UP-FRAMESHIFT PROTEINS AND THEIR DISTINCT ROLES IN HIV-1 RNA METABOLISM

By

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November, 2011

A thesis submitted to McGill University in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

ABSTRACT

HIV-1 co-opts host cell proteins at every step of its replication cycle to ensure proper replication. Our work identified that the HIV-1 genomic RNA is not a substrate for nonsense-mediated mRNA decay (NMD) even though it has multiple open reading frames as well as a long 3'UTR. We demonstrate that Up-frameshift protein 1 (UPF1) is involved in HIV-1 genomic RNA stability such that overexpression of UPF1 increases HIV-1 genomic RNA levels and Gag translation. Moreover, the role of UPF1 in HIV-1 is NMD-independent, is observed in both nuclear and cytoplasmic compartments and does not require binding to UPF2. Furthermore, the shuttling function of UPF1 is required for HIV-1 genomic RNA export since a UPF1 nuclear export mutant sequesters the genomic RNA in the nucleus and a nuclear localization mutant does not immunoprecipitate with the HIV-1 genomic RNA. UPF1's role in HIV-1 genomic RNA export is observed in both Rev-dependent and -independent conditions. In addition, UPF1 is found in complex with Rev, CRM1, Nup62 and DDX3, cellular proteins with already characterized roles in HIV-1 genomic RNA export. Lastly, we also identified UPF2 as a negative regulator, such that its binding to UPF1 results in the nuclear sequestration of the HIV-1 genomic RNA. We have identified a possible mechanism to explain how HIV-1 escapes the RNA quality control mechanism of NMD by co-opting UPF1 function for efficient HIV-1 genomic RNA export, stability and translation.

RÉSUMÉ

Le VIH-1 requiert plusieurs protéines cellulaires à chaque étape de son cycle de réplication pour assurer une réplication efficace. Notre travail a mené à l'identification que l'ARN génomique du VIH-1 n'est pas un substrat pour la dégradation des ARNm aberrants (NMD), même si elle a plusieurs cadres de lecture ainsi qu'une longue 3'UTR. Nous avons démontré que UPF1 est impliqué dans la stabilité de l'ARN génomique du VIH-1 car la surexpression de UPF1 a engendré une augmentation des niveaux d'ARN du VIH-1 et de Gag. Par ailleurs, le rôle de UPF1 est distinct de son rôle dans le mécanisme NMD, il est observé dans les compartiments nucléaires et cytoplasmiques et ne nécessite pas sa liaison à UPF2. De plus, la fonction navette de UPF1 est requise pour assurer l'exportation de l'ARN génomique du VIH-1 car le mutant NES d'UPF1 bloque son export et le mutant NLS n'immunoprécipite pas avec celui-ci. Ce nouveau rôle d'UPF1 est observé dans la présence et l'absence de Rev. De plus, UPF1 se trouve en complexe avec Rev, CRM1, Nup62 et DDX3, les protéines cellulaires déjà caractérisées comme étant impliquées dans l'exportation de l'ARN génomique du VIH-1. Enfin, nous avons également identifié UPF2 comme étant un régulateur négatif, de telle sorte que sa liaison à UPF1 engendre un blocage nucléaire de l'ARN génomique du VIH-1. Nous avons identifié un mécanisme possible démontrant comment le VIH-1 s'évade du mécanisme NMD en cooptant UPF1 pour faciliter l'exportation, la stabilité et la traduction de l'ARN génomique du VIH-1.

PREFACE AND CONTRIBUTIONS

This manuscript-based thesis comprises five chapters. The first chapter represents a literature review of the HIV-1 history, replication cycle and therapies, the UPF proteins and their diverse functions and roles. The following three chapters (Chapters 2, 3, 4) are published, in revision, and in preparation manuscripts respectively.

The manuscript presented in chapter 2 was published in *RNA* (14:914-927) entitled 'Unexpected roles of UPF1 in HIV-1 RNA metabolism and translation' by Lara Ajamian, Levon Abrahamyan, Miroslav Milev, Pavel Ivanov, Andreas E. Kulozik, Niels H. Gehring and Andrew J. Mouland. This work began during my Masters and was completed and published during my PhD. Most experiments were conducted by Lara Ajamian with help from Levon Abrahamyan and designed by Lara Ajamian, Levon Abrahamyan and Andrew J. Mouland. Miroslav Milev provided the Staufen1-TAP purification data (figure 1A-B) and Pavel Ivanov created many of the UPF plasmids used in the manuscript which were provided by Niels H. Gehring and Andreas E. Kulozik. This work was conducted in collaboration with Andreas E. Kulozik and Niels H. Gehring.

Chapter three entitled 'UPF1 shuttling is required for nucleocytoplasmic trafficking of the HIV-1 genomic RNA' by Lara Ajamian, Andreas E. Kulozik, Niels H. Gehring and Andrew J. Mouland is a manuscript in preparation. All

experiments were performed by Lara Ajamian and designed by Lara Ajamian and Andrew J. Mouland. We thank Kishanda Vyboh for technical help in performing some of the experiments. This work was conducted in collaboration with Andreas E. Kulozik and Niels H. Gehring.

Chapter 4, is a manuscript in preparation entitled 'UPF2 negatively regulates HIV-1 genomic RNA export' by Lara Ajamian, Kishanda Vyboh, Karen Abel, Andreas E. Kulozik, Niels H. Gehring and Andrew J. Mouland. All experiments were designed by Lara Ajamian in consultation with Andrew J. Mouland and performed by Lara Ajamian, Kishanda Vyboh and Karen Abel. This work was conducted in collaboration with Andreas E. Kulozik and Niels H. Gehring.

The last chapter (5) summarizes the work done during my PhD and future directions.

Other contributions (papers):

1- Bashar Ghoujal, Miroslav P Milev**, Lara Ajamian** and Andrew J Mouland. ESCRT-II interacts with Staufen1 in mammalian cells and influences HIV-1 expression**.** (manuscript in preparation).

2- Fernando Valiente-Echeverría[1,2#], Kishanda Vyboh[1,3#], Luca Melnychuk[1,3], **Lara Ajamian**[**1**,3], Andrew J. Mouland[1,2,3,4]. The HIV-1 Gag protein inhibits assembly of stress granules by interacting with eukaryotic

elongation factor 2 (eEF2). (Manuscript in preparation), (# equal contribution).

3- **Lara Ajamian**[1,2] and Andrew J Mouland[1,2,3]. Implications of RNA Helicases in HIV-1 replication: possible roles in latency. Current HIV Research 2011 Dec 1:9(8):588-94.

4- Heather Gordon [1], **Lara Ajamian**[1], William F. Rigby[2] and Andrew J Mouland [1.3]. Depletion of hnRNP A2/B1 overrides the nuclear retention of HIV-1 genomic RNA and promotes the formation of translationally deficient ribonucleoprotein complexes. (Manuscript in preparation).

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ATM	ataxia-telangiectasia mutated kinase
CA	capsid
CDK9	cyclin dependent kinase 9
CES	*cis* enhancing sequence
CRM1	chromosome region maintenance 1
CRS	*cis* repressor sequence
DIS	dimerization initiation site
DDX	dead box proteins
EIF5A	eukaryotic initiation factor 5A
EJC	exon junction complex
Env	envelope
FISH	fluorescence *in situ* hybridization
Gag	group-specific antigen
HAART	highly active antiretroviral therapy
HAX-1	HS1-associated protein X-1
HIV-1	human immunodeficiency virus type 1
hnRNP	heterogeneous nuclear ribonucleoprotein
hRIP	human Rev interacting protein
HTLV	human T-cell lymphotropic virus
IF	immunofluorescence

IMP1	insulin-like growth factor II mRNA binding protein
IN	integrase
INS	instability sequence
Kb	kilobase
LSCM	laser scanning confocal microscopy
LTR	long terminal repeat
MA	matrix
NC	nucleocapsid
Nef	negative factor
NES	nuclear export signal
NLS	nuclear localization signal
NMD	nonsense-mediated mRNA decay
NPC	nuclear pore complex
NUPs	nucleoporins
NXF1	nuclear RNA export factor 1
ORF	open reading frame
PBS	primer-binding site
PIC	pre-integration complex
Pol	polymerase
PPT	polypurine tract
Pr	protease
PTC	premature translation termination codon
Rev	regulator of virion expression
RNA	ribonucleic acid

RRE	Rev response element
RSV	Rous sarcoma virus
RSE	Rous sarcoma virus stability element
RT	reverse transcriptase
RTC	reverse transcription complex
Sam68	src-associated protein in mitosis 68kDa
SBS	Staufen1-binding site
SD	splice donor
SIV	simian immunodeficiency viruses
SMG	suppressor with morphological effect on genitalia
TAR	*trans*-activating response element
Upf	up-frameshift protein
UTR	untranslated region
VLP	virus-like particle
Vpr	viral protein R
Vpu	viral protein U

ACKNOWLEDGMENTS

First and foremost, I express my gratitude to my supervisor Dr. Andrew J. Mouland. His support, encouragement, guidance and patience have shaped me into an independent scientist. His helpful advice, positive outlook and confidence have resulted in the work presented in this thesis. It has been an honor working in his laboratory.

I would also like to thank our collaborators Pavel Ivanov, Niels H. Gehring and Andreas E. Kulozik for their generosity of critical reagents towards the project and their helpful discussions as well as PhD committee members Drs. Imed Gallouzi, Kostas Pantopoulos and Pascal Chartrand and my advisor Dr. Lorraine Chalifour. I thank you for your advice and guidance towards directing my project.

I am grateful to Levon Abrahamyan for training me and helping me to kick off the UPF1 project. I also thank Kim Hu for training me when I first arrived in the lab. Olivier, Bashar, Miroslav, Martin, Mukunth and Anne, the past members of the lab who have graduated, you made everyday memorable, especially Anne, my conference buddy! To my current labmates, Kishanda, Fernando, MJ, Luca, Karen and Christina. Without a doubt, you continue to make science fun and interesting. The everyday excitement and laughs have made it easier to go through long days. I express a special thanks to Kishanda, my “santa’s little helper”. Your help and encouragement will always be appreciated. I am also very thankful for the lifelong

friendships that I have made throughout this journey with Melody, Sonia, Patrick, Alex, Diane and Guerline.

I would like to thank the Department of Medicine, the Division of Experimental Medicine, and more particularly Dr. Hugh P.J. Bennett, Marylin Linhares and Dominique Besso, for their help and support. Thank you to the secretaries Bianca, Anna and Rita for their help and support. I also thank Dr. Mark Wainberg, Ceasar, Maureen and Sue for the access and the maintenance of the BCL3 facility. I extend my appreciation to the Canadian Institute for Health Research (CIHR) for awarding me a CIHR Doctoral Research Award. I also extend my gratitude to the Division of Experimental Medicine for providing the Dr. Price Award, the CSMB for the travel fellowships and the funding agencies CIHR and CANFAR for grants awards to my supervisor for my project, which has given me the opportunity to attend national and international conferences.

My parents, Hrair and Sona, I thank you for your love, support and inspiration. Your patience and faith in me has helped me accomplish my PhD and many other life accomplishments. To my sister Tamar, you always encouraged me to follow my dreams and be driven and to believe in myself. You have taught me: *"If you want to be successful, It's just simple. Know what you are doing. Love what you are doing. And believe in what you are doing (Will Rogers)";* and I therefore dedicate my thesis and accomplishments to my family.

Chapter 1

Chapter 1

Introduction

INTRODUCTION

1.1 The History of Viruses

Infectious diseases have influenced our human history from two different perspectives: one being that we have learned a great deal from them and the other being the unfortunate way in which these contagious diseases have decimated human kind.

Numerous modern breakthroughs in molecular biology are linked to studies from virology. The field of virology was launched in 1892 when Dimitri Ivanowski showed for the first time that something tiny, small enough to go through a filter, was able to cause mosaic disease in plants leading to the identification of the tobacco mosaic disease virus [1]. This was followed by the work of Peyton Rous, who was the first to show that a virus leads to cancer in chickens [2]. Moreover, bacteriophages, which are viruses adapted to bacteria, have shown that DNA is indeed hereditary [3]. Another example comes from human viruses, which have led to the discovery of reverse transcriptase as well as to the process of RNA splicing. One of the most important notions stemming from viral studies is the notion of vaccines. Edward Jenner, more than 200 years ago, performed the first vaccination against smallpox [4] and this was followed by Louis Pasteur and Jonas Stalk whose works led to the eradication (to some extent) of rabies and poliovirus respectively [5].

Unfortunately, throughout history many viral outbreaks of diseases have led to millions of deaths. Viruses have been classified as being epidemics, pandemics, and chronic infections. Some of these pandemics include, Ebola and Polio virus. The Spanish Flu (Influenza virus) and Malaria, a parasitic disease, have also made their mark on history by killing millions. HIV-1, an emerging virus, is also part of these devastating diseases which has led to million of deaths.

1.2 HIV-1: causative agent of AIDS

1.2.1 Historical Perspective and Epidemiology

The first recorded HIV positive sample was observed as far back as 1959 from a frozen plasma sample in Kinshasa, the Democratic Republic of Congo [6]. But studies conducted by the virologist Bette Korber showed that this sample dated back to 1931, where the virus (most likely the M subtype) transferred to humans from the Cameroon chimps. Other phylogenetic studies have shown that the virus made its way into the human population as far back as 1890 [7].

The Acquired Immunodeficiency Syndrome (AIDS) epidemic was first documented in the early 1980s mainly in the homosexual community and was termed “gay-related immune deficiency syndrome” (GRID). Before it was named “Human Immunodeficiency Virus Type 1” (HIV-1), it was also named “lymphoadenopathy-associated virus” (LAV), “human T-cell leukemia virus type-III” (HTLV-III) among others [8]. In 1983, Luc Montagnier and Françoise Barré-

Sinoussi, recipients of a Nobel Prize for their breakthrough, identified HIV-1 as being the causative agent of AIDS [9].

