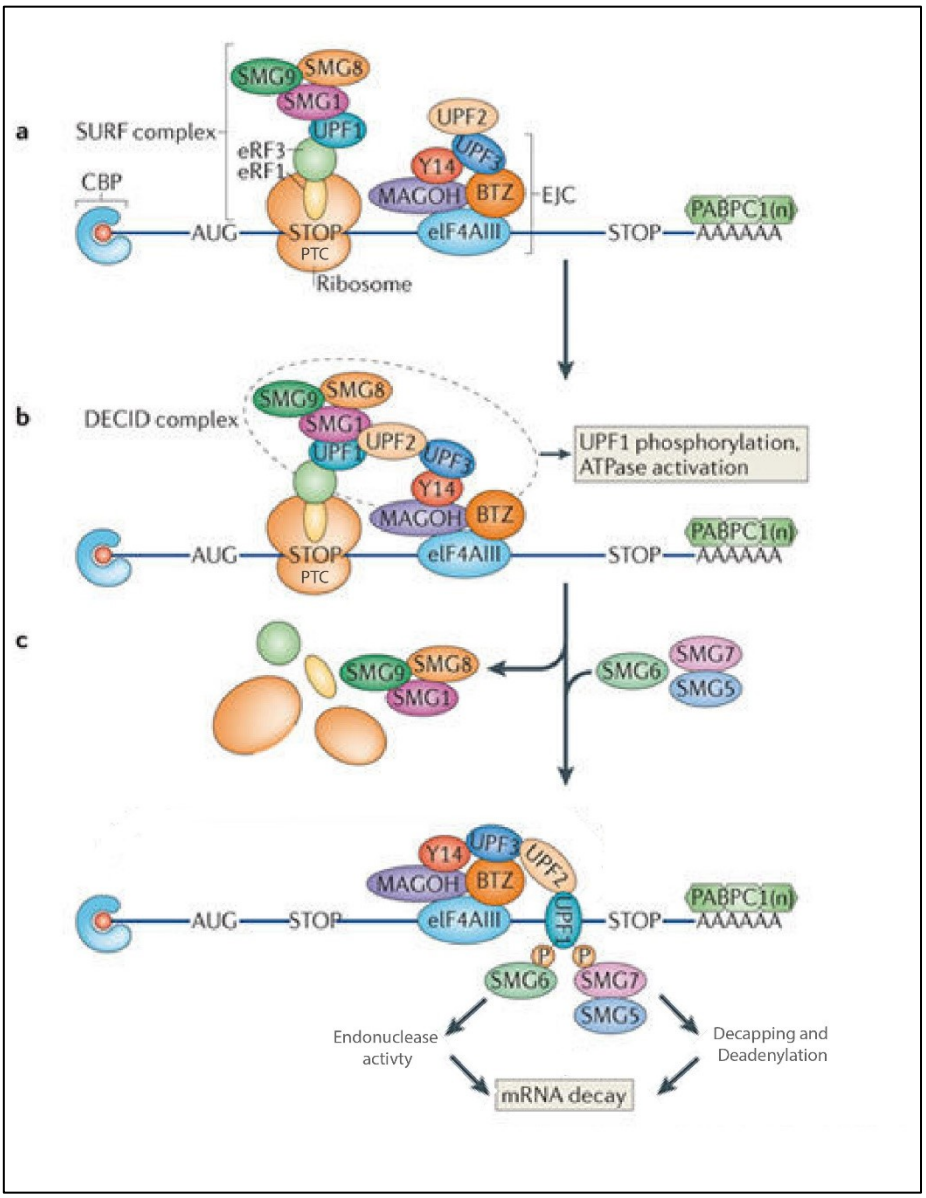

Figure 1.16: EJC-dependent NMD pathway

A) A PTC-containing mRNA that contains the cap-binding complex (CBP) and the poly-A binding protein complex (PABP1C) contains an EJC that is not displaced by the pioneer round of translation. The SURF complex assembles on the PTC. B) UPF1 interacts with UPF3B on the EJC via UPF2 to form the DECID. C) UPF1 is phosphorylated by SMG1 and recruits SMG5/7 or SMG6, resulting in the degradation of the target mRNA. (See text for details). Adapted and reprinted with permission from [309].

1.7.1.2.2. EJC-independent NMD pathways:

The other models of NMD are independent of the EJC. In the UPF1 3'-UTR sensing and potentiation model, UPF1 is thought to associate with transcripts that contain long 3'-UTRs in a length-dependent manner at the UTR itself. This interaction occurs irrespective of the mRNA sequence and results in the formation of a yet to be characterised mRNP that triggers the decay on that mRNA [310]. However, this model is disputed because UPF1 can also bind to non-NMD inducing UTRs [311-313]. Furthermore, some human mRNAs with long 3'-UTRs have been shown to evade NMD through the use of cis-acting elements such as AU-rich regions in the first 200 nucleotides [314]. The faux-3' UTR model also depends on the binding of UPF1 to long 3'-UTR but also takes into account the inefficient translation termination at PTCs as compared to normal translation termination codons [315]. Interestingly, it has been found that UPF1 in its steady state binds promiscuously to mRNAs, regardless of NMD fate [316]. Therefore, the marker for identifying NMD targets would be the phosphorylation of UPF1-bound mRNA rather than the binding of UPF1 to mRNA itself [317].

1.7.1.3. NMD in disease and treatment

NMD has been closely linked to human diseases, with one-third of inherited human diseases being caused by PTCs that are introduced by nonsense mutations, frameshift mutations or splicing errors [318, 319]. Two kinds of diseases can be caused by NMD misregulation. The first occurs when a PTC-deficient transcript is degraded, resulting in the absence of full length protein expression and a recessively inherited form of the disease. The second arises when a PTC-containing transcript is not degraded, resulting in the expression of defective truncated proteins and the dominantly inherited form of the disease. Examples of such diseases are β-thalassemia, Duchenne muscular dystrophy, spinal muscular atrophy, inherited blindness and neurocristopathic syndromes [320]. One potential therapeutic treatment of diseases that are due to in-frame nonsense (but not frameshift) mutations aims to promote translational read-through of PTC-containing transcripts, thus allowing some full-length protein to be produced. Some examples of such drugs are Ataluren, read-through compound (RTC)13, Amlexanox, synthetic

aminoglycosides and nonaminoglycosides, some whose efficacy are being evaluated in ongoing clinical trials [321-326].

1.7.1.4. HIV-1 and NMD

Viruses are obligate intracellular parasites that utilise the host cell machinery to ensure their gene expression. Many viruses also harbour long 3'-UTRs and PTCs in their ORFs and thus could be a target for NMD. Viruses have developed mechanisms to evade host RNA surveillance mechanisms to ensure their gene expression (reviewed in [251, 327]). For example, the full-length unspliced transcript of the retrovirus Rous sarcoma virus (RSV) contains a PTC in the Gag ORF but is still able to evade NMD by virtue of a cis-acting RNA element termed the RNA stability element (RSE) located downstream of the Gag PTC [328, 329]. The RSE forms a complex RNA secondary structure that binds to the polypyrimidine tract binding protein 1 (PTBP1) [329, 330]. The recruitment of PTBP1 to the proximity of a termination codon inhibits the recruitment of UPF1 and antagonizes NMD, thereby resulting in the stabilization of RSV full-length RNA [330]. Another retrovirus, the human T-lymphotropic virus type 1 (HTLV-1), has also been shown to downregulate NMD via the action of its two viral proteins Tax and Rex [331-333]. Tax was shown to bind to UPF1 and the translation initiation complex component INT6/eIF3E, which results in partial inhibition of NMD while Rex has an important role in stabilising viral transcripts [334]. A recent report also demonstrated that Tax can bind to UPF1 and inhibit UPF1's RNA-binding activity [333]. Tax can also prevent the translocation of UPF1 on mRNA to inhibit NMD [333] .

The unspliced vRNA of HIV-1 contains long 3'-UTRs, but is not subjected to NMD. Not only is HIV-1 capable of evading NMD, it also hijacks the NMD pathway for its own benefit by recruiting UPF1 to enhance vRNA stability, translation, nucleocytoplasmic export and specific infectivity of released virions [98, 335, 336]. In HeLa cells, UPF1 assembles into two distinct RNPs with the vRNA, the first in the nucleus containing UPF1, Rev, CRM1, DDX3, the nucleoporin p62 [98]. The formation of this nuclear mRNP promotes the stability of the vRNA and its nucleocytoplasmic export [98]. This effect may be exerted during the rapid, co-transcriptional association of UPF1 with vRNA during transcription [337].

Once in the cytoplasm, UPF1 assembles in another distinct RNP on the vRNA with Staufen1 and Gag, resulting not only in the increased stability of the vRNA, but also in its enhanced translation and increased levels of the Gag viral production [335]. UPF1 knockdown results in reduced vRNA and Gag levels and UPF1 overexpression enhances them [335]. Moreover, mutational analysis also demonstrated that the role of UPF1 in HIV-1 gene expression is mutually exclusive from its functions in NMD [335]. These UPF1-mediated effects also require the ongoing translation of the vRNA and the ATPase activity of UPF1 [335]. Additionally, UPF1 interacts with vRNA in an RNA length-dependent manner and this could contribute to its incorporation into progeny HIV-1 virions [310, 336-338]. The incorporation of UPF1 into viral particles is mediated through specific interactions with the NC domain of Gag [336]. Interestingly, UPF2 and its interacting partner UPF3AL are excluded from these specific UPF1/HIV-1 mRNPs and are negative regulators of vRNA export [98]. Using protein-protein docking studies, it was demonstrated that UPF2 is excluded from the nuclear HIV-1 RNPs through antagonistic interactions with the viral protein Rev [98]. In the cytoplasmic RNP, Staufen1 and UPF2 compete for the same UPF1-binding site. Additionally, UPF1 was also shown to be critical for early events of the HIV-1 replication cycle. A knockdown of UPF1 or the ectopic expression of ATPase activity mutants resulted in reduced viral entry and reverse transcription (RT) [336]. Therefore, HIV-1 positively regulates its gene expression by using the cellular UPF1 and demonstrates how the virus can hijack the host mRNA surveillance pathways for its own benefit. The various steps at which the NMD proteins have been demonstrated to affect vRNA metabolism are demonstrated in Figure 1.17.

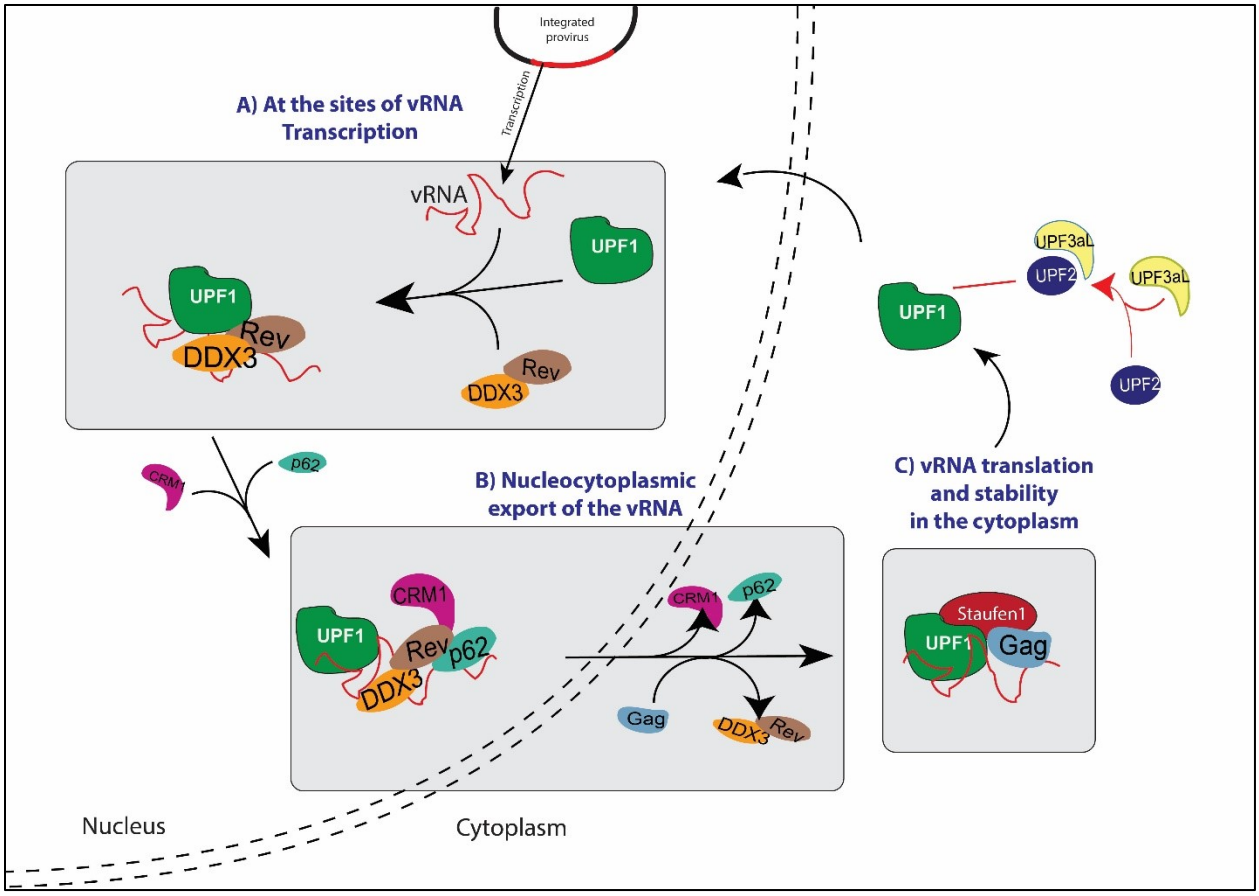

Figure 1.17.: UPF1 and vRNA metabolism

A) UPF1 is recruited co-transcriptionally to the vRNA with DDX3 and Rev, and increases vRNA stability in the nucleus [335, 337]. B) UPF1 forms a nuclear RNP in HeLa cells with the vRNA, Rev, CRM1, DDX3, DDX1 and Nup62 that promotes vRNA nucleocytoplasmic export [98]. C) Once in the cytoplasm, UPF1 forms a cytoplasmic RNP with Staufen1 and Gag to promote vRNA stability to ensure Gag synthesis [335]. UPF2-UPF3aL acts as a negative regulator of vRNA nuclear export by binding to UPF1 and sequestering it in the cytoplasm [98].

1.7.2. Staufen-mediated decay

1.7.2.1. Staufen proteins and functions

Staufen proteins belong to a family of RNA-binding proteins (RBPs) that are involved in multiple post-transcriptional regulatory processes. Their most characterised role is in RNA trafficking and localisation in both oocytes and somatic cells in vertebrates, as well as in invertebrates [339, 340] (reviewed in [341]). Staufen proteins contain multiple dsRNA-binding domains (dsRBDs) which are required to bind RNAs that contain double-stranded secondary structures. In humans, two Staufen orthologues, Staufen 1 and Staufen2, are encoded by two distinct genes. Staufen2 is primarily found in neuronal cells and plays roles in RNA biogenesis and trafficking [342, 343]. Staufen1 is more ubiquitously expressed in human cells and has numerous functions in RNA localisation, translation and decay [275, 344-346]. Staufen1 contains four dsRBDs and one microtubule binding domain that binds to tubulin [347] (Figure 1.18.). Staufen1 assembles into multiple RNPs and this characteristic is important in the ability of Staufen1 to exert its multiple functions [348]. At least 7% of cellular mRNAs were shown to be present in several Staufen1 RNPs such as the RNA transport RNPs, stress granules (SG) and APOBEC3G complexes amongst others [349-354]. Although Staufen1 is an SG component, it has been reported to modulate the host cell stress response. The N-terminal region of Staufen1 binds to polysomes and results in the stabilisation of the ribosomal subunits on the mRNA, thus inhibiting SG assembly. [350, 355]. An overexpression Staufen1 was shown to impair SG formation, whereas Staufen1 knockdown hinders their dissolution [350, 355].

Figure 1.18.: Staufen1 protein structure and domains

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1.7.2.2. Staufen-mediated decay mechanism and functions

Staufen1 is also involved in the degradation of transcripts by a translation-dependent host process known as Staufen-mediated decay (SMD) that involves UPF1 [275, 357]. During SMD, Staufen1 binds to the 3'-UTRs of targeted transcripts which results in the recruitment of UPF1 to elicit mRNA decay [246]. Staufen1 recognises dsRNA structures known as Staufen-binding sites (SBS) formed within the 3'-UTR of target mRNAs. The presence of primate-specific Alu short interspersed elements or base pairing between Alu elements can also trigger SMD [358-360]. SMD is implicated in the stability of about 1% of transcripts, and transcriptome studies demonstrated that these transcripts were upregulated upon Staufen1 depletion [361]. Staufen2 has also been demonstrated to elicit SMD in human cells [362]. SMD has reported roles in the regulation of numerous cellular functions such as myogenesis, adipogenesis, cell mobility and invasion, and stress-induced apoptosis [358, 359, 363-366]. Staufen1 and UPF2 compete for the same UPF1 binding site, and SMD and NMD have been reported to be competitive mRNA decay pathways [246, 363].

1.7.2.3. HIV-1 and Staufen1

HIV-1 has been known to recruit Staufen1 to form specific viral RNPs to promote virus production (reviewed in [252]). Staufen1 plays a role in various steps of the virus life cycle including Gag multimerisation, vRNA encapsidation and viral assembly [120, 122, 123, 367]. A role for Staufen1 in viral encapsidation was first described when it was determined that Staufen1 is incorporated into virions in a vRNA-dependent manner [339]. An overexpression of Staufen1 was demonstrated to increase vRNA encapsidation three-fold which resulted in a significant impairment of viral infectivity [339]. The N-terminal Staufen1 moiety is also required for efficient Gag multimerisation [367]. Staufen1, via its third dsRBD, interacts specifically with the zinc fingers of the NC domain of Gag in an RNA-independent manner [123]. This interaction results in the formation of an HIV-1 RNP containing Staufen1, Gag, UPF1 and the vRNA, amongst other host proteins [122, 123, 356, 367]. The formation of this RNP could promote vRNA encapsidation. The specific knockdown of Staufen1 resulted in a significant reduction in viral infectivity [122]. It was later described that during conditions of oxidative stress, HIV-1 can prevent the assembly of SGs, but promotes the assembly of another type of RNP, the Staufen1 HIV-1-dependent RNP (SHRNP) [120]. SHRNPs are high molecular-weight, detergent insoluble complexes that contain Staufen1, Gag, the vRNA and many other viral and cellular components [122, 341, 356, 368]. Staufen1, likely in the context of SHRNPs, interacts with the NC domain of Gag and enhances Gag assembly and vRNA packaging [120, 369]. SHRNPs are distinct from SGs and PBs and their formation can also be hypothesised to serve to prevent the degradation of the vRNA [120].

Staufen1 was also demonstrated to promote the translation of mRNAs that contain the HIV-1 *trans*-activating response region (TAR) at the 5'-end [346]. Staufen1 binds to the TAR region and is hypothesised to facilitate the nucleocytoplasmic transport of these transcripts, thus contributing to their interaction with the host translational machinery [346]. Another cellular protein called the TAR-RNA Binding Protein (TRBP), is known to bind to the TAR region and stimulate translation of TAR-containing mRNAs [370]. Sequence alignments revealed that the TRBP domain involved in TAR-binding is homologous to the second and third dsRBDs of Staufen1 [371]. Indeed, the binding of Staufen1 to TAR-RNAs upregulates their translation to levels comparable to those induced by TRBP binding [346]. Therefore, it is evident that HIV-1 recruits Staufen1 to promote various functions in viral gene expression, vRNA trafficking, and viral particle assembly. Staufen2 also plays a positive role in the HIV-1 life cycle by promoting the export of HIV-1 mRNAs containing an RRE via an RNA-independent interaction between Staufen2 and the viral protein Rev [372].

1.7.3. P-bodies and stress granules

The translation and decay of mRNAs play key roles in the control of eukaryotic gene expression and the non-translating mRNAs can accumulate in two types of mRNP granules: P-bodies (PBs), and stress granules (SGs) [238, 247-249]. In order to ensure their own gene expression, many viruses including HIV-1 have developed ways to circumvent the assembly of these granules by hijacking SG components [250, 252].

1.7.3.1. HIV-1 and P-bodies

PBs are cytoplasmic foci associated with the mRNA decay machinery which contain mRNA decapping enzymes (Dcp1/2), deadenylation factors (Ccr1, Caf1, Not1), the 5'-3' exonuclease XRN1, scaffolding proteins (Ge-1/Hedls), NMD-associated proteins (SMG5-6-7, UPF1), and translational control factors (CPEB, eIF4E-T, DDX6) (reviewed in [250]). The disruption of PBs was reported to result in enhanced viral production and infectivity, indicating that PB assembly can be detrimental to HIV-1 [373]. HIV-1 has evolved to counter the antiviral activity of PBs and in HIV-1 expressing cells, a dramatic decrease of PBs was observed around the SHRNPs, indicating that PBs are dissolved near the viral assembly sites [120]. HIV-1 also recruits some PB-components like UPF1, AGO2, MOV10 and DDX6 to facilitate virus production [121, 335, 374, 375].

1.7.3.2. HIV-1 and stress granules

In response cellular stress, such as viral infection, the host cell reprograms its translational machinery to inhibit viral gene expression by assembling translationally silent RNP complexes known as SGs [376, 377]. HIV-1 utilises the host cell machinery to facilitate its own gene expression, and its replication can be significantly decreased by an impediment to cellular mRNA translation. A report demonstrated that an SG-dependency factor GTPase activating protein (GAP) SH3 domain-binding protein 1 (G3BP1) binds to the vRNA and restricts viral replication in macrophages [378]. Hence, HIV-1 (and other viruses) have developed mechanisms to circumvent the cellular stress response (reviewed in [250, 379]). Two types of SGs that differ in morphology, composition, and mechanism of assembly have been described [380]. HIV-1 disrupts the canonical type I SG assembly in an eIF2α-phosphorylation (eIF2α-P) independent manner via an interaction between the N-terminal domain of the CA domain on Gag and the host eukaryotic elongation factor 2 (eEF2) [120, 381]. Gag could also mediate the disassembly of pre-existing SGs via an interaction with G3BP1 [381]. Moreover, HIV-1 is also capable of blocking the assembly of type II, noncanonical SGs by reducing the amount of hypophosphorylated 4EBP1 associated with the 5′ cap, potentially through an interaction with its target, eIF4E [382].

The mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol 3-kinase (PI3K) family that controls protein homeostasis via the regulation of translation, autophagy and proteasomal degradation [383]. mTOR is normally partitioned to the SGs during stress conditions [384, 385]. Since mTOR is a master regulator of translation, one mechanisms for viruses to evade the host stress response is to maintain mTOR activation. The inhibition of SGs by HIV-1 ensures the availability of the mammalian target of rapamycin (mTOR) for utilisation by the virus [381, 382]. HIV-1 infection results in the activation of mTOR and the virus commandeers mTOR-associated late endosome/lysosome trafficking, thus inhibiting stress-induced repositioning of the late endosomes/lysosomes [386]. This ensures viral particle assembly at the plasma membrane and efficient HIV-1 replication [386]. Thus, it is evident that HIV-1 has devised methods to hijack RNA granules and modulate P-body and SG assembly to prevent vRNA degradation and promote viral gene expression and assembly.

1.8. Objectives

The overall objective of the research presented in this thesis was to investigate the roles of the host mRNA decay proteins on HIV-1 vRNA metabolism and viral gene expression. In chapter 2, the results of our previously observed effects of the NMD proteins on vRNA metabolism (discussed in section 1.7.1.4.) to were applied to HIV-1 latency studies. This resulted in the characterisation of a novel effect of the NMD proteins on the post-transcriptional maintenance of viral latency in T cells. Our studies were then expanded to macrophages and an effect of the mRNA decay proteins UPF1, UPF2, SMG6 and Staufen1 on viral gene expression was demonstrated in chapter 3. Finally, in chapter 4, a novel function of Staufen1 in the rescue of viral gene expression during conditions of cellular stress was characterised. The research can be divided into three main aims:

- To determine the effects of the NMD proteins UPF1, UPF2 an SMG6 on HIV-1 gene expression in a latently-infected T cell line and in primary CD4+ T cells (Chapter 2).
- To determine the effects of the NMD proteins UPF1, UPF2, SMG6 and Staufen1 on HIV-1 gene expression in primary monocyte-derived macrophages (Chapter 3).
- To determine how the SMD protein Staufen1 modifies HIV-1 NC-induced stress granule assembly and rescues viral gene expression (Chapter 4).

The results generated from these studies could be applied towards the development of an HIV-1 cure or novel next-generation ARVs, as discussed in Chapter 5.

1.9. References

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Chapter 2

The RNA surveillance proteins UPF1, UPF2 and SMG6 affect HIV-1 reactivation at a post-transcriptional level

This chapter was adapted from the following manuscript:

Rao, S., Amorim, R., Niu, M., Temzi, A. & Mouland, A. J. (June 2018) "The RNA surveillance proteins UPF1, UPF2 and SMG6 affect HIV-1 reactivation at a post-transcriptional level". *Retrovirology. 28;15(1):42.*

2.1. Preface

In chapter 1, we described the previously characterised roles of the host mRNA decay proteins involved in nonsense mediated mRNA decay (NMD) on viral gene expression. This chapter presents data on the characterisation of the roles on these NMD proteins on the post-transcriptional regulation of HIV-1 latency in cells of the lymphoid lineage. Specifically, we investigated the effect of the NMD proteins UPF1, UPF2 and SMG6 on viral reactivation in a HIV-1 infected T cell model using fluorescence in situ hybridisation - Flow Cytometry (FISH-flow). The effect of UPF1 on vRNA expression in HIV-1 infected primary CD4+ T cells was also explored.

Author contributions: S.R., R.A. and A.J.M conceived the study and designed experiments. S.R. conducted most of the experiments and data analysis with contribution from R.A. M.N. and A.T. provided technical help. S.R. drafted the manuscript with the support and comments from R.A. and A.J.M. All authors read and approved the final manuscript.

Funding information: This study, S.R. and R.A. were supported by The Canadian HIV Cure Enterprise Team Grant HIG-133050 (to A.J.M.) from the Canadian Institutes of Health Research (CIHR) in partnership with Canadian Foundation for HIV-1/AIDS Research and International AIDS Society and from the Lady Davis Research Institute/Jewish General Hospital. R.A. was funded by a Conselho Nacional de Desenvolvimento Científico e Tecnológico Fellowship (Brazil). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Acknowledgements: We thank the late Mark Wainberg, Andreas Kulozik, Niels Gehring, Jens Lykke-Andersen, Oliver Muhlemann and Mark Fabian for generous provision of cells and reagents; Niels Gehring and Nada Hafez for helpful discussions; Daniel Kaufmann, Alan Cochrane, Amy Baxter and Lewis Liu for assay development; Christian Young for technical assistance; and Alessandro Cinti for critical reading of the manuscript.

2.2. Abstract

The ability of human immunodeficiency virus type 1 (HIV-1) to form a stable viral reservoir is the major obstacle to an HIV-1 cure and post-transcriptional events contribute to the maintenance of viral latency. RNA surveillance proteins such as UPF1, UPF2 and SMG6 affect RNA stability and metabolism. In our previous work, we demonstrated that UPF1 stabilises HIV-1 genomic RNA (vRNA) and enhances its translatability in the cytoplasm. Thus, in this work we evaluated the influence of RNA surveillance proteins on vRNA expression and, as a consequence, viral reactivation in cells of the lymphoid lineage. We used fluorescence in situ hybridisation - Flow Cytometry (FISH-flow) to characterise the roles of RNA surveillance proteins on viral reactivation in a latently infected model T cell line. UPF1 was found to be a positive regulator of viral reactivation, with a depletion of UPF1 resulting in impaired vRNA expression and viral reactivation. UPF1 overexpression also modestly enhanced vRNA expression and its ATPase activity and N-terminal domain were necessary for this effect. UPF2 and SMG6 were found to negatively influence viral reactivation, both via an interaction with UPF1. UPF1 knockdown also resulted in reduced vRNA levels and viral gene expression in HIV-1-infected primary CD4+ T cells. Overall, these data suggest that RNA surveillance proteins affect HIV-1 gene expression at a post-transcriptional level. An elucidation of the role of vRNA metabolism on the maintenance of HIV-1 persistence can lead to the development of novel curative strategies.

2.3. Introduction

The implementation of combination antiretroviral therapy (cART) to treat human immunodeficiency virus type 1 (HIV-1) has led the infection to be likened to a chronic condition, with patients on cART having near-normal life expectancy [1]. However, this therapy is not without its drawbacks, such as adverse side effects that lower the adherence rates, the development of drug resistance and its economic repercussions [2-4]. But one of the biggest disadvantages of this therapy is that it is not curative and an infected individual needs to be on cART for the entire duration of their lifetime to effectively suppress viremia. The major hurdle towards an HIV-1 cure is the property of virus to form a stable latent reservoir upon infection that is responsible for the rapid rebound of plasma viral loads when cART is discontinued [5]. This reservoir is primarily composed of resting memory CD4+ T cells along with monocytes and macrophages [6] in peripheral blood and other anatomical compartments such as the gut, lymph nodes and central nervous system. Latency in HIV-1 infection is defined as a reversibly non-productive state of infection which is characterised by the presence of infected cells that do not actively produce viral particles, but retain the ability to do so [7]. Latent cells harbour a replication competent proviral DNA integrated in their genomes [8]. Many research groups have studied the functional aspects of the maintenance of latency in cells by investigating the molecular mechanisms leading to a block at the level of transcription (reviewed in [6, 9, 10]). However, certain studies also highlight that co and post-transcriptional events can also contribute to the maintenance of latency in HIV-1 infected cells [11-13]. These include defective splicing of the genomic viral RNA (vRNA) [14], inhibition of nucleocytoplasmic export of vRNA [13, 15, 16] or an impediment to vRNA translation [17, 18]. Thus, in this work, we investigate the role of the RNA surveillance proteins on the post-transcriptional events that are involved in the maintenance of HIV-1 latency.

RNA surveillance is a host quality control mechanism that identifies and degrades unspliced, aberrantly spliced, intron-containing, upstream open reading frame-containing and premature termination codon (PTC)-containing mRNAs to prevent the accumulation of potentially toxic truncated proteins within the cell (reviewed in [19]). A central player in this mechanism is the Up Frameshift Protein 1 (UPF1), an RNA binding protein that has ATPase and RNA helicase activity

[20]. It is a multifunctional protein that has defined roles in DNA repair and replication [21, 22], RNA stability [23-25], telomere metabolism [21] and cell cycle progression [22] (reviewed in [26]). Its most characterised function, however, is its role in nonsense-mediated mRNA decay (NMD) during which UPF1 interacts with a family of proteins such as UPF2, UPF3A and UPF3B, a kinase SMG1 and an endonuclease SMG6 resulting in the degradation of aberrant mRNAs (reviewed in [19, 27]). Although NMD was previously implicated only in the degradation of aberrant mRNA, it is now widely accepted that NMD also targets up to 10% of other physiological mRNAs for degradation in response to cellular needs [19, 28-30], including transcripts that contain long 3'UTRs [31].

In order to promote their survival, viruses have evolved numerous strategies to either evade or manipulate the RNA surveillance pathways (reviewed in [32]). Retroviruses, despite containing long 3'UTRs that are recognised by UPF1, are capable of evading NMD by virtue of the presence of RNA stability elements in their genome [33] (reviewed in [34, 35]). In previous studies, our group has demonstrated that HIV-1 not only evades NMD, it also hijacks UPF1 to form an RNP that promotes vRNA stability and nucleocytoplasmic export in HeLa cells [36, 37]. This effect may be exerted during the rapid, co-transcriptional association of UPF1 with vRNA during transcription [38]. UPF2, another protein involved in NMD, has been shown to block nucleocytoplasmic export of the vRNA by binding to UPF1 and preventing its nucleocytoplasmic shuttle [37]. Once in the cytoplasm, UPF1 assembles in another distinct RNP on the vRNA resulting in not only the increased stability of the vRNA, but also in its enhanced translation leading to increased levels of the HIV-1 structural protein $pr55^{Gag}$ viral production [36]. Additionally, UPF1 interacts with vRNA in an RNA length-dependent manner and this could contribute to its incorporation into progeny HIV-1 virions [38-41]. Therefore, there is substantial evidence to show that UPF1 can affect vRNA metabolism at different levels.

In this study, we investigated the ability of UPF1 and its associated proteins UPF2 and SMG6 to influence HIV-1 gene expression and, as a consequence, viral reactivation at a post-transcriptional level by overexpression and siRNA-mediated knockdown studies in cells of the lymphoid lineage. We employed a fluorescence *in situ* hybridisation / flow cytometry (FISH-Flow) to monitor vRNA expression levels and viral protein production in a latently-infected T cell line. We observed that these proteins can modulate HIV-1 gene expression and thus the post-transcriptional maintenance of HIV-1 latency. We have also identified the domains responsible for these effects on viral reactivation by mutational studies. Importantly, we also demonstrate a direct effect of UPF1 on vRNA expression in primary HIV-1 infected CD4+ T cells.

2.4. Results

2.4.1. FISH-Flow can be used to monitor vRNA levels and viral reactivation in J-Lat cells

UPF1 has previously been demonstrated to affect vRNA metabolism at three distinct stages: overall vRNA stability, the nucleocytoplasmic export of the vRNA, and vRNA translation in the cytoplasm [36, 37]. Therefore, we employed the FISH-Flow technique using probes against the GagPol region of the vRNA in latently infected J-Lat 10.6 cells to monitor both the transcriptional as well as translational products of the HIV-1 provirus. This technique has previously been employed to assess ongoing HIV-1 replication, to quantify the size of the inducible latent reservoir in HIV-infected individuals, to determine the kinetics of latency reversal and to characterize the specific cell subpopulations of CD4+ T cells that transcribe HIV-1 RNA [17, 42-44] (reviewed in [45, 46]). Using this technique, it is possible to distinguish between cells that contain both vRNA and viral proteins, and cells that only contain untranslated vRNA, thus differentiating between the transcription-competent and translation-competent viral reservoir [45, 46]. Cells can then also be seeded on a coverslip to determine the sub-cellular localisation of the vRNA using laser scanning confocal microscopy (LCSM). This comprehensive analysis enables us to investigate how UPF1 influences viral reactivation and to distinguish between an effect on vRNA expression, export or translation. J-Lat 10.6 cells, a well-established model of studying HIV-1 latency and reactivation [47-49], and primary CD4+ T cells are used in this study. The J-Lat cells have a GFP reporter in the *nef* open reading frame of the virus to monitor viral gene expression and, thus, viral reactivation. The cells can be reactivated by treatment with phorbol myristate acetate (PMA) or TNFα (Supplementary Figure 2.8. A). To assess whether the FISH-Flow technique can be used in the J-Lat cell model to measure reactivation, cells were either mock treated with dimethyl sulfoxide (DMSO) or treated with PMA to reactivate the cells. PMA is a protein kinase C agonist and is a strong activator of cellular transcription and was the latency reversing agent of choice because it leads to maximal reactivation of the J-Lat 10.6 cells [50]. We also validated the PMA treatment did not affect the baseline expression levels of our proteins of interest: UPF1, UPF2 and SMG6 (Supplementary Figure 2.8. B-D). Jurkat cells were used as a negative, uninfected control to determine the specificity of the FISH-Flow technique. Upon treatment with PMA, 60.89 (±11.35) % of J-Lat cells produced GFP indicating viral protein

production and reactivation (Figure 2.1. A and B). Efficient GagPol mRNA staining was also observed in 63.78 (±15.16) % of PMA-treated cells. (PE channel, Figure 2.1. A and B). It is also important to note that 4.79 (±2.44) % of PMA-treated cells contained vRNA but not GFP, representing the transcription-competent viral reservoir as previously described [45, 46]. The 2.48 (±1.17) of PMA-treated cells that were GFP+ but did not contain vRNA represent the cells that are generating multiply-spliced transcripts but not full length transcripts, since the GFP codon is present on the *nef* open reading frame [47]. The uninduced J-Lat cells contained some residual vRNA and GFP production, with 2.59 (±1.76) % of cells expressing GFP and 0.27 (±0.11) % of cells expressing vRNA (Figure 2.1. A and B). Although the vRNA is the unspliced genomic viral RNA whereas GFP is generated from the multiply spliced viral RNA, GFP was used as a marker for viral reactivation rather than intracellular p24 due to the efficiency of measuring viral reactivation at a single cell level by Flow cytometry due to the stability of GFP. The levels of pr55Gag, coded for by the vRNA, can be measured by Western blot to further correlate effects vRNA transcription and translation, if necessary. The Jurkat cells did not show any vRNA+ cells, indicating that this technique is highly specific (Figure 2.1. A). Cells from each of these conditions were seeded onto coverslips and observed by laser scanning confocal microscopy (Figure 2.1. C) to view the subcellular localisation of the vRNA. Therefore, the FISH-Flow technique is an efficient method to monitor viral reactivation at the transcriptional and translational levels in J-Lat cells.