HIV belongs to the *Retroviridae* family of viruses and is classified in the Lentivirus genus which describes its main characteristic as a chronic, slow, and persistent viral disease. HIV is characterized as being a zoonotic infection for both viruses: HIV-1 and HIV-2 [10]. The cross-species transmission for HIV-1 and HIV-2 originate from the Simian Immunodeficiency virus (SIV) found in different nonhuman primates: chimpanzees and sooty mangabeys, respectively [11]. Even though both viruses (HIV-1 & HIV-2) are transmitted the same way and present the same symptoms, their main differences are in the rate of progression of the disease and their prevalence. HIV-1 is more common compared to HIV-2 and is the main leader in the epidemic as well as being a more aggressive virus, even though both strains lead to AIDS. Moreover, while HIV-1 is present worldwide, HIV-2 is found primarily in Western Africa [12, 13].

The 2010 Global Report on the AIDS epidemic from UNAIDS shows that in 2009 there were 33.3 million people worldwide living with HIV and there were about 2.6 million new infections (UNAIDS 2010). Unfortunately in 2009, there were about 7200 new infections per day of which 1200 represented infections of children under the age of 15 (UNAIDS 2010). HIV has affected every corner of the world. The region suffering the most from this pandemic is Sub-Saharan Africa where 22.5 million (68%) infected adults and children live with HIV and this is followed by Asia (4.9 million infected) (UNAIDS 2010). Between 2001

and 2009, while a decline in new infections of more than 25% was detected in most countries, including Sub-Saharan Africa, was observed; countries in Eastern Europe/Central Asia showed an increase (>25%) in HIV incidence (UNAIDS 2010).

The HIV classification system is divided into types and then subdivided them into groups and subtypes. As previously mentioned, the two types of HIV are HIV-1 and HIV-2 which were discovered in 1983 and 1986 respectively [9, 14]. HIV-1 is further classified into groups: M (main), O (outlier), N (non-M, non-O or new) and P (pending classification/identification); where the M group is the main source of the HIV-1 pandemic [15-19]. The M group comprises 9 subtypes (A-D, F-H and J-K) [20]. The strains of subtypes E and I are classified as circulating recombinant forms (CRFs) most likely due to the recombination of viruses [21-24]. Subtypes A and F are further classified into sub-subtypes: A1-A4 and F1-F2. HIV-2 is also subdivided into 8 groups (A to H) where the most prevalent groups are A and B [20]. The most common HIV-1 strain (North America) is categorized as group M-subtype B (roughly 90% of infections) and the majority of HIV-1 studies have been conducted on subtype B. However, a pressing question remains if the antiretroviral treatments are equally effective against all subtypes since subtype B represents about 10% of infections worldwide and subtype C accounts for about half of all new infections and is the most widespread of HIV-1 subtypes [19, 25-31].

1.2.2 Transmission, Symptoms and Pathogenesis

The main route of transmission is via sexual contact. Non-sexual contact such as blood transfusions, intravenous drug users as well as vertical transmission (mother-to-child) and medical mistakes can also lead to the spread of the virus. Regardless of the route of transmission, through mucosal surfaces or percutaneous, the pattern of infection as well as viral and host markers in regards to the different phases of HIV-1 infection are similar [32] [33]. HIV-1 infection can be separated into three phases: acute (primary infection), chronic (asymptomatic) and progression to AIDS (symptomatic). Following transmission, the virus crosses several mucosal barriers and remains undetected for a few weeks. During this phase of primary infection, the infected individual can develop a rash, fever, and flu-like symptoms. The viral RNA remains undetectable during this period of seroconversion where its levels increase with a correlating decrease in CD4+ T cells. During this phase, the immune response is able to replenish the depleted CD4+T cells. Viral RNA can be detected in the plasma via qualitative methods at a concentration of 1-5 copies/mL and quantitatively at 50 copies/mL [34, 35]. An immune response and latency, in mainly CD4+T cells, is established during this phase and there is a concomitant increase in viral load and a destruction of CD4+T cells. The chronic phase is an asymptomatic phase, lasting anywhere between 3 to 10 years, where the virus destroys the CD4+ T cells [36-38].

Latency (discussed in section 1.4.1.3), a characteristic of lentiviruses, is dependent on many cellular factors as well as the immune system and the genetic background of the infected individuals [36, 39]. These factors distinguish infected individuals as being rapid progressors, slow progressors, or long-term nonprogressors. In rare cases, individuals present with very low levels of CD4+T cells but do not progress to AIDS. The genetic background of individuals plays an important role in AIDS progression; and the chemokine receptor CCR5 is one example of a cellular factor distinguishing progressors from long-term non progressors (LTNP). A Δ32 deletion in CCR5, passed on genetically in the Black Death survivors, confers resistance to HIV-1 infection. The Bubonic Plague (*Yersinia Pestis)* and HIV-1 use CCR5 as an entry receptor. The presence of this CCR5Δ32 deletion in the CCR5 gene in about 10% of Europeans renders them resistant to HIV-1 infection or, if infected, leads to very slow progression to AIDS [40]. In addition, this Δ32 deletion in CCR5 led to the first human target, the CCR5 antagonist, Maraviroc [41] (discussed in section 1.3). Elite controllers (EC), which represent less than 1% of the HIV-1 infected individuals, are similar to LTNP. EC's in that they are able to control viral replication with a viral load below detection levels compared with LTNPs where viremia is detected [42, 43].

The infected individual is infectious at this chronic phase but less than in the acute phase. When the immune system cannot replenish the depleted immune cells, opportunistic infections and/or immunological disorders arise [44]. Some examples of opportunistic infections include candidiasis, cytomegalovirus, herpes virus as well as several malignancies such as kaposi sarcoma and lymphoma [45].

Once CD4+T cell levels become less that 200 cells/uL, the infected individual is in the last phase of HIV-1 infection, AIDS [32].

1.3 HIV Therapies: Present and Future

For many years following HIV-1 discovery, no effective treatment was available for infected individuals. In 1988, zidovudine (AZT), the first nucleoside reverse transcriptase inhibitor, was prescribed to patients giving hope to the HIV-1 treatment world [46]. But soon, studies showed that AZT monotherapy was ineffective due to the emergence of resistant mutations. In the 90s (1996), the use of 3 anti-retrovirals (HAART: highly active anti-retroviral therapy) showed the best outcome for patients [47, 48]. HAART is the combination of three different anti-retrovirals and showed tremendous benefits but was not without complications. Complications such as adverse side effects as well as the massive pill burden and adherence to treatment resulted in the rise of drug resistant mutations [49]. Fortunately, most of these issues have been successfully dealt with such as newer anti-retrovirals with fewer side effects and a one pill a day ATRIPLA regiment [50]. In addition, in August 2011, a new multi–drug was approved by the FDA called Complera. In light of these therapeutic strategies, the life expectancy of HIV-1 positive individuals has increased from 12 months to close to a normal lifespan.

To date there are more than 25 different anti-retrovirals approved for HIV-1 therapy (FDA website) [31, 49]. They are classified into six different groups (Table 1) and they all target multiple steps of the HIV-1 replication cycle.

Interestingly, through high throughput studies (HTS), the entry inhibitor Maraviroc was discovered. Maraviroc inhibits the CCR5 co-receptor during the step of attachment and entry of the HIV-1 viral life cycle. Since CCR5 viruses are most common throughout infection, targeting CCR5 became a great treatment of choice [51-53]. Maraviroc was therefore the first CCR5 antagonist approved and the first human cellular target.

Since the virus cannot survive without the host cell and is dependent on cellular proteins at each step of the HIV-1 life cycle, studying virus-host interactions can lead to identifying new therapeutic targets. In recent years, numerous screens identified cellular proteins that could be targeted to block HIV-1 replication [54-56]. However, there is a very low percentage of overlap among these screens; thus characterization of the role of these dependency factors is needed to identify potential candidates [57]. Nevertheless, these studies broaden the pool of potentially new anti-retrovirals, especially in identifying proteins involved in HIV-1 latency, the major barrier faced in eradicating HIV-1.

There are currently several ongoing HIV-1 vaccine trials in different phases. One of the first phase III HIV-1 vaccine trails is the AIDSVAX B/E Thailand trial. The largest HIV vaccine trial in Thailand opened the possibility for an effective vaccine against HIV-1 [58]. The trial began in October 2003 and participants were given a prime and boost vaccine of strains circulating mainly in Thailand. They showed that the participants who were given the vaccine demonstrated a 31% protection against HIV-1 infection [58]. Even though the vaccine was against

Inhibitors	Brand names (Generic names)	Approved
Nucleoside Reverse transcriptase inhibitors (NRTIs)	Combivir (lamivudine and zidovudine)	1997
	Emtriva (amtricitabine, FTC)	2003
	Epivir (lamivudine, 3TC)	1995
	Epzicom (abacavir and lamivudine)	2004
	Retrovir (zidovudine, azidothymidine, AZT, ZDV)	1987
	Trizivir (abacavir, zidovudine, lamivudine)	2000
	Truvada (tenofovir disoproxil fumarate, emtricitabine)	2004
	Videx EC (enteric coated didanosine, ddI EC)	2000
	Videx (didanosine, dideoxyinosine, ddI)	1991
	Viread (tenofovir disoproxil fumarate, TDF)	2001
	Zerit (stavudine, d4T)	1994
	Ziagen (abacavir sulfate, ABC)	1998
NonNucleoside Reverse transcriptase inhibitors (NNRTIs)	Edurant (rilpivirine)	2011
	Intelerence (Etravirine)	2008
	Rescriptor (Delavirdine, DLV)	1997
	Sustiva (Efavirenz, EFV)	1998
	Viramune (nevirapine, NVP)	1996
	Viramune XR (nevirapine, NVP)	2011
Protease Inhibitors	Agenerase (Ampronavir, APV)	1999
	Aptivus (Tipranavir, TPV)	2005
	Crixivan (Indinavir, IDV)	1996
	Invirase (Saquinavor mesylate, SQV)	1995
	Kaletra (Lopinavir and ritonavir, LPV/RTV)	2000
	Lexiva (Fosamprenavir Calcium, FOS-APV)	2003
	Norvir (Ritonavir, RTV)	1996
	Prezista (Darunavir)	2006
	Rayataz (Atazanavir sulfate, ATV)	2003
	Viracept (Nelfanavir mesylate, NFV)	1997
Fusion Inhibitors	Enfuvirtide, T-20	2003
Entry Inhibitors	Maraviroc	2007
Integrase inhibitors	Raltegravir	2007

TABLE 1: *Approved Anti-Retroviral Drugs.*

(FDAwebsite:www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm)

Thailand-specific HIV-1 strains and the protective effect was 31%, this still opens new avenues and direction for future vaccine development strategies [58]. Microbicides are another treatment of choice with promising outcomes, especially in countries where anti-retroviral therapies are limited and costly. Microbicides are gels/creams applied before sexual contact targeting mainly early events in HIV-1 infection [59-61]. Microbicides generated from anti-retrovirals (ARV) already approved, such as tenofovir or raltegravir, which target not only early (entry and fusion) steps of the HIV-1 replication cycle but as well as the reverse transcription, integration and protease cleavage steps, have shown protection [60]. In any case, resistant mutations can and do arise and a combination of microbicides would represent a better treatment choice [60].

Interestingly, a bone marrow transplant from a patient harbouring the Δ32CCR5 deleted gene was transplanted into an HIV positive patient. The patient seems 'cured' of HIV-1 such that he hasn't been taking any ARVs for more than three years and has undetectable virus [62]. Thus, attempts such as vaccines, microbicides and bone-marrow transplants have highlighted the possibility of decreasing infections and winning this war against HIV-1.

1.4 HIV-1 mature virions, genome and viral proteins

1.4.1 The HIV-1 particle structure

HIV-1 is characterized as having a spherical shape ranging from 110-140nm (immature: 120-140nm, mature: 110-130nm) in diameter [63, 64] (Figure 1). The virus is enveloped by a lipid bilayer acquired during the process of budding from the infected cell. The envelope glycoproteins, gp120 (surface protein) and gp41 (transmembrane protein), are anchored in the viral membrane in a trimeric conformation. Underlying the lipid envelope is a layer of the matrix protein p17 which surrounds a cone-shaped structure consisting of approximately 1500 molecules of capsid [65, 66]. This viral core consists of a variety of cellular and viral proteins as well as the 2 positive strand viral RNA molecules to which the nucleocapsid (NC) proteins are bound. The viral proteins identified in virus particles are the enzymes protease (PR), integrase (IN) and reverse transcriptase (RT) as well as the viral protein R (Vpr), the negative factor (Nef) and the viral infectivity factor (Vif) [67].

FIGURE 1: *The infectious HIV-1 virus particle.*

The HIV-1 virus is surrounded by a lipid bilayer membrane derived from the host membrane where the envelope proteins (gp120, gp41) are anchored. The viral CA comprises the 2 copies of the single stranded viral RNA to which the NC proteins, the viral enzymes RT and IN as well as accessory proteins bind to. This viral core is surrounded by the viral protein MA. Numerous cellular proteins are encapsidated in viral particles (not indicated). Env: envelope, RT: reverse transcriptase, IN: integrase, PR: protease, CA: capsid, MA: matrix, NC: nucleocapsid. Vif: viral infectivity factor, Vpr: viral protein R, Nef: negative factor (see text for more detail) [63-67].