2.4.2. UPF1 knockdown attenuates HIV-1 proviral reactivation

In previous studies conducted by our group, we observed that UPF1 knockdown lead to reduced vRNA stability in the nucleus and in the cytoplasm of cells [36]. Thus, we hypothesised that the depletion of UPF1 can reduce vRNA expression at a post-transcriptional level and thereby inhibit viral reactivation. To evaluate the effect of UPF1 levels on proviral reactivation, J-Lat cells were either transfected with a non-silencing siRNA (siNS) or with an siRNA against UPF1 (siUPF1). In each of these conditions, cells were either left uninduced (DMSO) or treated with PMA to reactivate the cells. The percentage of reactivation in the form of GFP production was monitored by flow cytometry and the cell lysates were subjected to Western blotting to validate UPF1 knockdown using antibodies against UPF1, $pr55^{Gag}$ and actin. Treatment of cells with siUPF1 resulted in a 68.9 (±29.9) % decrease in UPF1 protein levels as measured by Western blot, demonstrating the efficiency of siUPF1 treatment (Supplementary Figure 2.9. A). UPF1 knockdown had no significant effect on viral reactivation in the uninduced condition (Figure 2.2. A). However, upon reactivation with PMA, UPF1 knockdown lead to a 35.3 (±8.4) % decrease in viral reactivation as compared to the siNS condition (Figure 2.2. A), which correlated with reduced $pr55^{Gag}$ levels observed by Western blots (Figure 2.2. B). In order to determine if this decrease in viral reactivation was due to an effect on the vRNA levels or due to inefficient nucleocytoplasmic export or translation of the vRNA, we also conducted FISH-Flow analyses in each of the above reactions. The levels of vRNA were also quantified by RT-qPCR. Upon treatment with PMA, UPF1 knockdown lead to a 23.5 (±4.8) % decrease in the number of vRNA expressing cells as compared to the siNS treated cells (Figure 2.2. C and D) as well as a 72.6 (±0.1) % decrease in the levels of vRNA as quantified by RT-qPCR (Figure 2.2. E). Of these vRNA expressing cells, a knockdown of UPF1 also led to a 28.0 (±11.8) % decrease in per cell vRNA levels as measured median fluorescence intensity (MFI) of the vRNA channel (PE) as compared the vRNA in the siNS treated cells (Supplementary Figure 2.9. B). This is in accordance with our previous work where we demonstrated that a knockdown of UPF1 resulted in a decrease in vRNA stability [36]. The reduction in vRNA levels as quantified by RT-qPCR in the siUPF1 condition is more dramatic than the reduction of GFP production in the same condition, possibly due to increased stability of GFP as compared to the vRNA. It is also important to note that these detrimental effects of UPF1

knockdown on vRNA levels are specific to the vRNA, since no significant differences were observed in the % of cell expressing a housekeeping mRNA RPL13A and the MFI of the RPL13A mRNA channel measured by FISH-Flow, or in the relative levels of housekeeping mRNA GAPDH measured by RT-qPCR (Supplementary figures 2.9. C – E). However, in these experimental conditions, we can not differentiate between cells that have successful knockdown of UPF1 and non-transfected cells. Therefore, to partially overcome this caveat, we also stained the cells with a UPF1 mRNA probe and, using FISH-Flow analysis, we delineated between UPF1 high vs. UPF1 low cells (Figure 2.2. F). Using this gating strategy, it was observed that the UPF1 low population of the siUPF1-PMA treated cells showed a 50.5 (±31.07) reduction in the % of vRNA-expressing cells as compared to the UPF1 high population of the siNS-PMA condition (Figure 2.2. G). Of these vRNA expressing cells, a knockdown of UPF1 also led to a 1.66 fold reduction in the median fluorescence intensity (MFI) of the vRNA channel (PE) as compared the vRNA in the siNS treated cells (Figure 2.2. H). Since UPF1 has previously characterised roles in nuclear export [37], we determined if a knockdown of UPF1 resulted in increased nuclear retention of the vRNA. Cellular fractionation was performed and the vRNA present in whole cell, cytoplasmic and nuclear fractions were quantified by RT-PCR (Supplementary Figure 2.10. A and B). A decrease is vRNA levels was observed in all fractions, thus implying that in these experimental conditions, UPF1 is acting on vRNA expression rather than on nuclear export (Supplementary Figure 2.10. A and B). Taken together, these data suggest that a knockdown of UPF1 leads to attenuated HIV-1 proviral reactivation in J-Lat cells at a post-transcriptional level, by downregulating vRNA levels and thus, viral reactivation and protein production.

2.4.3. UPF1 overexpression enhances HIV-1 proviral reactivation by stabilising vRNA

UPF1 overexpression has been shown to enhance vRNA stability, nucleocytoplasmic export and translation in previous studies [36, 37]. Therefore, we hypothesised that UPF1 overexpression could enhance proviral reactivation. J-Lat cells were either mock transfected or transfected with FLAG-UPF1. They were then either left uninduced (DMSO) or reactivated with PMA. We employed the FISH-Flow technique using probes against the vRNA as well as UPF1 mRNA to gate for UPF1-overexpressing populations (Figure 2.3. A). The percentage of reactivation was monitored by flow cytometry and the cell lysates were subjected to Western blotting to validate UPF1 overexpression using antibodies against UPF1, $pr55^{Gag}$ and actin (Figure 2.3. B and C). UPF1 overexpression resulted in a 21.3 (±13.5) % increase in viral reactivation upon PMA treatment as compared to the mock-transfected condition (Figure 2.3. B). UPF1 overexpression also led to a 14.4 (±4.2) % increase in vRNA levels in the UPF1 overexpressing cells and compared to the mock transfected cells (Figure 2.3. D and E). UPF1 overexpression in uninduced condition shows no increase in % of vRNA cells as demonstrated by FISH-Flow (Figure 2.3. F), indicating that UPF1 alone is unable to activate transcription of the provirus and PMA is necessary for transcription to take place. UPF1 overexpression also does not result in a change in the % of vRNA+/GFP- cells as compared to mock treated cells (Supplementary figure 2.10. C). This implies that enhanced viral reactivation upon UPF1 overexpression is due to an effect on vRNA levels rather than an increase in the translation of the transcriptional-competent reservoir. Hence, UPF1 overexpression enhances proviral reactivation at a post-transcriptional level by modestly increasing the expression of the vRNA, thereby resulting in enhanced viral reactivation. This is consistent with our previous work where we demonstrated that an overexpression of UPF1 results in enhanced vRNA stability [36].

In order to determine which domain of UPF1 is responsible for enhancing vRNA expression, we either mock transfected cells, or transfected them with FLAG-UPF1 or other constructs of UPF1 that contain deletions in the N-terminal region (FLAG-UPF1-Δ20-150), deletions in the C-terminal (FLAG-UPF1-1-1074), mutations in the RNA helicase domain of UPF1 (FLAG-UPF1-RR857AA), mutations leading to a deficiency in UPF2 binding ability (FLAG-UPF1-LECY) or mutations in the ATPase region of UPF1 (FLAG-UPF1-DE). These cells were then treated with PMA and the % of

reactivation was monitored by flow cytometry (Supplementary Figure 2.11. A). The ability of UPF1 overexpression to enhance viral reactivation was lost when the FLAG-UPF1-Δ20-150 construct, which contains an N-terminal deletion, or the FLAG-UPF1-DE that has impaired ATPase activity, were used (Figure 2.3. G and H). The overexpression of these UPF1 mutants resulted in reactivation at levels comparable to the mock transfected cells treated with PMA. These results indicate that the N-terminal domain and ATPase activity of UPF1 are necessary for its mild effect on enhancing vRNA expression and are consistent with our previous work [36].

2.4.4. UPF2 overexpression attenuates HIV-1 reactivation via an interaction with UPF1

Previous work from our lab has demonstrated that UPF2 is excluded from the HIV-1 RNP and that its overexpression can block UPF1-mediated nucleocytoplasmic export of vRNA [37]. UPF2 is also known to bind UPF1 with a high affinity [51]. For these reasons, we hypothesised that when UPF2 is present in excess it can sequester UPF1 in the cytoplasm resulting in reduced UPF1 being bound to vRNA. J-Lat cells were either mock transfected or transfected with FLAG-UPF2 and cells were either left uninduced (DMSO) or treated with PMA. The percentage of reactivation in the form of GFP production was monitored by flow cytometry and the cell lysates were subjected to Western blotting to validate UPF2 overexpression using antibodies against UPF2, $pr55^{Gag}$ and actin. Upon reactivation with PMA, UPF2 overexpression resulted in a 25.95 (±16.8) % decrease in viral reactivation (Figure 2.4. A) and viral protein production (Figure 2.4. B). To differentiate between UPF2 overexpressing cells from the whole population and to see if it has any effect on vRNA levels, we conducted FISH-Flow using probes against UPF2 mRNA and vRNA (Figure 2.4. C). Upon reactivation with PMA, UPF2 overexpression led to a 57.36 (± 27.83) decrease in the percentage of vRNA expressing cells as compared to the mock transfected cells (Figure 2.4. D and E). Therefore, an overexpression of UPF2 resulted in a modest, albeit statistically significant ($p<0.05$) decrease in viral reactivation due to a reduction in vRNA expression.

In order to determine if this detrimental effect of UPF2 on vRNA levels is an indirect effect due to its binding to UPF1, we transfected cells with a mutant of UPF2 that does not bind to UPF1 [37, 52, 53] (FLAG-UPF2-1-1096) and compared the % of reactivation in the mock transfected cells, the UPF2 expressing cells and the UPF2-1-1096-expressing cells. It was observed that when UPF2 loses the ability to bind UPF1, there is a loss of its inhibitory effect on reactivation, with reactivation at levels comparable to the mock treated cells (Figure 2.4. F and H). We also co-transfected FLAG-UPF2 with either FLAG-UPF1 or with FLAG-UPF1-LECY that contains a mutation in the UPF2 binding site and monitored the % of reactivation. UPF1 coexpression is able to rescue the deleterious effect of UPF2 on viral reactivation, but not when it contains a mutation to the UPF2-binding site (Figure 2.4. G and H). This indicates that the deleterious effect of UPF2 on viral reactivation is a result of its binding to UPF1 which is sequestered and unable to exert a positive effect on vRNA expression, consistent with previous reports [37].

2.4.5. SMG6 overexpression is detrimental to HIV-1 proviral reactivation

UPF1 is an integral member of a network of proteins involved in NMD, including UPF2, UPF3A, UPF3B, SMG6, SMG5, SMG7 and SMG1. SMG6 is the endonuclease involved in the final step of the degradation of aberrant RNA in NMD [54, 55] and has a direct influence on RNA levels. Thus, to evaluate the roles of SMG6 in proviral reactivation, we either mock transfected J-Lat cells or transfected them with HA-SMG6 and either left them uninduced or reactivated them with PMA. The percentage of reactivation in the form of GFP production was monitored by flow cytometry (Figure 2.5. A) and the cell lysates were subjected to Western blotting to validate SMG6 overexpression using antibodies against SMG6, $pr55^{Gag}$ and actin (Figure 2.5. B). Overexpression of SMG6 resulted in a 21.2 (± 9.1) % decrease in reactivation (Figure 2.5. A). Furthermore, upon reactivation with PMA, FISH-Flow analyses revealed a small but significant decrease (7.6 ± 4.1%) in the percentage of vRNA expressing cells upon SMG6 overexpression as compared to the mock-transfected cells (Figure 2.5. C and D). Of the vRNA present upon SMG6 overexpression, there was a 1.25-fold decrease in the median fluorescence intensity (Figure 2.5. E). Thus, SMG6 is detrimental to vRNA expression and attenuates PMA-induced proviral reactivation.

SMG6 contains an exon junction binding domain (EBM) [56], a 14-3-3-like domain that binds to phosphorylated UPF1 [57] and a PilT N-terminus (PIN) domain [58] that possesses the endonuclease activity [58-60]. In order to determine which of these domains are responsible for the negative effect on vRNA levels, we transfected J-Lat cells with plasmids that express SMG6 with mutations in each of the aforementioned domains; HA-SMG6-mEBM, HA-SMG6-m14-3-3 and HA-SMG6-mPIN respectively. These cells were reactivated with PMA and the percentage of reactivation was monitored using flow cytometry. While the overexpression of HA-SMG6 and the exon junction binding mutant HA-SMG6-mEBM attenuated proviral reactivation, the overexpression of HA-SMG6-m14-3-3 and HA-SMG6-mPIN displayed reactivation levels similar to the mock transfected cells (Figure 2.5. F and G). Thus, these results demonstrate that both, the binding of SMG6 to phosphorylated UPF1 and its endonuclease activity are necessary for its inhibitory effect on vRNA levels.

2.4.6. SMG6 knockdown increases vRNA expression, but does not affect viral reactivation

To determine the effect of SMG6 depletion on HIV-1 proviral reactivation, we conducted siRNA mediated knockdown studies. J-Lat cells were either transfected with a non-silencing siRNA (siNS) or with siRNA against SMG6 (siSMG6) and cells were either left uninduced (DMSO) or treated with PMA to reactivate the cells. The percentage of reactivation in the form of GFP production was monitored by flow cytometry and the cell lysates were subjected to Western blotting to validate SMG6 knockdown using antibodies against SMG6, pr55Gag and actin. A knockdown of SMG6 did not have a significant effect on viral reactivation at the level of protein production (Figure 2.6. A and B). However, upon reactivation with PMA and using FISH-Flow using probes against vRNA, SMG6 knockdown resulted in a small but significant increase (6.9 ± 1.8 %) in the total number of vRNA expressing cells as compared to the siNS condition (Figure 2.6. C and D). This further illustrates that SMG6 is detrimental to vRNA levels.

2.4.7. UPF1 knockdown impairs vRNA expression in primary HIV-1 infected CD4+ T cells

UPF1 enhances vRNA stability and, as a consequence, viral reactivation in J-Lat cells. UPF2 and SMG6 are detrimental to vRNA expression, both, via interactions with UPF1. We also assessed the effects of UPF1, UPF2 and SMG6 overexpression on TNFα-induced reactivation of J-Lat cells and observed comparable results (Supplementary Figure 2.11. B). However, whether these effects of UPF1 on vRNA expression and $pr55^{Gag}$ expression were also observed in primary CD4+ T cells was yet to be determined. In order to address this question, we conducted shRNA-mediated knockdown of UPF1 in primary CD4+ T cells and observed the effects on vRNA levels and $pr55^{Gag}$ expression upon HIV-1 infection by FISH-Flow. Negatively selected CD4+ T cells from three donors were activated with phytohemagglutinin (PHA). They were then transduced with shUPF1-containing lentiviral particles. Lentiviral particles containing a scrambled sequence were used as a negative control (shNS). The cells were infected with HIV-1 24 h post transduction by spinoculation. Cells were collected 6 days post infection and FISH-Flow was conducted to monitor vRNA and intracellular $pr55^{Gag}$ levels. Cell lysates were also subjected to Western blotting to validate UPF1 knockdown (Figure 2.7. A). In humans, UPF1 has two isoforms and both isoforms are detected in primary CD4+ T cells [61] (Supplementary Figure 2.12. A). However, in J-Lat cells, only the larger one is expressed at high enough levels to be detected by the UPF1 antibody (Supplementary Figure 2.12. A). shUPF1 treatment in primary T cells resulted in a 53.8 (±4.5) % decrease in UPF1 protein levels as compared to the shNS-treated cells (Supplementary Figure 2.12. B). Results from three independent donors demonstrated that a knockdown of UPF1 resulted in a 45.16 (± 27.9) % decrease in vRNA levels as compared to the mock treated cells (Figure 2.7. B and C). This also corresponded with 20.1 (± 10.9) % reduced intracellular $pr55^{Gag}$ staining (Figure 2.7. D). Therefore, UPF1 also enhances vRNA levels and promotes viral gene expression in primary CD4+ T cells.

2.5. Discussion

The 'active viral reservoir' has been defined as the HIV-1 infected cells that contain viral RNA species but do not produce infectious viral particles [62, 63] and this highlights the post-transcriptional maintenance of HIV-1 latency. Latently-infected resting CD4+ cells T cells have been demonstrated to contain cell-associated unspliced and multiply spliced HIV-1 RNA [11, 64]. In these cells, the vRNA was sequestered within the nucleus and could be efficiently rescued through the overexpression of the host polypyrimidine tract binding protein (PTB), suggesting that latency can be reversed at a post-transcriptional level [64]. Two characterised primary T cell models of latency have also demonstrated a post-transcriptional block to HIV-1 reactivation, either by sequestration of the vRNA in the nucleus or splicing defects [14, 16, 65]. In addition, microRNAs have been implicated in the maintenance of HIV-1 latency (reviewed in [18]), providing another example of how post-transcriptional events can affect proviral reactivation. In the quest for an HIV-1 cure, the importance of investigating the contribution of post-transcriptional events and vRNA metabolism in the maintenance of HIV-1 latency is being recognised [66-68]. One HIV-1 cure strategy is the 'shock and kill' approach which involves reactivating the latent provirus by small molecules (shock) and then to eliminating the virus (kill) using intensive cART and/or immunomodulators [69]. Numerous compounds are under investigation as candidates for latency-reversing agents (LRAs) which promote the transcription of the provirus (reviewed in [70, 71]). So far, the use of LRAs have limited ability to decrease the size of the viral reservoir, with only two reports of successful reduction in reservoir size [7, 72, 73]. The shortcomings of current LRAs is highlighted in a recent study using FISH-Flow in which CD4+ T cells from HIV-1 infected patients were reactivated with the LRAs romidepsin or PMA/ionomycin and only 2-10% of cells that expressed vRNA produced viral proteins [17]. Therefore, the LRAs might be more effective if used in combination with drugs that affect vRNA metabolism at a post-transcriptional level. By modulating the activities of the RNA surveillance proteins or creating small molecules that mimic their activity, we can increase the stability of the vRNA to facilitate reactivation of these latent cells so that they are visible to the immune system and can be targeted by host immune responses and antiretrovirals. Alternatively, we can also

apply this study to create novel long-lasting antiretrovirals by designing small molecules to inhibit the binding of UPF1 to vRNA thereby decreasing vRNA stability and reducing viral production.

Using FISH-Flow, this study demonstrates that the RNA surveillance proteins UPF1, SMG6 and UPF2 can affect HIV-1 gene expression, and thus viral reactivation at a post-transcriptional level. Although the effects of UPF1, UPF2 and SMG6 overexpression on modulating viral latency are modest (Figure 2.3. B, 2.4. A and 2.5. A), these effects nevertheless provide novel evidence of the contribution of post-transcriptional events in viral reactivation from latency. Complete knockout of UPF1, UPF2 or SMG6 using CRISPR-cas9 gene editing strategies could result in more profound effects on viral replication. UPF1 was demonstrated to be a positive regulator of viral reactivation in the J-Lat 10.6 latent T cell model. Notably, we also demonstrate a direct effect of UPF1 on enhancing vRNA levels and viral gene expression in primary CD4+ T cells. The overexpression of the ATPase mutant of UPF1 (FLAG-DE-UPF1) did not lead to enhanced reactivation of HIV-1 in J-Lat cells (Figure 2.3. G and H), indicating that the ATPase activity is responsible for enhanced vRNA expression and viral reactivation. This is in concordance with our previous work where we showed that this UPF1 construct was unable to upregulate vRNA levels and enhance vRNA stability [36]. This ATPase mutant has impaired RNA-binding capacity [74]. To exert its positive effects on vRNA metabolism, UPF1 needs to be able to bind to the vRNA and subsequently lead to the assembly of distinct RNPs that promote vRNA stability, export and translation [37]. An impairment of RNA binding capability could lead to a dissociation of UPF1 from the vRNA, thereby providing another possible explanation why no enhanced viral reactivation was observed when the ATPase mutant of UPF1 was used.

The HIV-1 vRNA metabolism is controlled by numerous cis-acting RNA sequences [75], such as the cis-repressive sequences or instability sequences (INS) [76]. UPF1 contains two zinc fingers that have been implicated to bind to INSs [77] and thus, could promote vRNA stability. The FLAG-UPF1-Δ20-150 construct contains a deletion in the zinc finger motif [36] that could lead to impaired binding to the HIV-1 INS. In agreement with our previous studies where we demonstrate that an overexpression of FLAG-UPF1-Δ20-150 does not lead to enhanced vRNA expression levels [36]; here we demonstrated that, in the context of reversal from viral latency, an overexpression of FLAG-UPF1-Δ20-150 does not lead to enhanced proviral reactivation (Figure

2.3. G and H), most likely due to impaired binding of UPF1 to the vRNA due to the loss of a zinc finger motif.

We have also previously shown that UPF2 is excluded from HIV-1 RNPs through antagonistic interactions with the viral or host proteins such as Rev or Staufen1 [37]. The binding of UPF2 to UPF1 has been reported to induce a conformational change in UPF1 that stimulates its RNA helicase activity and dampens its RNA binding capability, thereby hampering its binding to the vRNA [78, 79]. UPF2 also binds to UPF1 with high affinity [80] and this could limit the availability of UPF1 to bind to the vRNA. Our data reinforce the hypothesis that UPF2 is detrimental to vRNA metabolism, as we observed that overexpression of UPF2 resulted in reduced vRNA expression and viral reactivation (Figure 2.4. A - E). This deleterious effect is likely a result UPF2 binding to UPF1 and its sequestration, since viral reactivation was restored to levels similar to control cells when the UPF2 mutant deficient in UPF1 binding was used (Figure 2.4. F to H). In accordance with our work, a previous report using an shRNA library in J-Lat 5AB cells showed that shRNAs against UPF1 were disenriched in the reactivated population as compared to the latent population, indicating that it exerts a positive effect on the reactivation of the HIV-1 provirus [81]; whereas shRNAs against UPF2 were enriched in the reactivated population, indicating that UPF2 promotes that maintenance of latency in J-Lat cells [81].

SMG6 is the endonuclease responsible for cleaving mRNAs that are targeted for NMD [54, 55]. Both SMG6 and UPF1 have been reported to be present at transcription sites [82] and SMG6 interacts with UPF1 in a phospho-dependent [57] and a phospho-independent manner [61]. Furthermore, because of its endonuclease activity, SMG6 could have a direct effect on UPF1-bound mRNA levels, such as the vRNA. Our observation that an overexpression of SMG6 results in a decrease of vRNA expression and, consequently, decreased viral reactivation, suggests that SMG6 is detrimental to vRNA stability (Figure 2.5. A – G). Using mutational studies, we identified that the binding of SMG6 via its 14-3-3 like domain to phosphorylated UPF1 as well its endonuclease activity via its PIN region is necessary to downregulate the viral reactivation (Figure 2.5. F and G).

Recent transcriptome analyses have demonstrated that UPF1 binds promiscuously to all cellular RNAs; both, canonically identified NMD targets as well as to non-NMD targets and long non-coding RNAs [39, 83-86]. The marker for a cellular NMD target has been revealed to be the RNA's binding to phosphorylated UPF1 [19, 87]. UPF1 interacts with the PIK-related protein kinase SMG1, SMG8, SMG9, and the two translation termination factors eRF1 and eRF3 to form a decay inducing complex called the SURF [88, 89]. The phosphorylation of UPF1 by SMG1 is necessary for mRNA decay and creates an N-terminal binding platform for SMG6 that cleaves the targeted mRNAs [54, 55, 57]. Hyperphosphorylated UPF1 has been also shown to attract downstream NMD machinery with higher affinity [90]. Therefore, we can speculate that in the context of the interaction between UPF1 and the vRNA, the hyperphosphorylation of UPF1 would be detrimental to vRNA stability due to increased recruitment of SMG6 and other mRNA decay factors. The ATP deficient UPF1 mutant FLAG-UPF1-DE has also been demonstrated to be hyperphosphorylated and assembles complexes with SMG6 on both target and non-target mRNAs [86]. This could provide another possible explanation why the overexpression of the ATPase defective UPF1 did not result in enhanced viral reactivation (Figure 2.3. G and H). Further investigation is required to elucidate the roles of the phosphorylation status of UPF1 on proviral reactivation.

In this manuscript, we provide evidence that the RNA surveillance proteins UPF1, UPF2 and SMG6 can affect vRNA expression and thus, the maintenance of HIV-1 latency. These findings can be applied to bolster the reactivation of the HIV-1 provirus to effectively decrease the size of the viral reservoir using a shock and kill approach or can be harnessed to create a novel set of antiretrovirals.

2.6. Materials and methods

2.6.1. Cell culture

J-Lat 10.6 cells (J-Lat full-length clone 10.6; NIH AIDS Reagent Program) are a Jurkat derived T-cell line that is latently infected with HIV-1 in which the *nef* sequence was replaced with a green fluorescent protein (GFP) coding sequence [47]. J-Lat latent proviruses were reactivated by adding 20ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) to the culture media for 24 h. In case of reactivation with TNFα, 10ng/ml TNFα (Sigma-Aldrich) was added to the culture media for 24 h. Reactivation of cells was quantified by measuring GFP expression by flow cytometry. All cell cultures were maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone) and 1 % penicillin/streptomycin (Life Technologies) at 37 °C and 5% CO_2. HEK293T cells were purchased from the American Type Culture Collection (ATCC). TZM-bl HeLa cell line was obtained from NIH AIDS Reference and Reagent Program. Both of these cells lines were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin (Invitrogen). PBMCs were isolated from leukophoresed blood collected from healthy donors. All subjects provided informed consent for participating in this study. The research ethics boards of the recruiting sites, the Centre Hospitalier de l'Universite de Montreal and McGill University Health Centre approved this study. PBMCs were isolated by density-gradient centrifugation using lymphocyte separation medium (Corning). CD4+ T cells were negatively selected using the EasySep human T cell enrichment kit according to manufacturer's protocol (StemCell). Negatively selected CD4+ T cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone) and IL-2 (Sigma-Aldrich). CD4+ T cells were activated by treating them with 10ug/ml PHA (Sigma-Aldrich) for 72 hours.

2.6.2. Antibodies

Mouse anti-p24, was obtained from NIH AIDS Reagents Program; rabbit antisera to UPF1 and UPF2 were generously supplied by Jens Lykke-Andersen (University of California, San Diego, CA, USA); rabbit anti-EST1A (SMG6) and mouse anti-actin were purchased from Abcam; rabbit anti-FLAG was purchased from Sigma-Aldrich; mouse anti-HA was purchased from Roche; mouse anti-GAPDH was purchased from Techni-science; mouse anti-nucleolin was purchased from Santa-

Cruz Biochemistry; KC57-FITC was purchased from Beckman Coulter; horseradish peroxidase-conjugated secondary antibodies were purchased from Rockland Immunochemicals.

2.6.3. Plasmids

The plasmids pCI-FLAG, FLAG-UPF1, FLAG-UPF1-Δ20-150, FLAG-UPF1-1-1074, FLAG-UPF1-RR857AA, FLAG-UPF1-LECY, FLAG-UPF1-DE, FLAG-UPF2 and FLAG-UPF2-1-1096 were described previously [36, 37, 52]. HA- SMG6, HA-SMG6-mEBM, HA-SMG6-m14-3-3 and HA-SMG6-mPIN were a kind gift from Dr. Oliver Muhlemann and are previously described [61]. pNL4.3 was obtained from NIH AIDS Reagents Program.

2.6.4. Gene silencing

Custom siRNA duplexes were synthesised by Qiagen. The target sequence for UPF1 was 5'-AAGATGCAGTTCCGCTCCATT-3' and for SMG6 was 5'-GCTGCAGGTTACTTACAAG-3'. The siNS used in this study is a commercially available non-silencing control duplex with target sequence 5'-AATTCTCCGAACGTGTCACGT'-3'.

2.6.5. Transfections

J-Lat or Jurkat cells were transfected with either 1 µg of plasmid DNA or 20 nM of siRNA per 1 x 10^6 cells using the Neon Transfection System (Thermo Fisher Scientific) according to manufacturer's protocols using the following electroporation parameters: three pulses of 1,350 V and 10 ms at a cell density of 1×10^7/mL. J-Lat cells were reactivated 24 hours after transfection. HEK293T cells were transfected using JetPrime transfection reagent according to manufacturer's protocol (Polyplus) using 1ul of Jetprime for 1ug of plasmid DNA.

2.6.6. Viral transduction

psPAX2, pMD2.G and the pLKO-shNS lentiviral control plasmid containing scrambled non-target shRNA used as a negative control was a kind gift from Dr. Marc Fabian (McGill University). pLKO-shUPF1 (TRCN0000022254) expression vector containing shRNA to UPF1 was obtained from the McGill genetic perturbation service. HEK293T cells were plated in 10cm-dishes plates and were co-transfected with either shNS or shUPF1 expressing lentivirus, psPAX2 and pMD2.G. Supernatants were collected 48 hr post-transfection, passed through a 0.45-µm filter (Pall) and supplemented with 5 µg/ml polybrene (Sigma-Aldrich). The viral particles were added to the

primary CD4+ T cells (1ml of supernatant per 10000000 cells) and incubated for 16 hours, following which they were infected with HIV-1.

2.6.7. HIV-1 virus production and infection

NL4.3 virus particles were prepared by transfection of HEK293T cells with HIV-1 NL4-3 provirus-encoding plasmid pNL4.3 using the JetPrime transfection reagent. The supernatants were collected 48 hours post transfection, filtered through a 0.45-µm filter (Pall) and centrifuged at 20,000 r.p.m. for 1 hour at 4°C to pellet the virus. Viruses were resuspended in RPMI and stored at -80°C. The multiplicity of infection (MOI) of viruses were quantified using the X-gal staining assay in TZM-bl cells as described in [91]. CD4+ T cells in RPMI were infected with an MOI of 0.5 NL4.3 viruses by spinoculation at 1800 r.p.m. for 45 minutes. Following spinoculation, the cells were washed and replenished with complete culture media. Cells were collected 6 days post infection.

2.6.8. Western blotting

Cells were lysed in NP40 lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40). Protein concentration on each cell lysate was quantified by Bradford assay. Equal amounts of protein (20 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Blocking was performed using 5% non-fat milk in Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TBST) for 1 hour at room temperature. Membranes were probed with the indicated primary and corresponding horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using Western Lightning Plus-ECL (PerkinElmer). Signal intensities were scanned by densitometry using ImageJ software (NIH, Bethseda, USA).

2.6.9. FISH-Flow

Cells were collected, fixed, permeabilized and subjected to the PrimeFlow RNA assay (Thermo Fisher Scientific) following the manufacturer's instructions and as described in [43, 92]. For intracellular $pr55^{Gag}$ staining in primary CD4+ T cells, KC57-FITC antibody (Beckman Coulter) was used in permeabilisation buffer from the kit at a dilution of 1:50 for 30 min at room temperature, followed by 30min at 4°C. For all samples, mRNA was labelled with a set of 40 probe pairs diluted 1:20 in diluent provided in the kit and hybridized to the target mRNA for 2 hr at 40°C. The probes

for GagPol, UPF1, UPF2 and SMG6 used had the following catalog numbers: GagPol HIV-1 VF10-10884, UPF1 VA1-3004200, UPF2 VA1-3007897 and SMG6 VA1-3001031. Positive control probes against the house-keeping gene RPL13A (VA1-13100) were included in each experiment. Samples were washed to remove excess probes and stored overnight in the presence of RNAsin. Signal amplification was then performed by sequential 1.5 hr, 40°C incubations with the pre-amplification and amplification mix. Amplified mRNA was labelled with fluorescently-tagged probes for 1 hr at 40°C. Gates were set on the uninfected Jurkat cells, unstimulated J-Lat control or uninfected primary CD4+ T cells where appropriate. Samples were acquired on a BD LSR Fortessa Analyzer. Analysis was performed using the FlowJo V10 software (Treestar).

2.6.10. Confocal Microscopy following FISH-Flow

Cells that underwent the FISH-Flow assay described above were seeded on 18 mm diameter coverslips and air dried. Coverslips were mounted in ProLong Gold Antifade Reagent with DAPI (Life Technologies). Laser scanning confocal microscopy was performed on a Leica DM16000B microscope equipped with a WaveFX spinning disk confocal head (Quorum Technologies) using a 63X objective lens. Images were acquired with a Hamamatsu ImageEM EM-charges coupled device (CCD) camera and image reconstruction was performed with the Imaris software (v. 8.4.1, Bitplane, Inc.).

2.6.11. RT-qPCR

For data presented in Figure 2.2. E, total RNA was extracted from cells using Aurum Total RNA Mini kits (Bio-Rad). RT-qPCR analysis of HIV-1 RNA levels was performed as previously described [93, 94]. For data presented in Supplementary Figure 2.9. E and 2.10. B , cellular fractionation was performed as described in [95]. RNA extraction from each fraction were performed using Trizol Reagent (Thermo Fisher Scientific) following manufacturer's instructions. cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA and primers were then added to GoTaq Green Master Mix (Promega). GAPDH was amplified using the primers GAPDH_1 forward 5'- TGACCACAGTCCATGCCATC-3' and GAPDH_1 reverse 5'-ATGATGTTCTGGAGAGCCCC-3' and HIV-1 vRNA using the primers pNL4-3_1 forward 5'-GGGAGCTAGAACGATTCGCA-3' and pNL4-3_1 reverse 5'-GGATGGTTGTAGCTGTCCCA-3'. The PCR products were visualised in a 1% agarose gel by staining the DNA with RedSafe Nucleic Acid

Staining Solution (iNtRON). Signals were captured using a Gel Doc System and intensities were normalised to the GAPDH signal.

2.6.12. Statistical analysis

All experiments were performed in triplicate, and the data are presented as the mean ± standard deviation (SD). A p-value of <0.05 in a student's t-test, one-way or two-way ANOVA test was considered statistically significant. GraphPad Prism 6 (Graphpad Software Inc.) was used to conduct statistical analyses and create graphs.

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2.8. Figures and figure legends

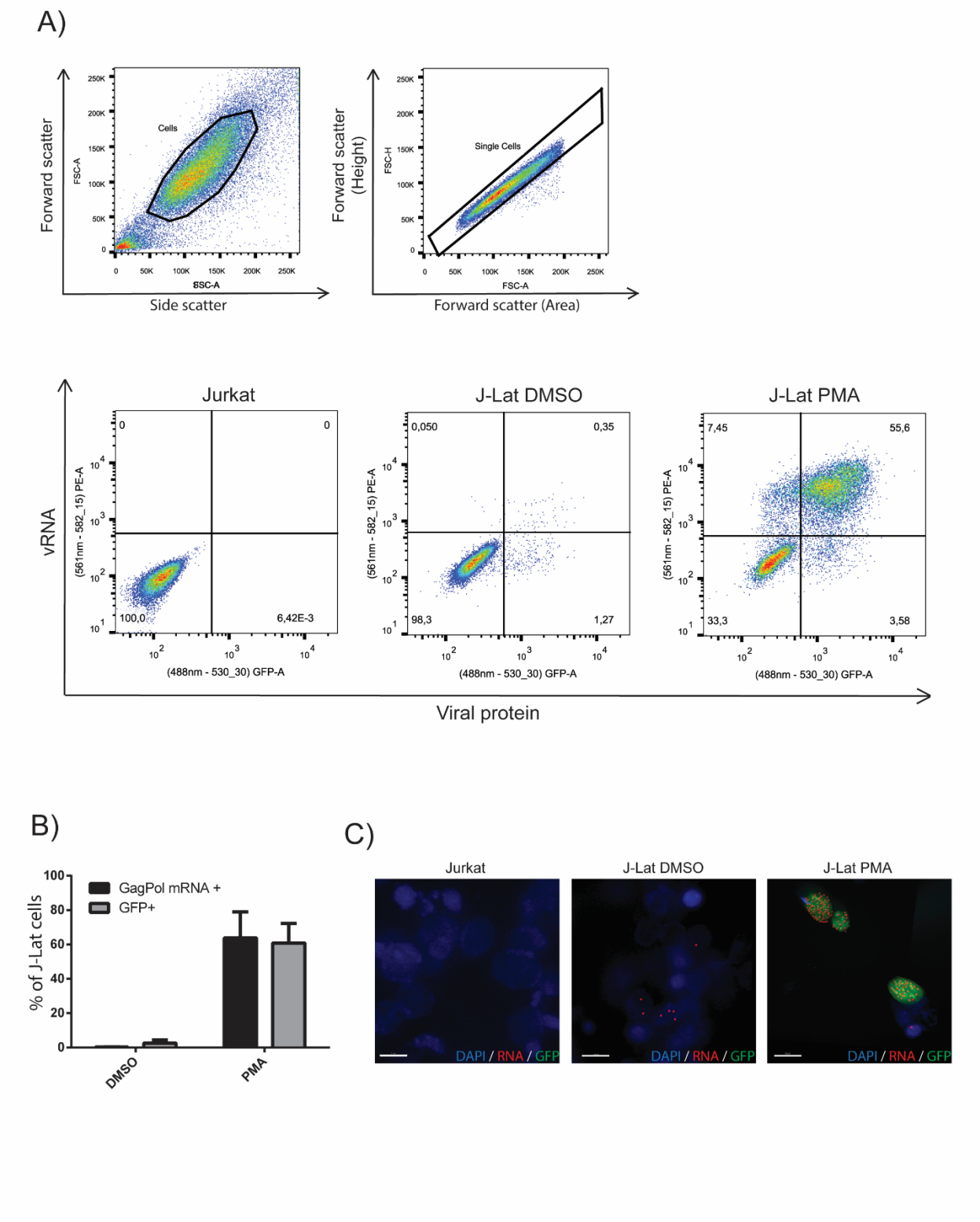

Figure 2.1.: Characterisation of FISH-Flow technique in J-Lat cells.

Figure 2.1.: Characterisation of FISH-Flow technique in J-Lat cells (continued).

J-Lat cells were either treated with DMSO or with PMA to reactivate the provirus. Jurkat cells were used as an uninfected negative control. **A)** Dot plots representing cells gated for size by forward and side scatter, for singlets by forward scatter height vs. area and finally for GFP expression and vRNA staining. **B)** The % of GFP+ and the % of vRNA-expressing cells were quantified. Error bars represent the standard deviation from three independent experiments. **C)** Representative images of cells in each of the above conditions imaged by confocal microscopy. In example images from sorted populations, DAPI is in blue, vRNA in red, and cells making viral protein produce GFP in green. Scale bars represent 10µm.

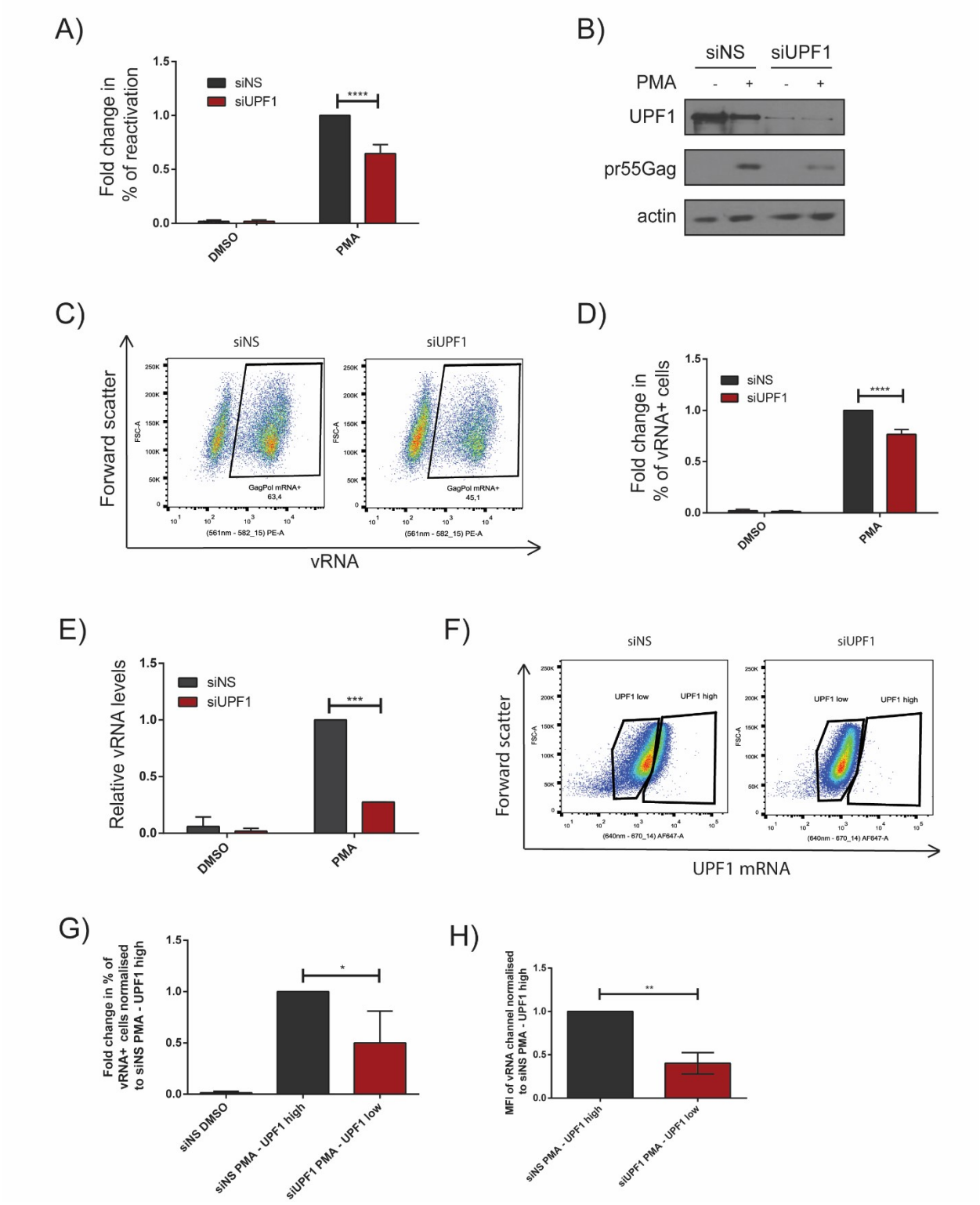

Figure 2.2.: UPF1 knockdown attenuates reactivation of HIV-1 in J-Lat cells.

Figure 2.2.: UPF1 knockdown attenuates reactivation of HIV-1 in J-Lat cells (continued).

J-Lat 10.6 cells were either transfected with siNS or siUPF1 and were uninduced (DMSO) or reactivated (PMA). **A)** Reactivation, monitored by GFP production, was quantified by Flow cytometry and the percentages of reactivation were normalised to the siNS-PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.0001$). **B)** Cell lysates were run on SDS-PAGE gels and UPF1 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **C)** Example dot plot depicting vRNA expression in siNS-PMA and siUPF1 PMA conditions using FISH-Flow technique and, **D)** the % of vRNA expressing cells were quantified and normalised to the siNS-PMA condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.0001$). **E)** Levels of vRNA were quantified using RT-qPCR and normalised to the siNS-PMA condition. Error bars represent the standard deviation from two independent experiments, each done in triplicate. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.001$). **F)** Gating strategy of cells separated into UPF1 low or high by detecting UPF1 mRNA levels by FISH-Flow. **G)** The % of vRNA expressing cells in each condition normalised to the siNS-PMA/UPF1 -high condition. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.05$). **H)** MFI of the vRNA signal were quantified. Asterisks represent statistically significant difference between groups (student's t-test; $p < 0.01$)

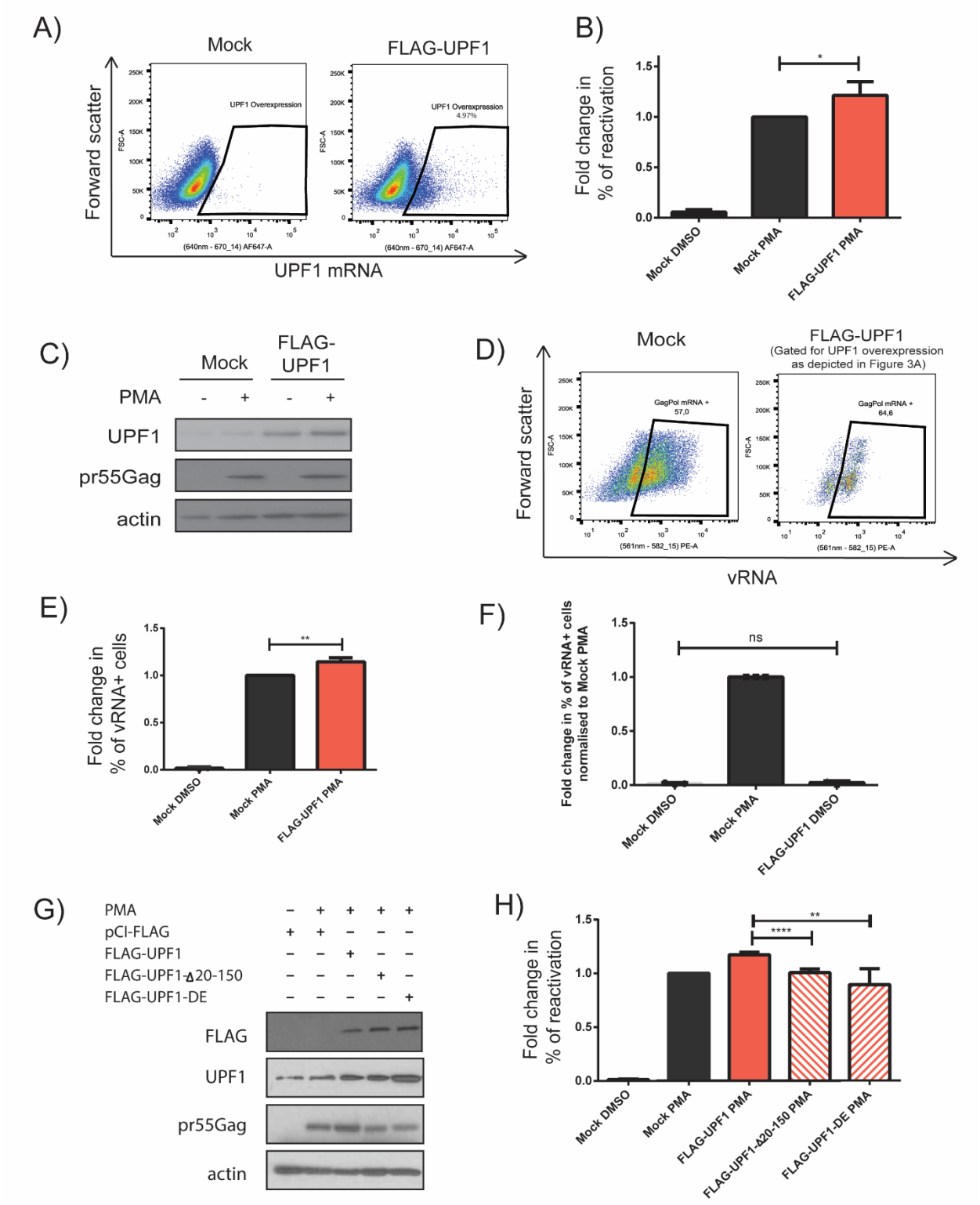

Figure 2.3.: UPF1 overexpression leads to enhanced reactivation of HIV-1 in J-Lat cells.

Figure 2.3.: UPF1 overexpression leads to enhanced reactivation of HIV-1 in J-Lat cells (continued).

J-Lat 10.6 cells were either mock transfected or transfected with Flag-UPF1 and were uninduced (DMSO) or reactivated (PMA). **A)** Gating strategy to detect UPF1 overexpressing cells by detecting UPF1 mRNA levels by FISH-Flow. **B)** Of the UPF1 overexpressing cells gated for in A), reactivation, monitored by GFP production, was quantified by flow cytometry and the percentages of reactivation were normalised to the mock-PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.05$). **C)** Cell lysates were run on acrylamide gels and UPF1 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **D)** Example dot plot depicting vRNA expression in mock transfected and UPF1 overexpressing populations using FISH-Flow technique. **E)** The % of vRNA expressing cells were quantified and normalised to the mock-PMA condition. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.01$). **F)** J-Lat cells were either mock transfected and uninduced (Mock DMSO), mock transfected and reactivated with PMA (Mock PMA) or transfected with FLAG-UPF1 and left uninduced (FLAG-UPF1 DMSO). The % of vRNA expressing cells were quantified. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p > 0.05$). **G)** J-Lat cells were mock transfected or transfected with FLAG-UPF1, FLAG-UPF1-Δ20-150 or FLAG-UPF1-DE and reactivated using PMA. Cell lysates were run on SDS-PAGE gels and UPF1 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **H)** Reactivation was quantified in FLAG-UPF1-Δ20-150 and FLAG-UPF1-DE expressing cells and the percentages of reactivation were normalised to the mock-PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.0001$ and $p < 0.05$ respectively).

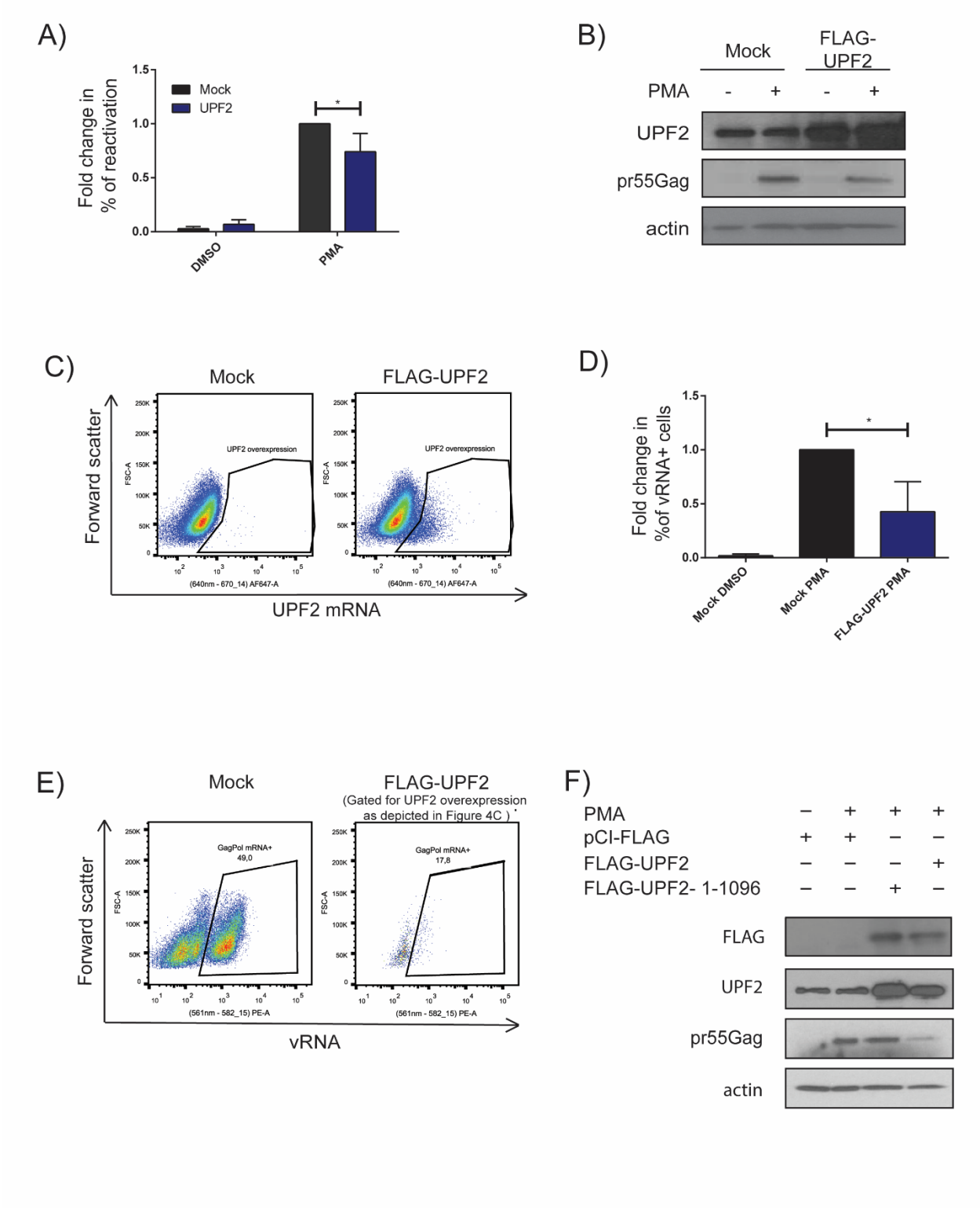

Figure 2.4.: UPF2 overexpression inhibits the reactivation of HIV-1 in J-Lat cells.

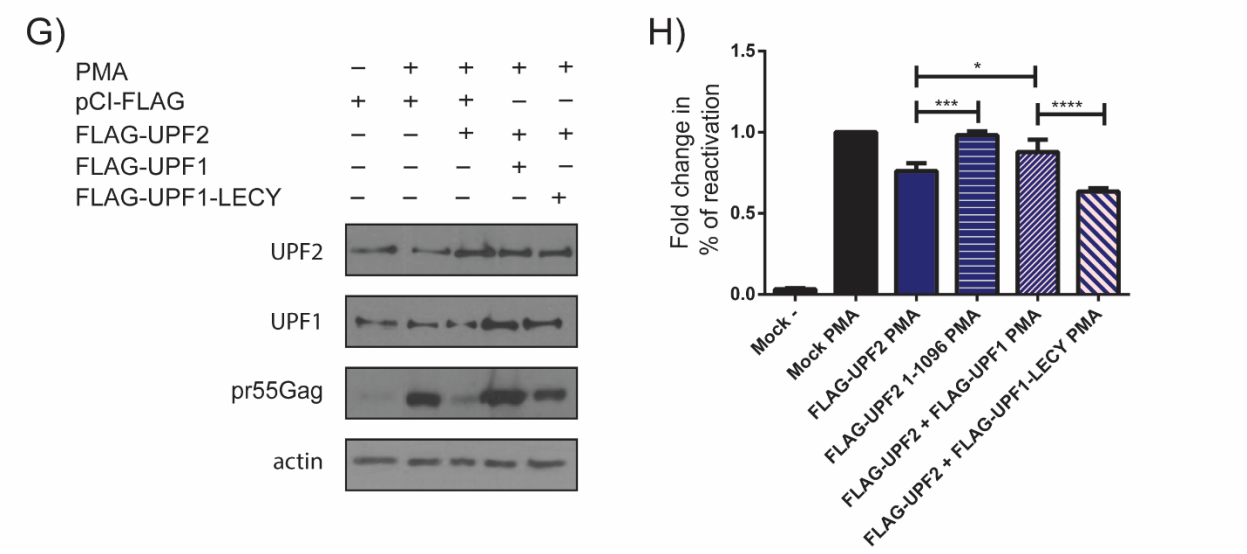

Figure 2.4.: UPF2 overexpression inhibits the reactivation of HIV-1 in J-Lat cells (continued).

J-Lat 10.6 cells were either mock transfected or transfected with Flag-UPF2 and were uninduced (DMSO) or reactivated (PMA). **A)** Reactivation, monitored by GFP production, was quantified by flow cytometry and the percentages of reactivation were normalised to the mock-PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.05$). **B)** Cell lysates were run on SDS-PAGE gels and UPF2 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **C)** Gating strategy to detect UPF2 overexpressing cells by detecting UPF2 mRNA levels by FISH-Flow. **D)** Of the UPF2-mRNA expressing cells gated for in C), the % of vRNA expressing cells were quantified. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.05$). **E)** Example dot plot depicting vRNA expression in mock transfected and UPF2 overexpressing populations using FISH-Flow technique. **F)** J-Lat cells were mock transfected or transfected with FLAG-UPF2 or FLAG-UPF2-1-1096. Cell lysates were run on acrylamide gels and UPF2 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **G)** J-Lat cells were mock transfected, transfected with FLAG-UPF2 or co-transfected with FLAG-UPF1 or FLAG-UPF1-LECY. Cell lysates were run on acrylamide gels and UPF2, UPF1 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **H)** Reactivation in the form of GFP expression was quantified in cells

transfected as in F) and G) cells and the percentages of reactivation were normalised to the mock-PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.0001$).

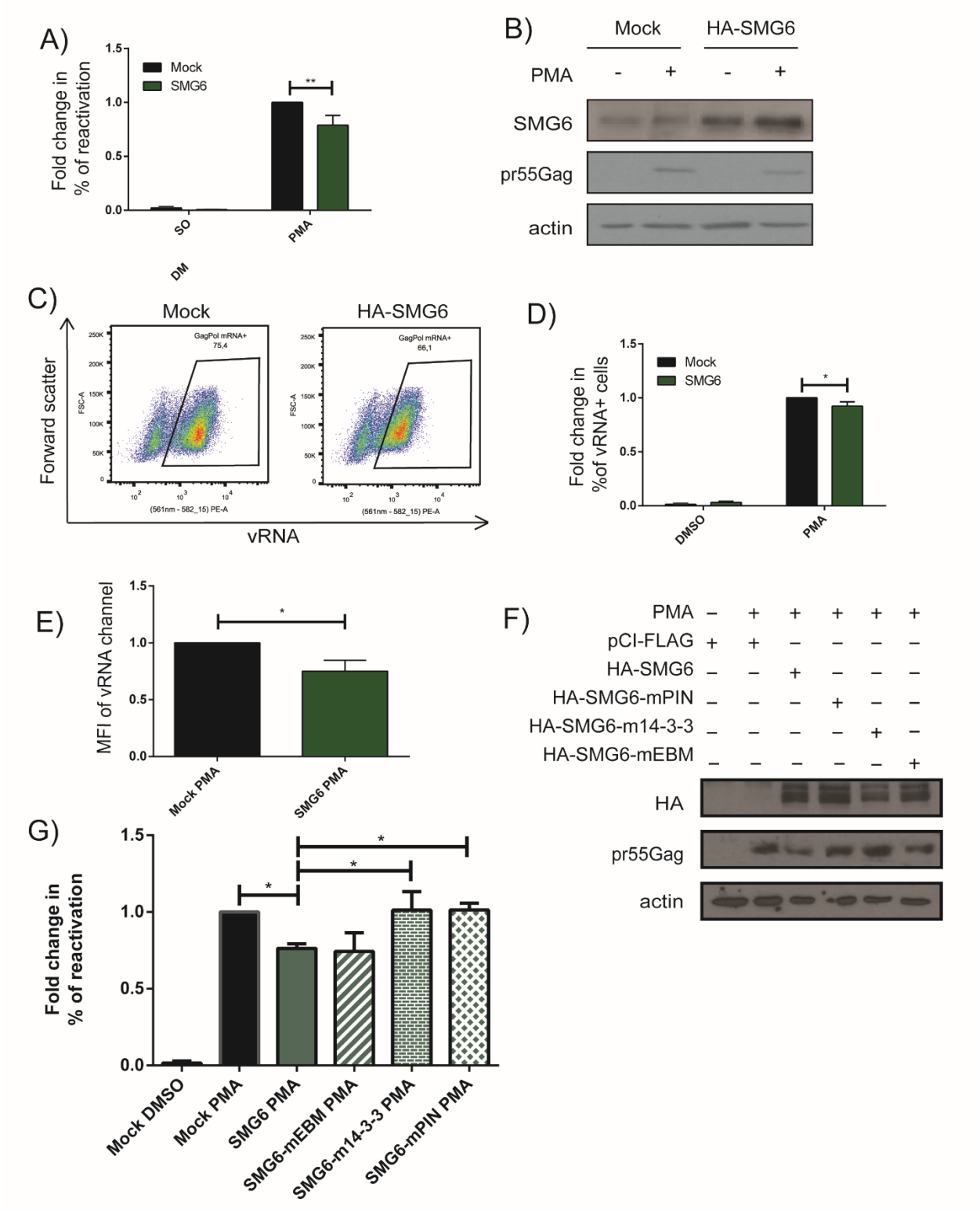

Figure 2.5.: SMG6 overexpression leads to attenuated reactivation of HIV-1.

Figure 2.5.: SMG6 overexpression leads to attenuated reactivation of HIV-1 (continued).

A) J-Lat 10.6 cells were either mock transfected or transfected with HA-SMG6 and were uninduced (DMSO) or reactivated (PMA). Reactivation, monitored by GFP production, was quantified by flow cytometry and the percentages of reactivation were normalised to the mock-PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.01$). **B)** Cell lysates were run on acrylamide gels and SMG6 and $pr55^{Gag}$ protein levels were detected by Western Blotting. **C)** Example dot plot depicting vRNA expression in mock PMA and SMG6 PMA conditions using FISH-Flow technique. **D)** The % of vRNA expressing cells were quantified. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.05$). **E)** MFI of the vRNA signal were quantified. Asterisks represent statistically significant difference between groups (student's t-test; $p < 0.05$). **F)** J-Lat cells were mock transfected or transfected with HA-SMG6, HA-SMG6-mEBM, HA-SMG6-m14-3-3 or HA-SMG6-mPIN and reactivated with PMA. Cell lysates were run on acrylamide gels and SMG6 and $pr55^{Gag}$ protein levels were detected by SDS-PAGE followed by Western Blotting. **G)** Reactivation in the above conditions was quantified and the percentages of reactivation were normalised to the mock PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.05$).

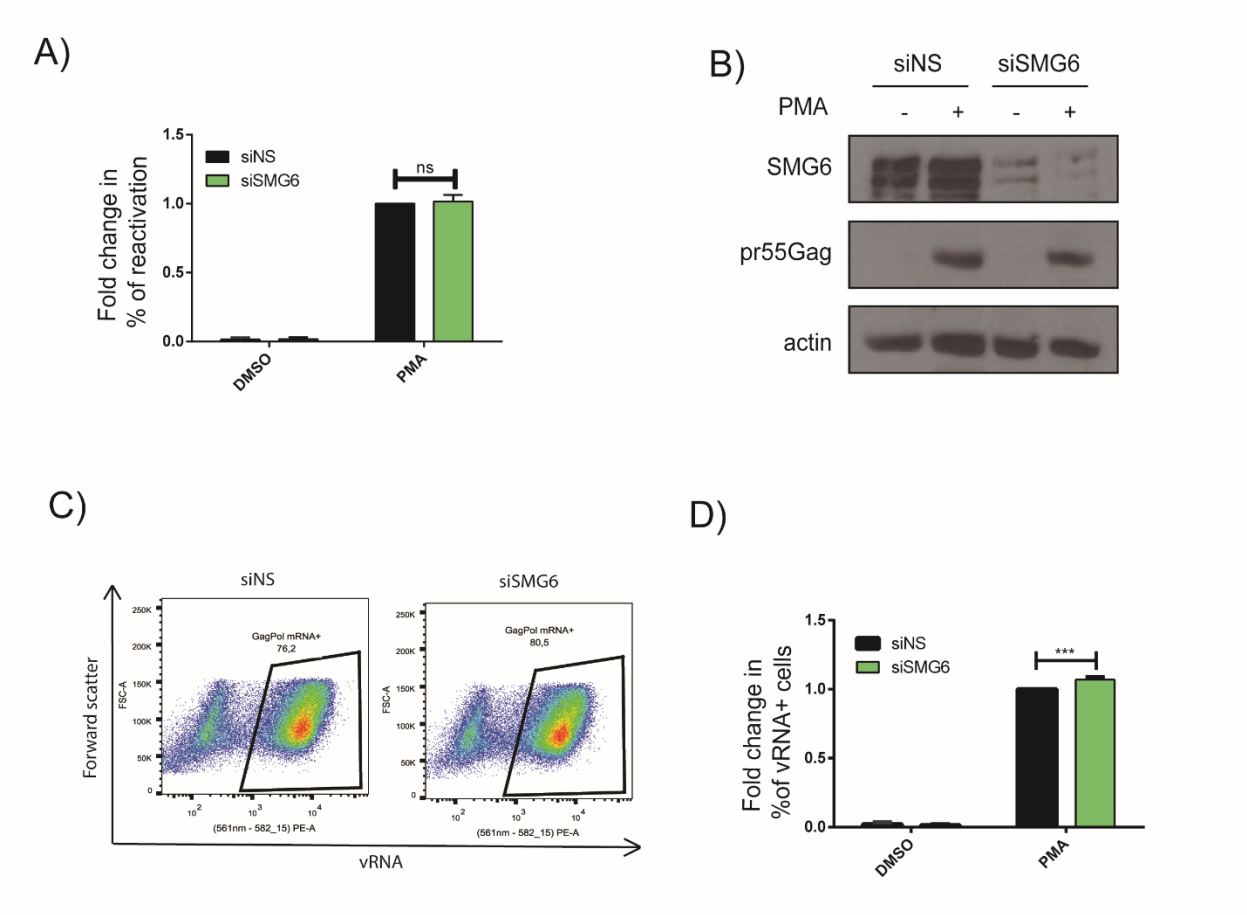

Figure 2.6.: SMG6 knockdown leads to increased vRNA levels, but not reactivation in J-Lat cells.

J-Lat 10.6 cells were either transfected either siNS or siSMG6 and were either uninduced (DMSO) or reactivated (PMA) **A)** Reactivation monitored by GFP production was measured by flow cytometry. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. (Two-way ANOVA; $p > 0.05$). **B)** Cell lysates were run on acrylamide gels and SMG6 and $pr55^{Gag}$ protein levels were detected by Western Blotting **C)** Example dot plot depicting vRNA expression in siNS PMA and siSMG6 PMA conditions using FISH-Flow technique and, **D)** The % of vRNA expressing cells were quantified. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.001$).

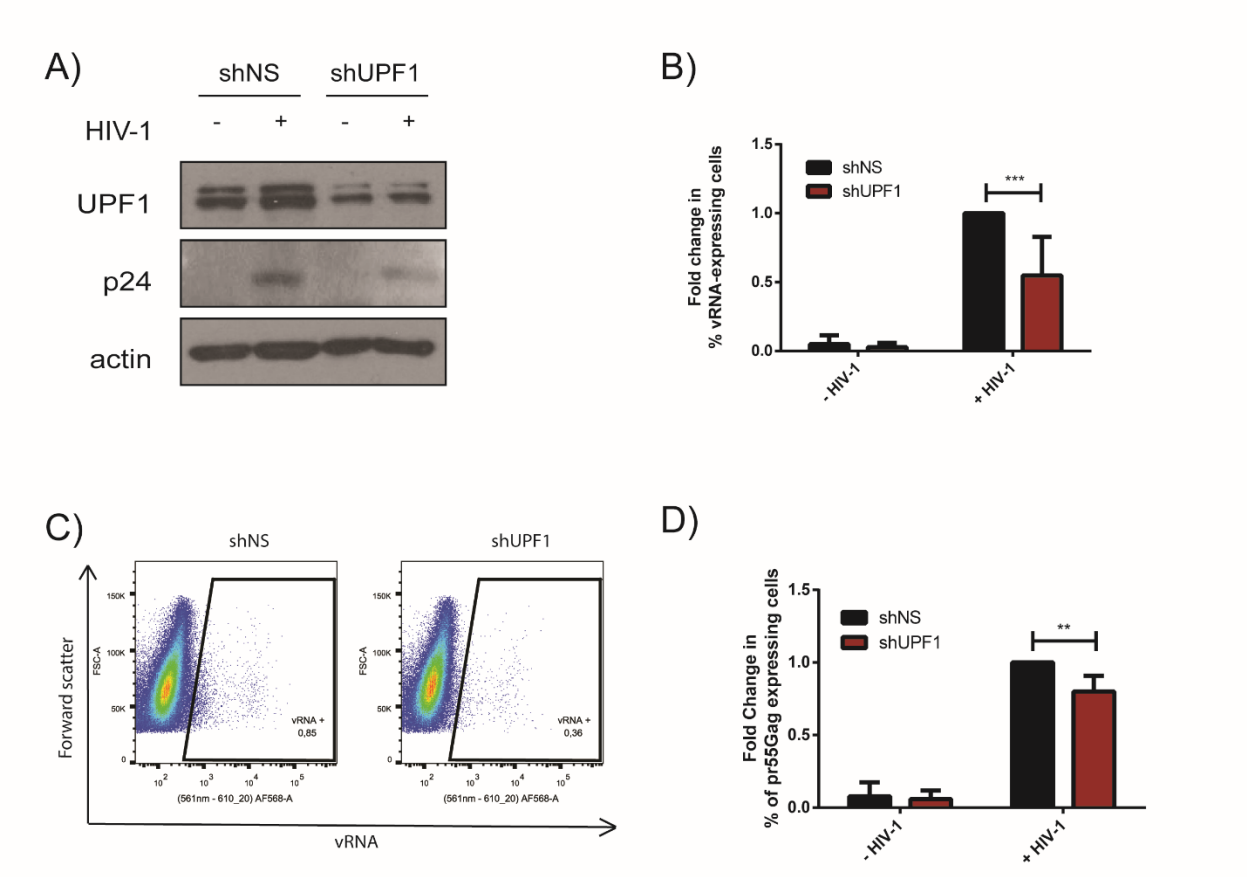

Figure 2.7.: UPF1 knockdown leads to reduced vRNA levels and Gag expression in primary HIV-1 infected CD4+ T cells.

Primary CD4+ T cells were either transduced with shNS or shUPF1-containing lentiviral particles and either left uninfected or infected with HIV-1. **A)** Cell lysates were run on SDS-PAGE gels and UPF2 and pr55^{Gag} protein levels were detected by Western Blotting. **B)** The % of vRNA expressing cells were quantified and normalised to shNS HIV-1-infected condition. Error bars represent the standard deviation from six independent experiments (three donors in duplicate) with at least 5000000 cells counted per experiment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.001$). **C)** Example dot plot depicting vRNA expression in HIV-1 infected shNS and shUPF1 conditions using FISH-Flow technique and, **D)** The % of Gag expressing cells were quantified and normalised to shNS HIV-1-infected condition. Error bars represent the standard deviation from nine independent experiments (three donors in duplicate) with at least 5000000 cells counted per experiment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.01$).

2.9. Supplemental figures and figure legends

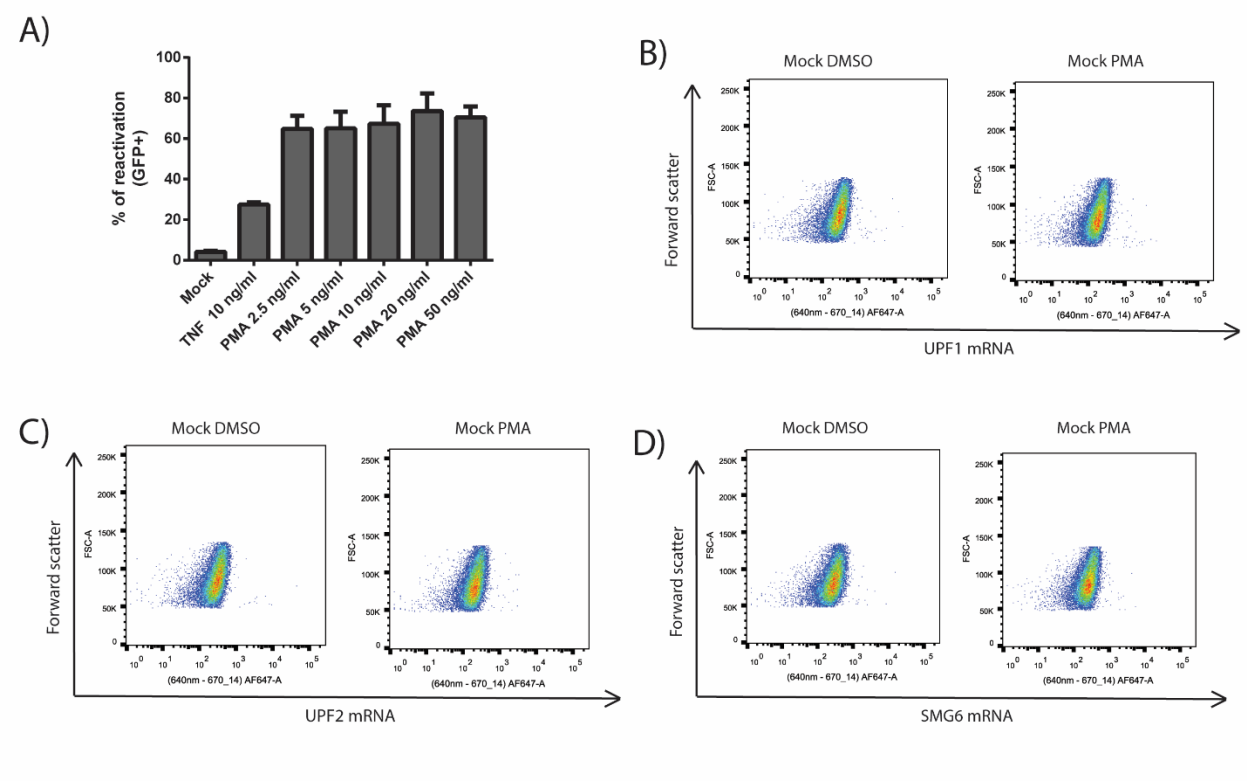

Figure 2.8.: The expression of UPF1, UPF2 and SMG6 mRNA is not significantly altered by PMA treatment.