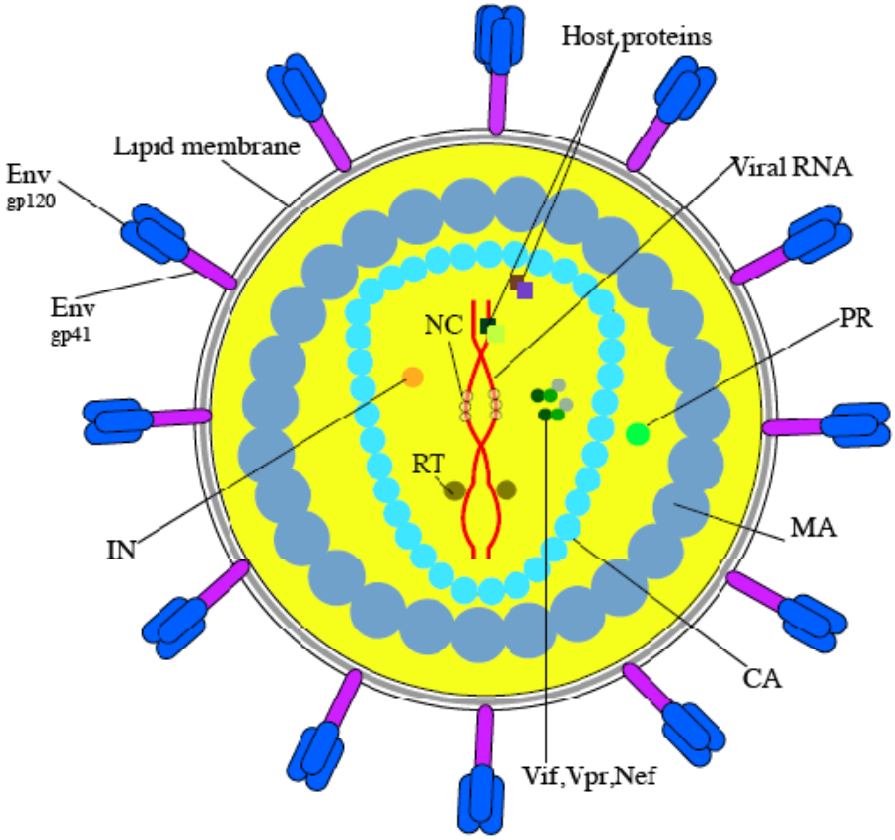

FIGURE 1*: The infectious HIV-1 virus particle.*

1.4.2 HIV-1 genome and viral proteins

The HIV-1 genome is 9.4kb long and has 9 open reading frames generating 15 viral proteins [68] (Figure 2). Non-coding regions termed 5'- and 3'- untranslated regions (UTR), where the major regulatory sequences are found, flank both ends of the viral RNA and surround the coding regions. Interestingly, the viral HIV-1 RNA resembles cellular RNAs such that it contains a 5'Cap (7mGpppG) and a poly (A) tail at its 3'end [69, 70].

Most of the regulatory sequences are found in the 5' or 3' UTR regions on the HIV-1 genome [71] (Figure 2A). The 5' UTR contains the transactivation response region (TAR) which is required for transcription and is a binding site for the viral protein Tat [72]. The primer binding site (PBS) follows the TAR region and this is where the $tRNA^{lys3}$ binds serving as primer and thus initiating the process of reverse transcription [73] [74]. Downstream of the TAR region, the dimerization initiation site (DIS), the splice donor (SD) sites and the SL3 stem-loop are present [68, 75].

Numerous regulatory regions are also located throughout the coding regions. The -1 frameshifting site is located at the *gag-pol* region mediating a -1 ribosomal frameshifting important for the production of the viral enzymatic proteins [76]. Two regions rich in purines called polypurine tract (PPT), located in the U3 region and the *pol* gene, are important to ensure proper reverse transcription [77, 78]. The Rev response element (RRE) is a structure important for efficient HIV-1

vRNA export to which the viral Rev protein binds [79]. Numerous sequences such as CRS ('Cis-Acting Repressive Sequences') or INS ('Instability Sequences') or CES ('Cis Enhancing Sequences') are located in the *gag, pol and env* genes. These sequences are AU rich regions and are important during the HIV-1 replication cycle where many cellular proteins bind to regulate stability, export and translation [75, 80-83].

There are three main coding regions: *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) (Figure 2B). Through the process of cleavage, different viral products are generated. Gag is the major structural protein of HIV-1 and is 55kDa. At the late stages of HIV-1 production, during the process of budding and maturation of virus particles, the viral protease functions to cleave Gag into the matrix (MA, p17, 17kDa), nucleocapsid (NC, p7, 7kDa), capsid (CA, p24, 24kDa), p6 (6 kDa) and the spacers SP1 and SP2 proteins. The viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) are generated from the *pol* coding region. Lastly, the *env* coding region generates the gp160 polyprotein which in turn is cleaved to produce both envelope proteins: the transmembrane protein gp41 (TM) and the surface protein gp120 (SU) [68, 84]. Their roles are described in more detail in Section 1.4.

The other six open reading frames generate the regulatory and accessory HIV-1 proteins (Figure 2B). The two regulatory viral proteins are the transactivator of transcription (Tat) and the regulator of virion expression (Rev) whose major roles are to stimulate transcription from the HIV-1 long terminal repeats (LTR) of the

FIGURE 2: *The HIV-1 genome.*

The HIV-1 genomic RNA (A) and provirus (B) are illustrated. (A) The genomic RNA has two untranslated regions (UTR) located at the extremities which contain many regulatory sequences. It also contains numerous *cis* acting sequences such as INS, CES, CRS, RRE and -1 frameshifting site. The HIV-1 genomic RNA also has a 5' Cap and 3' poly A tail. (B) The LTRs (Long Terminal Repeats) flank the viral and enzymatic proteins. The viral enzymes generated from the *pol* region are protease (PR), integrase (IN) and reverse transcriptase (RT). Matrix (MA), nucleocapsid (NC), Capsid (CA), p6, SP1 and SP2 are cleaved from the Gag precursor. The *env* coding region generated the envelope proteins gp120 and gp41. [68-78, 84].

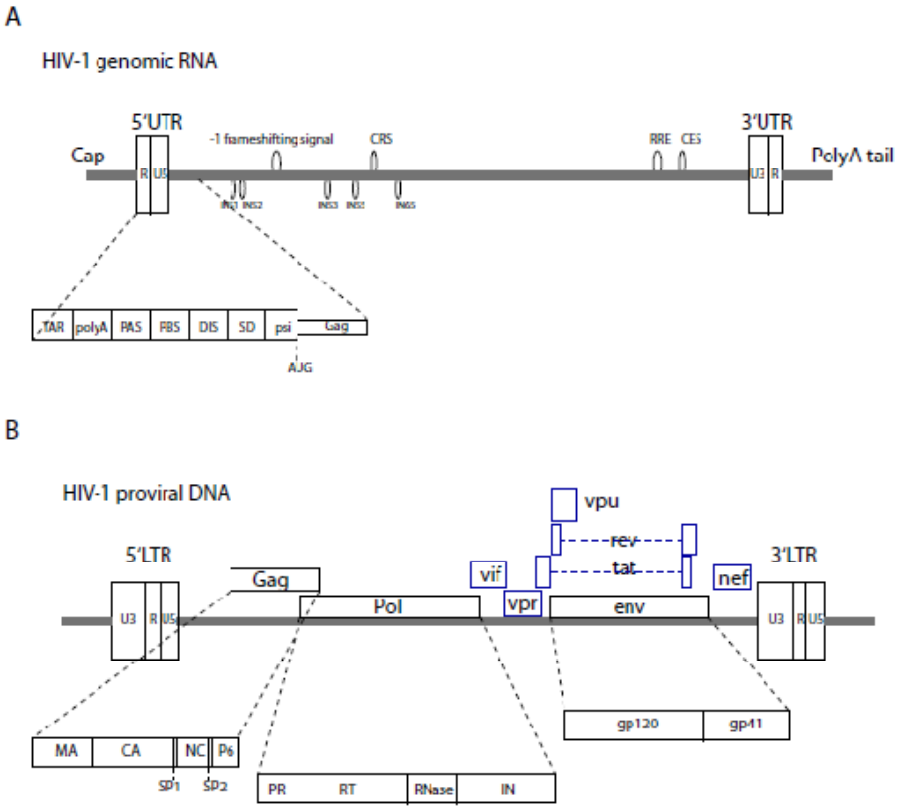

FIGURE 2: *The HIV-1 genome.*

integrated proviral genome and to export the unspliced and singly spliced transcripts into the cytoplasm respectively. The remaining four accessory genes are the virion infectivity factor (Vif), the viral protein R (Vpr), the negative factor (Nef) and the viral protein U (Vpu). Vif inhibits the cytidine deaminase APOBEC3G and is involved in virus infectivity [85]. Vpr is involved in HIV-1 nuclear import and is involved in arresting cells in the G2 phase of the cell cycle. Nef has been shown to be involved in down-regulating CD4 and MHC-1 as well as inhibiting apoptosis. Moreover, viruses deficient of Nef exhibit a slower disease progression. Lastly, Vpu not only decreases CD4 levels but also increases virus release by blocking tetherin function [68, 86-88].

1.5 HIV-1 replication

The HIV-1 replication cycle can be divided into two main stages (early and late) separated via the process of integration [68, 84] (Figure 3).

1.5.1 Early phases of HIV-1 replication

1.5.1.1 Attachment, fusion and entry

The early stages of the HIV-1 replication cycle begin with the process of recognition and binding of the mature infectious virus particles and its target cell. The viral proteins required for this process are the envelope proteins gp120 and gp41. Early studies pointed out that HIV-1 primarily infects CD4+ T cells

identifying CD4 as the major receptor that binds to the glycoprotein gp120. The CD4 receptor is found on the surface of T cell subsets but also on a variety of cells besides CD4+ T cells such as macrophages, dendritic cells, among others. The requirement of a co-receptor was soon discovered based on the fact that HIV-1 underwent variable infection efficiencies depending on the cell type. Soon, the co-receptors CXCR4 and CCR5 were identified explaining HIV-1 viral tropism [89, 90].

Once the virus meets its target cell, an interaction with CD4 and gp120 takes place leading to a conformational change exposing the co-receptor binding site. During this conformational step, the V3 loop (third variable loop) on gp120 is exposed dictating the choice of the co-receptor [91]. Thus, viral tropism is established early during HIV-1 replication [89]. Following binding of the co-receptors CXCR4 or CCR5, a series of conformational changes take place leading to the insertion of the hydrophobic region of gp41 into the target cellular membrane. This results in both the viral and cellular membranes to come in close proximity, allowing fusion of both membranes and release of the viral core into the target cell [68, 84, 92].

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, many other routes such as endocytosis, virological/immunological synapse as well as the involvement of other co-receptors such as DC-SIGN, CCR2/3, ICAM, LFA-1, Galectin-1 and gp340, among others, have been established [93, 94] [95, 96]. Studies showed

FIGURE 3*: The HIV-1 replication cycle.*

Following receptor/co-receptor binding (step 1,2) and fusion of viral and cellular membranes, the viral core is released into the cytoplasm (step 3) where uncoating takes place and the HIV-1 genomic RNA is reverse transcribed into the double stranded proviral DNA. The HIV-1 genome is found in a complex called PIC (step 4.5). The PIC complex translocates into the nucleus through NLS (nuclear localization signal) signals and interactions with the nuclear pore complex where it integrates into the host genome (step 6,7,8). Following integration, the late events in HIV-1 replication begin with the transcription of the integrated DNA generating the genomic RNA which gets multiply spliced into 2kb transcripts (step 9). These 2kb RNAs are exported into the cytoplasm through the NXF1 export pathway (step 10) where translation takes place generating the viral proteins Tat, Rev and Nef (step 11). Then Tat and Rev shuttle to the nucleus where Tat transactivates transcription. Rev binds to the unspliced and singly-spliced HIV-1 RNAs, stabilizes them and exports them through the CRM1 export pathway with the help of numerous cellular proteins (step 10). This is followed by synthesis of Gag and other viral proteins (step 11) and trafficking of viral and cellular proteins as well as the HIV-1 genomic RNA to the plasma membrane (step 12). The HIV-1 replication cycle is completed via the budding and maturation of virus particles (step 13). (see text for more details).