A) J-Lat cells were treated with TNF-alpha or different concentrations of PMA and the % of GFP positive cells were measured. Example dot plot depicting B) UPF1 mRNA, C) UPF2 mRNA and D) SMG6 mRNA expression in mock transfected cells with and without PMA addition using FISH-Flow technique.

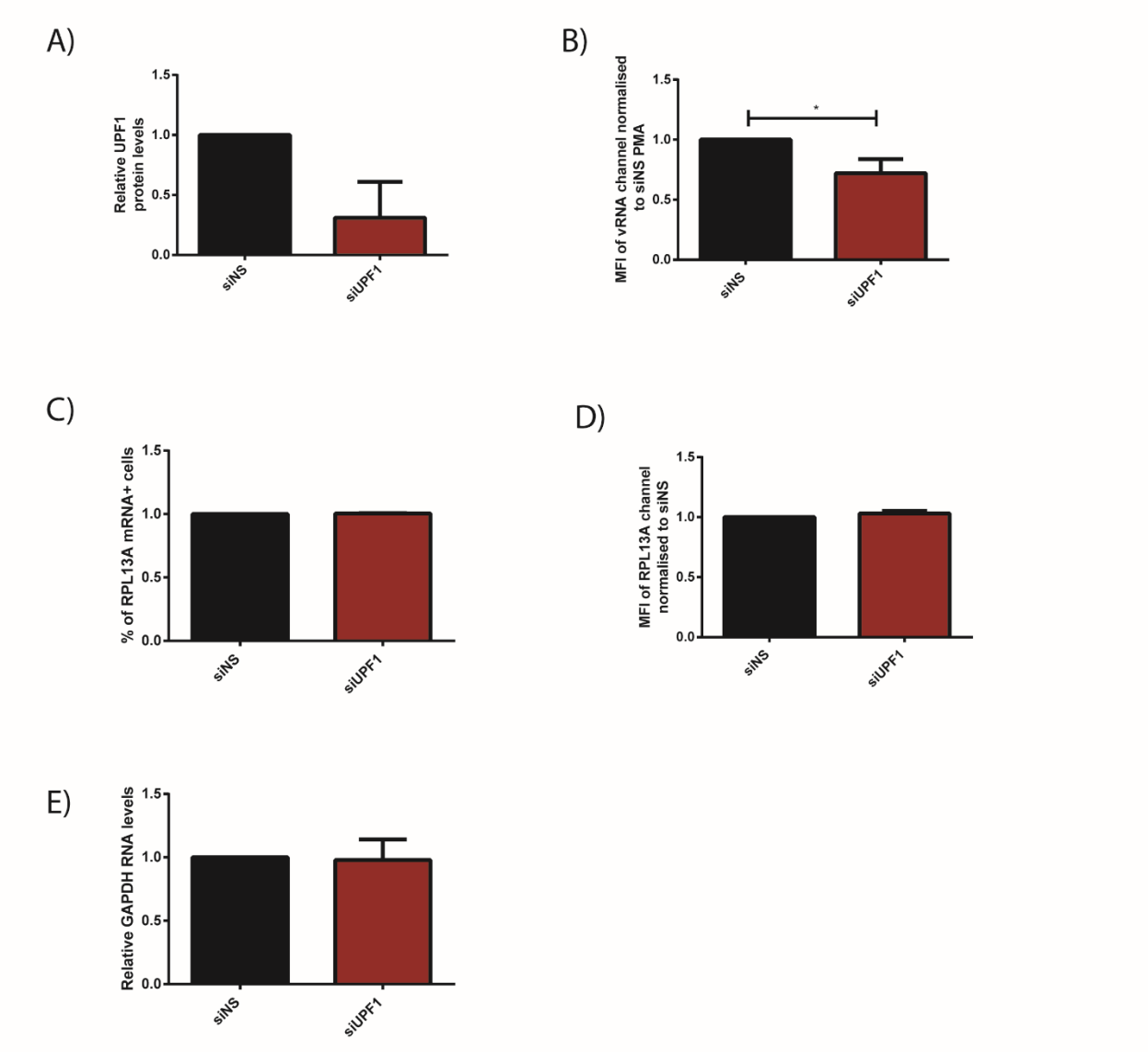

Figure 2.9.: UPF1 knockdown does not affect housekeeping mRNA levels.
J-Lat 10.6 cells were either transfected with siNS or siUPF1 and were uninduced (DMSO) or reactivated (PMA). A) Quantification of UPF1 protein expression by densitometry analysis of Western blots. B) MFI of the vRNA signal were quantified. Asterisks represent statistically significant difference between groups (student's t-test; $p < 0.05$). C) The % of RPL13A mRNA expressing cells were quantified. D) MFI of the PRL13A signal were quantified. E) Relative GAPDH mRNA levels as measured by RT-PCR. For all graphs, error bars represent the standard deviation from three independent experiments.

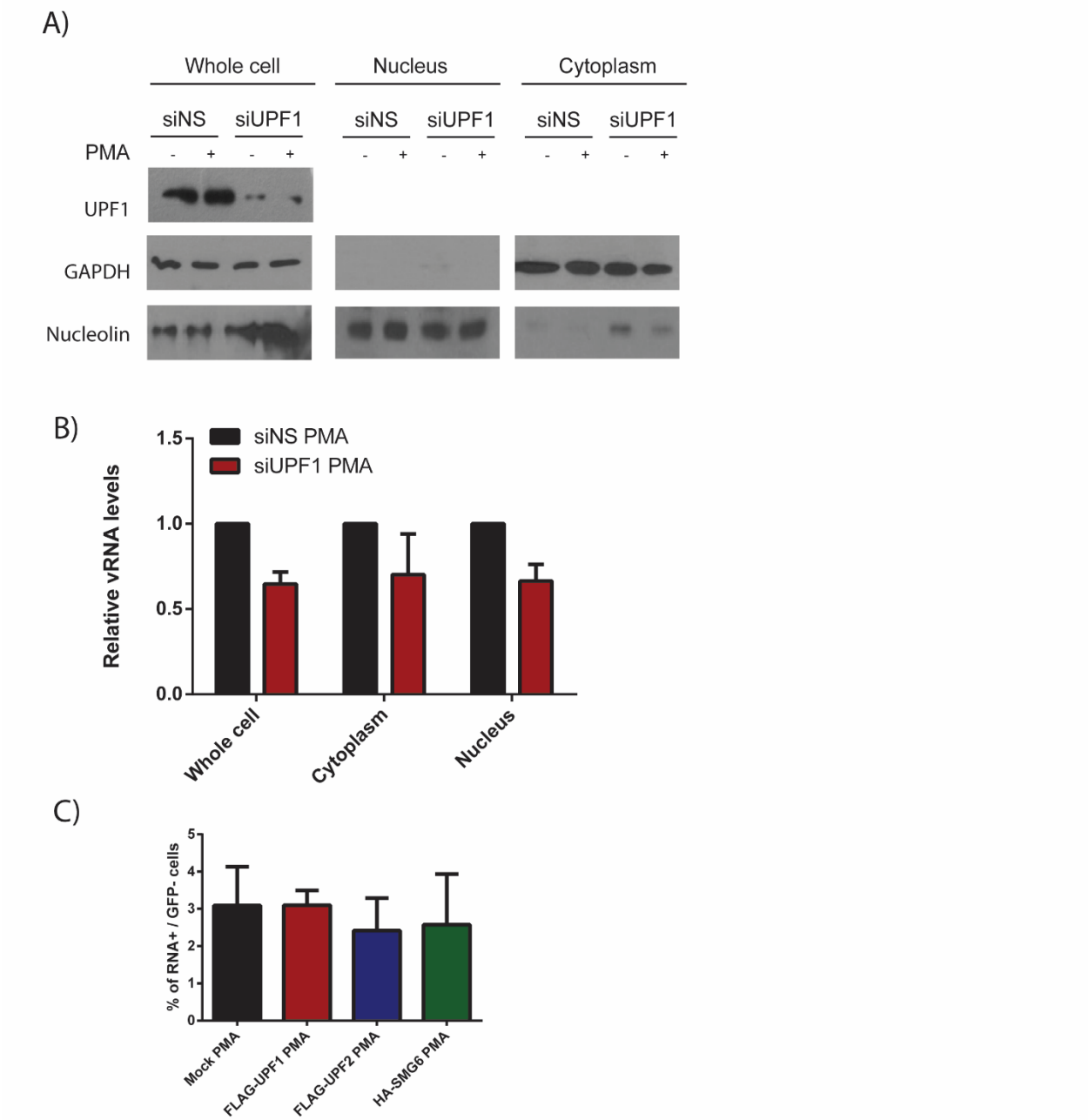

Figure 2.10.: UPF1 knockdown affects overall vRNA levels

A) Cellular fractionation was performed in siNS or siUPF1 treated conditions, with and without PMA treatment. The fractions were run on SDS-PAGE gels and GAPDH and nucleolin protein levels were detected by Western Blotting to confirm fractionation. B) The relative amounts of vRNA in each fraction were quantified by RT-PCR and normalised to levels of GAPDH mRNA. Error bars represent the standard deviation from three independent experiments. C) J-Lat cells were mock transfected, transfected with FLAG-UPF1, FLAG-UPF2 or HA-SMG6 and reactivated with PMA. The % of vRNA+/GFP- cells was quantified. Error bars represent the standard deviation from three independent experiments.

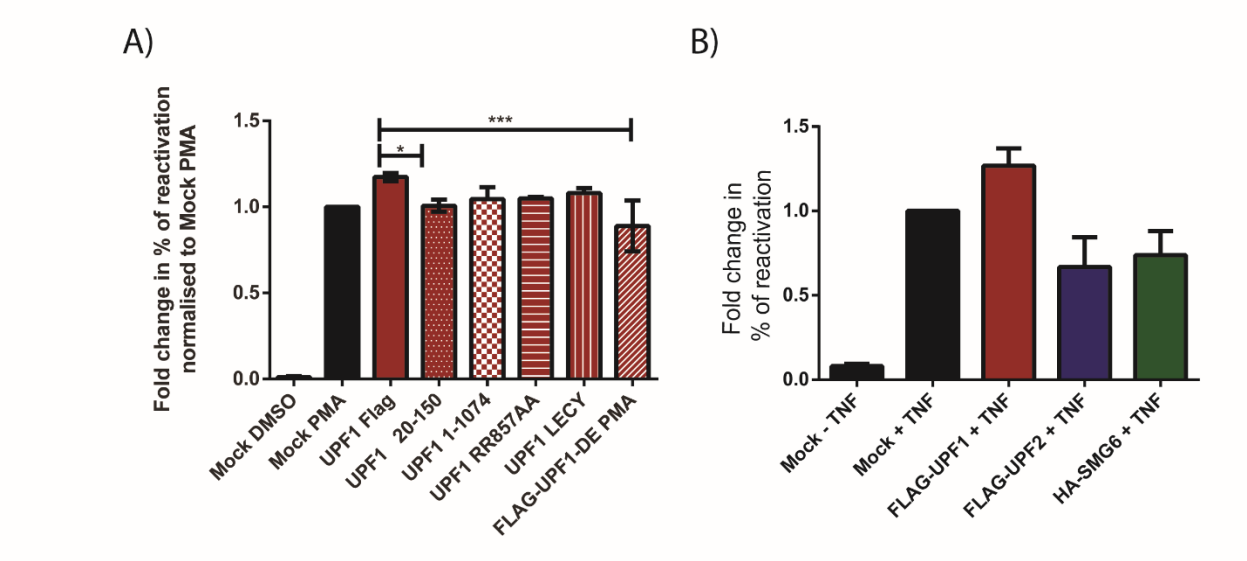

Figure 2.11.: Not all UPF1 constructs enhance viral reactivation and UPF1, UPF2 and SMG6 overexpression affect TNFα-induced reactivation.

A) J-Lat cells were mock transfected, transfected with FLAG-UPF1 or with FLAG-UPF1 mutants and reactivated with PMA. Reactivation in the above conditions was quantified and the percentages of reactivation were normalised to the Mock PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment. Asterisks represent statistically significant difference between groups. B) J-Lat cells were mock transfected, transfected with FLAG-UPF1, FLAG-UPF2 or HA-SMG6 and reactivated with TNFα. Reactivation in the above conditions was quantified and the percentages of reactivation were normalised to the Mock PMA reactivated condition. Error bars represent the standard deviation from three independent experiments with at least 10000 cells counted per treatment.

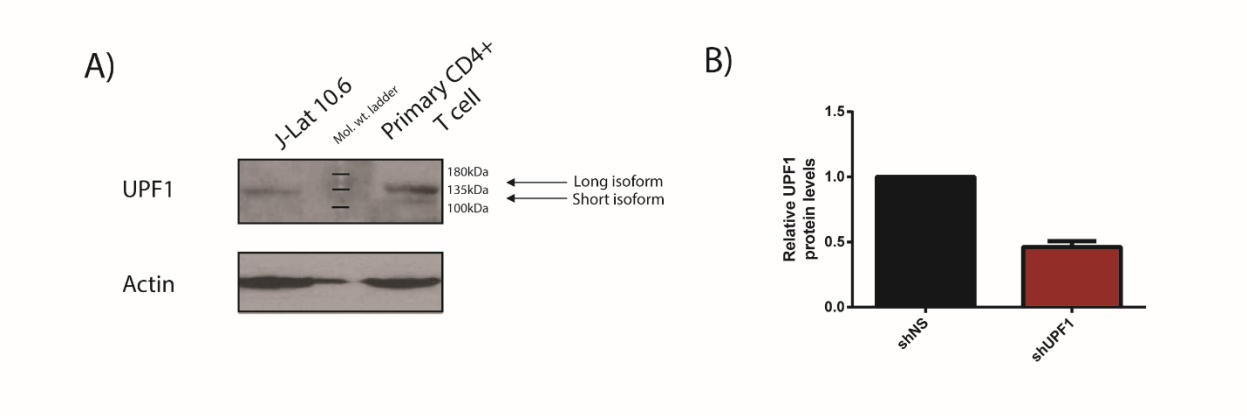

Figure 2.12.: Detection of both isoforms of UPF1 in primary CD4+ T cells and efficiency of shUPF1 transduction.

A) Equal amounts of cell lysates from J-Lat 10.6 and primary CD4+ T cells were subjected to Western blotting and probed for UPF1 and actin. B) Primary CD4+ T cells were either transduced with shNS or shUPF1-containing lentiviral particles. Quantification of UPF1 protein expression by densitometry analysis of Western blots.

Chapter 3

The host mRNA decay proteins influence HIV-1 replication and viral gene expression in primary monocyte-derived macrophages

This chapter was adapted from the following manuscript:

Rao, S.*, Amorim, R.*, Niu, M., Breton, Y., Tremblay, M.J., & Mouland, A. J. (Manuscript submitted). “The host mRNA decay proteins influence HIV-1 replication and viral gene expression in primary monocyte-derived macrophages”. (* These authors contributed equally)

3.1. Preface

In Chapter 2, we identified a role for the NMD proteins UPF1, UPF2 and SMG6 in vRNA stability in cells of the lymphoid lineage. In this chapter, we extend our studies to cells of the myeloid lineage. Specifically, we investigate the effect of the NMD proteins UPF1, UPF2 and SMG6, as well as the SMD protein Staufen1, on viral gene expression in primary monocyte-derived macrophages.

Author contributions: S.R., R.A. and A.J.M conceived the study and designed experiments. S.R. conducted the experiments presented in Figure 2, 3 and 4; R.A. conducted HSA separation and western blotting presented in Figure 1, 2 and 4; Y.B. and M.J.T. provided essential reagents, expertise and methodologies; M.N. prepared and maintained primary cell cultures; S.R., R.A. and A.J.M. jointly drafted the manuscript. All authors revised and approved the final manuscript.

Funding information: This study, S.R. and R.A. were supported by The Canadian HIV Cure Enterprise Team Grant HIG-133050 (to A.J.M.) from the Canadian Institutes of Health Research (CIHR) in partnership with Canadian Foundation for HIV-1/AIDS Research and International AIDS Society. M.N was funded by the Lady Davis Research Institute/Jewish General Hospital. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Acknowledgements: We thank Alan Cochrane, Brendan Bell, Niels Gehring, Jens Lykke-Anderson for the generous provision of reagents, cell lines and helpful discussions; Mario Legault, Jean-Pierre Routy, the Fonds recherche en Santé-Québec and all the blood donors for generous provision of human cells; and Anne Gatignol and Elodie Rance for RT assays.

3.2. Abstract

Macrophages play multiple roles in HIV-1 pathogenesis, as they can not only be directly infected by the virus but also help its dissemination and contribute to the activation of the inflammatory response. Although most studies investigating the regulation of HIV-1 gene expression are focused on CD4+ T cells, the contribution of host proteins on vRNA metabolism in macrophages remains largely unexplored. Mammalian cells harbour RNA quality control and degradative machineries that target aberrant mRNAs for clearance from the cell to avoid ectopic gene expression (immune heterogeneity), such as nonsense-mediate mRNA decay. The role of the host mRNA decay pathways in macrophages in the context of HIV-1 infection is yet to be elucidated. Therefore, we characterized the roles for key host mRNA decay proteins UPF1, UPF2 and SMG6 in HIV-1-infected primary monocyte-derived macrophages (MDMs). Steady-state expression levels of the NMD proteins were significantly downregulated in HIV-1-infected MDMs. Moreover, the NMD auxiliary proteins UPF2 and SMG6 restricted HIV-1 gene expression by directly influencing viral genomic RNA levels. *Staufen1*, a gene also involved in host mRNA decay and that acts at several HIV-1 replication steps, enhanced HIV-1 gene expression in MDMs. These results provide novel evidence for a role for NMD in the restriction of viral replication, to a similar level achieved by SAMHD1, in HIV-1-infected macrophages. The identification of categorized host mRNA decay proteins capable of influencing HIV-1 replication in MDMs can serve as potential targets for broad-spectrum antiviral therapeutics.

3.3. Introduction

Macrophages are cells of the myeloid lineage that are an important component of the innate immune response. They recognise and phagocytose invading pathogens and serve many roles in tissue development, homeostasis and repair [1]. They are present in most tissues in the body and arise from the terminal differentiation of infiltrating monocytes [2]. Examples of tissue-resident macrophages are the alveolar macrophages in the lung, Kupffer cells in the liver and the microglial cells of the central nervous system [3].

During human immunodeficiency virus type 1 (HIV-1) infection, macrophages play multiple roles in viral pathogenesis (reviewed in [4-6]). Macrophages express the host cell receptors CD4 and CCR5 required for HIV-1 entry and thus, can be directly infected by HIV-1 [7, 8]. They can also be infected by the selective capture and engulfment of HIV-1 infected T cells [9]. Macrophages promote the dissemination and cell-to-cell transmission of HIV-1 via the assembly of virological synapses at the sites of contact with T cells [10-12]. Furthermore, they directly contribute to HIV-1 pathogenesis via the activation of inflammatory pathways resulting in the cognitive dysfunction, respiratory dysfunction, cardiovascular disease microbial translocation in the intestine associated with HIV-1 infection (reviewed in [5]).

The ability of HIV-1 to form a stable viral reservoir upon infection is the major obstacle towards an HIV-1 cure [13]. Most studies on HIV-1 latency have focused on CD4+ T cells. However, the contribution of cells of the myeloid lineage to the maintenance of HIV-1 latency is recently being recognised [14]. Macrophages have been proposed to be a long-lived component of the HIV-1 viral reservoir [5, 15-17] as they have a longer life-span than CD4+ T cells and also possess self-renewing properties [18]. During HIV-1 infection, macrophages are more resistant to the cytopathic effects of the virus and display increased telomerase activity which contributes to their increased longevity [19, 20]. In recent *in vivo* studies using humanised mouse models, tissue-resident macrophages were able to sustain and propagate HIV-1 infection independently of CD4+ T cells [21]. In follow up studies using the same humanized myeloid-only mouse model, HIV-1 infection was rapidly suppressed by antiretroviral treatment (ART) [17]. However, viral

rebound was observed in a third of the mice following the discontinuation of ART, thus representing the first direct evidence of HIV-1 persistence in tissue macrophages *in vivo [17].*

One of the strategies to cure HIV-1 infection is the "kick and kill" approach. This strategy involves the use of latency-reversing agents (LRAs) to stimulate virus production from latently-infected cells; followed by their elimination by the host immune system, cytopathic effects of virus production or ART [22]. These LRAs have been demonstrated to induce viral production in CD4+ T cells [23]. However, LRA treatment in macrophages was found to decrease viral release due to the activation of autophagy and the degradation of intracellular viral proteins [24]. Moreover, in a study evaluating the efficacy of a combination of two LRAs (i.e. byrostatin and JQ1), monocytic cells were more efficiently reactivated than lymphoid cells [25]. This highlights cell-type differences between T cells and macrophages during HIV-1 infection and underlies the need for a greater understanding of the role of host cell proteins that control HIV-1 gene expression in macrophages.

mRNA surveillance pathways are host quality control mechanisms that degrade aberrant mRNA to prevent the accumulation of potentially toxic truncated or misfolded proteins. Examples of these pathways include the nonsense-mediated mRNA decay (NMD) and Staufen-mediated decay (SMD) that involve the host cell proteins UPF1, UPF2, SMG6 and Staufen1, amongst others [26, 27]. In previous work from our group, we have demonstrated that not only is the HIV-1 genomic RNA (vRNA) able to evade mRNA surveillance, HIV-1 has also been demonstrated to hijack UPF1 to promote vRNA stability and ensure viral gene expression by assembling an HIV-1 dependent ribonucleoprotein complex (RNP) with the host cell protein Staufen1 [28, 29]. UPF2 has been demonstrated to be excluded from this RNP and is detrimental to the nucleocytoplasmic export of the vRNA [30]. Furthermore, UPF2 and SMG6, the endonuclease involved in the final step of the degradation of aberrant RNA in NMD, are detrimental to vRNA stability in a UPF1-dependent manner (Chapter 2). These effects were observed in a latently-infected T cell model of HIV-1 as well as in primary HIV-1-infected CD4+ T cells (Chapter 2). Staufen1 has also been demonstrated to play a role in various steps of virus assembly including vRNA translation, Gag multimerisation and vRNA encapsidation [31-35]. Whether these effects

of the host mRNA decays proteins are also observed in cells of the myeloid lineage remains to be elucidated.

In this study, we have characterised the effects of the host mRNA decay proteins UPF1, UPF2, SMG6 and Staufen1 on HIV-1 replication in primary monocyte-derived macrophages (MDMs). We observed that these proteins had profound effects on HIV-1 replication in MDMs. The identification of novel host proteins capable of restricting HIV-1 replication in MDMs can pave the way for novel targets for therapeutic intervention.

3.4. Results

3.4.1. The expression of UPF1, UPF2 and SMG6 is decreased in HIV-1-infected MDMs

During the process of NMD, UPF1 interacts with a network of proteins, including UPF2 and SMG6. Previous work from our group has demonstrated that these proteins have differential effects on vRNA metabolism [28, 30] (Chapter 2). We hypothesized that RNA surveillance proteins can also impact HIV-1 replication in primary MDMs. To determine whether the expression of these proteins are modulated during HIV-1 infection, we assessed the levels of UPF1, UPF2 and SMG6 expression in HIV-1-infected primary MDMs using an HIV-1 reporter construct NL4-3-Bal-IRES-HSA [36-38]. This R5-tropic molecular clone of HIV-1 encodes all viral genes and, additionally, the murine heat-stable antigen (HSA), a cell surface reporter that allows the detection of cells that are productively infected with HIV-1 [38]. To generate primary monocyte-derived macrophages (MDMs), primary monocytes were isolated from PBMCs by the adherence method and differentiated into MDMs by a 3-day treatment with M-CSF. All the data presented in this manuscript were generated using MDMs from at least three independent donors, unless indicated otherwise. After 3 days of resting post-differentiation, cells were then infected with NL4-3-Bal-IRES-HSA virus. Cells were collected 6 days post-infection, incubated with anti-HSA antibody and sorted through magnetic separation as depicted in Figure 3.1. A and described in [36]. Whole-cell lysates were obtained from HSA-positive (infected cells) and HSA-negative (bystander cells), and the expression levels of UPF1, UPF2 and SMG6 were quantified by Western blotting. As expected, $pr55^{Gag}$ expression was detected only in the HSA-positive population, indicating that HIV-1 infected and bystander cells were efficiently separated (Figure 3.1. B). Importantly, significantly lower expressions of the NMD proteins UPF1, UPF2 and SMG6 were detected in the HIV-1 infected cells (Figure 3.1. B and C), with a decrease of 0.71 (± 0.09) log for UPF1, 0.63 (± 0.10) log for UPF2 and 0.71 (± 0.15) log for SMG6. This data indicates that the expression of these NMD proteins is either downregulated during HIV-1 replication, or that the population with higher expression of the NMD proteins is refractory to productive HIV-1 infection. The expression of the NMD proteins UPF1, UPF2 and SMG6 is modulated during HIV-1

infection in macrophages, thereby implying that these proteins play a role during HIV-1 replication.

3.4.2. UPF2 and SMG6 restrict HIV-1 replication and viral gene expression in primary MDMs

Since we observed lower levels of UPF1, UPF2 and SMG6 in HIV-1-infected primary MDMs, we sought to determine the roles of these proteins during HIV-1 replication. We performed siRNA-mediated depletion of these NMD proteins in primary MDMs and evaluated the effects on HIV-1 gene expression. Cells were either transfected with a non-silencing siRNA (siNS) or with siRNA against UPF1 (siUPF1), UPF2 (siUPF2) or SMG6 (siSMG6) and were infected with NL4-3-Bal-IRES-HSA virus after 24 hours. siRNA-mediated silencing was repeated 2 days after infection to maintain gene knockdown. Cells were collected 6 days post-infection and whole cell lysates were analysed by Western blotting. Following siRNA transfection, UPF1, UPF2 and SMG6 expression are efficiently reduced by at least 70% in all cases (Figure 3.2. A-C). Interestingly, we observed no significant change in $pr55^{Gag}$ levels in cells transfected with siRNA against UPF1 (Figure 3.2. A and D). However, a 1.54 (± 0.37) and 1.47 (± 0.35)-fold increase in $pr55^{Gag}$ levels was observed in cells silenced for UPF2 and SMG6, respectively (Figure 3.2. B and C, respectively, and D), suggesting that these proteins are detrimental for HIV-1 replication in MDMs.

To quantify the effect of UPF1, UPF2 and SMG6 on the ability of cells to be productively infected by HIV-1, cells were treated with the siRNAs as described above and the percentage of infected cells was monitored by flow cytometry using antibodies against the HSA tag. A siRNA against SAMHD1 (siSAMHD1) was used as a positive control. SAMHD1 is a well-characterized HIV-1 restriction factor in macrophages and we expect that the MDMs depleted of SAMHD1 are more conducive to productive HIV-1 infection [39]. The experiments were conducted on MDMs from 5 independent donors in triplicate. Consistent with the effects observed on $pr55^{Gag}$ levels by Western blotting (Fig 2A-D), no significant difference was found in the percentage of infected cells between siNS and

siUPF1 transfected cells (Fig. 2E and F). An increase in the percentage of infected cells was observed in cells depleted of UPF2, with a 2.14 (± 0.85) - fold increase in the percentage of cells productively-infected with HIV-1 in the siUPF2 treated cells as compared to the siNS condition (Fig. 2E and F). This augmentation is comparable to cells transfected with siRNA against SAMHD1, which demonstrated a 2.48 (± 1.01) – fold change in the percentage of productively-infected cells (Fig 2E and F). Furthermore, a knockdown of SMG6 also resulted in a 1.77 (± 1.02) - fold increase in the percentage of infected cells as compared to the siNS condition. These data are consistent with our previous observations that UPF2 and SMG6 are detrimental to vRNA metabolism and viral gene expression [30] (Chapter 2). These observations reveal that UPF2 and SMG6 impair productive HIV-1 replication in primary MDMs and restrict viral gene expression.

3.4.3. UPF2 and SMG6 affect vRNA expression in primary HIV-1 infected MDMs

Since the silencing of UPF2 and SMG6 led to higher levels of intracellular $pr55^{Gag}$ and increased percentages of productively infected cells, we next sought to determine the stage of viral replication where UPF2 and SMG6 restrict viral replication. We first validated that the virus being produced from these UPF2- and SMG6- depleted cells are not defective. Primary MDMs were transfected with control siRNAs (siNS) or siRNAs against UPF2 and SMG6 and infected with NL4-3-Bal-IRES-HSA virus as described above. At 6 days post infection, virus production was quantified by the reverse transcriptase (RT) activity in the cell supernatant and the results were normalized to the percentage of infected cells in each condition. We observed no statistically significant differences between the treatments (Figure 3.3. A). To confirm that silencing of these proteins has no effect on the infectivity of the viral progeny, we also measured the production of infectious viral particles in the supernatant of each condition using an X-gal staining assay in TZM-bl cells as described in [40]. No significant differences were observed in the infectivity of the viruses in the supernatants of all treatments (Figure 3.3. B). These findings suggest that the NMD proteins UPF2 and SMG6 have no detrimental effect on late stages of viral

replication (i.e., budding and maturation) and do not influence the infectivity of the progeny viruses.

We then distinguished whether the effects of UPF2 and SMG6 on HIV-1 replication were observed at a stage before the integration of the proviral DNA into the host genome or at a post-integration stage. Proviral DNA integration in control and UPF1-, UPF2- and SMG6-silenced MDMs was measured using a combined Alu-HIV-1 PCR as described in [41]. We observed no statistically significant differences between the amounts of integrated provirus across all conditions described (Figure 3.3. C and D). This suggests that UPF2 and SMG6 restrict HIV-1 replication in primary MDMs and a post-integration stage.

The NMD proteins are known to directly influence mRNA levels [42]. We evaluated whether NMD was inhibited upon siRNA-mediated knockdown of the NMD proteins UPF1, UPF2 and SMG6 and whether UPF2 and SMG6 knockdown could affect intracellular vRNA expression. In MDMs transfected with siNS, siUPF1, siUPF2 and siSMG6, the levels of Gas5 mRNA, which is normally subjected to NMD, were measured by semi-quantitative RT-PCR [43]. Intracellular vRNA expression in each condition was also quantified as described in [30, 31]. Gas5 mRNA levels were increased upon knockdown of UPF1 and UPF2, indicating that NMD is inhibited upon depletion of these proteins (Figure 3.3. E). Although a modest increase in Gas5 mRNA was observed upon SMG6 knockdown, the levels were not comparable to the increase observed upon UPF1 and UPF2 knockdown (Figure 3.3. E). This could be because although SMG6 is involved in the degradation of aberrant mRNA during NMD, the mRNA could also be degraded via a SMG6-independent pathway involving the proteins SMG5 and SMG7 [26, 44]. No statistically significant difference was observed in the vRNA levels of cells silenced for UPF1 as compared to control cells (Figure 3.3. E and F). However, the MDMs depleted of UPF2 and SMG6 presented a 1.74 (± 0.62)- and 1.91 (± 0.66)- fold increase in the expression of intracellular vRNA (Figure 3.3. E and F). This data suggests that the NMD proteins UPF2 and SMG6 inhibit viral gene expression by directly influencing vRNA expression.

UPF2 is known to bind UPF1 with a high affinity [45] and we have previously demonstrated that the detrimental effects of UPF2 on vRNA metabolism is directly

related to its binding to UPF1 [30]. Since the silencing of UPF2 led to an increased percentage of infected cells and intracellular $pr55^{Gag}$ in primary MDMs (Fig 3.2 D and 3.2. E), we next determined whether this effect was dependent on UPF1. We transfected primary MDMs from one donor in three independent experiments with control siRNA (siNS), siUPF1 or siUPF2 alone or siUPF1 and siUPF2 combined and quantified the percentage of infected cells by detecting the expression of the HSA tag by flow cytometry. We observed that, in the doubly-silenced cells, the proportion of productively infected cells is comparable to control cells (Figure 3.3. G), indicating that the deleterious effect of UPF2 on viral replication in primary MDMs depends on UPF1.

3.4.4. Staufen1 enhances HIV-1 gene expression in primary MDMs

In addition to NMD, mammalian cells harbour another UPF1-dependent RNA surveillance pathway called Staufen-mediated decay (SMD), in which the mRNA degradation process is mediated by the binding of Staufen1 to the 3'-untranslated region (3'-UTR) of target mRNAs (reviewed in [27]). Staufen1 has been previously demonstrated to bind to the vRNA in the cytoplasm, facilitate translation initiation of the vRNA and be selectively packaged into HIV-1 virions [32, 35, 46]. Therefore, we next sought to determine if these effects of Staufen1 on HIV-1 replication are also observed in primary MDMs. Cells were either transfected with a non-silencing siRNA (siNS) or with siRNA against Staufen1 (siStaufen1) and subsequently infected with NL4-3-Bal-IRES-HSA virus. Silencing was repeated 2 days after infection to maintain gene knockdown. Cells lysates collected 6 days post-infection and analysed by Western blotting and the percentage of infected cells was monitored by detection of the HSA tag by flow cytometry. We observed that silencing of Staufen1 led to a significant decrease in intracellular $pr55^{Gag}$ (54.7 ± 0.1%) (Figure 3.4. A and B). The knockdown of Staufen1 also resulted in a 63.02 (± 19.05) % decrease in the percentage of infected cells as compared to the mock-treated cells (Figure 3.4. C and D). In order to determine whether this effect was due to a reduction in vRNA stability or a defect in vRNA translation, we conducted semi-quantitative RT-PCR from whole cell lysates in the above described conditions. No significant difference in the intracellular

levels of vRNA between siNS and siStaufen1-transfected cells was observed (Figure 3.4. E). This suggests that Staufen1 enhances the translation of the vRNA in primary MDMs, similarly to what was observed in other cell types [31, 35].

3.5. Discussion

The cellular mRNA quality control pathways have been hypothesised to be a conserved form of intrinsic antiviral immunity [47-49]. Recent evidence indicates that these pathways can restrict viral infections in mammalian cells by different mechanisms. The NMD proteins UPF1, SMG5 and SMG7 were found to restrict the replication of Semliki Forest virus (SFV) and Sindbis virus (SINV) of the *Togaviridae* family and the genomic RNA of SFV was found to be a substrate for NMD [43, 48, 50]. To ensure viral gene expression, members of the *Retroviridae* family such as Rous Sarcoma Virus (RSV) and Human T-lymphotropic Virus Type 1 (HTLV-1) have devised mechanisms to inhibit NMD [51-55]. In our previous work, we have demonstrated that HIV-1 also subverts NMD and hijacks UPF1 to promote vRNA stability, nucleocytoplasmic export and translation [28, 30]. It is important to note that these effects of UPF1 on the vRNA are independent of its function in NMD and the expression of an NMD-null UPF1 construct also resulted in enhanced vRNA stability and translation [28]. UPF1 was also demonstrated to promote vRNA stability and viral gene expression in primary CD4+ T cells (Chapter 2).

Interestingly, in primary MDMs, the knockdown of UPF1 had no significant effect on viral replication. However, the NMD proteins UPF2 and SMG6 were demonstrated to restrict HIV-1 gene expression in primary MDMs by downregulating vRNA levels (Figure 3.3. E and F). The observation that UPF2 and SMG6 are detrimental to viral gene expression is consistent with our previous work [28, 30] (Chapter 2). Specifically, a knockdown of UPF2 resulted in a 2.14-fold increase in HIV-1 gene expression (Figure 3.2. E). This is comparable to the 2.48-fold increase in HIV-1 gene expression observed due to the knockdown of SAMHD1, a well characterised HIV-1 restriction factor [39]. UPF2, in contrast to several of the other NMD components, has not been associated with non-NMD functions [56]. In cells that were depleted of UPF2, NMD was indeed downregulated, as demonstrated by the increase in the levels of an endogenous mRNA targeted by NMD (Figure 3.3. E). Importantly, the impairment of NMD by UPF2 knockdown also correlated with an increase in vRNA levels and viral gene expression (Figure 3.2. E and 3.3. F). This implicates a novel function for NMD in the downregulation of vRNA in MDMs and is supported by the fact that the knockdown of SMG6, another protein involved in NMD, also resulted in increased vRNA levels and viral gene expression. Moreover, HIV-1 infected MDMs presented significantly lower

levels of the NMD proteins UPF1, UPF2 and SMG6 (Figure 3.1. B and C), thus implying that NMD is detrimental to viral gene expression in primary MDMs.

However, the question of why UPF1, the central player involved in NMD, did not have an effect on viral replication remained outstanding. We hypothesise that this is due to the multifaceted nature of UPF1. Although the most characterised role of UPF1 is its role in NMD, UPF1 also has defined roles in DNA repair and replication [57, 58], RNA stability [59-61], telomere metabolism [57] and cell cycle progression [58] (reviewed in [62]). In the context of HIV-1 infection, the previously demonstrated effect of UPF1 on the enhancement of vRNA stability and viral gene expression is independent of UPF1's roles in NMD [28]. Therefore, on one hand, UPF1-knockdown could result in increased vRNA levels and gene expression due to impaired NMD as seen in UPF2- and SMG6- depleted MDMs. But on the other hand, the knockdown of UPF1 could also result in reduced levels of UPF1 that stabilises the vRNA in an NMD-independent manner. This duality of UPF1 function is highlighted by studies that demonstrate that UPF1 binds promiscuously to all cellular RNAs; both, canonically identified NMD targets as well as to non-NMD targets and long non-coding RNAs [42, 63-66]. Cellular mRNAs bound to phosphorylated UPF1 are more likely to be subjected to NMD that those mRNAs bound to non-phosphorylated UPF1 [67, 68].

The binding of UPF2 to UPF1 induces a conformational change in UPF1 that facilitates its phosphorylation by the kinase SMG1 [69-71]. This conformational change also impairs UPF1's RNA-binding capacity which could hinder the binding of UPF1 to the vRNA [70]. Furthermore, UPF2 also binds to UPF1 with high affinity [72] and this could limit the availability of UPF1 to bind to the vRNA. During HIV-1 infection, we have previously demonstrated that UPF2 is excluded from HIV-1 RNPs through antagonistic interactions with the viral or host proteins such as Rev and Staufen1 [30]. In the MDMs, a knockdown of UPF2 resulted in increased viral gene expression and we postulate that this is because of two additive mechanisms. Firstly, a reduction in cellular NMD (Figure 3.3. E) could lead to increased vRNA levels and gene expression (Figure 3.2. E and F). Secondly, a depletion of UPF2 could result in increased levels of hypophosphorylated UPF1 that is capable of binding to and stabilising the vRNA. This is supported by our double-knockdown

experiments where a depletion of both UPF1 and UPF2 did not have any significant effect on viral gene expression (Figure 3.3. G).

In this work, we highlight the cell-type differences that exist between T cells and MDMs in vRNA metabolism, with the vRNA in T cells being able to evade NMD to a better extent than in MDMs. It would be interesting to further characterise the contribution of the phosphorylation of UPF1 to the differential regulation of RNA quality control pathways in T cells and macrophages, and its subsequent effect on HIV-1 gene expression.

The depletion of Staufen1 in primary MDMs resulted in decreased levels of intracellular $pr55^{Gag}$ and viral gene expression with no changes in steady-state vRNA levels (Figure 3.4. B – E). This data is consistent with previous reports from our group [31] and suggests a role for Staufen1 in translational derepression. We and others have shown that Staufen1 also plays a role in the vRNA metabolism and viral gene expression in primary MDMs, most likely by the assembly of a distinct HIV-1 RNP in the cytoplasm with the vRNA, $pr55^{Gag}$ and UPF1 as we and others have shown [28, 29, 31, 32, 73, 74].

In this work, we have determined the roles of the host mRNA decay proteins on viral gene expression and identified a novel function for the NMD proteins UPF2 and SMG6 in the restriction of HIV-1 vRNA expression in primary MDMs. We also emphasise that although some proteins such as UPF2, SMG6, and Staufen1 have similar effects on vRNA metabolism across different cell types, other proteins such as UPF1 behave differently in cells of the lymphoid and myeloid lineage. It is imperative to address these differences when designing novel therapeutics to treat HIV-1 infection.

The current antiretroviral drugs have different effects in macrophages as compared to T cells (reviewed in [19]). Moreover, in the context of HIV-1 curative therapies, the effect of LRAs in macrophages have not been effectively characterised and may have off-target effects such as the induction of autophagy [24]. The antifungal drug amphotericin B has been reported to reactivate HIV-1 in a model cell line for the HIV-1 latency in macrophages, but not in T lymphocytes, highlighting another example of how reactivation from latency is different in T cells and macrophages. In this work, we identified novel targets to modulate HIV-1 gene expression in both

T cells and macrophages. For example, novel small molecule inhibitors can be used to mimic the activities of UPF2 and SMG6 to impair viral gene expression. The binding of Staufen1 to the vRNA can also be hindered using vRNA mimics to sequester Staufen1 and prevent HIV-1 gene expression. These strategies would lead to the development of novel broad-spectrum antiretrovirals or a functional HIV-1 cure. Conversely, novel drugs could be generated to either mimic Staufen1 activity on the vRNA or to block the binding of UPF2 to UPF1, thus paving the way for a novel class of post-transcriptional LRAs that are effective across both lymphoid and myeloid components of the HIV-1 reservoir.

3.6. Materials and methods

3.6.1. Cell culture

PBMCs were isolated from leukophoresed blood collected from healthy donors. All subjects provided informed consent for participating in this study. The research ethics boards of the recruiting sites, the Centre Hospitalier de l'Universite de Montreal, and McGill University Health Centre approved this study. PBMCs were isolated by density-gradient centrifugation using lymphocyte separation medium (Corning). Human monocytes were differentiated into monocyte-derived macrophages (MDMs) in 150 mm dishes (Sarstedt) by incubation at 37°C and 5% CO_2 for 3 days in RPMI-1640 culture medium (Life Technologies) supplemented with M-CSF (25 ng/mL) (Sigma-Aldrich) and 10% human AB serum (Sigma-Aldrich). Following this period, culture medium was replaced by fresh culture without M-CSF for additional 3 days, then incubated with Accutase Solution (Sigma-Aldrich) for 60 to 90 minutes and detached with a cell scraper. Cells were plated at 5×10^5 cells/mL in 12-well plates (Corning). In each experiment, cells from at least three different donors were used unless otherwise stated. HEK293T cells were purchased from the American Type Culture Collection (ATCC). TZM-bl cells were obtained from NIH AIDS Reference and Reagent Program. Both cells lines were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin (Invitrogen).

3.6.2. Antibodies

Mouse anti-p24, was obtained from NIH AIDS Reagents Program; rabbit antisera to UPF1 and UPF2 were generously supplied by Jens Lykke-Andersen (University of California, San Diego, CA, USA) [30]; rabbit anti-EST1A (SMG6) and mouse anti-actin were purchased from Abcam; mouse anti-CD24 (henceforth referred as anti-HSA) biotin conjugated clone M1/69 was purchased from BD Biosciences; mouse anti-CD24 (henceforth referred as anti-HSA) PE conjugated clone M1/69 was purchased from eBioscience; rabbit anti-Staufen1 was produced and purified at the McGill University Cell Imaging and Analysis Network (Montréal, Québec, Canada); horseradish peroxidase-conjugated secondary antibodies were purchased from Rockland Immunochemicals.

3.6.3. Virus production and infection

NL4.3-Bal-IRES-HSA virus particles were prepared by transfection of HEK293T cells with HIV-1 NL4.3-Bal-IRES-HSA encoding plasmid [38] using the JetPrime transfection reagent. The supernatants were collected 48 hours post-transfection, filtered through a 0.45-µm filter (Pall) and centrifuged at 20,000 r.p.m. for 1 hour at 4°C to pellet the virus. Viruses were resuspended in RPMI and stored at -80°C. Viral titer was quantified using the X-gal staining assay in TZM-bl cells as described in [40]. Primary MDMs in RPMI culture medium were infected with an MOI of 1.0 by 2 hours at 37°C and 5% CO_2. Following infection, culture media was supplemented with human AB serum (Sigma-Aldrich) at a final concentration of 10%. Cells were collected 6 days post infection.

3.6.4. Gene silencing

To perform the siRNA transfection in the primary MDMs, 1 µL Lipofectamine 2000 (Life Technologies) was added to 50 µL of RPMI-1640. Each individual siRNA was used at a final concentration of 20 nM diluted in 50 µL of RPMI-1640 into each well of a 12-well cell culture plate. After 20 minutes of incubation at room temperature, 400 µL of cell suspension containing 5 x 10^5 cells were added to the mixture containing the Lipofectamine 2000 and siRNAs complexes. Cells were incubated at 37 °C in the presence of 5% CO_2 for 2 h before adding 500 µL of RPMI-1640 medium supplemented with 20% human serum (10% final concentration). The medium was replaced 24 h after transfection, when infection was performed. Custom siRNA duplexes were synthesised by Qiagen. The target sequence for UPF1 was 5'-AAGATGCAGTTCCGCTCCATT-3', for UPF2 was 5'-AAGTTGGTACGGGCACTC-3', for SMG6 was 5'-GCTGCAGGTTACTTACAAG-3', and for Staufen 1 was 5'-AAATAGCACAGTTTGGAAACT-3 [32]. The siNS used in this study is a commercially available non-silencing control duplex with target sequence 5'-AATTCTCCGAACGTGTCACGT'-3'.

3.6.5. Cell separation

Cells were separated into virus-infected and uninfected bystander cells using the EasySep Biotin Selection kit (StemCell Technologies) as described in [36, 38]. Briefly, cells were detached by

treatment with Accutase Solution for 60 min and washed in DPBS. Next, cells were incubated with the biotinylated anti-HSA antibody biotin-conjugated at a final concentration of 3 µg/mL and separation was performed followed by 5 rounds of magnetic separation of 5 minutes each in 0.5% BSA.

3.6.6. Flow cytometry

Flow cytometry analysis was performed with 5 x 10^5 cells that were incubated anti-HSA PE-conjugated antibody diluted 1:400 in DPBS for 60 min at 37°C. Cells were then detached by treatment with Accutase Solution for 60 min and washed in DPBS. Finally, cells were washed, fixed in 4% paraformaldehyde for 30 min and analysed on a BD LSR Fortessa Analyzer. Analysis was performed using the FlowJo V10 software (Treestar).

3.6.7. Nucleic acid extraction and RT-PCR

Intracellular DNA and RNA extraction were performed using Trizol Reagent (Thermo Fisher Scientific) following manufacturer's instructions. For RNA samples, cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA and primers were then added to GoTaq Green Master Mix (Promega). GAPDH was amplified using the primers GAPDH_1 forward 5'- TGACCACAGTCCATGCCATC-3' and GAPDH_1 reverse 5'-ATGATGTTCTGGAGAGCCCC-3', HIV-1 vRNA using the primers pNL4-3_1 forward 5'-GGGAGCTAGAACGATTCGCA-3' and pNL4-3_1 reverse 5'-GGATGGTTGTAGCTGTCCCA-3', and Gas5 using the primers Gas5 forward 5'-GCACCTTATGGACAGTTG-3' and Gas5 reverse 5'-GGAGCAGAACCATTAAGC-3'. For DNA analysis, DNA and primers were added to the GoTaq Green Master Mix (Promega). GAPDH was amplified using the primers GAPDH_S forward 5'-GCTGATGCCCCCATGTTCGT-3' and GAPDH_AS reverse 5'-CAAAGGTGGAGGATGGGTGT-3' and alu-HIV-1-LTR using the primers Alu forward 5'-TCCCAGCTACTCGGGAGGCTGAGG-3' and M661 reverse 5'-CCTGCGTCGAGAGATCTCCTCTG-3'. The PCR products were visualised in a 1% agarose gel by staining the DNA with RedSafe Nucleic Acid Staining Solution (iNtRON). Signals were captured using a Gel Doc System and intensities were normalised to the GAPDH signal.

3.6.8. Reverse-transcriptase assay

RT activity in cell supernatants was analysed as described in [75]. Briefly, 5 µL of viral supernatant were added to 50 µL of supplemented RT cocktail and incubated at 37°C for 2 h. 5 µL of each reaction mixture were spotted onto DEAE filter paper (Whatman). The membranes were washed and read using a Microbeta scintillation counter (PerkinElmer).

3.6.9. Infectivity assay

Viral titer in cell supernatants was quantified using the X-gal staining assay in TZM-bl cells as described in [40]. Briefly, different dilutions of supernatants of each condition were added to TZM-bl cells seeded onto 96-well plates (Corning). After 48 h, cells were fixed with 1% paraformaldehyde, washed and treated with X-Gal for the detection of β-galactosidase.

3.6.10. Western blotting

Cells were lysed in NP40 lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40). Protein concentration on each cell lysate was quantified by Bradford assay. Equal amounts of protein (20 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Blocking was performed using 5% non-fat milk in Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TBST) for 1 hour at room temperature. Membranes were incubated with the indicated primary and corresponding horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using Western Lightning Plus-ECL (PerkinElmer). Signal intensities were scanned by densitometry using ImageJ software (NIH, Bethseda, USA).

3.6.11. Statistical analysis

All experiments were performed with at least three donors (unless indicated otherwise) in three independent experiments, and the data are presented as the mean ± standard deviation (SD). A p value of <0.05 in a student's t-test, one-way or two-way ANOVA test was considered statistically

significant (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$). GraphPad Prism 6 (GraphPad Software Inc.) was used to conduct statistical analyses and create graphs.

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3.8. Figures and figure legends

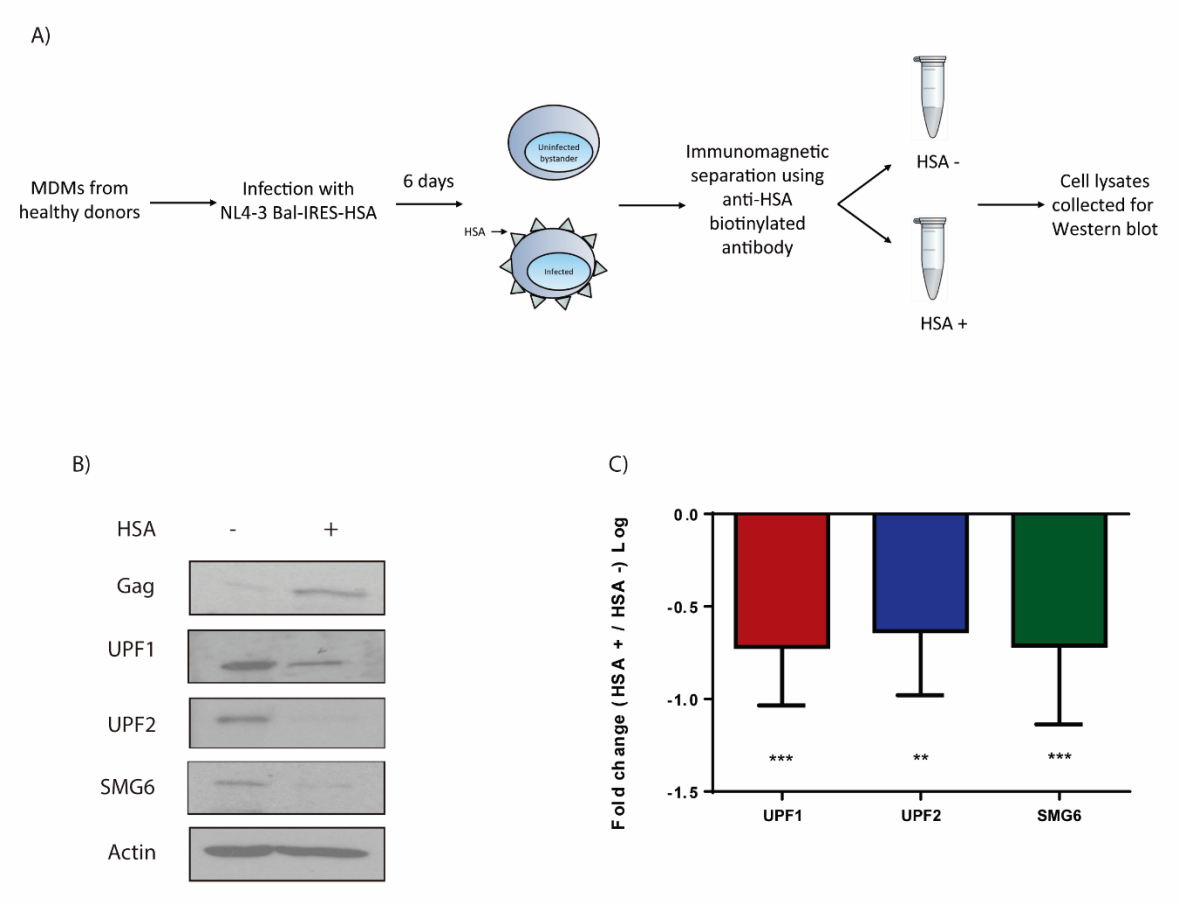

Figure 3.1.: UPF1, UPF2, and SMG6 expression are reduced in HIV-1 infected MDMs. Human monocytes were differentiated into MDMs and infected with an MOI of 1.0 of NL4.3-Bal-IRES-HSA virus. 6 days after infection, cells were collected, incubated with anti-HSA antibody and sorted through magnetic separation as described in [36]. **A)** Schematic of the sorting strategy to separate HSA-negative from HSA-positive cells. **B)** Cell lysates were run on SDS-PAGE gels and UPF1, UPF2, SMG6, $pr55^{Gag}$ and actin protein levels were detected by Western Blotting. **C)** Fold changes in expression levels of each protein between bystander and HIV-1 infected cells. Error bars represent the standard deviation from three independent experiments with cells from three different donors each. Asterisks represent statistically significant difference between bystander and infected cells (One-way ANOVA; ** $p \leq 0.01$ and p *** $p \leq 0.001$).

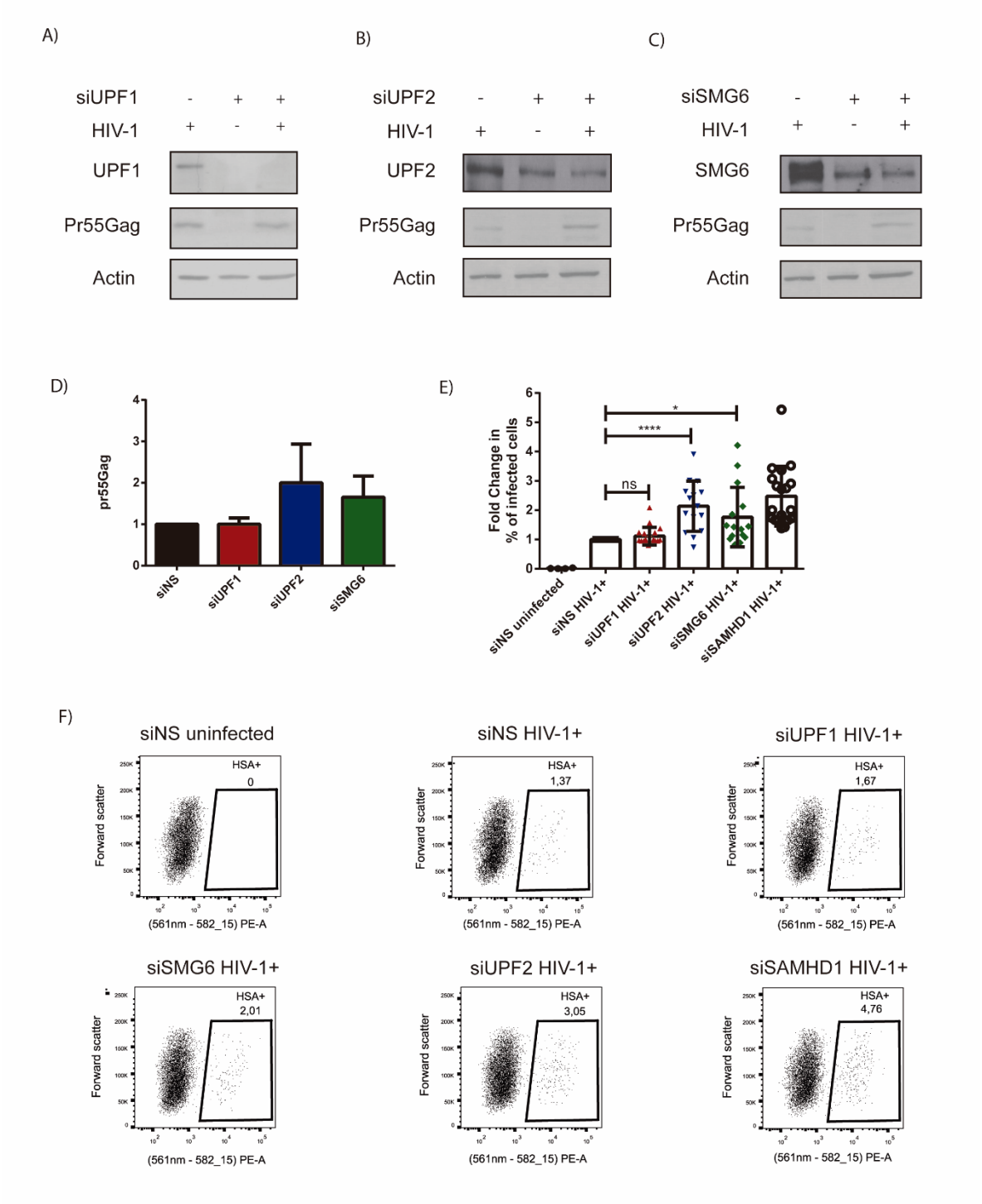

Figure 3.2.: UPF2 and SMG6 knockdown enhance HIV-1 viral gene expression and replication in primary MDMs.

Figure 3.2.: UPF2 and SMG6 knockdown enhance HIV-1 viral gene expression and replication in primary MDMs (continued).

Human monocytes were differentiated into MDMs and then transfected with the indicated siRNAs. 24 h later, cells were infected with NL4-3-Bal-IRES-HSA virus at an MOI of 1.0 and kept in culture for 6 days. Cells silenced for **A)** UPF1, **B)** UPF2 or **C)** SMG6 were collected, lysates were run on SDS-PAGE gels and protein levels were detected by Western blotting. **D)** Fold change in the levels of $pr55^{Gag}$ normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each. **E)** Cells silenced for UPF1, UPF2, SMG6 or SAMHD1 were collected, incubated with anti-HSA antibody and analysed by flow cytometry. Fold change in the HSA expression was normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from 5 different donors each. Asterisks represent statistically significant difference between groups (One-way ANOVA; ns: not significant, * $p \leq 0.05$ and p **** $p \leq 0.0001$). **F)** Representative dot plot depicting HSA expression in siNS, siUPF1, siUPF2, siSMG6 and siSAMHD1 transfected primary MDMs.

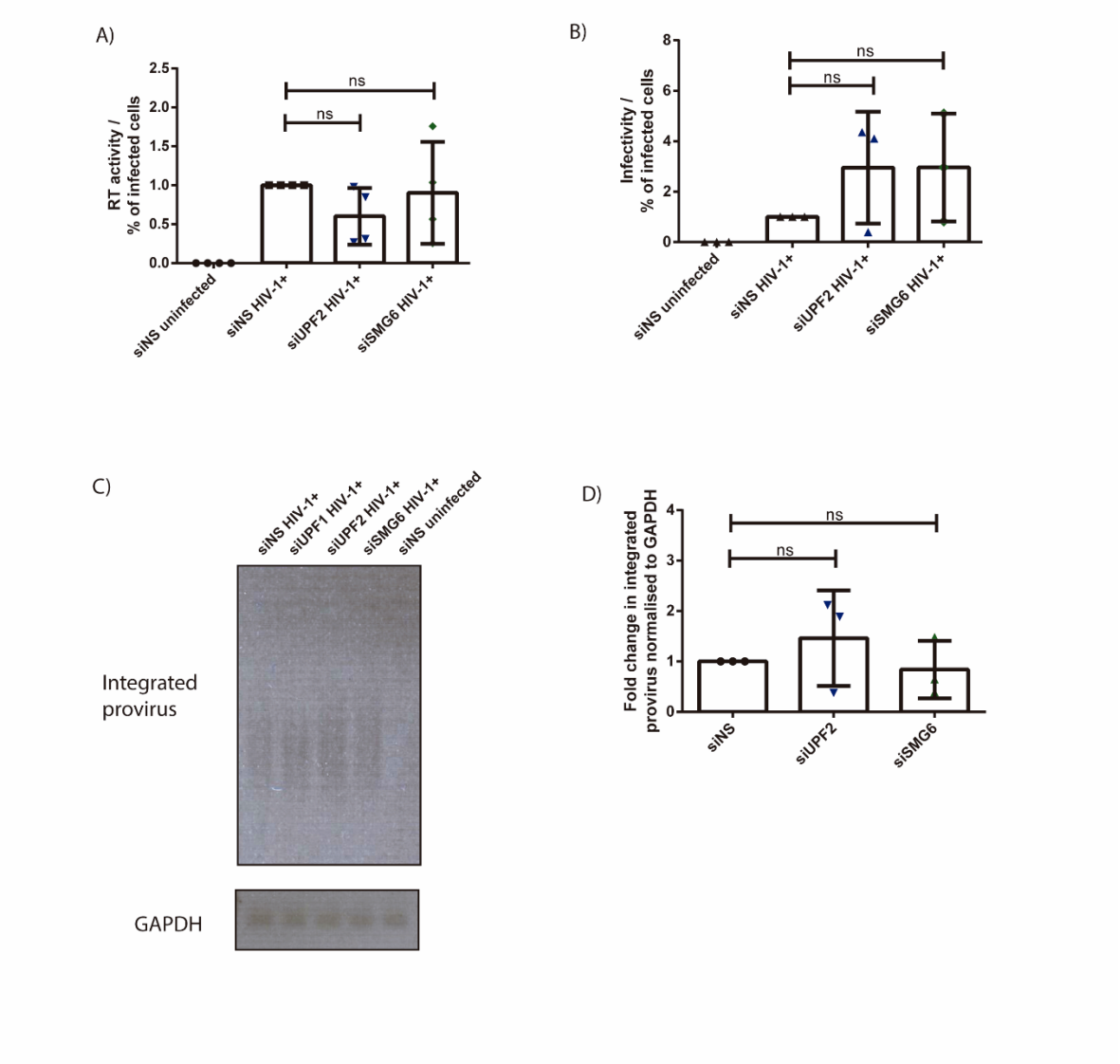

Figure 3.3.: UPF2 and SMG6 knockdown enhance HIV-1 vRNA expression in primary HIV-1 infected MDMs.

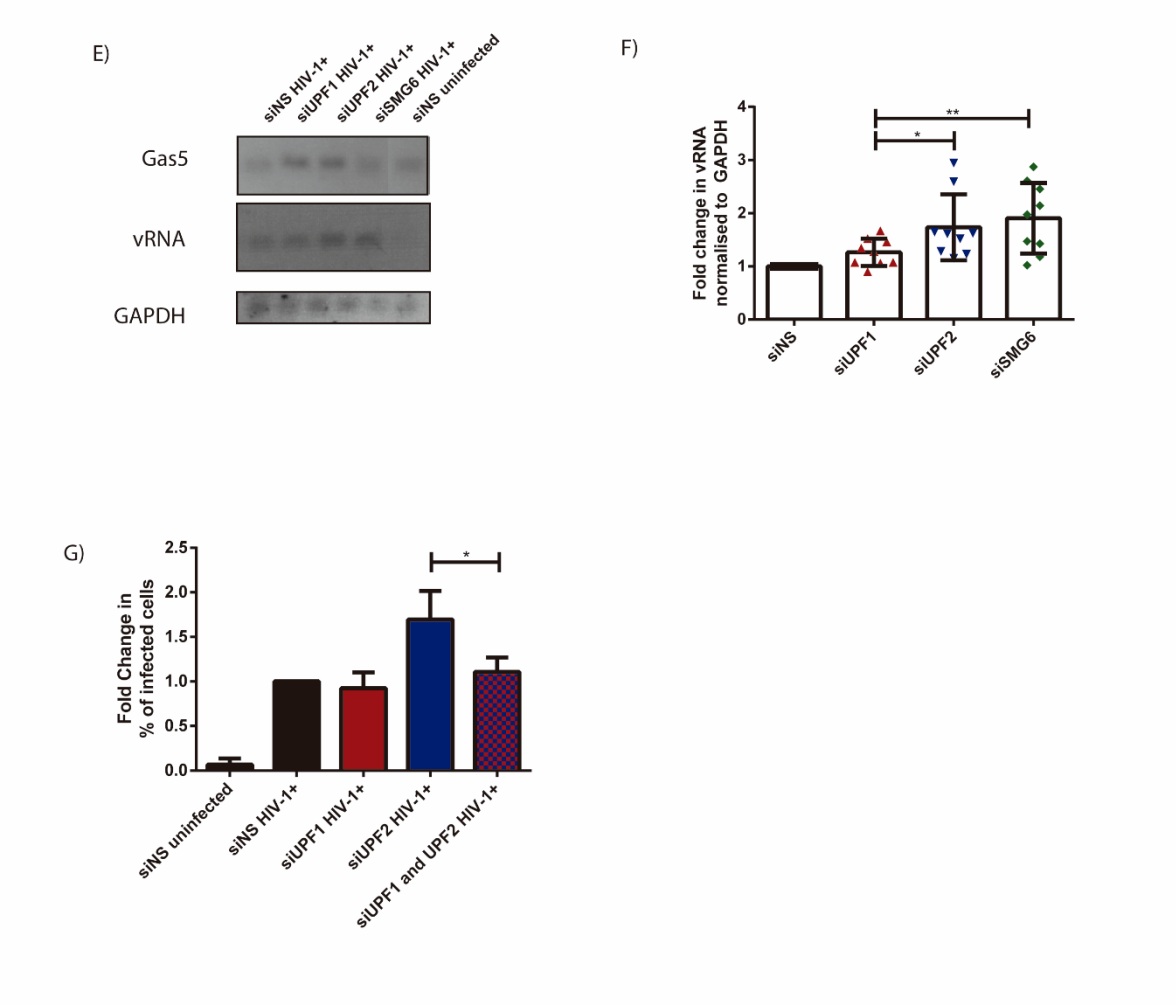

Figure 3.3.: UPF2 and SMG6 knockdown enhance HIV-1 vRNA expression in primary HIV-1 infected MDMs (continued).

Human monocytes were differentiated into MDMs and then transfected with control siRNA (siNS) or siRNAs against UPF1, UPF2 or SMG6. 24 h later, cells were infected with NL4-3-Bal-IRES-HSA virus at an MOI of 1.0 and kept in culture for 6 days. **A)** RT activity in cell supernatants was analysed and fold change in the RT activity were normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each (One-way ANOVA; ns: not significant). **B)** Viral titer in cell supernatants was quantified using the X-gal staining assay in TZM-bl cells and fold change in viral titer were normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each (One-way ANOVA; ns: not

significant). **C)** Integrated proviral DNA was measured using a combined Alu-HIV-1 PCR and PCR products were visualized in a 1% agarose gel and DNA staining **D)** Fold change in the levels of integrated proviral DNA visualized in C and normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each (One-way ANOVA; ns: not significant). **E)** The NMD target Gas5 mRNA and vRNA levels were measured by RT-PCR and PCR products were visualized on a 1% agarose gel and DNA staining. **F)** Fold change in the levels of vRNA visualized in E and normalized to the siNS HIV-1 + condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each (One-way ANOVA; ns: not significant, * $p \leq 0.05$ and p ** $p \leq 0.01$). **G)** Cells were transfected with siNS, siUPF1, siUPF1 or siUPF1 and siUPF2 combined, infected and after 6 days were collected, incubated with anti-HSA antibody and analysed by flow cytometry. Fold change in the HSA expression was normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from one donor. Asterisks represent statistically significant difference between groups (One-way ANOVA; * $p \leq 0.05$).

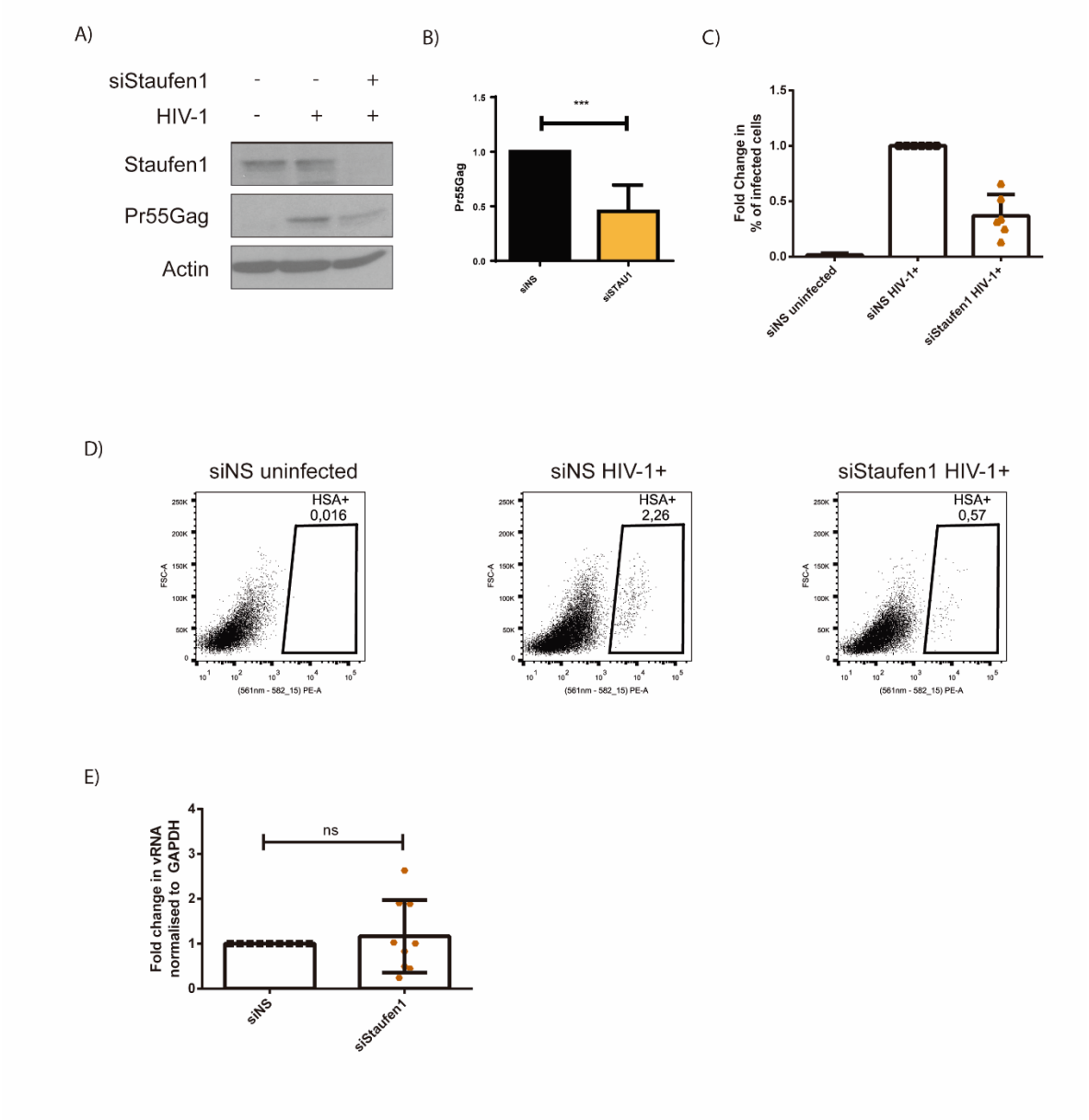

Figure 3.4.: Staufen1 knockdown impairs HIV-1 viral gene expression and replication in primary HIV-1 infected MDMs.