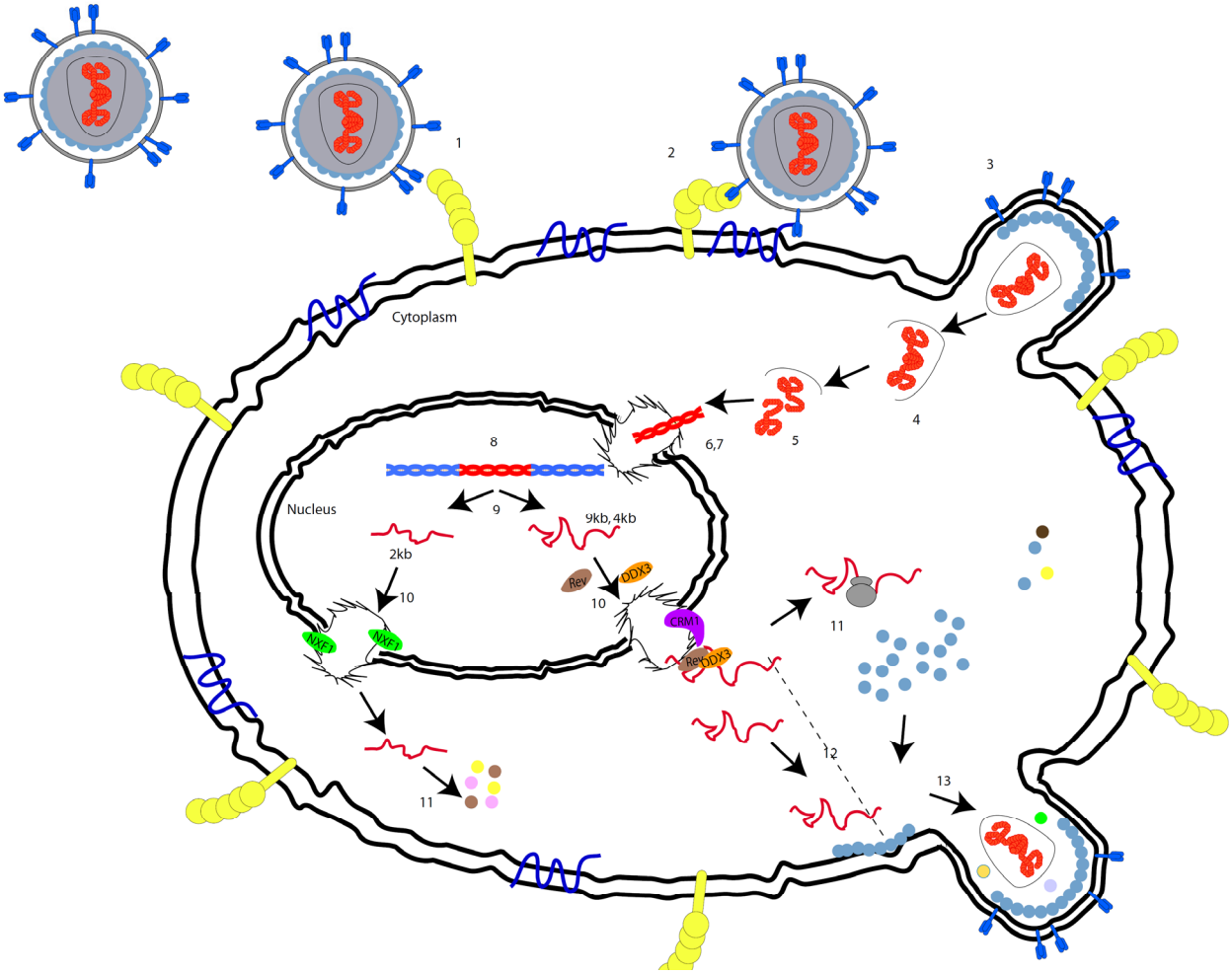

FIGURE 3: *The HIV-1 replication cycle.*

that HIV-1 transmission in T cells occurs mainly via cell-cell spread. In addition, interactions not only between the viral envelope and the receptor/co-receptors but also interactions between LFA-1 and ICAM aid in the formation of virological synapses [97].

1.5.1.2 Uncoating and reverse transcription

The release of the viral core into the cytoplasm is followed by the process of uncoating. During uncoating, the outer core made of several capsid proteins is disassembled while other interactions are maintained. This step is not very well understood but studies have pointed out the possible involvement of cellular proteins such as MAP kinases, Pin1, cyclophilin A and the viral proteins Vif and Nef during uncoating [88]. This process of uncoating exposes the genomic RNA as part of a new complex termed the RTC (reverse transcription complex). The interactions that are maintained through the process of uncoating form the RTC and are required for the process of reverse transcription. These interactions include the viral enzymes RT and IN, the viral protein products of Gag, MA and NC, the accessory proteins Vif and Vpr, and several cellular proteins as well as $tRNA^{lys3}$ as well as the two copies of the ssRNA HIV-1 genome [98].

The viral enzyme RT is important for the process of reverse transcription. This step, characteristic of retroviruses, is where the viral genomic RNA is converted into the double stranded proviral DNA which is integrated into the host genome. The reverse transcriptase enzyme has two characteristics: it is a DNA/RNA

dependant DNA polymerase and has RNAseH activity [99]. During the process of reverse transcription, the $tRNA^{lys3}$ binds to the PBS site on the genomic RNA and serves as a template for the RT enzyme generating the (-)DNA strand [74]. This is followed by the degradation of the (+) strand RNA via the RNAse H activity of the RT enzyme [99]. Subsequently, the (-)DNA strand is then used as a template for the (+)DNA strand synthesis. As a result, the double-stranded proviral DNA is synthesized [68, 100]. Reverse transcription is completed by the formation of a 99 nucleotide DNA flap, important during nuclear PIC import [101] (section 1.4.1.3).

1.5.1.3 Nuclear import and integration

Following reverse transcription, the double-stranded linear DNA is part of a new complex called PIC**.** The PIC comprises a variety of cellular and viral proteins important for the step of nuclear import. The viral proteins MA, Vpr, and the viral enzymes RT and IN form the PIC which also contains a variety of cellular proteins [102]. Due to the large size of the PIC, it is not able to diffuse/translocate into the cytoplasm and thus the presence of the viral proteins MA, Vpr and IN, which harbour nuclear localization signals (NLS), aid in this process of nuclear import [103]. Moreover, the central DNA flap generated during reverse transcription assists for proper translocation into the nucleus [101]. Numerous cellular proteins also compose the PIC such as BAF, INI1 and LEDGF/p75 among others [104, 105]. Following a series of interactions between the PIC and the nuclear pore complex (NPC), transportins, importins, the microtubules, and the dynein motor, the PIC complex enters the nucleus [106-110].

The last step in the early phase of the replication cycle is integration of the proviral DNA into the host genome via the viral enzyme integrase and multiple cellular proteins [104]. The process of integration is divided into two chemical reactions: 3' end processing and strand transfer. The step of 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 [111]. In the nucleus, the second chemical reaction, strand transfer, takes place where IN binds to the target DNA, cuts and a 5 base pairs are inserted, and with the help of cellular repair enzymes closes the gap and integration is complete [111]. Many cellular proteins are required during integration such as LEDGF/p75 and INI1 [104].

1.5.1.4 Latency

Latency is one of the principal barriers faced in eliminating HIV-1. Many immunological cells contribute to HIV-1 latency but resting CD4+ T cells are the major reservoir since they have the longest half-lifes of 44 months [112]. HIV-1 latency can be a pre- and post-integration events but the one that results in long term latency is post-integration of the double stranded proviral DNA into the host genome [113, 114] [115].

Viral latency is dependent on a variety of factors. Latency can be established due to the absence of cellular factors required for transcription or the presence of repressors of transcription. Moreover, the site of integration, being one of high

transcription, is important to ensure replication. Some examples of transcriptional repressors are the histone deacetylase HDAC1 as well as the cellular proteins Ying Yang 1 (YY1), Late SV40 factor (LSF) and C-promoter binding factor-1 (CBF-1) which reduce histone acetylation [115] [116, 117]. Histone methylation and mutations in Tat or TAR have also been linked to latency [118-120]. Studies have also shown that the multiply spliced HIV-1 transcripts are also present in the nucleus of latently infected cells indicating a defect in export [115] [121].

MicroRNAs (miRNAs) and RNA interference (RNAi) have also been linked to latency such that in latency, several cellular miRNAs are upregulated in resting CD4+T cells [115] [122]. Some of these microRNAs identified as being upregulated are mir-28, -125b, -150, -223 and -382. Since their roles are to block translation or degrade mRNA transcripts, the increase in their expression could promote latency. Moreover, these miRNAs were shown to inhibit HIV-1 by targeting their 3'end. Viral miRNAs from Nef and TAR were also involved in establishing latency [123, 124]. Interestingly, a number of RNA helicases were altered in expression following reactivation of latency hinting that they may be involved [115] [125]. Some of these helicases that were altered in expression are involved in the late stages of HIV-1 replication. For example, the expression of DDX3 and DDX1 was altered, among others, indicating that proteins involved in HIV-1 RNA export could have roles in latency. There have been many attempts to eliminate latent reservoirs but nevertheless, latency still remains one of the major obstacles for HIV-1 eradication [115].

1.5.2 Late phases of HIV-1 replication

1.5.2.1 Transcription

Following integration of the proviral DNA into the host genome, transcription takes place and this depends on a variety of factors. Tat is the viral protein required for efficient transcription, but due to its absence at the beginning of transcription, the cellular machinery is required [126]. This Tat-independent transcription is initiated by the cellular RNA polymerase II (RNApolII). RNApolII stimulates transcription by binding to the HIV-1 5'LTR region and generates the HIV-1 full length mRNA which is subject to splicing to generate the multiply spliced 2kb HIV-1 RNAs. They are exported into the cytoplasm via the NXF1 export pathway [127]. Once in the cytoplasm, translation takes place generating the regulatory proteins Tat and Rev and the accessory protein Nef. Tat and Rev shuttle back into the nucleus where the Tat-dependent transcription begins [68, 84, 128].

Tat-dependent transcription is initiated by Tat binding to TAR and recruiting important transcription factors such as cyclin T and CDK9. The phosphorylation of the CTD domain of RNA polymerase II leads to increased polymerase activity, transcription, and stability [129-131]. This burst in transcription coupled with alternative splicing generates more than forty different mRNAs which are classified into the three main HIV-1 RNAs; 9kb unspliced RNAs, 4kb singly-spliced RNAs and the multiply-spliced 2kb RNAs [132-134].

HIV-1 alternative splicing is a controlled process generating more than 40 different mRNAs. HIV-1 mRNA splicing involves core splicing signals located at 5'splicing, 3'splicing and at the branch point signal [134, 135]. Generally, splicing takes place at 3'ss sites located before the open reading frames (ORFs) of each viral protein. The strength or weakness of each splicing site explains why not all alternatively spliced HIV-1 mRNAs are present at similar abundances. Several cellular and viral proteins are involved in HIV-1 splicing. HnRNP A1 and SR proteins are examples of cellular proteins that affect splicing by downregulating or stimulating splicing respectively [134, 135]. In addition, splicing efficiency has also been linked to the viral proteins Tat, Rev and Vpr [136, 137].

1.5.2.2 Nuclear export and Translation of viral mRNAs

The unspliced and singly-spliced RNAs contain a *cis*-acting sequence called Rev responsive element (RRE) [138]. This sequence is 250 nucleotides in length, located in their *env* gene to which the viral protein Rev binds, oligomerizes (8 molecules of Rev per viral RNA) and exports these intron-containing RNAs into the cytoplasm [139, 140] (Figure 4). Rev binds to the HIV-1 vRNA via its NLS region and through its NES it interacts with the export protein CRM1 (chromosome region maintenance 1) and with RanGTP [141-143]. The gradient formed across the nuclear membrane, RanGTP concentration highest in the nucleus while RanGDP concentration is highest in the cytoplasm, directs this nuclear export complex towards the cytosol [144]. Once the complex reaches the

cytoplasm, RanGTP is exchanged for RanGDP and the exported 9kb and 4kb RNAs are release where they undergo translation [84, 141, 144].

Numerous cellular proteins play a role in Rev-mediated HIV-1 RNA export (Figure 4, Table 1). Some proteins are termed as positive regulators of export while others inhibit Rev-dependent export (Table 2). Sam68 (Src associated protein in mitosis of 68kDa) was shown to be a Rev cofactor, and synergistically stimulated HIV-1 viral RNA export. Studies also demonstrated that Sam68 can also replace Rev function [145, 146]. Two RNA helicases, DDX3 and DDX1 were shown to be required for Rev-mediated export and they both immunoprecipitated with Rev and CRM1 [147, 148]. In addition, DDX1 was shown to be involved in the proper localization of Rev in astrocytes [149]. EIF5A (eukaryotic translation initiation factor 5A) was identified as being an adaptor protein involved in Rev-mediated export [143, 150, 151]. Many nucleoporins have also been characterized as playing crucial roles in HIV-1 vRNA export such as the nucleoporins Nup98, Nup214, Nup358, Nup153 and Nup62 [152]. Moreover, the cellular protein ATM (ataxia-telangiectasia-mutated kinase), which has several roles in the HIV-1 replication cycle including integration, is also involved to activate Rev [153] [154]. Some of these proteins involved in HIV-1 viral RNA export have also been linked as being involved in HIV-1 translation [155, 156]. Following nuclear export, other cellular proteins ensure proper release of the HIV-1 viral RNA from the nuclear periphery such as hRIP (human Rev-interacting protein) and hnRNPA2 (heterogeneous nuclear ribonucleoprotein A2) [157-159]. Inhibitors of HIV-1 RNA export have also been identified such as IMP1 (insulin-like growth factor II mRNA binding protein) and Hax-1

FIGURE 4: *The host proteins involved in HIV-1 genomic RNA export.*

Following transcription (1), the generated genomic RNA is multiply spliced into 2kb RNAs which are exported into the cytoplasm via the NXF1 export pathway. Sam68 has been shown to participate in Rev-independent viral RNA export (2). Tat, Rev and Nef are produced where Tat and Rev re-enter the nucleus and Tat generates the unspliced and singly spliced RNAs which contain an RRE RNA sequence. Rev binds to this sequence and exports these 9kb and 4kb transcripts into the cytoplasm via the CRM1 export pathway (4). Numerous cellular proteins participate in Rev-mediated export such as DDX1/3 and Sam68. Sam68 has also been shown to be involved in trafficking the HIV-1 genomic RNA to translation sites (5). HnRNPA2 and hRIP are important for trafficking the viral RNA from the perinuclear region to the plasma membrane (6). (see text and table 2 for more details).