Figure 3.4.: Staufen1 knockdown impairs HIV-1 viral gene expression and replication in primary HIV-1 infected MDMs (continued). Human monocytes were differentiated into monocyte-derived macrophages (MDMs) and then transfected with control siRNA (siNS) or siRNA against Staufen1. 24 h later, cells were infected with NL4-3-Bal-IRES-HSA virus at an MOI of 1.0 and kept in culture for 6 days. **A)** Cells were collected, lysates were run on SDS-PAGE gels and protein levels were detected by Western Blotting. **B)** Fold change in the levels of $pr55^{Gag}$ normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each (One-way ANOVA; p *** $p \leq 0.001$). **C)** Cells were collected, incubated with anti-HSA antibody and analysed by flow cytometry. Fold change in the HSA expression was normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each. **D)** Representative dot plot depicting HSA expression in siNS and siStaufen1 transfected primary MDMs. **E)** vRNA was measured by RT-PCR and fold change in the levels of vRNA were normalized to the siNS condition. Error bars represent the standard deviation from three independent experiments with cells from three different donors each (One-way ANOVA; ns: not significant).

Chapter 4

HIV-1 NC-induced stress granule assembly and translation arrest are inhibited by the dsRNA binding protein Staufen1

This chapter was adapted from the following manuscript:

Rao, S., Cinti, A., Temzi, A., Amorim, R., You, J. C., & Mouland, A. J. (February, 2018). "HIV-1 NC-induced stress granule assembly and translation arrest are inhibited by the dsRNA binding protein Staufen1". *RNA, 24, 2, 219-236.*

4.1. Preface

In Chapter 3, we demonstrated that the host mRNA decay protein Staufen1 is a positive regulator of viral gene expression in primary monocyte-derived macrophages. This chapter provides data on the further characterisation of Staufen1 on viral gene expression and its modulation of the host cell stress response. Specifically, we investigate the effects of Staufen1 on stress granule assembly and its subsequent effect on virus production during HIV-1 nucleocapsid-induced translation arrest.

Author contributions: S.R., and A.J.M conceived the study and designed experiments with input from A.C. S.R. conducted most of the experiments and data analysis. A.C. conducted microscopy analysis for Figure 4.2. A.T. and R.A. assisted with some western blot and microscopy analysis presented in Figure 4.1. E. and Figure. 4.6. A-G. S.R. drafted the manuscript with the support and comments from A.C. and A.J.M. All authors read and approved the final manuscript.

Funding information: This study was supported by Canadian Institutes of Health Research (CIHR) Grants MOP-38111 & MOP-56974 (to AJM) and by The Canadian HIV Cure Enterprise Team Grant HIG-133050 (to AJM) from the CIHR in partnership with Canadian Foundation for HIV-1/AIDS Research and International AIDS Society.

Acknowledgements: We thank the late Mark Wainberg, Guy Lemay, Anne Gatignol, Marc Fabian, Tamiko Nishimura, Nancy Kedersha and Robert Gorelick for generous provision of reagents; Maureen Oliviera and Ilinca Ibanescu for assay development; Hugues de Rocquigny for helpful discussions; and Fernando Valiente-Echeverria and Luc DesGroseillers for critical reading of the manuscript.

4.2. Abstract

The nucleocapsid (NC) proteins is derived from the N-terminal portion of the HIV-1 Gag precursor polyprotein, $pr55^{Gag}$. NC possesses key functions at several pivotal stages of viral replication. For example, an interaction between NC and the host, double-stranded RNA-binding protein Staufen1 was shown to regulate several steps in the viral replication cycle, such as Gag multimerisation and genomic RNA encapsidation. In this work, we observed that the overexpression of NC leads to the induction of stress granule (SG) assembly. NC-mediated SG assembly was unique as it was resistant to the SG blockade imposed by the HIV-1 capsid (CA), as shown in earlier work. NC also reduced host cell mRNA translation, as judged by a puromycylation assay of *de novo* synthesized proteins and this was recapitulated in polysome profile analyses. Virus production was also found to be significantly reduced. Finally, Staufen1 expression completely rescued the blockade to NC-mediated SG assembly, global mRNA translation as well as virus production. NC expression also resulted in the phosphorylation of protein kinase R (PKR) and eIF2α and this was inhibited with Staufen1 coexpression. This work sheds light on an unexpected function of NC in host cell translation. A comprehensive understanding of the molecular mechanisms by which a fine balance of the HIV-1 structural proteins NC and CA act in concert with host proteins such as Staufen1 to modulate the host stress response will aid in the development of new antiviral therapeutics.

4.3. Introduction

The HIV-1 Nucleocapsid (NC) is a highly versatile, 9 kDa protein that is intricately associated with the HIV-1 genomic viral RNA (vRNA), exerting an effect at both early and late steps of the HIV-1 replication cycle from reverse transcription (RT) and DNA integration to vRNA selection, packaging and assembly (reviewed in [1]). It is a product of the proteolytic processing of the precursor Gag polyprotein ($pr55^{Gag}$, referred to as Gag herein) and contains two CCHC zinc finger domains flanked by basic residues, all of which contribute to both sequence and non-sequence specific nucleic acid binding activity. NC also possesses chaperone activity that facilitates the rearrangement of nucleic acids into thermodynamically more stable structures [2-5]. NC recruits numerous host proteins to facilitate its functions and these include the double-stranded (ds) RNA-binding protein Staufen1, a host factor that is involved in mRNA trafficking and translation [6-9]. In our previous work, we have shown that Staufen1 regulates several events in the HIV-1 replication cycle by assembling large HIV-1-dependent ribonucleoprotein complexes (SHRNPs) and via its interactions with NC, affects various steps of virus assembly including Gag multimerisation, vRNA encapsidation [10-13]. Staufen1 also has been reported to play a role in modulating the cellular stress response [14-17].

To counteract conditions of stress, such as that of viral infection, the host mounts a cellular stress response that leads to the assembly of translationally silent ribonucleoprotein (RNP) complexes known as stress granules (SGs) [18, 19]. Since viruses are obligate intracellular parasites that utilise the host cell machinery to facilitate their own gene expression, their replication can be markedly affected by an impediment to cellular mRNA translation. Therefore, viruses have developed the capability to circumvent this innate antiviral host cell response by numerous mechanisms (reviewed in [20, 21]). Two types of SGs have been described that differ in morphology, composition, and mechanism of assembly [22]. In our previous work, we have shown that HIV-1 disrupts the canonical type I SG assembly in an eIF2α phosphorylation (eIF2α-P) independent manner via an interaction with the eukaryotic elongation factor eEF2 with the capsid (CA) domain on the Gag polyprotein [10, 23]. We also demonstrated that Gag is able to block the assembly of type II, noncanonical SGs by reducing the amount of hypophosphorylated 4EBP1 associated with the 5’ cap potentially through an interaction with its target, eIF4E [24].

Interestingly, a recent study has reported that the expression of the HIV-1 NC alone leads to the assembly of SGs [25].

In this study, we have characterised NC-induced SGs and have elucidated the mechanism by which they assemble. Here, we demonstrate that NC induces the assembly of SGs, and although the composition resembles that of type I, canonical SGs, they can not be dissociated by HIV-1 Gag expression [10, 23]. We also show that Staufen1, a host protein that has roles in stabilising polysomes and SG dynamics [14], is capable of inhibiting NC-induced SG assembly. We also demonstrate that Staufen1's F135 amino acid residue in its third dsRNA binding domain (dsRBD3) is critical for this activity. We also demonstrate that NC expression leads to the phosphorylation of protein kinase R (PKR) and eIF2α resulting in hindered host cell mRNA translation and this impairs viral production; this can also be rescued by Staufen1 co-expression. This work sheds light on an unexpected function of NC on host cell mRNA translation and the mechanism by which it operates in concert with the host protein Staufen1 to modulate the host stress response.

4.4. Results

4.4.1. NC induces the assembly of SGs containing TIAR1, G3BP1, eIF3, PABP and poly(A) mRNAs

SGs are associated with silenced transcripts and many viruses are known to subvert the function of these RNA granules for their replicative advantage [26]. As NC expression has been recently demonstrated to lead to the assembly of SGs [25], we set out to quantify and describe this NC induced assembly of SGs. HeLa cells were either mock transfected with RLuc or transfected with NC-RLuc, fixed and SG assembly was monitored by indirect immunofluorescence of Ras-GAP SH3 domain-binding protein 1 (G3BP1) and TIA-1-related RNA-binding protein (TIAR1). SGs were detected in 66.2 (SD ±2.7) % of NC-expressing cells, in striking contrast compared to the 5.1 (SD ±4.2) % in the RLuc-transfected cells (Figure 4.1. A and B).

Two distinct types of SGs have previously been characterised [22] that differ in their mechanism of assembly, localization, as well as in composition. The canonical, type I SGs, such as those induced by Arsenite and Pateamine A, are larger and contain the eukaryotic initiation factors eIF4G, eIF4E and eIF3, amongst several other components. In contrast, type II SGs, which are induced by Selenium, are smaller in size and do not contain eIF3. To determine which type of SGs are induced by NC, we performed indirect immunofluorescence analyses on NC-RLuc-transfected HeLa cells and probed them for eIF3 along with another SG marker Poly-A Binding Protein (PABP). We observed that eIF3 is present in the NC induced SGs, indicating that they are likely to be the canonical, type I stress granules (Figure 4.1.C) [22, 24]. Thus, these newly characterised NC-SGs contain G3BP1, TIAR1, eIF3 and PABP.

Recent studies have demonstrated that some stresses such as ultraviolet irradiation and rocaglamide A (RocA) treatment assemble SG-like foci that do not contain poly(A) mRNAs [27]. In order to determine if the NC-induced SGs are bona fide SGs that contain polyadenylated mRNAs [28], we conducted FISH for poly(A) mRNA with an oligo(dT) probe in mock transfected and NC-expressing cells. It was observed that in the NC-expressing cells, the poly(A) mRNAs colocalised with the SG marker TIAR, indicating that NC expression leads to the assembly of bona fide SGs that contain mRNAs (Figure 4.1.D).

NC is composed of an N-terminal basic region, two CCHC type zinc fingers (ZFs) and a basic linker region between the ZFs. In a previous study, it was observed that a loss of the ZFs of NC led to

impaired SG assembly as compared to the wild type NC [25], suggesting that the NC ZFs contribute to SG assembly. In order to test if the presence of a ZF from another virus can also elicit a stress response, we transfected cells with the plasmid pSV-S4 that encodes the Reovirus σ3 protein, which is a dsRNA binding protein that is a component of the reovirus outer capsid and contains CCHC type zinc fingers similar to NC [29]. The expression of this protein did not lead to SG induction, indicating that merely the presence of CCHC-type zinc fingers alone does not lead to SG assembly, and that this activity is specific to NC (Figure 4.1.E).

4.4.2. Neither Gag nor CA disassembles NC-induced SGs

In our previous work, we have shown that Gag disassembles preformed type I SGs, irrespective of eIF2α phosphorylation, by interacting with the eukaryotic elongation factor eEF2 via the Gag capsid (CA) domain [23]. Therefore, we sought to determine if full length Gag or CA dissociates NC-induced SGs. HeLa cells were transfected with either Gag-GFP or CA-GFP plasmids alone or with NC-RLuc. 24 h later cells were left untreated or treated with arsenite and SGs were visualised by indirect immunofluorescence. Although Gag and CA were able to efficiently inhibit arsenite-induced SGs, neither Gag nor CA was capable of dissociating NC-induced SGs (Figure 4.2.A – D). These results suggest that the NC-induced SGs are of a different nature than the ones induced by arsenite, pateamine A or selenite that Gag is able to dissociate [23, 24].

4.4.3. NC-induced SG assembly and translation arrest are inhibited by Staufen1

Staufen1 is a dsRNA binding protein that affects HIV-1 at multiple stages of its life cycle coinciding with many of the NC-associated functions in Gag multimerisation and assembly, as well as in vRNA encapsidation [9, 12, 13, 30, 31]. It exerts many of these functions by interacting with the zinc fingers of NC via its dsRBD3 domain, as shown in our previous work [9, 12, 13]. As a known interacting partner of NC with previously defined roles in the modulation of the stress response [14], we therefore hypothesised that Staufen1 may be able to counteract NC-induced SG assembly. When HeLa cells were co-transfected with NC-RLuc and Staufen1-YFP, SGs were present only in 11.6 (SD ±5.6) % of co-transfected cells, as compared to the 55.6 (SD ±6.1) % of SG containing cells observed in the cells transfected with NC-RLuc only (Figure 4.3.A and B). To determine the mechanism of Staufen1-mediated disruption of NC-induced SGs, we co-transfected HeLa cells with NC-RLuc and Staufen1-F135A-YFP, that possesses a point mutation in the dsRBD3 domain which reduces Staufen1's capacity to bind both NC and RNA [13, 32]. Under this condition, SGs were observed in 58.6 (SD ±8.6) % of co-transfected cells, at levels comparable to the NC expressing cells alone (Figure 4.3.A and B).

To determine if *de novo* synthesis of proteins was reduced by NC expression, *de novo* synthesized proteins were labelled with puromycin in tissue culture. The puromycylation technique has been shown to be a valid alternative to the use of radioisotopes for measuring quantitative changes in protein synthesis in cell culture [33, 34]. HeLa cells transfected with RLuc, NC-RLuc or NC-RLuc and Staufen1-YFP were incubated with puromycin and then analysed for the amount of *de novo* puromycin-labelled proteins by Western blotting (Figure 4.3.C and E). As a positive control, RLuc-transfected cells were treated with emetine, a translation inhibitor (Figure 4.3.C and E). The results demonstrated that NC induced a 2-fold decrease in puromycin-labelled peptides, while coexpression of Staufen1 restored the protein synthesis to a level similar to mock transfected cells (Figure 4.3.C and E).

To confirm that NC-induced SG assembly has an effect on host cell translation and whether translation can be rescued by Staufen1 co-expression, we performed polysome profile analyses of cell lysates derived from cells that were either mock-transfected (RLuc-N1), transfected with NC-RLuc, NC-RLuc and Staufen1-YFP or Staufen1-F135A-YFP. An increase in the levels of RNA

present in the polysome-free fractions implies an inhibition in host cell translation. As compared to mock-transfected cells, the expression of NC induced an increase in absorbance in polysome-free gradient fractions corresponding to the 40S, 60S ribosomal subunits and 80S ribosomes of the profile (Figure 4.3.D and F), thus indicating that in the presence of NC, there are increased free ribosomal subunits and monosomes. The presence of Staufen1 partially reversed the effects of NC expression on polysome profiles, but this ability, was lost when the Staufen1-F135A construct was coexpressed (Figure 4.3.D and F). These findings show that the proportion of free ribosomal subunits and monosomes was increased in the presence of NC, and this is relieved by Staufen1 coexpression, therefore indicating that NC reduces cellular mRNA translation.

4.4.4. NC and Staufen1 interact *in situ* and *in vitro*

To further characterize the nature of the binding between Staufen1 and NC in host cells, we used a proximity ligation assay (PLA). This assay produces distinct countable spots that represent a single-molecule protein interaction ~40 nm apart [35, 36]. In cells co-transfected with Staufen1-YFP and NC-RLuc, we confirmed a close localization between Staufen1 and NC (103.3, SD ± 16 spots per cell) (Figure 4.4.A and B), whereas there was little signal detected upon transfection of NC-RLuc together with Staufen1-F135A-YFP (19 ± SD 9.0 spots per cell), at levels that were comparable to the background PLA signal (22.1 ± SD 14.6 spots per cell) (Figure 4.4.A and B). These data indicate that Staufen1 is in close proximity to NC *in situ*, likely mediated via its dsRBD3. To determine if Staufen1 and NC interact by direct association and to precisely characterize the Staufen1 binding site on NC, we conducted *in vitro* GST-pull down assays. Full length GST-tagged recombinant Staufen1 (D2-5), individual dsRBDs (D3, D3-4, D4 and D5; where D = dsRBD) as well as a dsRBD3 construct with point F135A mutation (DM3), used as a negative control, were incubated on GST-Spintrap columns (Figure 4.4.C). Recombinant, wild type NC or recombinant mutated NC in one (CCHC-SSHS) or both (SSHS-SSHS) Zinc Fingers were added to the columns and eluted after washing (Figure 4.4.C). These assays are only qualitative, not quantitative as the expression levels of the recombinant Staufen1 proteins differed due to differences in solubility. As shown in Figure 4.4.D, wild type NC directly bound to the full length Staufen1 (D2-5) as well as to the D3, D3-4 and D4 dSRBD truncations, but not to the D5, DM3 or GST only constructs. Furthermore, the binding of NC to D3 was lost when the two Zinc Finger mutants of NC were used (Figure 4.4.D). These data confirm the previously characterized binding of the Staufen1 dsRBD3 to the Zinc Fingers of NC [13], but also identify a novel Zinc Finger-independent binding site for Staufen1 via its dsRBD4. Taken together, these experiments indicate that Staufen1 is able to directly bind NC, both, *in situ* and *in vitro* in an RNA-independent manner and that this binding could lead to the sequestration of NC and a block to NC-induced SG assembly.

4.4.5. NC is found in a complex with SG components

To characterize a possible mechanism behind the NC-mediated SG assembly, we sought to determine the ability of NC to interact with components of SGs, by performing co-immunoprecipitation (co-IP) assays. HeLa cells were transfected with NC expressors that contained mutations in either the N terminal region (NC-R7-YFP), the first Zinc Finger (ZF) (NC-C15-YFP), the second ZF (NC-C49-YFP) or both ZFs (NC-C14-C49-YFP) or mock transfected with GFP. As shown in Figure 4.5.A, using anti-GFP beads, we demonstrated that TIAR1 and Staufen1 specifically interacted with NC-YFP, but not GFP alone, as well as with all the NC mutants tested (Figure 4.5.A). Additionally, the interactions were not dependent on RNA, as TIAR1 and Staufen1 still co-immunoprecipitated in the presence of RNAse, albeit to lower levels. This indicates that the binding of TIAR1 and Staufen1 to NC is enhanced in the presence of RNA, although RNA is not necessary for it (Figure 4.5.A). To determine if G3BP1 is a binding partner of NC, a U2OS cell line that constitutively expresses GFP-tagged G3BP1, was transfected with NC-RLuc. G3BP1-GFP was pulled down using anti-GFP beads and NC was found to specifically co-immunoprecipitate with it, even after RNase treatment (Figure 4.5.B). Taken together these results indicate that NC is capable of associating with a number of SG components even after RNase treatment, and suggest that the interaction with these factors could promote NC-induced SG assembly.

A depletion of G3BP1 has been demonstrated to hinder the assembly of phospho-eIF2α dependant SGs [37]. In order to determine if G3BP1 is required for the assembly of NC-induced SGs, cells were either treated with non-silencing siRNA (siNS) or siRNA against G3BP1 (siG3BP1). The knockdown of G3BP1 was validated by western blot of cell lysates (Figure 4.5.C). They were either mock transfected or transfected with NC-RLuc and the assembly of SGs was determined by indirect immunofluorescence of the SG marker TIAR (Figure 4.5.D). It was observed that a knockdown of G3BP1 resulted in a significant decrease in the percentage of NC-induced SG assembly with only 40.42 (SD ±10.96) % of cells displaying SG assembly as compared to 70.22 (SD ±10.35) % of SG positive cells in the siNS treated cells (Figure 4.5.E). Thus, NC-induced SG assembly is impaired by the depletion of G3BP1.

4.4.6. NC expression leads to the phosphorylation of eIF2α by activating PKR

The phosphorylation of eIF2α is triggered by conditions of stress, thus blocking translation initiation and regulating SG assembly [28]. However, the formation of type I SGs is either eIF2α phosphorylation-dependent or -independent [38]. To determine if NC-induced SG assembly is linked to the eIF2α phosphorylation status, cell lysates from mock transfected cells (pcDNA3.1) or from cells expressing NC (NC-RLuc) were analysed by Western blots using antibodies against total and phosphorylated forms of eIF2α. An 8-fold increase in the amount of phosphorylated eIF2α was observed in the NC-expressing cells as compared to the mock-transfected cells (Figure 4.6.A and B). Furthermore, the coexpression of Staufen1 with NC significantly reduced the phosphorylation of eIF2α to levels comparable with mock-transfected cells (Figure 4.6.A and B). In order to determine the mechanism of eIF2α activation, we monitored PKR activation levels in the above conditions. PKR is an interferon-induced protein that senses dsRNA and its activation leads to the phosphorylation of eIF2α [39]. PKR was activated in the NC expressing cells, but was inactive in mock and NC/Staufen1 co-expressing conditions (Figure 4.6.C). In order to determine if Staufen1-F135A could also inhibit NC-mediated PKR and eIF2α phosphorylation, we either mock transfected cells or transfected them with NC-RLuc, NC-RLuc and Staufen-YFP, or NC-RLuc and Staufen1-F135A-YFP. For each condition, indirect immunofluorescence was used to quantify the phosphorylation of eIF2α (Figure 4.6.D) and PKR (Figure 4.6.F). A significant increase in the fluorescence intensity of both the P-eIF2α (Figure 4.6.E) and P-PKR (Figure 4.6.G) was observed upon NC expression. This phosphorylation was reduced to levels comparable to wildtype upon NC/Staufen1 coexpression, but not in the NC/Staufen1-F135A coexpressing condition (Figure 4.6.E and G). Therefore, Staufen1, but not Staufen1-F135A, is capable of preventing NC-induced activation of PKR and eIF2α.

The phosphorylation of eIF2α can be carried out by four kinases: PERK (PKR-like ER kinase), GCN2 (general control non-derepressible-2), HRI (heme-regulated inhibitor) and PKR [40]. In order to ascertain that NC-mediated phosphorylation of eIF2α is via the activation of PKR and not another kinase, we knocked down PKR using an shRNA (shPKR) via lentiviral transduction and measured eIF2α phosphorylation. An shRNA with a scrambled sequence was used as a negative control (shNS). In the shNS condition, NC expression resulted in a significant increase in ratio of

phosphor/total eIF2α (Figure 4.6.H and I). However, upon knockdown of PKR, no significant increase in eIF2α phosphorylation was observed in the NC-expressing cells (NC-RLuc) as compared to the mock treated cells in the same condition (RLuc-N1) (Figure 4.6.H and I). Thus, the NC-induced phosphorylation of eIF2α is dependent on the activation of PKR.

4.4.7. Staufen1 rescues the NC-mediated reduction of viral production

In order to determine if the inhibition of global translation by NC can affect viral production, we transfected cells with either pNL4.3 alone, or co-transfected them with NC. The virus contained in the supernatants of these cells was then quantified by p24 ELISA. It was observed that an expression of NC led to a 10 (SD ±0.3)-fold reduction of viral production as compared to the cells expressing pNL4.3 alone (Figure 4.7.A). To determine if Staufen1 could rescue NC-induced inhibition of viral production, expression vectors encoding either Staufen1 or Staufen1-F135A were co-transfected with NC and pNL4.3. Staufen1 expression rescued viral production to levels comparable to pNL4.3 alone, whereas Staufen1-F135A was unable to do so (Figure 4.7.A). Cell lysates from the above conditions were analysed by Western blotting and decreased levels of Gag were observed in the NC-transfected cells as compared to pNL4.3 alone. Gag expression was rescued by Staufen1 coexpression, but not by F135A-Staufen1 coexpression (Figure 4.7.B). These results indicate that the decreased viral release is likely a result of the inhibition of mRNA translation thus resulting in reduced synthesis of Gag (Figure 4.7.B).

4.5. Discussion

In this study, we have used NC as a tool to understand how HIV-1 modulates gene expression and have demonstrated a detrimental effect of NC expression on mRNA translation. Based on our results, we hypothesize that NC induces SG assembly by one of two ways. The first is linked to NC's molecular chaperone activity whereby it catalyses the rearrangement of nucleic acids to more thermodynamically stable structures [3, 41, 42]. The interferon (IFN)-inducible PKR is a dsRNA sensor and is a key player in the innate antiviral immune response [43, 44]. Its activation leads to the phosphorylation of the eIF2α, thereby preventing translational initiation and inducing SG assembly [39]. When NC is overexpressed it could aggregate cellular mRNAs [45-47], thereby activating PKR (Figure 4.6.A and C). HIV-1 proteins like Tat have evolved countermeasures to block PKR activation by recruiting PKR Activator (PACT), adenosine deaminase acting on RNA (ADAR) 1 and TAR RNA Binding Protein (TRBP) [48-50]. However, in our experimental conditions NC was present in isolation, and therefore PKR activation could not be subverted. NC could then associate to SG components G3BP1 and TIAR1 (Figure 4.5.A and B), leading to SG assembly and the suppression of global host cell mRNA translation. The second mechanism of NC-induced SG assembly might also be a result of its nucleic acid binding property [51]. We observed an increased in the abundance of polysome-free mRNAs in NC-expressing cells (Figures 4.3.D and F). This suggests that NC is either preventing the attachment of the ribosomal subunits to the mRNA, probably due to steric hindrance as a result of its own binding to the mRNA; or NC is stalling the ribosomes due to NC's binding and aggregation of mRNA [45, 46]. Furthermore, the presence of the low complexity (LC) and intrinsically disordered (ID) regions in a protein can also promote SG assembly [52, 53]. NC has been revealed to be a highly disordered protein [54] and this could contribute to its ability to induce SG assembly.

HIV-1 has developed strategies to subvert the host cellular stress response. In our previous work, we have shown that the capsid (CA) domain of Gag blocks SG assembly in an eIF2α phosphorylation-independent manner via an interaction with the eukaryotic elongation factor eEF2. This interaction is stabilized by a Gag–Cyclophilin A association and inhibits a later stage of SG assembly [23]. However, the NC-induced SGs are formed in part due to a dissociation or

disruption of the attachment of the ribosomal subunits themselves, or an impediment to their translational initiation, steps upstream to eEF2 function. It is likely that for this reason, a co-expression of either CA or Gag with NC was unable to inhibit SG assembly (Figure 4.2.A and B).

Staufen1 is a host protein that has been reported to suppress SG assembly by binding the ribosomal subunits and stabilising polysomes [14, 55-57]. It also has been implicated in preventing the activation of PKR and the subsequent phosphorylation of eIF2α during hepatitis C virus infection [16]. As a known interacting partner of NC with previously characterised roles in the modulation of the stress response, we hypothesised that Staufen1 could block NC induced PKR activation. Indeed, the co-expression of Staufen1 can prevent NC-induced activation of PKR and downstream phosphorylation of eIF2α (Figure 4.6.A - C). Staufen1 alone, unlike TRBP for instance, is not able to subvert PKR activity during HIV-1 infection [10, 48, 58]. However, when in isolation or when bound to NC, the resulting suppression of PKR activation by Staufen1 is remarkable. The Staufen1-F135A can neither bind NC nor RNA. In this condition, NC is free to interact with cellular mRNAs and allows assembly of NC-induced SGs. Interestingly, the co-expression of the dsRBD3 binding mutant, F135A-Staufen1, with NC had little suppressive activity on PKR. This indicates that the efficient binding of NC to Staufen1 may be required to prevent PKR activation, or that the dsRBD3 and the ability to bind RNA is responsible for PKR downregulation by Staufen1. Staufen1's ability to interact with RNA and stabilise polysomes by binding to ribosomes via its N-terminal domain [14] may augment its ability to block NC-induced SG assembly. Staufen1 can prevent the dissociation of attached ribosomal subunits and facilitate mRNA translation, acting at a stage downstream of eIF2α phosphorylation. However, if this was the only method of Staufen1-mediated disruption of NC-SGs, then an increase in P-eIF2α would be observed in the NC/Staufen1 co-expressing cells. But our results show that Staufen1 can prevent eIF2α phosphorylation (Figure 4.6. A, B, D and E) and is therefore also acting upstream of polysome stabilisation, probably by binding and sequestering NC. The F135A mutation in Staufen1 impairs RNA binding capability and this may hinder Staufen1's ability to stabilise polysomes, thereby exacerbating its inability to inhibit NC-induced SG-assembly. Staufen1 co-expression relieves the NC-induced global translation block as shown by polysome profile and

puromycylation assays resulting in enhanced virus production (Figures 4.3.E and 4.7.A and B). Overall, NC induces the assembly of SGs by activating PKR and destabilising polysomes. Staufen1 disrupts NC induced SG assembly by binding and sequestering NC and by binding to RNA and stabilising polysomes. The model for Staufen1's blockade of NC-induced SG assembly is depicted in Figure 4.8.

In our earlier work, we demonstrated that Staufen1 interacted with HIV-1 Gag precursor via the NC domain using a variety of *in vitro* and biophysical analyses. In this paper, we now show a direct association between NC and Staufen1 (Figure 4.4.D), a type of study that has largely been hampered by the solubility of recombinant Staufen1 proteins. Indeed, the full length Staufen1 remains poorly soluble, but the data presented herein (Figure 4.4.D) indicate a rather selective association to the third dsRNA binding domain, as we have shown earlier [13]. The results (Figure 4.4.D) also suggest an additional binding interaction between the dsRNA binding domain 4 and NC. The association of Gag to Staufen1 via the NC domain was shown to impact virus assembly [12, 13], Gag and vRNA trafficking [30], vRNA encapsidation [10] and an antiviral stress response [10]. These roles are likely to be coupled, such that the dsRNA binding protein Staufen1 likely functions by contacting the vRNA directly or as a component of a larger ribonucleoprotein as we and others have shown [10, 59-61]. A direct role has yet to be substantiated for Staufen1 in encapsidation [9] but recent work in other viruses supports a role in this late step of virus assembly [16].

During the late stages of the viral replication cycle, the detrimental effects of NC on host cell translation highlight the importance of timely Gag polyprotein processing. There is significant evidence that Gag polyprotein processing, and hence the generation of mature NC protein, takes place concomitantly or only shortly after budding (reviewed in [62, 63]). That is, under normal conditions of the viral life cycle during viral assembly, free NC is largely absent from the producer host cell. The premature precursor processing and the appearance of NC in the cytoplasm correlates with defects in virus assembly and production [64], but also contributing to these parameters would be the marked decrease in mRNA translation, marked by the assembly of SGs.

These observations are consistent with our previous work, where the presence of SGs in HIV-1 expressing cells decreased virus production and infectivity ([23]; Figure 4.7.A and B), while that more recently, it was shown that G3BP1 can bind the HIV-1 vRNA in the cytoplasm of macrophages to inhibit viral replication [65]. Furthermore, it has been demonstrated that the binding of the NC protein to the vRNA causes a rearrangement in vRNA secondary structure from the long distance interaction (LDI) to the branched structure with multiple hairpins (BMH) conformation, thus promoting dimer formation during virion assembly and reducing vRNA translation [66].

Staufen1 was shown to suppress SG assembly during oxidative stress [14] but it also assembles with Gag and vRNA to favour the assembly of another type of RNP, the Staufen1 HIV-1-dependent RNP (SHRNP) [10]. SHRNPs are high molecular weight, detergent insoluble complexes containing Staufen1, amongst many other viral and cellular components [11, 59]; [67, 68]. Staufen1, likely in the context of SHRNPs, enhances Gag assembly and vRNA packaging [10, 30], roles ascribed to the interaction of Staufen1 with the NC domain of Gag. Therefore, we speculate that the effects of Staufen1 on the rescue of NC mediated translational arrest, viral assembly and vRNA packaging are linked. This is supported by the recovery of viral production and Gag mRNA translation upon coexpression of Staufen1 following NC's inhibitory effects on host cell gene expression (Figure 4.7.A and B). Indeed, the link between translation and packaging has been explored in earlier work [69, 70] and by studying whether translatable pools of vRNA were packageable or not [71, 72].

This work sheds light on a novel function of NC on cellular mRNA translation and highlights how a tightly regulated balance of the HIV-1 proteins, NC and CA, act in concert with host proteins such as Staufen1 to modulate the host stress response to ensure viral gene expression. An elucidation of the molecular mechanisms of viral pathogenesis can identify novel targets for antiviral therapeutic interventions.

4.6. Materials and methods

4.6.1. Plasmids. The construction of pCMV-NC-RLuc, pCMV-NC-YFP, pCMV-NC-R7-YFP, pCMV-NC-C15S-YFP, pCMV-NC-C49S-YFP, pCMV-NC-C15S-C49S-YFP, CA-GFP pCMV-Staufen1-YFP, pCMV-Staufen1-F135A-YFP was described previously [11-13, 23]. pcDNA3.1 was purchased from Invitrogen and pEGFP-C1 from Clontech. pGag-GFP was obtained from NIH AIDS Reference and Reagent Program. pSV-S4 was provided by Dr. Guy Lemay (Université de Montréal, Montréal, Québec) [29].

4.6.2. Antibodies. A rabbit anti-Staufen1 antiserum generated to the full-length recombinant protein was produced and purified at the McGill University Cell Imaging and Analysis Network (Montréal, Québec, Canada). Hybridoma cell lines producing mouse anti-σ3 (4F2) have been described before [73] and were a kind gift from Dr. Guy Lemay (Université de Montréal, Montréal, Québec). Anti-Staufen1 was used for Western blotting at a dilution of 1:1,000; rabbit or mouse anti-G3BP1 (Santa Cruz Biotechnology) were used for indirect immunofluorescence microscopy at a dilution of 1:1,000 and for Western blotting at a dilution of 1:10,000; goat anti-eIF3 (Abcam) was used for indirect immunofluorescence microscopy at a dilution of 1:500; goat anti-TIAR1 (Santa Cruz Biotechnology) was used for indirect immunofluorescence microscopy at a dilution of 1:500 and for Western blotting at a dilution of 1:2,000; mouse anti-PABP (Sigma-Aldrich) was used for indirect immunofluorescence microscopy at a dilution of 1:200; rabbit anti-RLuc (MBL) was used for indirect immunofluorescence microscopy at a dilution of 1:500 and for Western blotting at a dilution of 1:1,000; mouse anti RLuc (Abcam) was used for indirect immunofluorescence microscopy at a dilution of 1:500; mouse anti-σ3 (4F2) was used for indirect immunofluorescence microscopy at a dilution of 1:2; rabbit anti-phospho-eIF2α (Ser51) [24] (Cell Signaling Technology) was used for indirect immunofluorescence microscopy at a dilution of 1:200 and for Western blotting at a dilution of 1:1,000; mouse anti-eIF2α (Cell Signaling Technology) was used for Western blotting at a dilution of 1:1,000; rabbit anti-P-PKR (Abcam) was used for indirect immunofluorescence at a concentration of 1:300 and for Western blotting at a dilution of 1:1,000; mouse anti-PKR 71-10 [74] was used for Western blotting at a concentration of 1:1000 and was provided by Dr. Anne Gatignol (McGill University); rabbit anti-

GST (Sigma-Aldrich) was used for Western Blotting at a concentration of 1:2,000; goat anti-NC, a kind gift from Dr. Robert Gorelick (National Cancer Institute, Frederick, MD, USA; ACVP #77, lot R196099), was used for Western blotting at a dilution of 1:1,000 [75]; mouse anti-GFP (Sigma) was used for Western blotting at a dilution of 1:10,000; mouse anti-actin (Abcam) was used for Western blotting at a dilution of 1:10,000 and mouse anti-GAPDH (Abcam) was used for Western blotting at a dilution of 1:5,000. Horseradish peroxidase-conjugated secondary antibodies were purchased from Rockland Immunochemicals, while AlexaFluor secondary antibodies were from Life Technologies.

4.6.3. Cell culture and transfection conditions. HeLa cells, HEK293T cells and U2OS cells were maintained in DMEM (Life Technologies) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Hyclone). Cells were transfected with 1 µg of total DNA per 4 x 10^5 cells, unless indicated otherwise, using JetPrime (PolyPlus transfections) according to the manufacturer's instructions. If more than one plasmid was used to transfect cells, the amount of each plasmid used per transfection reaction was constant. 24 h after transfection, cells were fixed or lysed. For siRNA transfection, 20nM of siRNA was used to tranfect 150,000 cells using Lipofectamine 2000 (Invitrogen) reagent according to manufacturer's instructions. Cells were treated with 500 mM arsenite (Sigma) for 1h and with 1 µM Emetine (Sigma) for 50 min [76].

4.6.4. siRNAs. siRNA duplexes were purchased from QIAGEN-Xeragon. siNS is commercially available nonsilencing control duplex (QIAGENXeragon) and siG3BP1 is an siRNA targeting G3BP1 (SI00300265).

4.6.5. Viral transduction. psPAX2, pMD2.G, pLKO-shPKR#2 (TRCN0000196400) expression vector containing shRNA to PKR (target sequence GCTGAACTTCTTCATGTATGT) and a lentiviral control vector containing scrambled non-target shRNA that was used as a negative control were kind gifts from Dr. Marc Fabian (McGill University). 2,000,000 HEK293T cells were plated in 6-well plates one day prior to transfection. HEK293 were co-transfected with either scrambled shRNA (shNS) or shPKR expressing lentivirus, psPAX2 and pMD2.G. Supernatants were collected 48 hr

post-transfection, passed through a 0.45-µM nitrocellulose filter, supplemented with 5 µg/ml polybrene, and applied to HeLa cells at ~40% confluency. Cells were selected with puromycin (10 µg/ml, Sigma-Aldrich) for 2 days following which they were transfected with plasmids of interest.

4.6.6. Western blotting. Cells were collected after transfection, washed with DPBS (Corning) and lysed in ice-cold lysis buffer [100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40, protease and phosphatase inhibitor cocktail (Roche)]. Cell lysates were quantified by the Bradford assay (Bio-Rad) and 20 µg of lysates were denatured in Laemmli sample buffer and incubated for 5 min at 95°C. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad)**.** Membranes were blocked with 5 % non-fat milk in Tris-buffered saline pH 7.4 and 0.5 % Tween 20 (TBST) and then incubated with primary antibodies. After washes with TBST, the membranes were incubated with horseradish peroxidase conjugated secondary antibodies (Rockland Immunochemicals) and detect using Western Lightning Plus-ECL reagent (Perkin-Elmer). Signal intensity and densitometry analyses were conducted using ImageJ (NIH).

4.6.7. Immunofluorescence and imaging analyses. After transfection cells were washed once in Dulbecco's phosphate-buffered saline (DPBS) (ThermoFisher Scientific) and fixed with 4 % paraformaldehyde for 20 min. Cells were then washed with DPBS, incubated in 0.1 M glycine for 10 min, washed with DPBS, incubated in 0.2% Triton X-100 for 5 min and washed in DPBS. Primary antibodies were applied for 1 h at 37 °C, and then washed for 10 min in DPBS followed by secondary antibodies for 1 h. Cells were washed for 20 min in DPBS before being mounted on glass slides using ProLong Gold Antifade Reagent with DAPI (Life Technologies). Negative isotype-matched antibody were used to control staining specificity. Poly(A) mRNAs were detected by in situ hybridization assay. Briefly, cells were fixed with 4% PFA, treated with 0.1M glycine and permeabilized with 0.5% Triton-X, washed 2X with DPBS and hybridized with Cy5-conjugated oligo-dT(40) probe (0.2 µM) overnight at 37°C. Subsequently, cells were further processed for immunofluorescence for other proteins of interest. Confocal laser scanning microscopy was performed using a Leica DM16000B microscope equipped with a WaveFX spinning disk confocal

head (Quorum Technologies), and images were acquired with a Hamamatsu ImageEM EM-charge coupled device camera. Scanning was performed and digitized at a resolution of 1,024 1,024 pixel. Filter sets and laser wavelengths were described earlier [23, 77]. Image processing and analyses were performed by Imaris software (version 8.4.1 Bitplane/Andor) or by MetaXpress software (Molecular Devices). All imaging experiments were performed at least three times. The observed phenotypes were representative of n > 100 cells per condition in each experiment. SGs were defined as large G3BP1 or TIAR1 foci measuring >0.5 μm and a cell was deemed as SG positive if it exhibited at least three or more SGs [78]. For fluorescence intensity quantitation, the fluorescence intensity of each cell was determined using the ImageJ program (NIH) and then normalized to the mock transfected control.

4.6.8. Immunoprecipitation (IP) assays. HeLa cells were transfected with pCMV NC-YFP, wild type and NC Zinc Fingers mutants or pCMV-GFP, U2OS cells stably expressing G3BP1-GFP were transfected with pCMV NC-RLuc and 24 h later cells were solubilized with NP-40 lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCL, 0.5 mM EDTA and 0.5 % NP-40). For immunoprecipitation, 500 μg of protein lysates were incubated with 25 μL of GFP-beads (Life Technologies) for 1 h at room temperature. Beads were washed with NP40 lysis buffer three times before being eluted with 1X Laemmli sample buffer. Samples were resolved by SDS-PAGE and probed using antibodies against GFP, Staufen1 and TIAR1 by Western blot analysis.

4.6.9. *In situ* protein-protein interaction assay (DuoLink®). HeLa cells were transfected with NC-RLuc + pEGFP-E1, pCMV NC-RLuc + pCMV Staufen1 or pCMV NC-RLuc + pCMV Staufen1-F135A-YFP and, 24 h later, processed for *in situ* Proximity Ligation Assay (PLA) using the DUOLINK II In Situ kit (Duolink) following the manufacturer's instructions as previously described [23, 79]. Primary antibodies were mouse anti-RLuc and rabbit anti-GFP, which were detected using the DuoLink ® II Detection Reagent Red, Duolink® II PLA probe anti-Mouse Minus and DuoLink® II PLA probe anti-rabbit Plus. The NC-RLuc + pEGFP-C1 condition was used to measure background PLA signals for the above antibody combination. Imaging was performed as described above. The Spots Tool on Imaris software was used to quantify the number of spots per cell [23, 79].

4.6.10. *In vitro* binding assay. To generate Staufen1 - Glutathione S-transferases (GSTs) recombinant proteins, the hemagglutinin (HA)-tagged Staufen1 cDNA was PCR amplified from pcDNA3-RSV-Staufen1-HA [80] with the primers described in Table 4.1. The resulting PCR products were digested with *EcoRI* and *XhoI* (New England Biolabs) and cloned in the pGEX-4t-2 vector and transformed into *E. coli* BL21 cells. The colonies that contained the plasmid + insert were grown in LB broth and 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the bacterial culture to induce the expression of the GST fusion protein and cells were solubilized with NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA and 0.5 % NP-40) 6 hours after the addition of IPTG. These cell lysates were incubated in GST SpinTrap columns (GE Healthcare) for 30 min at room temperature. Columns were washed 6 times with TEN100 buffer (20mM Tris pH 7.4, 0.1 mM EDTA and 100 mM NaCl) to remove unbound proteins and subsequently incubated with 2 µg of recombinant NC protein for 2 h at 4°C. Captured complexes were washed 3 times with TEN100 buffer and elution was performed using Elution Buffer (50 mM Tris-HCl, pH8 and 10 mM glutathione). Samples were resolved by SDS-PAGE and probed using rabbit polyclonal antibodies against Staufen1 and NC by Western blot analysis.

4.6.11. Measurement of protein synthesis. Protein synthesis during NC expression was measured by the incorporation of puromycin into peptide chains [33, 34, 76]**.** Briefly, pCMV NC-RLuc, pCMV NC-RLuc + pCMV Staufen1-YFP and pCMV-RLuc transfected HeLa cells were incubated with 10 µg/ml puromycin (MilliporeSigma) for 10 min before cell lysis. Cell extracts were blotted with anti-Puromycin antibody (12D10, MilliporeSigma) and puromycin incorporation was assessed by summating the immunoblot intensity of all protein bands and subtracting background [76].

4.6.12. Polysome profile analysis. Polysome profile analysis experiments were performed as described [23, 81, 82]. Continuous sucrose density gradients (5% to 50% w/v) were prepared in buffer containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.6), 100 µg/mL cycloheximide, 1X protease inhibitor and 100 units/ml RNase Out (Invitrogen). Gradients were prepared in 5 mL

polyallomer tubes by gently layering 2.2 mL of 5% sucrose in buffer over 2.2 mL of 50% sucrose in buffer. Tubes were then sealed and turned on their sides to generate a continuous gradient overnight at 4 °C. HeLa cells were mock transfected or transfected with NC-RLuc, NC-RLuc + Staufen1 YFP or NC-RLuc + Staufen1 F135A-YFP. 24 h post-transfection, cells were incubated with 100 µg/ml cycloheximide in growth media for 5 min and then washed twice with ice-cold PBS containing 100 µg/ml cycloheximide. Cells were scraped and collected by centrifugation al 200 *x g* for 5 min at 4°C. Supernatant was removed and the cells were resuspended and lysed in hypotonic Buffer (5 mM Tris-HCl (pH 7.5), 1.5 mM KCl, 2.5 mM $MgCl_2$, 1X protease inhibitor, 200 units/ml RNase Out, 2 mM DTT, 150 µg/mL cycloheximide, 0.5% Triton X-100 and 0.5% SDS). Cell lysates were spun at 16,000 × *g* for 5 min at 4 °C, and supernatants were transferred to a new pre-chilled tubes. 500 µL of sample lysate (containing equal quantities of material as normalized by spectrophotometry, λ = 260 nm) was layered gently on to the gradients and ultracentrifuged in a Beckman Ti55 swing rotor at 222 000 × *g* for 2 h at 4 °C. Continuous OD_{254} readings for gradients were read from the bottom and fractions were collected using an ISCO fractionator (Teledyne, ISCO), as described in [23, 82, 83].

4.6.13. Quantification of virus in supernatants. Cells were transfected as described above and 48 h after transfection using 12 µg total DNA per 10 cm dish with each plasmid present in equal amounts**.** Culture supernatants were harvested and passed through a 0.2 µm filter (VWR) to remove cellular debris and centrifuged at 20,000 rpm for 1h. The pellet containing the virus was resuspended in 200 µl RPMI and the levels of p24 were determined by enzyme-linked immunosorbent assay (ELISA) (PerkinElmer).

4.7. References

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4.8. Tables

Staufen1 Domain	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
D2-5	GGAATTCTCGGAGGTGCTTATCCCCCGAGG	CCGCTCGAGGCAGGCAGGGGCGGTAACTTC
D3 or DM3	GGAATTCTGGAGCCCCTGCCAGAGAGGCTG	CCGCTCGAGGCAGGCAGGGGCGGTAACTTC
D3-4	GGAATTCTGGAGCCCCTGCCAGAGAGGCTG	CCGCTCGAGGGGTTTGGTGGGCTGCCGC
D4	GGAATTCTGAAGAAGTTACCGCCCCTGCC	CCGCTCGAGGGGTTTGGTGGGCTGCCGC
D5	GGAATTCTCCCCGAGGTCGCCCAGGCTG	CCGCTCGAGCCCACACACAGACATTGGTCCG

Table 4.1.: Primers used to amplify Staufen1 domains

4.9. Figures and figure legends

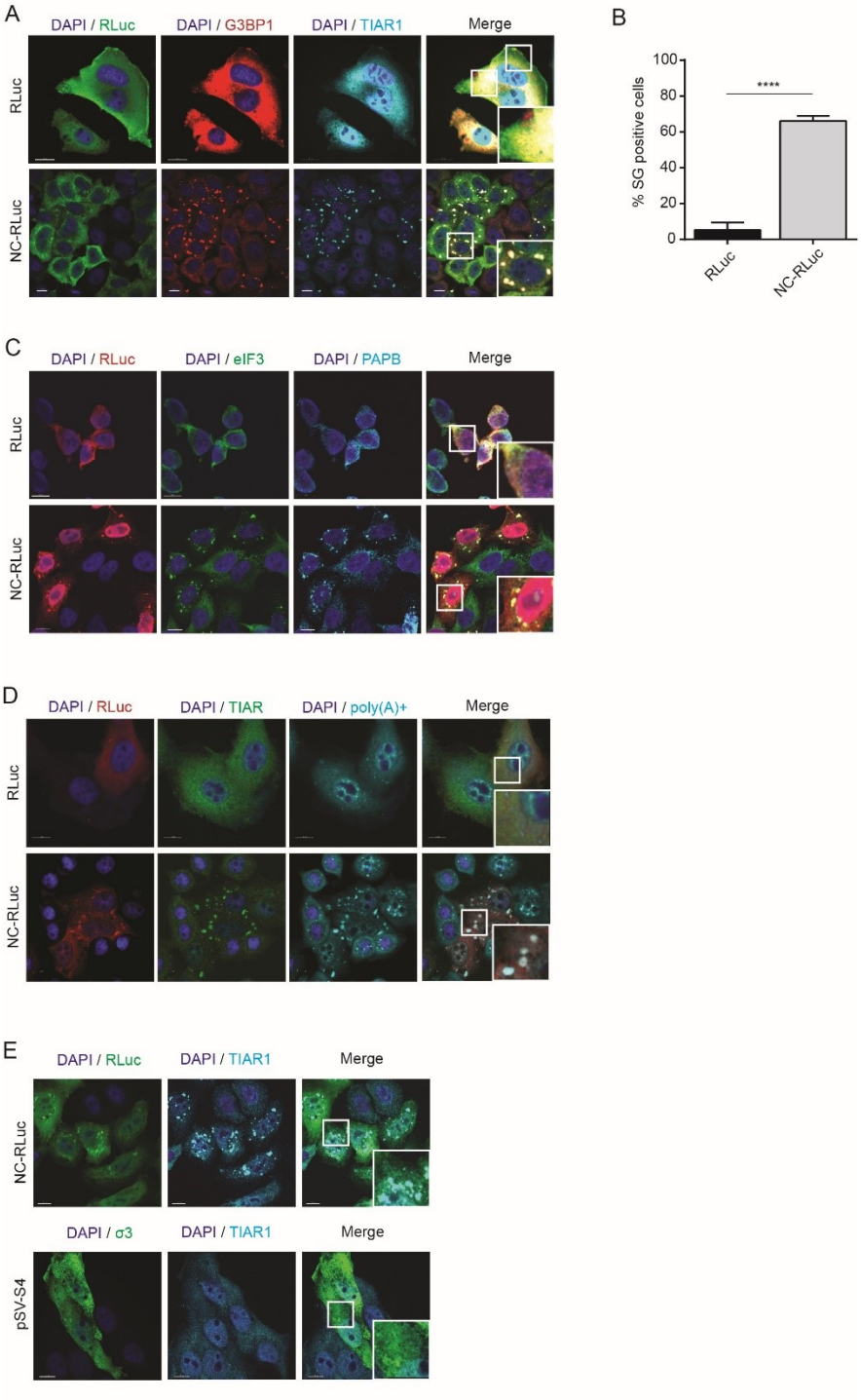

Figure 4.1.: NC expression induces assembly of SG containing G3BP1, TIAR1, PABP, eIF3 and poly(A) mRNAs.

Figure 4.1.: NC expression induces assembly of SG containing G3BP1, TIAR1, PABP, eIF3 and poly(A) mRNAs (continued). (A) HeLa cells were transfected with RLuc or NC-RLuc and 24 h later were stained for RLuc (green), G3BP1 (red) and TIAR1 (cyan). Scale bars are 10 µm. (B) Quantification of HeLa cells containing SGs transfected with RLuc or NC-RLuc from A. Error bars represent the standard deviation from three independent experiments with at least 150 cells counted per treatment. Asterisks represent statistically significant difference between RLuc and NC-RLuc-expressing cells (Student t-test; $p < 0.001$). (C) HeLa cells transfected as in A were stained for RLuc (red), eIF3 (green) and PABP (cyan). Scale bars are 10 µm. (D) HeLa cells transfected as in A were stained for RLuc (green), TIAR (red) and poly(A) mRNAs (cyan). Scale bars are 10 µm. (E) Expression of CCHC-type zinc finger on a dsRNA binding protein does not lead to SG assembly. HeLa cells where transfected with pSV-S4 to express the Reovirus σ3 protein (which contains CCHC-zinc fingers). SG assembly was then monitored by staining the cells for TIAR1 (cyan).

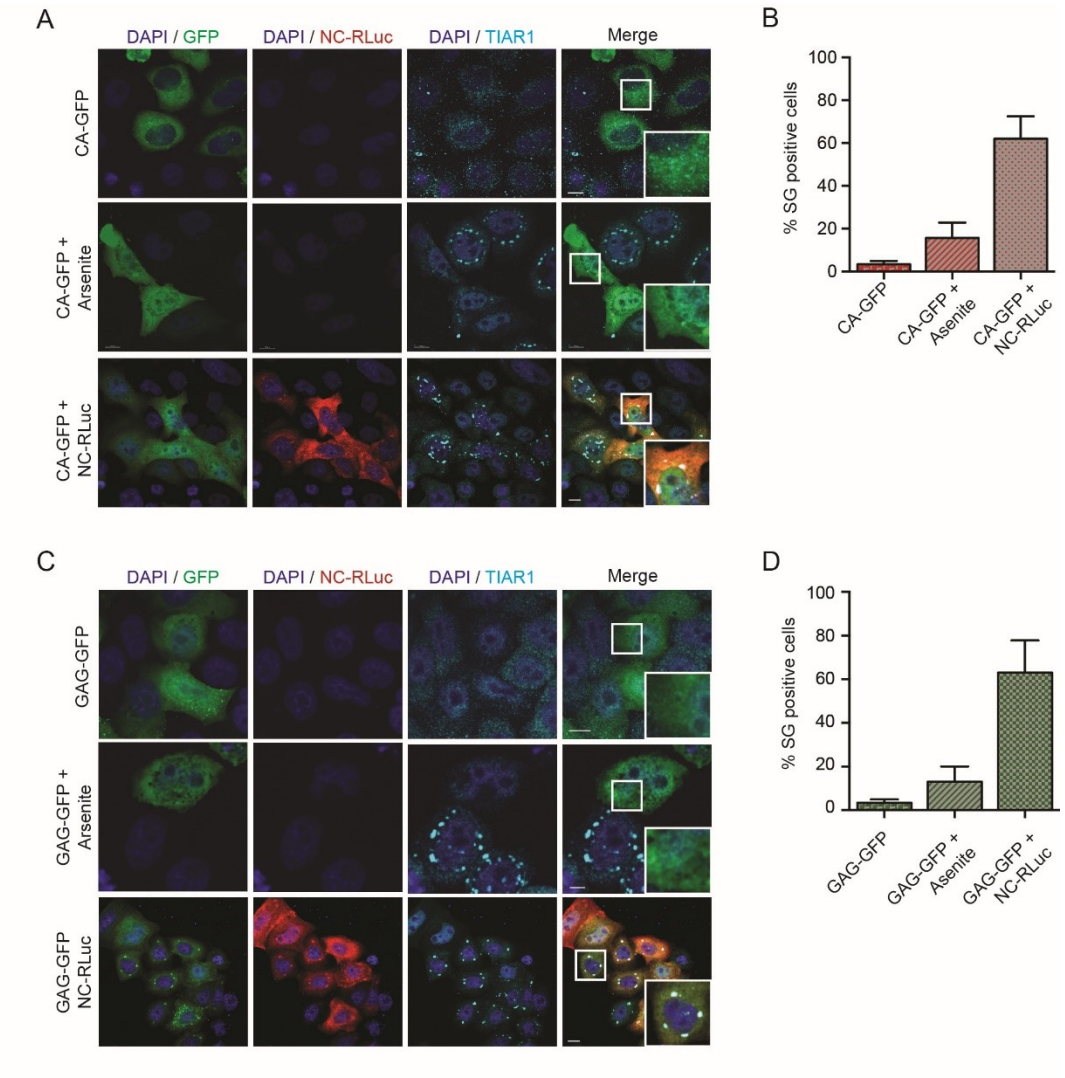

Figure 4.2.: Gag and CA block Arsenite-induced SGs but cannot disrupt NC-induced SGs. (A) HeLa cells were transfected with CA-GFP and CA-GFP + NC-RLuc. 24 h later cells were either untreated or treated with Arsenite and stained for RLuc (red) and TIAR1 (cyan). Scale bars are 10 µm. (B) Quantification of HeLa cells containing SGs from A. Only CA and NC expressing cells were considered for the quantification. Error bars represent the standard deviation from three independent experiments. (C) HeLa cells were transfected with GAG-GFP and GAG-GFP + NC-RLuc. 24 h later cells were either untreated or treated with Arsenite and stained for RLuc (red) and TIAR1 (cyan). Scale bars are 10 µm. (D) Quantification of HeLa cells containing SGs from C. Only Gag and NC expressing cells were considered for the quantification. Error bars represent the standard deviation from three independent experiments.

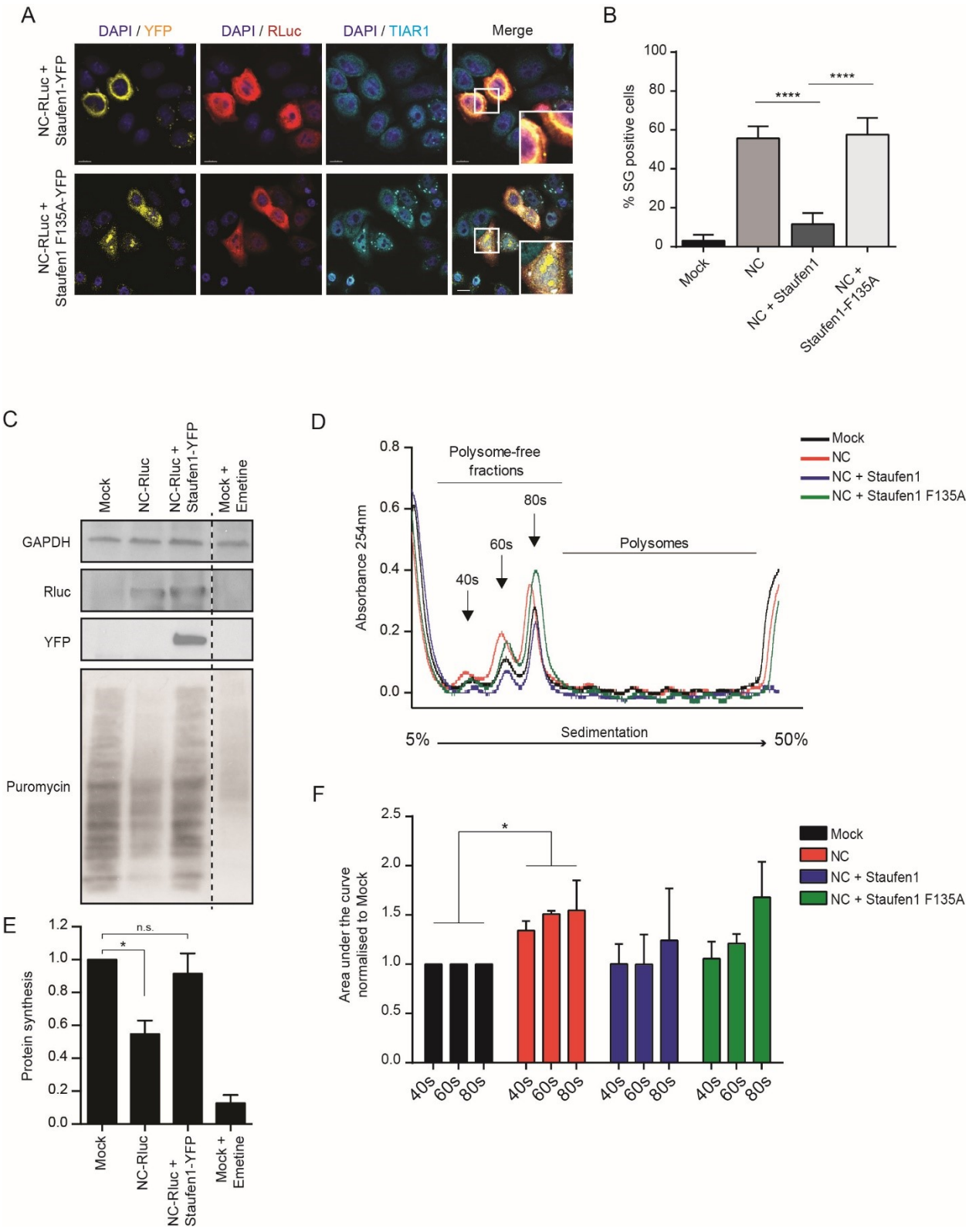

Figure 4.3.: Staufen1 rescues NC-induced SG assembly and translation arrest.

Figure 4.3.: Staufen1 rescues NC-induced SG assembly and translation arrest (continued).

(A) HeLa cells were co-transfected with NC-RLuc and Staufen1-YFP or Staufen1-F135A-YFP and 24 h later were stained for RLuc (red) and TIAR1 (cyan). Scale bars are 10 μm. (B) Quantification of HeLa cells containing SGs from A. Error bars represent the standard deviation from three independent experiments with at least 150 cells counted per treatment. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.001$). (C) Measurements of protein synthesis by puromycylation technique were performed by incubating mock, NC-RLuc or NC-RLuc + Staufen1-YFP -transfected HeLa cells with medium containing puromycin as described in Materials and Methods. As positive control mock-transfected cells were incubated with 1 μM Emetine 1 hour before the puromycin treatment. HeLa extracts were separated by denaturing electrophoresis and analyzed by western blot with antibody to puromycin (12D10). GAPDH immunoblot is shown as a loading control. (D) HeLa cells were mock-transfected or transfected with NC-RLuc, NC-RLuc + Staufen1-YFP or NC-RLuc + Staufen1-F135A-YFP and 24 h later polysomes fractionation and profiling was conducted. (E) Quantification of the puromycin-labelled peptides from C, values were normalized against mock cells extracts. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.05$) (F) Area under the curve corresponding to 40s, 60s and 80s peaks from D were quantified using GraphPad Prism 6. Error bars represent the standard deviation from three independent experiments. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.05$).

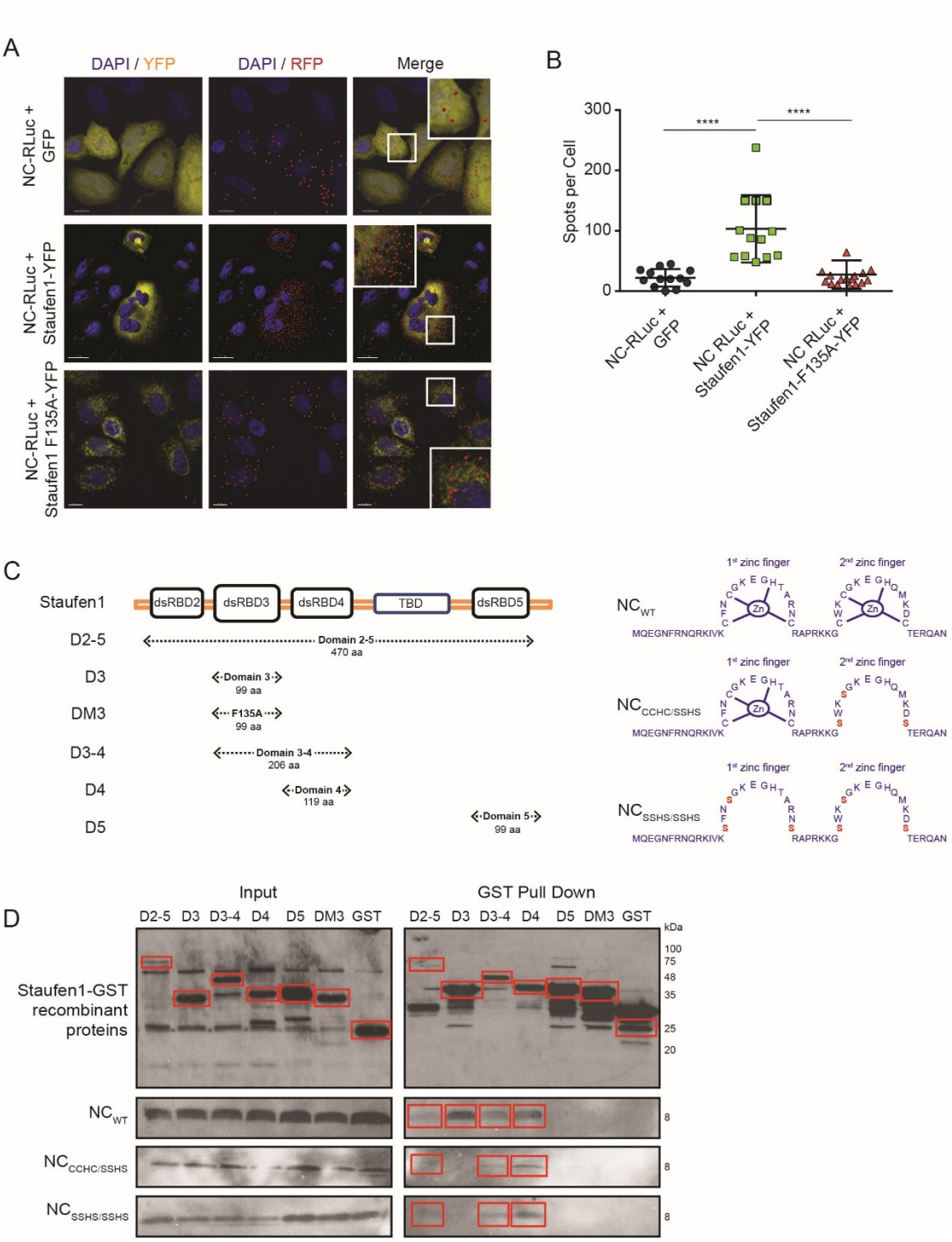

Figure 4.4.: NC and Staufen1 interact *in situ* and *in vitro.*

Figure 4.4.: NC and Staufen1 interact *in situ* and *in vitro* (continued). (A) HeLa cells were co-transfected with NC-RLuc and GFP or Staufen1-YFP or Staufen1-F135A-YFP and 24 h later were incubated with primary mouse and rabbit antibodies against RLuc and GFP. Coverslips were subsequently incubated with anti-mouse and anti-rabbit PLA probes. Each red signal corresponds to a single interaction event between NC and Staufen1. Nuclei were stained with DAPI (blue). Images shown are representative of >50 cells analyzed from 2 independent experiments. (B) The graph indicates the number of dots per cell. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.001$). (C) Representation of Staufen1 and NC mutants used in GST pull down assays. (D) GST-Staufen1 mutants were incubated with GST SpinTrap columns in the presence of absence of NC mutants. After washing extensively, the proteins bound to the beads were detected by Western blotting using anti-GST and anti-NC antibodies. Blot depicting GST tagged recombinant Staufen1 is a representative blot from three independent experiments using different NC constructs.

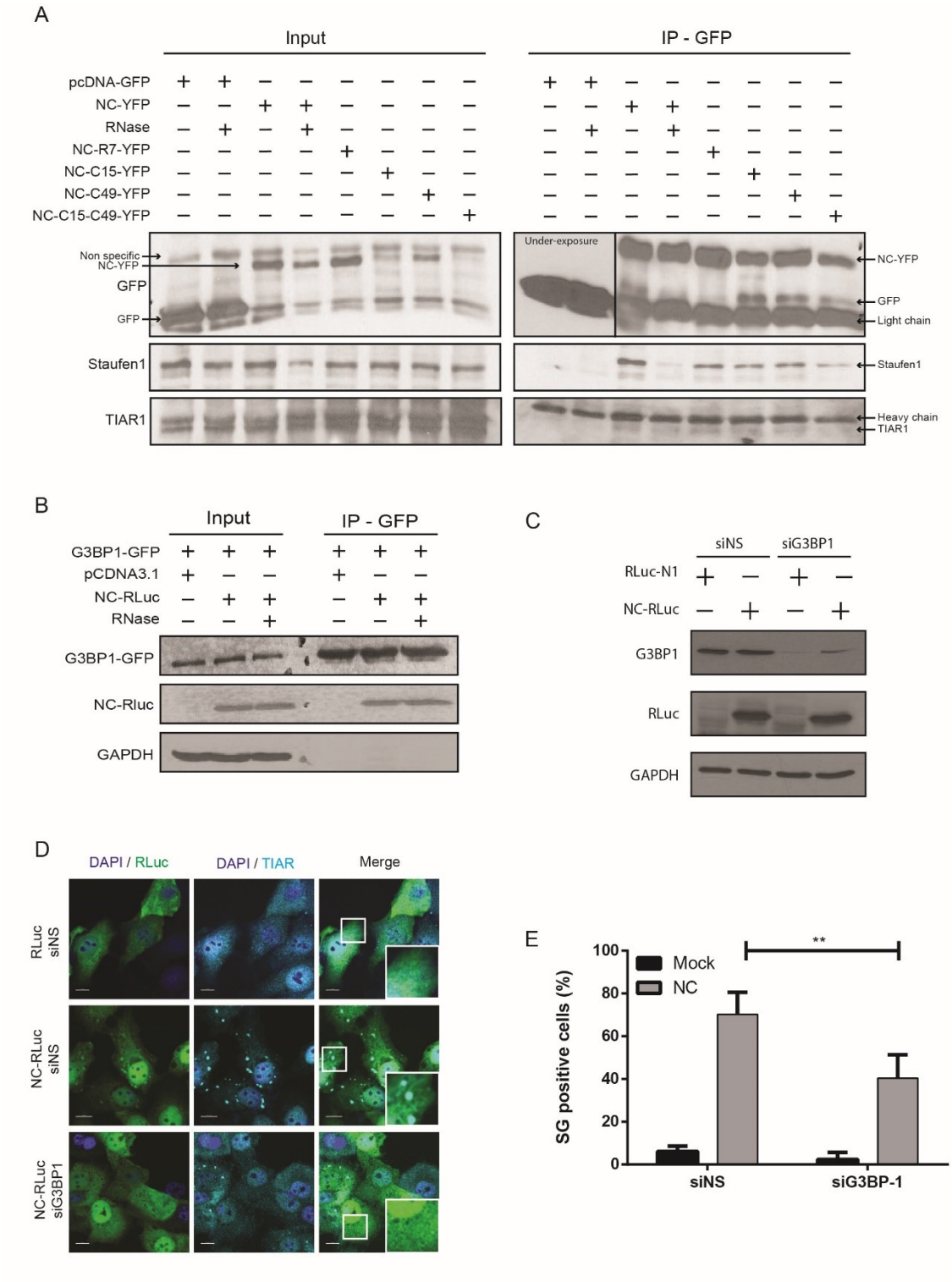

Figure 4.5.: NC co-immunoprecipitates with multiple SG markers.

Figure 4.5.: NC co-immunoprecipitates with multiple SG markers (continued). (A) HeLa cells were transfected with pEGFP-C1 or different NC-YFP mutants for 24 h. Cell lysates were collected, treated with RNase when indicated and subjected to anti-GFP immunoprecipitation. NC-associated proteins were processed for Western blotting and probed for GFP, Staufen1 and TIAR1. Representative blots from three independent experiments are depicted. (B) U2OS cells stably expressing G3BP1-GFP were transfected with pcDNA3.1 or NC-RLuc for 24 h. Cell lysates were collected, treated with RNase when indicated and subjected to anti-GFP immunoprecipitation. G3BP1-associated proteins were processed for Western blotting and probed for GFP, RLuc and GAPDH. Representative blots from three independent experiments are depicted. (C) HeLa cells were transfected as indicated and cell lysates were processed for Western blotting and probed for G3BP1, RLuc and GAPDH. (D) Cells transfected as depicted were stained for RLuc (green) and TIAR1 (cyan). Scale bars are 10 µm. (E) Quantification of HeLa cells containing SGs from D. Error bars represent the standard deviation from three independent experiments with at least 100 cells counted per treatment. Asterisks represent statistically significant difference between groups (Two-way ANOVA; $p < 0.01$).