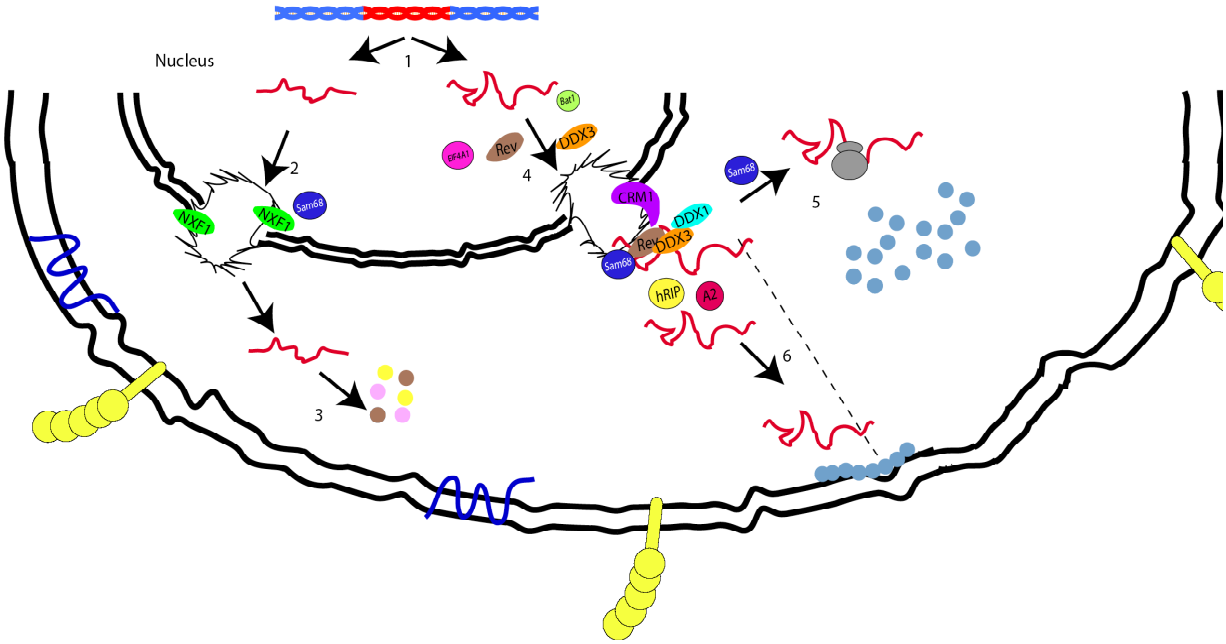

FIGURE 4: *The host proteins involved in HIV-1 genomic RNA export.*

Host proteins	Functions	References
CRM1 (chromosome region maintenance 1 or exportin 1)	- Binds to Rev, DDX3/1, RanBP1 - Is in a trimeric complex with RanGTP	[160]
DDX3 (DEAD box protein 3)	- Shuttling protein, found at the nuclear pore - Shuttling function required for Rev-mediated export - Binds CRM1, Rev - Increased in expression following reactivation from latency	[125, 147]
DDX1 (DEAD box protein 1)	- Shuttling protein - Binds CRM1, Rev and RRE - Involved in maintaining proper cellular Rev localization (astrocytes) - Increased in expression following reactivation from latency	[125, 148, 149]
Nucleoporins	- Nup98,nup62,nup358,nup214, nup153	[152]
Sam68 (Src associated protein in mitosis of 68kDa)	- Can replace Rev function - Acts synergistically with Rev - Binds to RRE	[145, 146, 155]
eIF5A (eukaryotic translation initiation factor 5A)	- Knock down or export mutants block Rev-mediated export, thus may act as an adaptor for Rev/CRM1 - Binds Rev	[143]
hRIP (human Rev-interacting protein)	- Rev cofactor - Involved in Rev-mediated export and release of HIV-1 genomic RNA from the perinuclear region	[157]
Hax-1 HS1-associated protein X-1)	- Binds Sam68, and interacts with Rev - Blocks Rev binding to RRE - Sam68 overexpression overcomes inhibition imposed by Hax-1 - Affects Rev localization	[161]
IMP-1 (insulin-like growth factor II mRNA binding protein)	- Binds Rev - Its overexpression relocalizes Rev to the cytoplasm	[162]
ATM (ataxia-telangiectasia-mutated kinase)	- Increases Rev-dependent export by activating Rev	[154]
Pur-A	- Binds Rev and RRE - Involved in RRE-mediated export	[163]
Matrin3	- Increases Rev-dependent export - Binds Rev in an RRE-dependent manner	[164]

TABLE 2: *List of cellular proteins involved in HIV-1 Rev-mediated export.*

(HS1-associated protein X-1) result in the redistribution of Rev leading to a block in HIV-1 viral RNA export thus classifying them as negative regulators [161, 162]. In the cytoplasm, translation of the HIV-1 viral RNAs takes place. The 9kb HIV-1 RNAs produce $pr55^{Gag}$ (55kDa) and $pr160^{GagPol}$ (160kDa), while the singly-spliced 4kb RNAs code for the envelope proteins and the accessory proteins Vif, Vpr and Vpu [68, 84, 128]. The multiply-spliced 2kb RNAs generate the regulatory proteins Tat and Rev and the accessory protein Nef [68, 84, 128]. Numerous cellular proteins have been characterized as being involved in this posttranscriptional process, such as DDX3, Sam68, eIF5A, who are also implicated in HIV-1 genomic RNA export.

1.5.2.3 Assembly, Budding and Maturation

The major structural protein of HIV-1, $pr55^{Gag}$, is the key player in the process of assembly. Alone, it can form VLPs (virus like particles). The process of assembly is a multistep highly regulated process which begins during the step of translation where the matrix (MA) of Gag gets myristoylated. The amino terminal (N)-myristoylation of $pr55^{Gag}$ targets it to the plasma membrane. More specifically, this modification inserts Gag into the lipid bilayer [68, 84, 165]. This process is very rapid such that studies have shown that $pr55^{Gag}$ reaches the plasma membrane in about 5-10 minutes following its synthesis [166, 167]. Myristoylation and membrane anchoring is followed by the multimerization of Gag via the capsid (CA), nucleocapsid (NC) and the spacer peptide 1 (SP1) domains of $pr55^{Gag}$ where about 2500 Gag molecules form the sphere-like shape

of HIV-1 particles [168-170]. Interactions between HIV-1 viral RNA and $pr160^{GagPol}$ to the NC and CA regions of $pr55^{Gag}$ respectively lead to their incorporation into budding virus particles. The selectivity of HIV-1 genomic RNA in virus particles is thought to be mediated by the encapsidation signal (Psi, ψ). This *cis* acting sequence, no less than 100 nucleotides in length, binds to the NC region of Gag [75, 171]. Two single-stranded RNA molecules linked together by the dimer initiation site (DIS) are incorporated per virion. Interestingly, studies have shown that the morphology of budding viruses in the presence of ψ-deficient HIV-1 RNAs is normal but cellular RNAs are packaged unspecifically [171]. Since the HIV-1 genomic RNA and cellular RNAs have the same features, 5'Cap and 3'polyA tail, this highlights that the presence of the ψ signal is an advantage and a recognition sequence for its encapsidation over cellular RNAs [171, 172]. Many cellular proteins are incorporated into virus particles through their association with $pr55^{Gag}$ or with the genomic RNA. The envelope, the only viral protein which enters the endoplasmic reticulum (ER), moves to the plasma membrane. Once all the viral components are incorporated, the plasma membrane undergoes an outward budding caused by the CA core [68, 84, 173]. HIV-1 budding requires the p6 domain of $pr55^{Gag}$, more specifically the sequences PTAP and YPLTSL in its L (late) domain which recruit the ESCRT (endosomal sorting complex required for transport) machinery for efficient budding [174].

Maturation, which begins during the process of budding, of virus particles is mediated via the viral enzyme protease which causes a stepwise cleavage process of the HIV-1 Gag viral proteins leading to a major morphological change and infectious virus particles [68, 84, 173].

1.6 The up-frameshift (UPF) proteins and their roles in cell metabolism

1.6.1 UPF proteins

The human UPF proteins were first identified and characterized in the yeast *Saccharomyces Cerevisiae*. Their roles have been intensely studied in the RNA quality control mechanism nonsense-mediated mRNA decay (NMD) discussed in Section 1.6.2 [175-178].

UPF1, which is 1118 amino acids, is an ATP-dependent RNA helicase belonging to the SF1 family of RNA helicases [175, 179, 180] (Figure 5). The cellular abundance of UPF1 is about $4\pm2X10^6$ molecules per HeLa cell, the most abundant of UPF proteins, which corresponds to approximately one UPF1 molecule per 3 ribosomes [175]. It is monomeric in function and has been shown in polysome and ribosome free fractions [181]. UPF1 knock out mice are embryonically lethal thus making it an essential survival gene [182]. The similarity between the conserved region (mid-region of the protein) between yeast (853 amino acids) and human UPF1 is 58%. This conserved region has two zinc fingers rich in cysteine and histidine (CH region) and the seven helicase motifs [179]. The zinc fingers are suggested to be sites for RNA interaction, between UPF1 and ribosomes as well as binding sites for instability (INS) RNA sequences and cellular proteins (eg UPF2, Staufen) [176, 183]. The CH region in yeast has a RING-like structure, and identified UPF1 as being a E3 ubiquitin ligase leading to its self-ubiquitination; and this activity required UPF3b [184]. UPF1 also interacts with eRF1/3

(eukaryotic translation release factor), a process important during NMD (reviewed in Section 1.5.2) and its ATPase activity spans two helicases motifs (Ia and II) [176, 177, 179]. UPF1 has a nuclear export (NES) and a nuclear localization (NLS) signal located between amino acids 55-416 and 596-697 respectively [185].

The main difference between yeast and human UPF1 proteins are the phosphorylation/dephosphorylation sites which are located at both ends and are missing in yeast UPF1. The C-terminal 83 amino acids are enriched in serine/glutamine and serine/glutamine/proline which are phosphorylated by the SMG1 (phosphatydylinositol 3-kinase related protein) kinase. The N-terminal 63 amino acids form a proline/glycine rich region that interacts with the SMG5 to dephosphorylate UPF1 [136, 179]. These sites are important for protein-protein and protein-RNA interactions. UPF1 has 28 S/T/Q sites mainly clustered at the C-terminal of the protein (14 sites) [181, 186]. UPF1 can be phosphorylated by numerous PIKKs (phosphoinositide-3-kinase-related protein kinase) (e.g., SMG1, ATM, ATR) and it is mainly dephosphorylated by the PP2A/SMG5/6/7 complex [136, 176, 177, 181, 186-188]. UPF2 and UPF3b were shown to be involved in UPF1 phosphorylation. Studies have shown that the cycle of phosphorylation and dephosphorylation of UPF1 is important for maintaining RNA/DNA stability [189, 190]. UPF1 is mainly cytoplasmic but it shuttles via CRM1 and there are numerous studies that characterize a nuclear role of UPF1 [185]. UPF1

FIGURE 5: *The structure of the UPF proteins.*

The structure of UPF1, UPF2, UPF3b and UPF3b are shown. They all harbour an NES and NLS signal. UPF1 contains two zinc fingers and several helicase domains. UPF2 has three MIF4G (middle portion of eIF4G) sites. UPF3b and UPF3a contain RRM (RNA recognition motif) motifs. UPF3b exon 8 skipping lacks residues 270-282 and UPF3a exon 4 skipping lacks residues 117-149.(see text for more detail) [178, 179, 185, 191, 192].

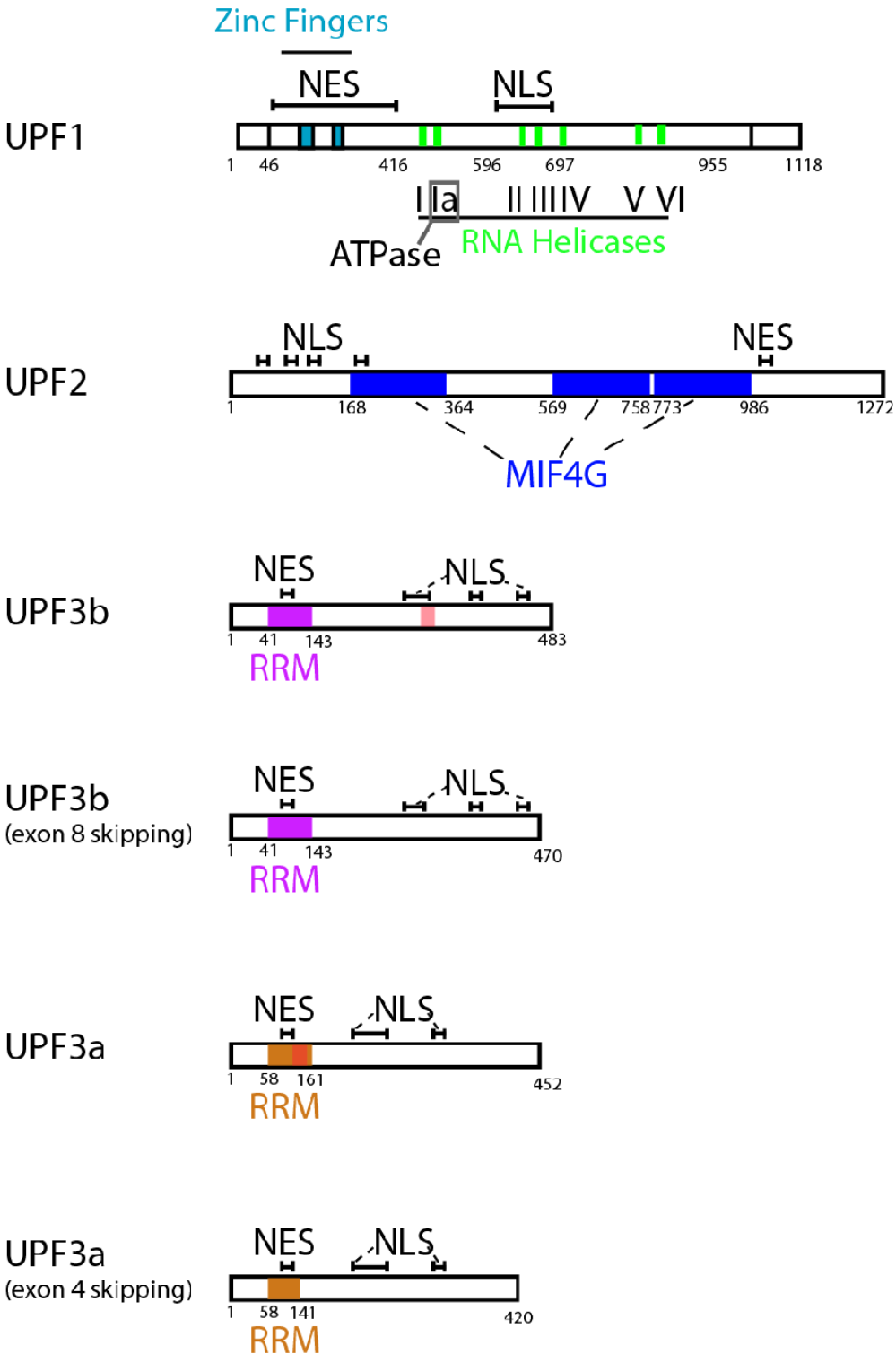

FIGURE 5: *The structure of UPF proteins.*

is involved in DNA replication as well as cell cycle progression because reduction in UPF1 expression leads to an S-phase arrest. UPF1 also associates with chromatin and DNA polymerase delta [189, 190, 193] and directly interacts with RNAPII [194]. It is also involved in histone mRNA degradation and *Xist* mRNA regulation [137, 195]. UPF1 is also involved in telomere maintenance because UPF1 protein decrease results in telomere damage by causing TERRA (telomeric repeat containing-containing RNA) accumulation [194, 196]. Yeast-two hybrid studies have also shown that UPF1 and the nucleoporin Nup100/116 (hNup98) interact [197], a nucleoporin involved HIV-1 genomic RNA export.

UPF2 is a phosphoprotein of 1272 amino acids localized to the nuclear periphery [176, 177, 192, 198, 199]. UPF2 is less abundant than UPF1 in HeLa cells, about $2\pm1X10^5$ molecules [175]. It contains three eIF4G-like (MIF4G: middle portion of eIF4G) domains and more than one NLS domain (amino acids 1-120). It also has an NES signal at the C-terminal of the protein. UPF2 interacts with UPF3b via the last MIF4G domain and with UPF1 via its C-terminus (amino acids 1084-1272) [192]. The N-terminal of UPF2 also interacts with UPF1 (amino acids 94-133) and contains serine residues which are phosphorylated, most probably via SMG1 [192, 200]. Nevertheless, the C-terminal of UPF2 is the major UPF1 binding region [201]. UPF2 also binds RNA in vitro; most likely this interaction happens during the process of splicing. UPF2 is involved in UPF1 phosphorylation, a process important for NMD and UPF1 binding to RNAs [176, 177]. It was also shown that UPF2 binding to UPF1 decreases UPF1's ability to bind single stranded RNA [198].

UPF3, the least conserved of the yeast UPF proteins, contains two genes: UPF3a (452 amino acids) and UPF3b (483 amino acids) [175, 192]. They show 60% similarity with their N-termini being highly conserved (86%) while their C-termini display differences. Interestingly, in S. cerevisiae, there is only one isoform of UPF3. These phosphoproteins are present at low levels, compared to UPF1 in HeLa cells, $8\pm2X10^4$ for UPF3a and $4\pm2X10^4$ for UPF3b [175, 192]. They are mainly nuclear proteins and contain NES and NLS signals. Both UPF3 isoforms interact with UPF2 via the N-terminal RBD domain and they are both recruited to RNAs during the process of splicing like UPF2 [176, 177, 191]. Therefore, UPF3 proteins as well as UPF2 bind spliced mRNAs. A recent study demonstrated that they both compete for binding to UPF2 and that there was a unidirectional regulation between UPF3b and UPF3a such that the association of UPF3b to UPF2 results in a decrease in UPF3a protein levels but this has no effect on its RNA [202]. When UPF2 binds UPF3a and not UPF3b, UPF3a protein levels are stabilized [202].

Both UPF3 isoforms have different roles in NMD. UPF3a only weakly triggers NMD when compared to the effect elicited by UPF3b [191]. UPF3a lacks an important residue in its C-terminal domain that is crucial in UPF3b-mediated NMD [176, 191]. Both UPF3b and UPF3a undergo alternative splicing generating exon8 (amino acids 270-282) and exon4 (117-149) skipping respectively [192]. These different isoforms result in the formation of different complexes. Both isoforms of UPF3a, UPF3aL and UPF3aS (exon 4 skipping isoform) are found in different UPF1 complexes [136]. UPF3aL preferentially associates with a

complex containing phosphorylated UPF1 (P-UPF1) and UPF2 which is called a post-phosphorylation complex and another complex called the pre-dephosphorylation complex formed with UPF3aS, PP2A, P-UPF1 and SMG5/7 [136, 203].

1.6.2 RNA surveillance mechanisms: Staufen-mediated decay (SMD) and nonsense-mediated decay (NMD)

Numerous regulation pathways ensuring proper gene expression have evolved in order to ascertain that deleterious proteins are not expressed. Nonsense-mediated mRNA decay (NMD) is one such RNA surveillance mechanism that degrades pretermination codon (PTC) containing transcripts. DNA and RNA transcripts can both harbour PTCs arising from mutations or transcriptional errors respectively. Interestingly, only 3-10% of cellular mRNAs are regulated by NMD [176, 177, 204].

The exon-junction complex (EJC) complex is about 350kDa and comprises at least 10 different proteins which get deposited 20-24 nucleotides upstream of exon-exon junctions following splicing. Furthermore, EJCs were discovered from the simple fact that normal stop codons, which are located in the final intron, do not undergo NMD and how intronless genes do not go through NMD. Thus, it was discovered that during the process of splicing, the spliceosome leaves behind a complex (EJC) of proteins which serves as the second signal for NMD and that

this complex is present on mRNA transcripts in the cytoplasm thus linking nuclear and cytoplasmic events [176, 177, 205].

The EJC is a network of cellular proteins (Figure 6) [176, 177, 205, 206]. The EJC core proteins are Y14, MAGOH, eIF4AIII and BTZ (Barents, MLN51 or CASC3). EIF4AIII is an RNA helicase mainly localized in the nucleus. It is a shuttling protein whose role is to stabilize the EJC core and links the EJC to RNAs. Following mRNA splicing, both Y14 and MAGOH, which are mainly nuclear, are deposited next to exon-exon junctions and stay on mRNAs following their nucleocytoplasmic export [176, 177, 205]. BTZ binds RNAs with or without eIF4AIII and also interacts with Staufen in neuronal cells. This tetrameric complex of eIF4AIII, MAGOH, Y14 and BTZ is surrounded by an outer shell of proteins including Acinus, RPNS1, SAP18, Pinin and Aly/REF. All outer shell proteins are shuttling proteins with the exception of Acinus and Pinin which are strictly nuclear. This outer shell is then surrounded by transiently interacting factors such as UPF1, 2, 3a/3b, Srm160, UAP56 and p15/NXF1 [176, 177, 205].

The three main players in NMD are the UPF proteins. A pretermination codon is seen as nonsense when it is located more than 50-55 nucleotides upstream of the last exon junction as well as being in frame with the initial ATG. During the pioneer round of translation, ribosomes travel along the mRNA and displaces EJCs until it reaches a stop codon. Once the ribosome reaches the stop codon, if there is no EJC following this, then normal termination of translation occurs. [176, 177, 183, 198, 205, 207, 208].

FIGURE 6: *The exon junction protein complex.*

The EJC protein complex has an inner core containing the proteins Y14, Magoh, eIF4AIII and MLN51. This is surrounded by an outer shell of Acinus, Pinin, Aly/REF, Sap18 and RNPS1. The UPF proteins, Srm160, NXF1/p15 and UAP56 are loosely attached around the outer shell. (see text for more detail) [176, 177, 205, 206].

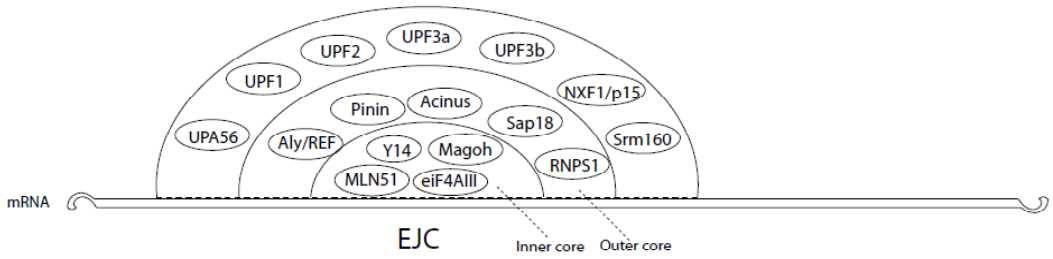

FIGURE 6: *The exon junction protein complex.*

It is expected that NMD should take place during the pioneer round of translation since erroneous protein translation is not desired. Interestingly, non-mammalian NMD does not take place at this step and thus deleterious protein translation still occurs. In the case where a PTC is present, following transcription, the main core and outer shell proteins bind to the newly transcribed RNAs before they are exported into the cytoplasm. During the process of export, UPF3a/b, followed by UPF2 are recruited to the EJC. The presence of the EJC stalls the ribosome on the mRNA, resulting in the recruitment of UPF1 (and SMG1) to the termination factors eRF1/3 to form the SURF complex comprised of the translation termination factors eRF1 and eRF3, UPF1 and the kinase SMG1 [176, 177, 183, 198, 205, 207, 208]. This recruitment of UPF1 to the aberrant mRNA forms a link between the ribosome and the EJC through the interaction between UPF1/SMG1 and UPF2 resulting in a decay-inducing complex (DECID) which in turn phosphorylates UPF1 via the kinase SMG1. It is the recruitment of UPF1 to the PTC containing mRNA via UPF2 and its phosphorylation that targets the PTC-mRNA for decay via NMD [176, 177, 183, 198, 205, 207, 208].

One of the differences between UPF3a and UPF3b is that UPF3a only weakly triggers NMD [191]. Also, UPF2, UPF3b and splicing independent NMD branches have also been characterized. In these alternative branches of NMD, recruitment of UPF1 to the aberrant mRNA via different factors results in their degradation [176, 208]. Moreover, it was shown that the one point mutation of R844C in UPF1 abolished NMD [209]. In addition, T-cell receptor is an exception to the classical NMD pathway such that NMD is elicited even though

the PTC is located less than 50-55 nucleotides upstream of the exon-exon junction.

It was recently shown that NMD also targets endogenous RNAs which do not contain a PTC but nevertheless contain some features making them substrates for this RNA quality control mechanism [176, 208]. Interestingly, not all transcripts containing PTCs undergo NMD such as when a PTC is located very close to the initiation start or termination codons [176, 208].

NMD has also been linked to human diseases. [176, 204]. Disease associated mutations are estimated to be linked to about 30% of mRNAs harbouring PTCs. PTCs can also be inherited in the case of genetic diseases. Thus, a link between nonsense mutation and genetic disease as well as cancer has been established. The link between NMD and disease occurs when a PTC-deficient or a PTC-containing transcript is degraded or not respectively. The presence of a PTC, regardless if it is through point mutation in an intron or exon or a frameshift mutation, is the synthesis of truncated proteins. β-Thalassemia, a haemoglobin production disorder, is an example where NMD exerts a protective function by degrading the truncated actin protein whose presence can be detrimental. On the other hand, NMD degradation of the dystrophin gene is an example of detrimental NMD effects because it leads to a more severe disease outcome. Cancer and NMD have also been linked such that PTCs in the tumour suppressor genes WT1, p53 and BRCA1 leads to their degradation [176, 204].

It has long been a debate if NMD occurs only in the cytoplasm since this process was termed as being translationally-dependent [210]. Interestingly, by using the live cell imaging technique, the Mühlemann group recently showed that NMD does indeed occur in the nucleus at transcription sites [210]. Moreover, the same group showed in another recent study that NMD factors are autoregulated such that the NMD factors which have a long 3'UTR elicit NMD; thus indicating that the NMD pathway undergoes autoregulation [211]. A recent link between NMD and the microRNA pathway was made [212]. The miRNA miR-128, mainly expressed in the brain, represses NMD by decreasing UPF1 and MNL51 levels [212].

UPF1 is also involved in Staufen-mediated mRNA decay (SMD). Unlike NMD, SMD degrades transcripts that contain Staufen1 binding sites (SBS) (19 base pairs), which are located in the 3'UTR of selective mRNAs [213]. Briefly, when an mRNA contains a SBS site, Staufen1 binds and recruits UPF1 [213]. The recruitment of UPF1, like in NMD, triggers the decay of these SBS containing transcripts. Transcriptome studies have shown that about 1% of transcripts were upregulated when Staufen1 was deleted resulting from the inhibition of this process [213] [183]. This degradation mechanism is also translational-dependent. Staufen1 and UPF2 compete for the same binding site on UPF1 [183]. This mutually exclusive binding confirms that NMD and SMD pathways compete [183].

UPF1 also participates in other RNA quality control mechanisms such as with ARE-mediated decay and was shown to associate with the nuclear and cytoplasmic exosomal complex[176, 204, 214]. The involvement of the UPF proteins in different nuclear and cytoplasmic mRNA quality control mechanisms shows that the UPF proteins, especially UPF1, are involved in maintaining cell survival [176, 204, 214].

1.6.3 UPFs and Retroviruses

Retroviruses rely on different host cell machineries and cellular proteins at different stages of the replication cycle. Therefore, NMD and the proteins involved in NMD can be seen as a threat to retroviruses subjecting their genomic RNAs for degradation. Retroviruses, like RSV (Rous Sarcoma Virus) and HIV-1, contain RNA features such as long 3'UTR and several open reading frames making them ideal NMD substrates [215-219]. However, these retroviruses have found ways to counteract this mRNA quality control mechanism.

The presence of a PTC in the *gag* open reading frame of the RSV unspliced genomic RNA renders it susceptible to NMD and degradation in a UPF1- and translation-dependent manner [219]. Interestingly, the RSV genomic RNA contains a stability element called RSE (RSV stability element) which protects the RSV genomic RNA from becoming an NMD substrate [215, 216, 218]. It is yet to be determined if one or more cellular proteins which renders the genomic RNA immune to this RNA quality control mechanism [215, 216, 220].