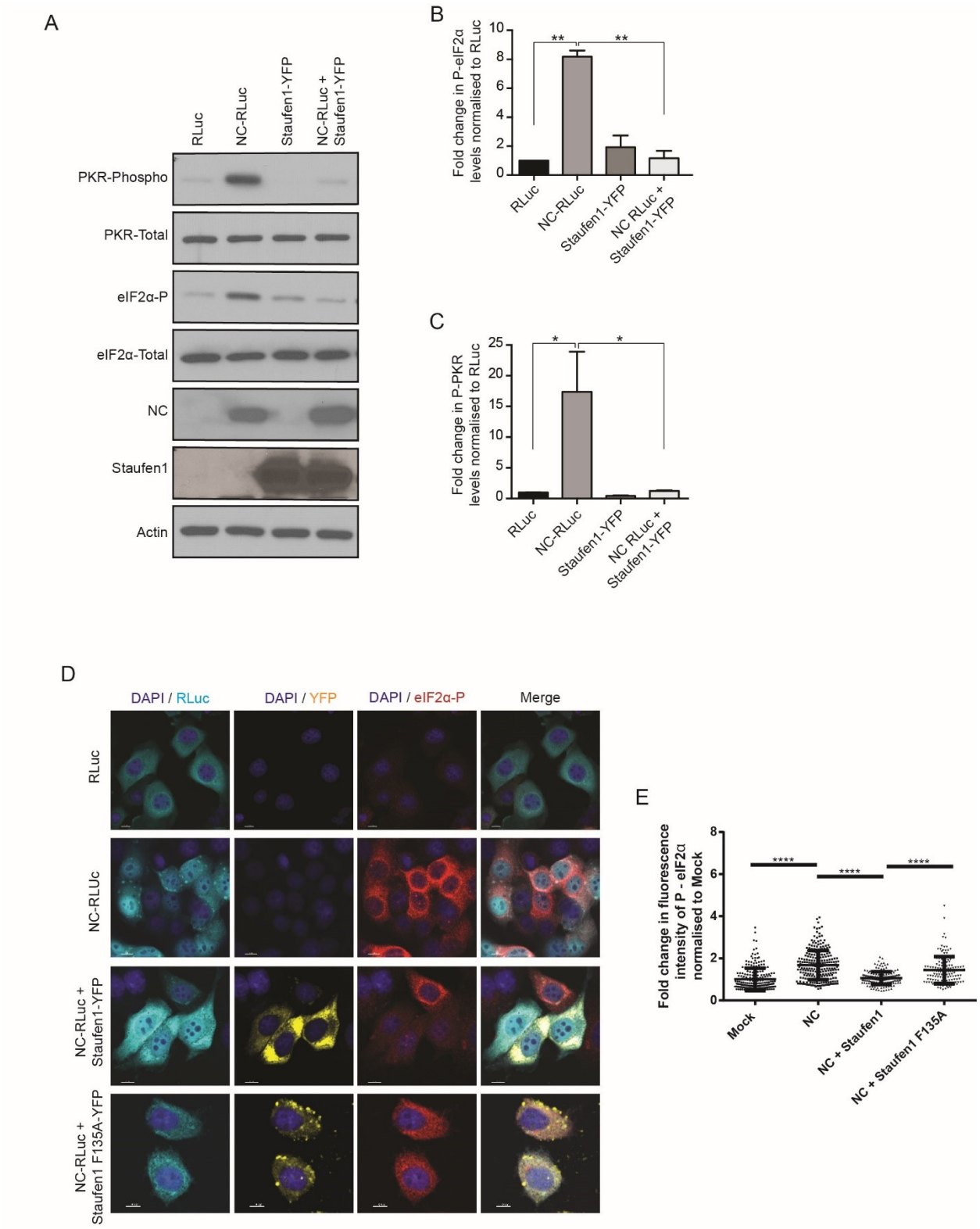

Figure 4.6.: NC induces PKR activation and eIF2α phosphorylation.

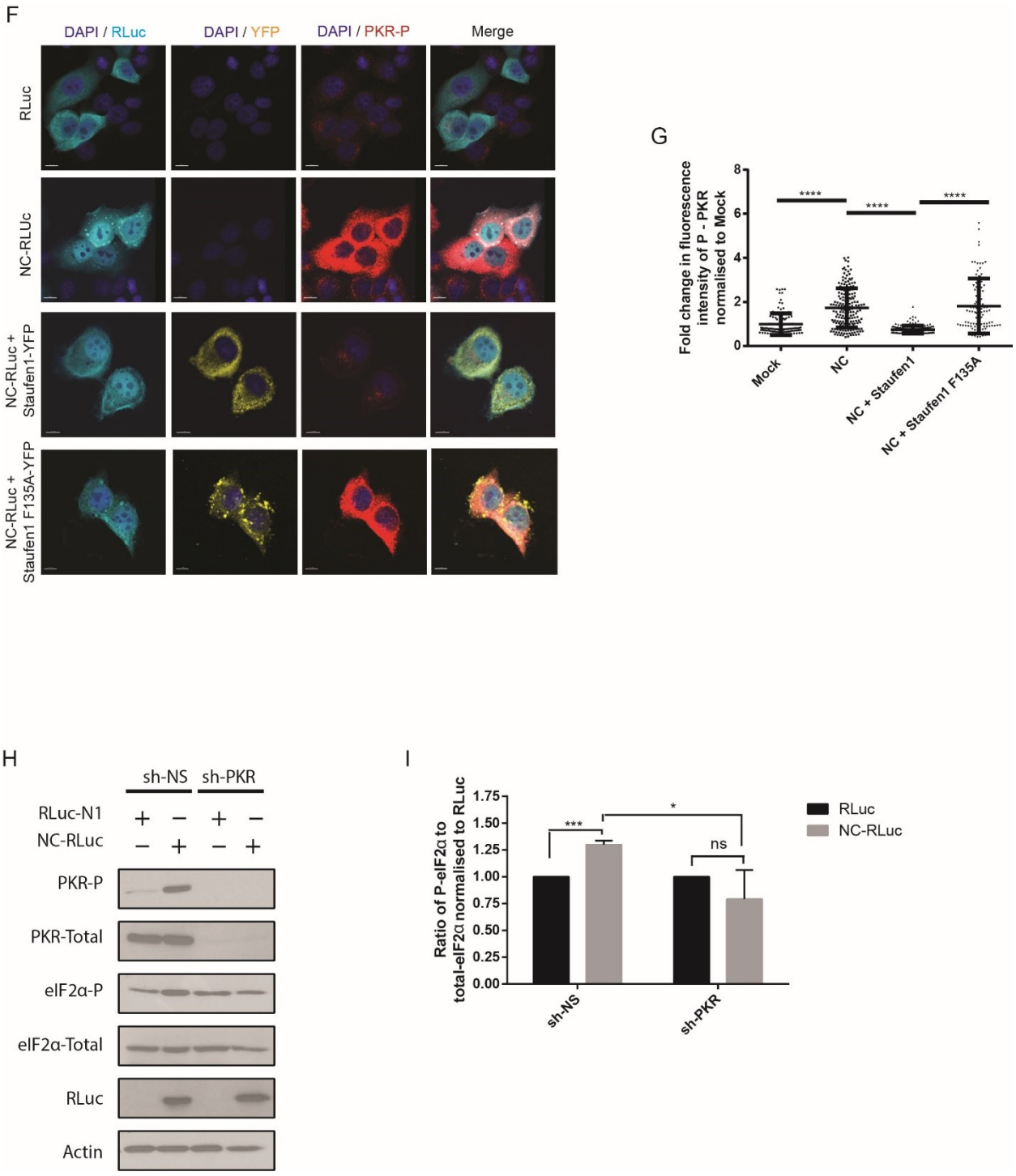

Figure 4.6.: NC induces PKR activation and eIF2α phosphorylation (continued).

Figure 4.6.: NC induces PKR activation and eIF2α phosphorylation (continued). (A) HeLa cells were transfected as indicated and 24 h later cell lysates were subjected to SDS-PAGE, immunoblotted and probed to investigate eIF2α and PKR phosphorylation. (B) Densitometry quantification of P-eIF2α was determined by ImageJ analysis. Values presented in the graph are normalized against the total amount of eIF2α in the cell lysate and represent fold change with the RLuc-transfected cells being arbitrarily set to 1. Error bars represent the standard error of the mean from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.01$). (C) Densitometry quantification of P-PKR was determined by ImageJ analysis. Values presented in the graph are normalized against the total amount of PKR in the cell lysate and represent fold change with the RLuc-transfected cells being arbitrarily set to 1. Error bars represent the standard error of the mean from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.05$) (D) Cells were transfected as indicated and stained for RLuc (cyan) and P-eIF2α (red). Images shown are representative of >150 cells analyzed from 3 independent experiments. Scale bars represent 10μm. (E) Quantification of the integrated density of p-eIF2α signal in cells from E from by ImageJ analysis. Each dot represents fluorescence intensity of a cell normalised to the mean fluorescence intensity of the mock transfected condition (arbitrarily set to 1). Error bars represent the standard error of the mean of cells from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.0001$).

Figure 4.6.: NC induces PKR activation and eIF2α phosphorylation (continued). (F) Cells were transfected as indicated and stained for RLuc (cyan) and P-PKR (red). Images shown are representative of >150 cells analyzed from 3 independent experiments. Scale bars represent 10µm. (G) Quantification of the integrated density of p-PKR signal in cells from F from by ImageJ analysis. Each dot represents fluorescence intensity of a cell normalised to the mean fluorescence intensity of the mock transfected condition (arbitrarily set to 1). Error bars represent the standard error of the mean of cells from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.0001$). (H) Cells were transfected as indicated and cell lysates were subjected to SDS-PAGE, immunoblotted and probed to investigate eIF2α and PKR phosphorylation. (I) Densitometry quantification of P-eIF2α was determined by ImageJ analysis. Values presented in the graph are normalized against the total amount of eIF2α in the cell lysate and represent fold change with the RLuc-transfected cells being arbitrarily set to 1. Error bars represent the standard error of the mean from three independent experiments. Asterisks represent statistically significant difference between groups (One-way ANOVA; * $p < 0.05$, *** $p < 0.001$).

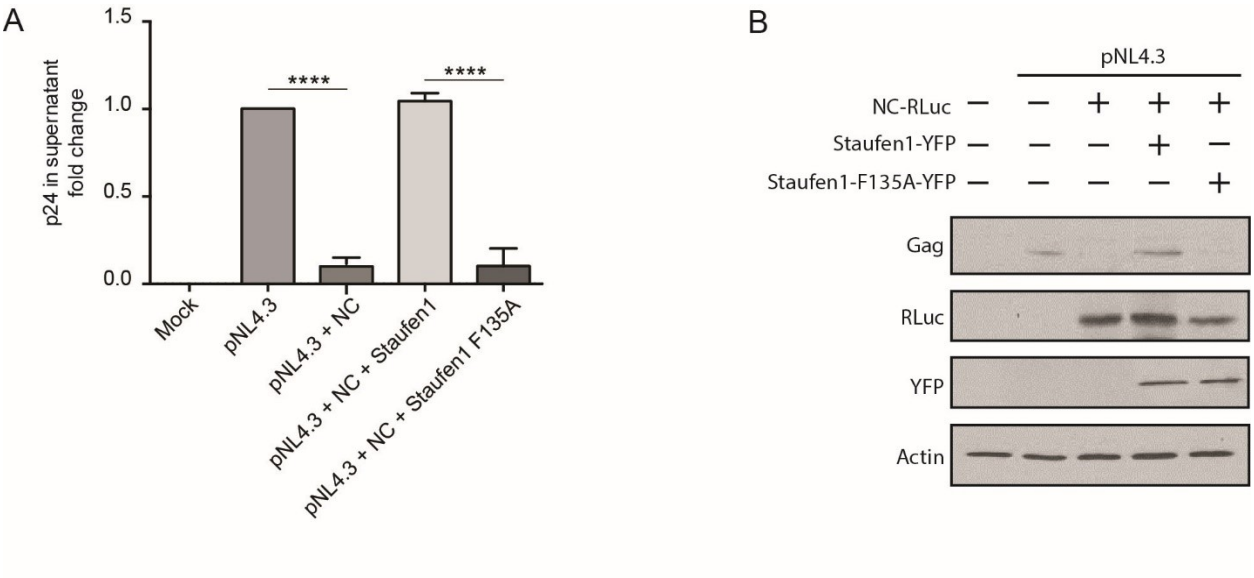

Figure 4.7.: NC-mediated reduction of viral production is rescued by Staufen1. (A) HIV-1 p24 in the supernatant of transfected HeLa cells was quantified via ELISA 48 h after transfection. Asterisks represent statistically significant difference between groups (One-way ANOVA; $p < 0.001$). B) Cell lysates were subjected to SDS-PAGE, immunoblotted and probed to investigate Gag production.

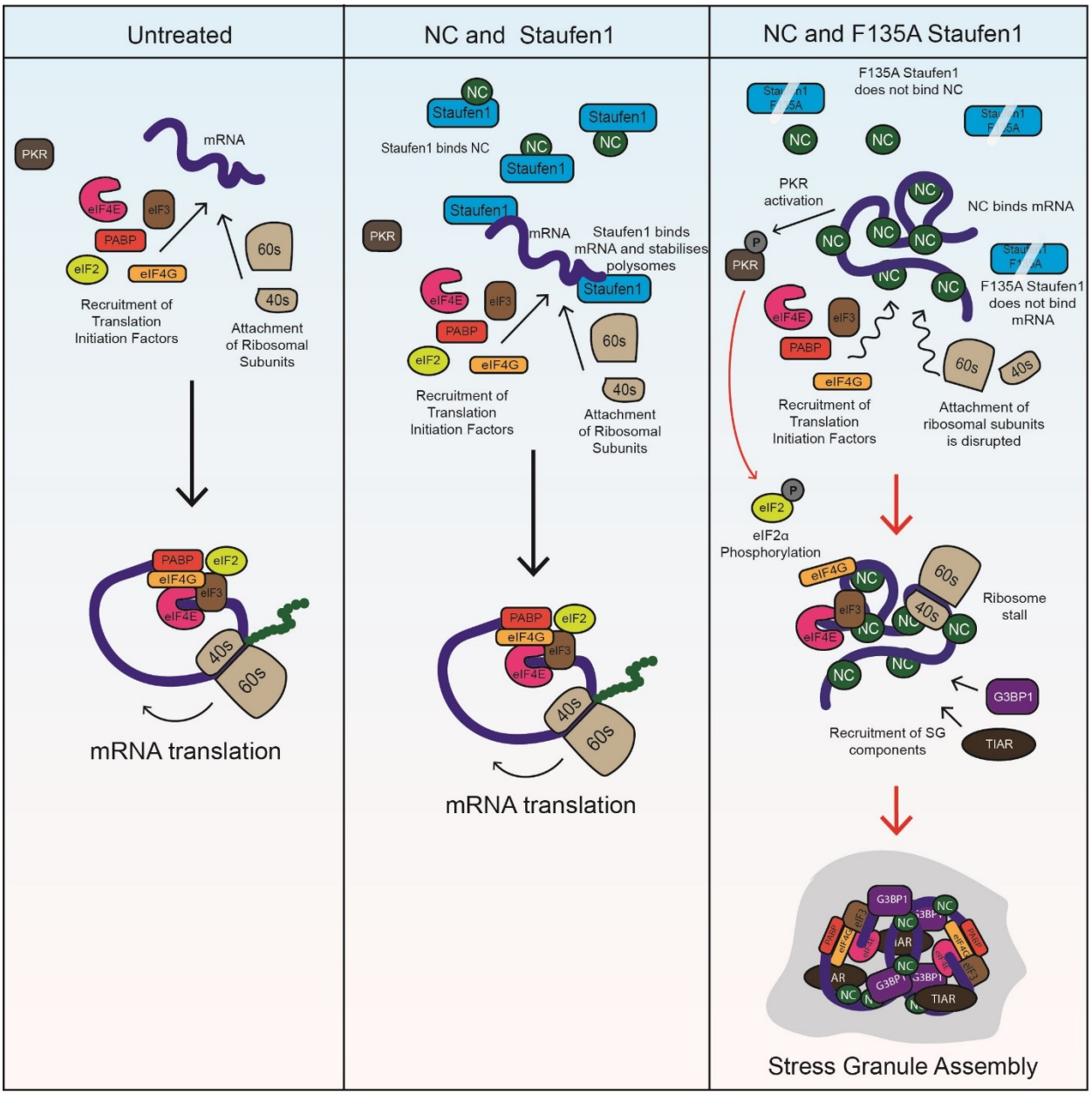

Figure 4.8.: Model of NC-induced SG assembly. Under untreated conditions, host cell translation progresses as normal. When NC is overexpressed, it binds cellular mRNAs, aggregates nucleic acids and leads to PKR activation. NC also prevents ribosomal translocation, thereby leading to SG assembly. Staufen1 can bind and sequester NC as well as stabilise polysomes and disrupt NC-induced SG assembly; but not if it contains an F135A mutation by virtue of which it loses its ability to bind NC and RNA.

Chapter 5

Discussion

5.1. Summary of main findings and claims to original scholarly contributions:

The research presented in this thesis identifies novel roles for the host mRNA decay proteins UPF1, UPF2, SMG6 and Staufen1 in vRNA metabolism during HIV-1 infection. This work makes important contributions to the field of post-transcriptional control of viral gene expression. A summary of the main, novel research findings are as follows:

- The NMD proteins UPF1, UPF2 and SMG6 influence the maintenance of HIV-1 latency at a post-transcriptional level in a latently-infected T cell model of HIV-1 infection.
    - UPF1 promotes viral reactivation by enhancing vRNA stability and, thus, viral gene expression. The ATPase activity and N-terminal portion of UPF1 mediate this activity.
    - UPF2 is detrimental to vRNA stability and viral reactivation. UPF2's role as a negative regulator of proviral reactivation is dependent on its ability to bind to UPF1.
    - SMG6 attenuates viral reactivation by impairing vRNA stability. SMG6's endonuclease region and ability to bind phosphorylated UPF1 influences this activity.
- UPF1 enhances vRNA stability and expression during HIV-1 infection in primary CD4+ T cells.
- In primary monocyte-derived macrophages (MDMs), UPF1 has no detectable effect on vRNA levels and HIV-1 gene expression. This highlights the cell type differences that exist between T cells and macrophages with regards to the roles of host proteins during HIV-1 infection.
- UPF2 and SMG6 restrict HIV-1 gene expression in primary MDMs by directly influencing vRNA expression. This implicates a novel role for nonsense mediated mRNA decay in the restriction of HIV-1 replication in cells of the myeloid lineage.
- Staufen1 promotes HIV-1 gene expression in primary MDMs by enhancing the translation of the vRNA.
- Staufen1 also rescues host cell translation and viral gene expression during HIV-1 nucleocapsid (NC) - induced translation arrest in HeLa cells.
- Staufen1 prevents NC-mediated activation of PKR, phosphorylation of eIF2α and stress granule assembly. The ability of Staufen to bind to either NC or RNA via its third dsRBD is necessary for its rescue of host cell translation.

5.2. Outstanding questions and future work

This research has resulted in the identification of novel roles for the host mRNA decay proteins on viral gene expression and on the maintenance of viral latency, as summarised in the previous section. However, some questions that arise from the research presented in this work require further investigation. These outstanding questions and strategies to address them are discussed in this section.

- **How does the phosphorylation of UPF1 contribute to its effect on vRNA metabolism?**

  UPF1 has been demonstrated to promiscuously bind to all cellular RNAs; both, canonically identified NMD targets as well as to mRNAs that are not subjected to NMD [1-5]. The marker for a cellular NMD target has been revealed to be the mRNA's binding to phosphorylated UPF1 [6, 7]. We can hypothesise that in the context of the interaction between UPF1 and the vRNA, the hyperphosphorylation of UPF1 would be detrimental to vRNA stability due to increased recruitment of SMG6 and other mRNA decay factors to elicit NMD. This is supported by our observations that hyperphosphorylated ATP-deficient UPF1 construct FLAG-UPF1-DE is unable to enhance vRNA stability [5, 8]. Furthermore, UPF2 is known to facilitate the phosphorylation of UPF1 by the kinase SMG1 and is excluded from HIV-1 RNPs [8-10]. In this thesis, we also demonstrate that UPF2 is detrimental to vRNA stability in both T cells and macrophages. Moreover, we show that the binding of SMG6 to phosphorylated UPF1 is detrimental to vRNA stability and viral gene expression. The phosphorylation of UPF1 can be hypothesised to be detrimental to UPF1's roles in stabilising the vRNA. A fine balance of hyperphosphorylated UPF1 that promotes NMD and hypophosphorylated UPF1 that promotes vRNA stability could determine UPF1's roles in vRNA metabolism.

  It would therefore be interesting to evaluate the contribution of the phosphorylation of UPF1 to vRNA metabolism and to characterise UPF1 function in HIV-1 infection based on its phosphorylation status. We can accomplish this by overexpressing UPF1 constructs that contain mutations in the phosphorylation sites of UPF1 and observe the effects on vRNA metabolism. We could also manipulate the levels of SMG1, the kinase responsible for the phosphorylation of UPF1, and observe the effects on viral gene expression. These studies

could lead to the identification of small molecule kinase inhibitors as post transcriptional latency reversing agents towards an HIV-1 cure.

- **Why does UPF1 have distinct roles on viral gene expression in T cells versus macrophages?**
  The work presented in this thesis demonstrates a differential effect of UPF1 on vRNA expression in primary CD4+ T cells and MDMs. We hypothesise that this observation is due to inherent cell specific differences in the phosphorylation of UPF1 and the regulation of NMD between cells of the myeloid versus lymphoid lineage. To confirm this, we need to evaluate the baseline levels of UPF1 phosphorylation and NMD activity in macrophages as compared to T cells. Western blotting using an anti-phospho-[S/T]Q antibody that detect levels of phosphorylated UPF1 or 2-dimensional gel electrophoresis can be used to evaluate the differential levels of phosphorylation of UPF1 between cell types, as demonstrated in [11-13]. It would then be necessary to evaluate whether the vRNA is bound to phosphorylated UPF1 and is subjected to NMD in MDMs, but not in T cells. This can be evaluated using immunoprecipitation of phosphorylated UPF1, followed by RNA sequencing to identify NMD targets during HIV-1 infection in different cell types (as described in [14]). Another alternative would be to conduct cross-linking Immunoprecipitation assays (CLIP) to pull down the vRNA, followed by the quantification of phosphorylated UPF1 co-immunoprecipitation. Quantitative phosphoproteomic analysis following HIV-1 infection as described in [15] could also be employed to characterise the viral modulation UPF1 phosphorylation in different cell types. These studies would result in the understanding of the differential regulation of NMD in macrophages and T cells and how this contributes to viral gene expression. This kind of research can aid in the development of novel broad spectrum antiretrovirals that are effective on all cell types of the viral reservoir.

- **Does the binding of SMG6 to the vRNA result in the endonucleolytic cleavage and subsequent degradation of the vRNA?**
  In our work, we demonstrated that the detrimental effect of SMG6 on vRNA stability was dependent not only on the ability of SMG6 to bind phosphorylated UPF1, but also on its endonucleolytic activity. It would be interesting to determine whether the binding of SMG6

to the vRNA is capable of inducing endonucleolytic cleavage and degradation of the vRNA. We can answer these questions by performing tethered functional assays that employ a construct of SMG6 that contains an MS2 tag and a vRNA that contains an MS2-binding site and then monitor the levels of SMG6-bound vRNA (described in [16]). Since SMG6 is recruited to mRNA via the binding to phosphorylated UPF1, we could also perform tethering experiments in UPF1-depleted conditions to evaluate the dependency of SMG6-mediated vRNA degradation on UPF1. This would aid in the elucidation of the mechanism of action of SMG6-mediated inhibition of viral gene expression and the development of novel SMG6-directed antiretroviral therapies.

- **Does the presence of intrinsically disordered domains on HIV-1 NC contribute to liquid-liquid phase separation and stress granule assembly?**

  In this research, we have characterised NC-induced stress granule (SG) assembly and its dissociation by Staufen1. Proteins that are largely prevalent in SGs contain intrinsically disordered domains (IDDs) [17, 18]. The presence of IDDs and low complexity regions (LC) on these SG-nucleating proteins promotes liquid-liquid phase separation and contributes to SG assembly [18-20]. NC is a highly disordered protein [21] and this could contribute to its ability to induce SG assembly. It would be interesting to characterise if the IDDs on NC contribute to liquid-liquid phase separation by using *in vitro* liquid droplet formation assays as described in [22]. This will result in a further understanding of the mechanisms of NC-induced SG assembly and its implications in other steps of the viral life cycle that involve cleaved HIV-1 NC such as viral maturation and reverse transcription. These studies could lead to the development of novel NC-directed antiretrovirals.

- **What is the role of NC-induced translation arrest in reverse transcription?**

  NC has a characterised role in reverse transcription (RT) and the molecular chaperone activity of NC is responsible for this function [23, 24]. When a virus infects a host cell, the vRNA is capped and polyadenylated and has all the signature elements to be translated. However, this vRNA is also coated with NC molecules at a frequency of about one NC molecule per six RNA nucleotides [25]. There is some evidence of early viral genes Tat and Nef being translated

pre-integration, but this is from the cDNA product of RT that was then subsequently transcribed and translated [26, 27]. However, there is no evidence yet of the incoming genomic vRNA being translated. In our work, we have shown that NC can inhibit host cell translation. We propose a novel role for NC in RT whereby it hinders the translation of the incoming genomic vRNA, thereby facilitating its reverse transcription. To characterise this, we can treat HIV-1 infected cells with integrase inhibitors in mock treated, NC-depleted or NC-overexpressed conditions and then monitor the amount of genomic vRNA by RT-PCR, the amount of one LTR and two LTR proviral reverse transcription products by PCR and host cell translation using puromycylation assays or polysome profile analysis. This could aid in the development of novel antiretrovirals that target NC function during reverse transcription.

5.3. Overall significance

The research presented in this thesis makes significant contributions to the fields of HIV pathogenesis as well as to overall RNA metabolism. The potential to apply this research to develop novel strategies for HIV treatment is discussed in the following sections.

5.3.1. Towards an HIV-1 Cure

As a consequence of the current drawbacks of cART (discussed in section 1.5.1.), one of the main priorities in the field of HIV research is the development of curative HIV treatments. Our studies have demonstrated that post-transcriptional events governed by the host mRNA decay proteins can significantly influence viral gene expression. This effect also extends to the realm of the maintenance of viral latency. Since the presence of a latent viral reservoir is the main obstacle towards the development of an HIV cure, we propose that our findings can be applied towards the two main HIV cure strategies: the sterilising cure and the functional cure (discussed in section 1.5.3.).

5.3.1.1. Post-transcriptional latency reversing agents for a sterilising HIV cure

The sterilising HIV cure involves the "kick and kill" strategy that employs latency reversing agents (LRAs) to reactivate the latent provirus, followed by elimination of the infected cells using ARVs and immunotherapy (discussed in section 1.5.3.2.). The current LRAs activate the transcription of the provirus and have been linked to an increase in cell associated vRNA; but they have limited to no reduction in the size of the viral reservoir in clinical trials [28-30]. This shortcoming can be linked to the post-transcriptional events that contribute to gene expression. Although the proviral DNA is transcribed into vRNA, efficient metabolism of the vRNA is necessary to ensure gene expression. The research presented in this thesis demonstrates that the host mRNA decay proteins can influence the maintenance of viral reactivation by modulating the post-transcriptional regulation of viral gene expression. Moreover, our studies have identified roles for these proteins in cells of both the lymphoid and myeloid lineage. We propose that this research can be applied to create a novel class of post-transcriptional LRAs in both the T cells as well as macrophages that make up the viral reservoir.

An attractive target for the development of these post-transcriptional LRAs is UPF2 function. In this research, UPF2 has been demonstrated to be detrimental to viral gene expression in both T cells and macrophages. UPF2's function has been linked to UPF1-binding in both instances. Therefore, we can use small molecule inhibitors generated by structure-based drug design or molecular modelling strategies to prevent UPF1-UPF2 binding. Alternatively, novel drugs to sequester intracellular UPF2 could also be employed. This could promote viral reactivation and ensure viral gene expression by two additive mechanisms. Firstly, this could result in increased UPF1 that can assemble HIV-1 specific RNPs to ensure the stability of the vRNA [8, 10]. Secondly, the inhibition of UPF1-UPF2 binding could prevent the induction of a conformational change in UPF1 that results in a loss of UPF1 RNA-binding capability and its enhanced phosphorylation [9, 31]. Both events are hypothesised to be detrimental to the stabilising effect of UPF1 for the vRNA. Furthermore, our results imply that the vRNA is subjected to NMD in macrophages. A depletion of UPF2 or inhibition of its binding to UPF1 could promote vRNA expression in macrophages by inhibiting NMD.

The NMD protein SMG6 was also demonstrated to be detrimental to vRNA stability and viral gene expression in T cells as well as macrophages. It could also serve as an attractive target for a post-transcriptional LRA, by using small molecule drugs to prevent SMG6 binding to UPF1 or to sequester intracellular SMG6. Another strategy that could be used to promote viral gene expression is inhibiting the phosphorylation of UPF1 using small molecule SMG1 kinase inhibitors, thus preventing NMD and promoting vRNA expression. Small molecule NMD-null UPF1 mimics can also be employed to promote viral reactivation. These post-transcriptional LRAs can be used for 'the kick' in combination with current LRAs to ensure efficient viral reactivation and gene expression, followed by 'the kill' with immunotherapy and ARVs. Therefore, the research presented in this thesis can be applied towards a sterilising HIV cure. A schematic of the "kick and kill" strategy with the added function of the post-transcriptional LRAs is depicted in Figure 5.1.

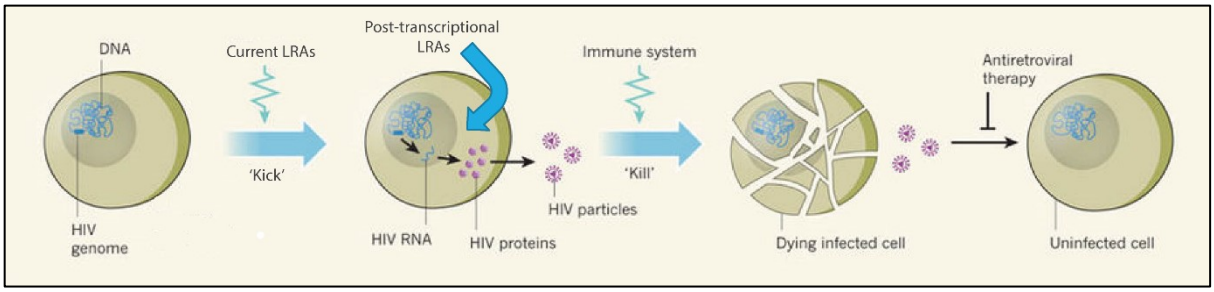

Figure 5.1.: The use of post-transcriptional LRAs in the 'kick and kill' HIV cure strategy

The integrated HIV provirus is reactivated using a combination of LRAs to promote proviral transcription, as well as with post-transcriptional LRAs to ensure viral gene expression and protein production. The infected cell is now recognised and eliminated by the host cell immune system or by cytopathic effects of virus production. Viral spread to uninfected cells is prevented by ARVs. Adapted and reprinted with permission from [32].

5.3.1.2. Longer lasting ARVs for a functional HIV cure

Another strategy suggested for an HIV cure is called the "block and lock" strategy in which the HIV-1 infected cells are locked in a state of deep latency [33, 34]. Our studies have identified targets to inhibit the post-transcriptional regulation of viral gene expression and could be applied towards the development of a functional HIV cure. These latency promoters would have to be longer lasting that the current ARVs and prevent rebound of viremia after treatment interruption. The first strategy towards the development of drugs to inhibit virus production would be to use small molecules that mimic UPF2 activity. These drugs would inhibit viral gene expression by binding and sequestering UPF1, as well as by modulating the activity of UPF1 to elicit NMD of the UPF1-bound vRNA. SMG6 mimics could also be used and would have a similar mechanism of action. Furthermore, these drugs would be effective in cells of both the lymphoid and myeloid lineage.

RNA therapy in the form of vRNA mimics could also be used to inhibit viral gene expression. This would entail the delivery of RNA that could bind to UPF1 and other host factors such as Staufen1 to act as a molecular sponge. This would result in the sequestration of host proteins necessary for efficient vRNA metabolism and an inhibition of viral gene expression. Molecules to promote the hyperphosphorylation of UPF1 could also be designed and employed to prevent vRNA stability and gene expression.

In our studies on the effect of NC on host cell translation, we observed that the expression of NC induced the assembly of stress granules that could not be dissociated by HIV-1 capsid (CA) or $pr55^{Gag}$. This provides an attractive target for therapeutic intervention in the form of a novel class of ARVS, the protease accelerators. This new class of drugs could prevent viral gene expression by prematurely activating the HIV-1 protease, thus resulting in the accumulation of NC in host cells before or during viral assembly. The use of these novel ARVs would lead to the assembly of NC-induced SGs that could not be dissociated by CA or $pr55^{Gag}$, the shutdown of host cell translation and reduction of viral production. The rescue of NC-induced host cell translation arrest could be inhibited by using drugs that inhibit the binding of Staufen1 and NC. Apart from enhancing vRNA translation, Staufen1 also plays a role in $pr55^{Gag}$ multimerisation, vRNA encapsidation and viral assembly; functions ascribed to Staufen1's binding to the NC domain on

pr55^{Gag} [35-38]. Therefore, drugs that inhibit Staufen1-NC binding would also have a detrimental effect on viral production by impairing viral assembly.

It is imperative to evaluate the selective toxicity of any potential novel ARVs. Toxicity studies using comprehensive *in vitro* assays first need to be conducted, followed by a thorough evaluation in humanised mouse models of HIV-1 infection. If a potential drug candidate has pharmacological properties conducive to use in humans, its antiviral efficacy can be determined in clinical trials.

For any potential antiretroviral drug described in this section to be a suitable candidate for a functional HIV cure, it needs to have a long half-life and prevent the rebound of viremia following treatment interruption. To evaluate the potential of these novel drugs for a functional cure, the pharmacokinetics of the drug must first be evaluated in primary cell models of HIV-1 latency or mouse models [39]. Following drug treatment and subsequent cessation, the time to rebound of viremia in latently infected mouse models must also be evaluated. The efficacy of drugs with the ability to prevent rebound viremia to be used a functional cure can be determined by conducting monitored treatment interruption studies in HIV-1 infected individuals. The potential of novel 'latency promoters' to contribute to a functional HIV-1 cure are depicted in Figure 5.2. Since HIV-1 has also been demonstrated to develop drug-resistance to current ARVs [40], it is important to continue to develop next-generation HIV therapies and even if these drugs are not able to prevent the rebound of viremia following treatment interruption, they can still be added to the arsenal of drugs that are currently being used to target HIV-1 infection.

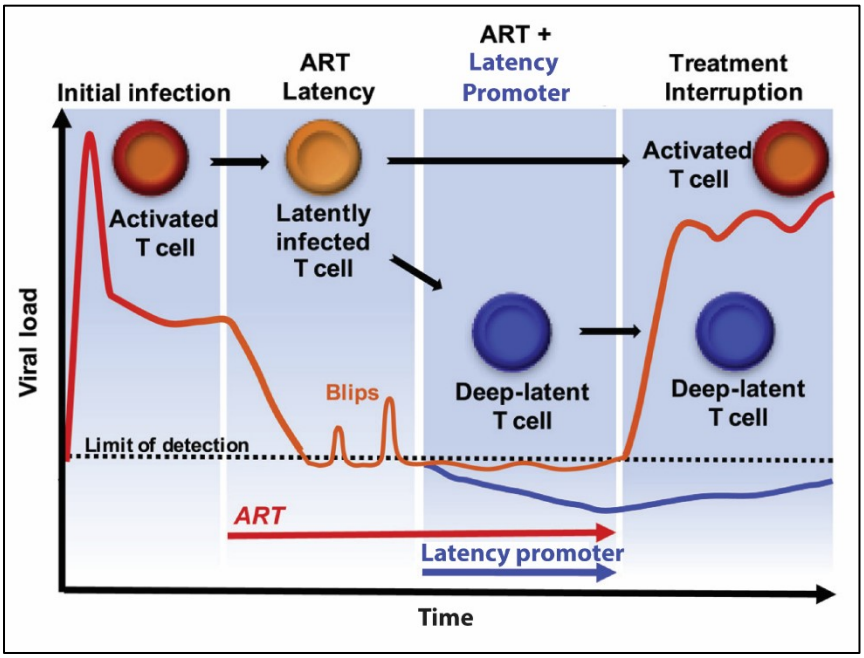

Figure 5.2.: The use of latency promoters in the 'block and lock' HIV cure strategy

Latency promoters that inhibit viral gene expression can be used along with cART to induce a state of deep latency in host cells, thus preventing viral rebound following treatment interruption. Figure adapted and reprinted with permission from [34].

5.4. Overall conclusion

In this work, we have characterised the roles of the host mRNA decay proteins UPF1, UPF2, SMG6 and Staufen1 on the regulation of HIV-1 gene expression and observed that they have profound effects on vRNA metabolism. We demonstrated a novel function for the NMD proteins UPF1, UPF2 and SMG6 on the post-transcriptional maintenance of HIV-1 latency in T cells. We also identified a role UPF2 and SMG6 in the restriction of HIV-1 replication in primary monocyte-derived macrophages. Moreover, we identify cell-specific differences in the effect of the NMD proteins in the regulation of viral gene expression in T cells as compared to macrophages. This highlights the importance of accounting for the contribution of macrophages in the development of novel antiretroviral therapies. We have also identified a novel role for the SMD protein Staufen1 in the rescue of viral gene expression during HIV-1 nucleocapsid-induced translation arrest. Thus, the research presented has the potential to be applied towards the development of a sterilising or functional HIV cure, thereby significantly improving the lives of the 37 million HIV-infected individuals across the globe.

5.5. References

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