A recent proteomic study of cellular proteins bound to the nuclear HIV-1 genomic RNA immediately following transcription identified UPF1 [164]. Interestingly, this points to a nuclear role of UPF1 in HIV-1 genomic RNA regulation (discussed in Chapters 2 and 3). Generally SF1 RNA helicases are encoded by (+) single stranded viruses [221]. UPF1 is one such SF1 RNA helicase whose functions might be co-opted by HIV-1 to evade NMD.

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Chapter 2

Chapter 2: research hypothesis and specific aims

It was initially hypothesized that Staufen1 might be involved in HIV-1 RNA metabolism. However, studies from our laboratory identified that Staufen1 was not implicated in HIV-1 RNA decay but at other levels including Gag RNA translation and RNA encapsidation.

In 2003, Staufen1 was found in neuronal RNA trafficking granules where it co-localized and co-immunoprecipitated with Barentsz [1]. Barentsz, a component of the exon-junction complex (EJC), was shown to be involved in nonsense-mediated decay (NMD), such that knockdown of Barentsz resulted in the stabilization of pretermination codon (PTC)-containing RNAs, demonstrating that Barentsz is an important factor, like UPF1, in nonsense-mediated mRNA decay (NMD) [2].

In light of these new results, we thought that there might be link between NMD and HIV-1 because the HIV-1 genomic RNA might also be a substrate for NMD because it contains multiple open reading frames and a long 3'UTR. We therefore turned some of our attention to the NMD protein, UPF1, and its role in HIV-1 replication. Remarkably, Staufen and UPF1 were found to associate in a bimolecular complex in a RNA quality control mechanism termed Staufen1-mediated decay (SMD).

The first results showed that Upf1 depletion influenced HIV-1 genomic RNA levels and formed the basis of the study of Upf1 function during HIV-1 replication described in this chapter.

Chapter 2

Unexpected roles of UPF1 in HIV-1 RNA metabolism and translation

Unexpected roles for UPF1 in HIV-1 RNA metabolism and translation

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Published in :RNA (2008), 14:914-927.

ABSTRACT

The HIV-1 ribonucleoprotein (RNP) contains the major structural protein, $pr55^{Gag}$, viral genomic RNA, as well as the host protein, Staufen1. In this report, we show that the nonsense-mediated decay (NMD) factor UPF1 is also a component of the HIV-1 RNP. We investigated the role of UPF1 in HIV-1-expressing cells. Depletion of UPF1 by siRNA resulted in a dramatic reduction in steady-state HIV-1 RNA and $pr55^{Gag}$. $Pr55^{Gag}$ synthesis, but not the cognate genomic RNA, was efficiently rescued by expression of an siRNA-insensitive UPF1, demonstrating that UPF1 positively influences HIV-1 RNA translatability. Conversely, overexpression of UPF1 led to a dramatic up-regulation of HIV-1 expression at the RNA and protein synthesis levels. The effects of UPF1 on HIV-1 RNA stability were observed in the nucleus and cytoplasm and required ongoing translation. We also demonstrate that the effects exerted by UPF1 on HIV-1 expression were dependent on its ATPase activity, but were separable from its role in NMD and did not require interaction with UPF2.

INTRODUCTION

A principal player in nonsense-mediated mRNA decay (NMD), a process by which RNAs that harbor premature termination codons are cleared from cells, is upframeshift protein 1, or UPF1. UPF1, an ATP-dependent RNA helicase of the SFI superfamily, is required for NMD and for efficient translation termination at nonsense codons in eukaryotes. UPF1 is also involved at various levels of RNA fate such as RNA splicing, transport, translation, and mRNA turnover [3-7]. UPF1 is a component of several functional ribonucleoprotein (RNP) complexes in cells. In NMD, for example, UPF1 is recruited by UPF2, which is, in turn, recruited by UPF3 to make up an NMD RNP complex on RNA. UPF1 is also a component of the SMG-1-UPF1-eRF1-eRF3 (SURF) RNP complex [8] that is functionally important during the activation of NMD by linking the terminating ribosome to downstream exon junction complexes. This RNP is also important for RNA degradation via NMD and translational activity. Recent results have uncovered a role for an additional RNP complex that is formed when UPF1 and the double stranded (ds) RNA-binding protein Staufen1 interact on an RNA. Staufen1 can bind a structured RNA sequence in the 3'-UTR of an mRNA and can recruit UPF1 to shunt an mRNA into a decay pathway called Staufen1-mediated decay (SMD) [9].

Staufen1 has roles in RNA trafficking, transport, and translation. It is found in neuronal RNA trafficking granules that contain RNA and many types of proteins

including motor proteins, translation factors, and several proteins involved in the fate of RNA in the cell [10]. Barentsz (Btz) is one such co-resident protein in RNA trafficking granules or RNPs, and it was demonstrated that Staufen1 and Btz interact [1]. Btz was later found to be a component of the exon junction complex (EJC), a complex that is deposited upstream of exon–exon junctions prior to splicing of primary transcripts. Furthermore, in experiments in which Btz was knocked down by siRNA, Btz, like UPF1, was found to be an important factor for NMD [2]. These results point toward tight functional links between EJC proteins in splicing, RNA trafficking, and RNA metabolism [11, 12].

HIV-1 RNA metabolism is controlled by a variety of cis-acting sequences in its 9-kb HIV-1 genomic RNA [13]. It was first noted that the genomic HIV-1 RNA has an AU-rich codon bias and a rare codon usage [14]. Multiple purine-rich sequences named cis-repressive sequences or instability sequences (CRS/INS) were also identified in the genomic RNA [15]. These sequences are bound by hnRNP A1, polypyrimidine tract binding protein (PTB), poly(A)-binding protein (PABP), and polypyrimidine tract-binding protein-associated splicing factor (PSF) to regulate HIV-1 gene expression at the post-transcriptional level [16, 17]. Tethering of other proteins such as the K homology splicing regulatory protein to HIV-1 RNA can also elicit RNA destabilization [18].

Rev is the key mediator of nucleocytoplasmic transport of the unspliced, genomic, and singly spliced HIV-1 RNAs via a specific interaction with the Rev-responsive element (RRE) in these RNAs [19]. This is achieved via the CRM1-dependent

pathway [20]. The Rev–RRE interaction counteracts the activities of the CRS/INS by conferring stability to viral transcripts and also promotes polysomal loading in the cytoplasm [15, 21]. Rev can also inhibit splicing of genomic RNA [22], but in its absence, HIV-1 genomic RNA is retained in the nucleus and is completely spliced to generate 2-kb RNAs. The post-transcriptional regulation of HIV-1 gene expression implicates cis-acting RNA sequences and the activities of viral and host cell RNA-binding proteins. HIV-1 may coopt the functions of these proteins, and this may offer the virus a replicative advantage and/or the ability to enter a state of low or latent expression in the host [23].

The association of Staufen1 to proteins belonging to the EJC and implicated in NMD was intriguing to our group. Our observation that the abundance of UPF1 was enhanced in the HIV-1 RNP led us to examine the function of UPF1 during HIV-1 gene expression. In this study, we depleted cells of UPF1 by siRNA and show that while UPF1 knockdown resulted in suppression of NMD as expected, there was also a catastrophic decrease in HIV-1 RNA and pr55Gag expression. We rescued $pr55^{Gag}$ expression using an siRNA-insensitive UPF1 expression construct, but HIV-1 mRNA levels could not be rescued, indicating that UPF1 enhances HIV-1 mRNA translatability. Conversely, overexpression of UPF1 led to enhanced levels of $pr55^{Gag}$ and steady-state HIV-1 RNA. These effects were found in both nucleus and cytoplasm and were found to be dependent on ongoing translation of the genomic (or gag) mRNA. Finally, we demonstrate that UPF1's role in HIV-1 expression is separable from its role in NMD. This study identifies

novel functions for UPF1 in the maintenance of HIV-1 RNA stability and protein synthesis.

RESULTS

Identification of UPF1 in the HIV-1 RNP

In order to characterize cellular binding partners of Staufen1, we generated a cell line that stably expresses a 75-kDa TAP-tagged Staufen1 protein following transfection of a CMV/Staufen1-TAP construct that is described in detail elsewhere [24]. Of 12 independent clones, two expressed Staufen1-TAP at high levels (Fig. 7A, #11,#12). A control stable cell line was also clonally expanded that expressed the TAP tag alone (Fig. 7A, TAP control cell line). The expression of TAP in these cell lines was confirmed using mouse anti-Protein A antisera (M. Milev and A.J. Mouland, unpubl.). Staufen1-TAP clone #11 was selected because Staufen1 was expressed at levels similar to those observed for endogenous Staufen1^{55kDa}. We investigated the binding partners of Staufen1 using the TAP purification method as described previously [24]. TAP- and Staufen1-TAP-expressing cell lines were transfected with HIV-1 proviral DNA and processed for TAP purification at 40 h post-transfection. Western blot analysis was performed to identify Staufen1-TAP before affinity purification (Staufen1-TAP) and post-elution to identify the fusion protein between Staufen1 and the remaining calmodulin binding domain (Staufen1-CBD) to the TEV protease cleavage site. The 75-kDa Staufen1-TAP was well expressed in cells (Fig. 7B). Staufen1-CBD was identified in the Staufen1-TAP extracts to demonstrate efficient affinity purification. Previously, we showed that Staufen1 principally binds the precursor,

55-kDa Gag, $pr55^{Gag}$ [25]; therefore, we validated this assay by probing the Staufen1-TAP affinity eluates for Gag proteins. An anti-p24 antiserum was used to detect $pr55^{Gag}$ as well as the mature Gag proteins, pr41 and p25/p24. $Pr55^{Gag}$ and mature Gag products were detected in cell lysates, but only $pr55^{Gag}$ was detected in the eluates. This result validated the assay and demonstrates the selectivity of this virus–host interaction [25]. The results are representative of five experiments.

We then determined if we could identify UPF1 in the HIV-1 RNP complex. UPF1 was abundantly expressed in cell lysates, and a strong band corresponding to UPF1 (130 kDa) was detected in the eluates derived from Staufen1-TAP-expressing cells, but not from TAP control cell lines. This was confirmed by mass spectrometry of the eluates (M. Milev and A.J. Mouland, in prep.). We also probed for a binding partner of UPF1, UPF2, but we could not detect it even though UPF3 was detectable in the Staufen1 eluates by Western blot analyses (data not shown).

The presence of UPF1 in the $pr55^{Gag}$ complex was then confirmed by immunoprecipitation, and effects of UPF1 depletion on the interaction were examined. Cells were mock transfected, transfected with HIV-1 proviral DNA, pNL4-3 both with a nonsilencing, control siRNA (siNS) or transfected with pNL4-3 and siRNA to UPF1 (siUPF1) to deplete UPF1 from cells (Fig. 7C). In cell lysates, a UPF1 signal was found in siNS-transfected cells (in both mock and pNL4–3 transfected), but it was dramatically reduced in UPF1-depleted cells.

$Pr55^{Gag}$ expression was abundant in siNS-treated cells, but decreased by 75% in UPF1-depleted cells (see additional data in Fig. 8). Glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) served as a loading control, and the amount of GAPDH in the input samples did not differ. $Pr55^{Gag}$ was immunoprecipitated from cell lysates, and the immunocomplexes were fractionated on SDS-PAGE gels followed by Western blotting for UPF1, $pr55^{Gag}$, and GAPDH. A prominent UPF1 signal was observed in the $pr55^{Gag}$ immunoprecipitate from pNL4-3/siNS-treated cells, but not from mock-transfected or UPF1- depleted cells (Fig. 7C). $Pr55^{Gag}$ levels were decreased in siUPF1 conditions, and this was also reflected by the negligible signal for UPF1 in the immunoprecipitate. The absence of GAPDH in the Gag immunoprecipitate demonstrated that the interaction was selective. Based on the loading and signal intensities, we calculated that ~0.1% and 5% of cellular UPF1 and Gag, respectively, was found in the Staufen1-TAP eluates. The abundance of $pr55^{Gag}$ in Staufen1-TAP eluates is consistent with our live-cell interaction analyses in which a small proportion of Gag was shown to interact with Staufen1 [25].

Laser scanning confocal microscopy (LSCM) was performed to identify the cellular locations of UPF1 and Gag in HIV-1-expressing cells. In mock-transfected cells, UPF1 exhibited diffuse, cytoplasmic staining (Fig. 7D, top panels) and there was no detectable signal for Gag. When HIV-1 was expressed by transfection of a proviral DNA construct, UPF1 staining was again mostly diffuse in the cytoplasm, and Gag was found in the cytoplasm as well as at the cell periphery, presumably representing assembly sites for HIV-1. We calculated that

~10%–20% of the UPF1 signal colocalized with Gag when analyzing entire cells. However, in almost 70% of cells with detectable Gag expression, UPF1 was also found to colocalize with Gag at the cell periphery in the form of small punctae (Fig. 7D, bottom panels). Sixty-five percent of the UPF1 in this region of the cell strictly overlapped with the Gag. These results put a proportion of UPF1 and Gag in the same cellular locale and would explain why UPF1 coimmunoprecipitates with Gag. The result also suggests that UPF1 may have a continued role during virus assembly.

UPF1 depletion results in decreased pr55^{Gag} synthesis and a catastrophic loss of HIV-1 RNA

To assess the function of UPF1 in HIV-1-expressing cells, siRNA was used to deplete cells of UPF1. Stable HeLa cell lines were used that contain a T-cell receptor-β (TCR-β) minigene with either a natural termination codon (PTC-, 3C1) or a premature termination codon (PTC+, 7C3) in the second VDJ exon (Fig. 8A; [26]). In order to confirm the sensitivity to ongoing translation of the TCR-β NMD substrate [27, 28], both cell lines were mock transfected or treated with cycloheximide (CHX). The TCR-β and *gapdh* mRNAs were analyzed by Northern blotting. As shown in Figure 8A, the TCR-b mRNA lacking a PTC was abundantly expressed in 3C1 cells. However, in PTC+ cells, levels of the TCR-β mRNA were increased only when cells were treated with CHX to block translation, demonstrating the dependence of NMD on ongoing translation. UPF1

expression levels were constant in these cell lines, showing that the NMD effect of CHX treatment was not caused by impaired UPF1 expression.

These cell lines were employed to explore the effects of UPF1 on HIV-1 expression and to have a homologous control for UPF1 function in NMD. 7C3 cells were mock transfected with siNS or siRNA to UPF1 (siUPF1) without proviral DNA (Mock-siNS, Mock-siUPF1), or with proviral pNL4-3 DNA (Fig. 8B, lanes 3–8) and Flag DNA (empty vector) and siNS or siUPF1 (Flag-siNS or Flag-siUPF1), $UPF1^{WT}$, and siNS or siUPF1 ($UPF1^{WT}$-siNS or $UPF1^{WT}$ -siUPF1) or $UPF1^{Rescue}$ and siNS or siUPF1 ($UPF1^{Rescue}$-siNS or $UPF1^{Rescue}$-siUPF1). UPF1, UPF2, $pr55^{Gag}$, and GAPDH expression levels were assessed by Western blot analysis. UPF1 knockdown was efficient and routinely ranged from 85% to 98% in most experiments (Fig. 8B, lanes 2,4,6) even when UPF1 was supplied *in trans* (Fig. 8B, lane 6). When cells were transfected with siUPF1, a significant reduction in $pr55^{Gag}$ was observed (Fig. 8B, cf. lanes 3,4). Cells either expressing $UPF1^{WT}$ or the siRNA-insensitive, $UPF1^{Rescue}$ *in trans* led to a threefold to fourfold up-regulation of $pr55^{Gag}$ expression levels (Fig. 8B, lanes 5,7). In cells depleted of UPF1, however, $UPF1^{Rescue}$ expression modestly rescued $pr55^{Gag}$ synthesis by 60% (±10%, SD; seven experiments) relative to $UPF1^{Rescue}$-siNS-transfected cells, indicating that UPF1 expression was important for the observed effects on $pr55^{Gag}$ expression (Fig. 8B, lane 8). The dramatic effects of UPF1 depletion on $pr55^{Gag}$ synthesis were reflected by a corresponding 20-fold decrease in virus production (data not shown). These effects on HIV-1 gene expression were absolutely identical in 3C1, parental HeLa, and other human epithelial cell

types (data not shown). Similar results were obtained using another set of siRNAs providing further evidence that the observed results were not due to offtarget effects.

When TCR-β and HIV-1 RNA levels were quantitated by Northern analyses, PTC-containing TCR-β mRNA levels responded accordingly such that they were up-regulated when cells were depleted of UPF1 (Fig. 8C, lanes 2,4,6,8). In contrast, HIV-1 mRNA levels were dramatically down-regulated to near undetectable levels (Fig. 8C, lanes 4,6; Fig. 15).

Overexpression of UPF1 by expression of $UPF1^{WT}$ or $UPF1^{Rescue}$, up-regulated HIV-1 RNA severalfold, corresponding to UPF1's effects on $pr55^{Gag}$ synthesis (Fig. 2B, lanes 5,7). $UPF1^{Rescue}$ was not able to efficiently rescue the HIV-1 RNA levels, despite the modest 60%–70% rescue of TCR-β NMD-mediated degradation (Fig. 2C, lane 8). In contrast, a modest rescue was obtained for $pr55^{Gag}$ synthesis (Fig. 8B, lane 8), suggesting that UPF1 influenced HIV-1 mRNA translation efficiency. These results are representative of at least 10 experiments. Increasing doses of $UPF1^{Rescue}$ in transfections partially rescued $pr55^{Gag}$ levels (Fig. 16).

A more detailed analysis was performed to evaluate the importance of UPF1 expression at several time points following transfection. Cells were transfected with either the empty vector Flag or $UPF1^{Rescue}$ in the presence of siNS or siUPF1. Protein and RNA analyses were performed at 6, 12, 24, and 30 h post-

transfection. UPF1 expression was stable, and $pr55^{Gag}$ and HIV-1 RNA levels increased with time in siNS- and Flag-transfected cells (Fig. 9A, lanes 1–4). There was a time-dependent knockdown of UPF1 mediated by siUPF1, and this resulted in dramatically reduced levels of steady-state HIV-1 mRNA and $pr55^{Gag}$ expression (Fig. 9A, lanes 5–8). Overexpression of $UPF1^{Rescue}$ (in the presence of siNS) greatly enhanced UPF1 levels and enhanced $pr55^{Gag}$ and steady-state HIV-1 genomic RNA levels 3.4-fold and fivefold, respectively (±10%, SD in both cases) (Fig. 9A, lanes 9–12). However, while the rescue of UPF1 expression in siUPF1-depleted cells was efficient and resulted in enhanced $pr55^{Gag}$ synthesis, there was not a corresponding change in the abundance of the levels of steady-state HIV-1 RNA (Fig. 9A, lanes 13–16). The line graph shown in Figure 9B summarizes the relative changes in HIV-1 RNA at each time point. The relative rescue ratios presented in Figure 9C show that despite an almost 80% rescue of $pr55^{Gag}$ levels (relative to maximal expression levels obtained with $UPF1^{Rescue}$-siNS), the levels of HIV-1 RNA only reached 10%–20% at the latest time points studied. These results demonstrate that UPF1 expression almost completely rescues $pr55^{Gag}$ synthesis, but it is not sufficient to efficiently rescue defects at the RNA level.

Effects on HIV-1 expression require ongoing translation of gag mRNA

We then determined if the observed effects of UPF1 depletion required ongoing HIV-1 RNA translation. We approached this question by expressing proviral HIV-1 DNAs that express RNAs that are programmed to either terminate translation immediately following *gag* RNA translation initiation or at a

downstream site. We mock transfected cells (no proviral DNA), or transfected cells with wild-type pNL4-3, pNL4-XX (a proviral DNA harboring two PTCs in the *gag* ORF: the first preventing *gag* RNA translation initiation at the bona fide initiator AUG and the other programming translation termination following reinitiation at a downstream AUG) or with HxBRUΔp6 (harboring a PTC in the C-terminal p6 region following the ribosomal frameshifting site) (Fig. 10). In each condition, cells were treated with either siNS or siUPF1. UPF1 knockdown was extremely efficient, reaching >90% in all cases (Fig. 10, even-numbered lanes). siUPF1 had the expected negative effects on $pr55^{Gag}$ synthesis and steady-state RNA levels in cells expressing wild-type pNL4-3 (Fig. 10, lane 4). $Pr55^{Gag}$ synthesis in cells expressing pNL4-XX was aborted due to the in-frame stop codons. In cells expressing HxBRUΔp6, UPF1 depletion had little effect on the synthesis of the Gag gene product expressed from this DNA construct, prGagΔp6. In pNL4-XX- and HxBRUΔp6-expressing cells, steady-state mRNA levels were not modulated when UPF1 was depleted from cells (Fig. 10, lanes 6,8). These results indicate that the translation of the *gag* ORF or the completion of the translation of this *gag* ORF is necessary for the UPF1-mediated stabilization of HIV-1 RNAs. This result was corroborated by the observation that Gag expression was also negatively influenced by UPF1 depletion with the use of the pNL4-X proviral DNA in which translation of the wildtype AUG is blocked, but in which gag translation reinitiates at a downstream AUG ([29]; L. Ajamian and A.J. Mouland, unpubl.). Thus, PTCs within the *gag* ORF are not recognized by the NMD machinery and are required for the destabilization of the RNA when

UPF1 is depleted. These results also demonstrate that UPF1 depletion has no effect on LTR-mediated transactivation.

RNA destabilizing effects of UPF1 depletion in the nucleus

We next explored the cellular compartment in which the effects of UPF1 were being exerted. We took advantage of the nuclear retention of the HIV-1 genomic RNA when the regulatory protein Rev is not expressed. siNS- or siUPF1-treated cells were mock transfected, transfected with a wildtype HIV-1 proviral DNA (HxBRU), or transfected with a Rev- proviral DNA, pcMRev(-). Cells were harvested, and protein and RNA expression levels were monitored by Western and Northern analyses. Efficient knockdown of UPF1 resulted in decreased $pr55^{Gag}$ expression as shown earlier [the expression of pcMRev(-) does not lead to any appreciable $pr55^{Gag}$ synthesis] (Fig. 11A). Analysis of steadystate mRNA levels using a pol-specific cDNA probe revealed that, even during the expression of the pcMRev(-) HIV-1, steady-state levels of genomic RNA were efficiently knocked down by 85% as a result of UPF1 depletion (Fig. 11B). When the completely spliced 2-kb RNAs were assessed by Northern blot analysis, they were found to be relatively unaffected by siUPF1 conditions in the Rev- context.

In order to ensure that the genomic RNA was confined to the nucleus in these experiments, FISH analysis was performed. An identical probe sequence to that used for Northern analysis of the 9-kb RNA was used to identify genomic RNA by FISH. In nontransfected cells, there was no detectable signal for HIV-1

genomic RNA. In cells treated with siNS exhibiting expression of HIV-1, genomic RNA had a punctate cellular distribution as shown in our earlier work (Levesque et al. 2006). In pcMRev(-)-expressing cells, genomic RNA was confined to the nucleus and was abundantly expressed in siNS-treated cells, while in UPF1-depleted, pcMRev(-)-expressing cells, the RNA was confined to the nucleus, and the apparent RNA staining intensity was dramatically reduced (Fig. 11C). Since genomic RNA levels were significantly reduced in the absence or presence of Rev expression, we can conclude that both cytoplasmic and nuclear HIV-1 RNA are stabilized by UPF1 expression. Consistently, overexpression of UPF1 enhances steady-state levels of genomic RNA in the Rev- background (data not shown). It remains to be shown if the effects on nuclear RNA stability are due to common mechanisms involving UPF1.

UPF1 enhances HIV-1 RNA expression independently of its NMD function

UPF1 is involved in the regulation of a number of cellular processes besides its central function in NMD. To identify the regions of UPF1 that are required for the stimulation of HIV-1 RNA expression, we overexpressed a number of UPF1 mutants and analyzed their effects on HIV-1 $pr55^{Gag}$ and RNA levels (Table 3). As shown earlier, overexpression of $UPF1^{WT}$ led to a dramatic increase of HIV-1 RNA (Fig. 12, lane 2). In contrast, a severalfold overexpression of UPF3b had little effect on steady-state HIV-1 RNA levels (Fig. 12, lane 3), indicating that the effects on HIV-1 genomic RNA are specific to UPF1. To our surprise, the RNA helicase *trans*-dominant negative (TDN) mutant UPF1(R844C) that is well

established to be ineffective in NMD in mammalian cells [30], stimulated HIV-1 RNA expression to similar levels achieved with $UPF1^{WT}$ (Fig. 12, lane 4), indicating that UPF1 enhances HIV-1 RNA expression independently of its NMD function. In contrast, N-terminal ($UPF1^{\Delta 20–150}$, $UPF1^{\Delta N40}$) and C-terminal ($UPF1^{1–1074}$) truncations and deletions of UPF1 were unable to enhance levels of HIV-1 RNA expression (Fig. 12, lanes 5–7).

We attempted to further map the determinants for UPF1 up-regulation using UPF2 binding mutants ($UPF1^{RVD}$, $UPF1^{DI}$) and with a mutant with a similar mutation in the UPF2-binding region ($UPF1^{RVD}$) that retains its ability to bind UPF2 (Table 3). All of these mutants up-regulated $pr55^{Gag}$ expression levels (and RNA levels) (data not shown) similar to that obtained using intact UPF1 proteins (Fig. 13). Therefore, the association of UPF1 with UPF2, an event that is important for NMD though not strictly required (Table 3; [31]), was not required for the enhancement in HIV-1 RNA and $pr55^{Gag}$ expression levels.

We then performed experiments to establish if the effect of UPF1 on HIV-1 expression was due to the ATPase activity of UPF1 using a well-characterized ATPase mutant whose expression also blocks NMD. We overexpressed TDN, an additional helicase *trans*-dominant mutant, $UPF1^{RRAA}$, and the ATPase mutant, $UPF1^{DE}$. $UPF1^{WT}$, TDN, and $UPF1^{RRAA}$ up-regulated $pr55^{Gag}$ severalfold (Fig. 14) to levels that quantitatively correlated with genomic RNA levels (data not shown). The overexpression of $UPF1^{DE}$, however, only led to a 10% increase in $pr55^{Gag}$

levels on average from three experiments (Fig. 14), which indicated the importance of UPF1 ATPase activity in HIV- 1 expression.

DISCUSSION

Using complementary biochemical approaches including tandem affinity chromatography, immunoprecipitation, and confocal microscopy, we demonstrated that UPF1 is a component of the HIV-1 RNP, which also includes $pr55^{Gag}$, genomic RNA, and the host protein Staufen1. UPF1 coeluted with HIV-1 $pr55^{Gag}$ from a Staufen1-TAP column. Further analysis revealed that UPF1 coimmunoprecipitated with $pr55^{Gag}$, and also a proportion of cellular UPF1 colocalized with $pr55^{Gag}$ at the plasma membrane, assembly sites for HIV-1. A strong correlation between HIV-1 expression and this localization pattern was found in almost 70% of HIV-1-expressing cells, while in cells that did not have detectable HIV-1 expression, UPF1 was dispersed in the cytoplasm. These results suggest that UPF1 acts in the context of the HIV-1 RNP and that HIV-1 coopts UPF1 function in cells.

The most important finding of this study lies in the identification of a novel effect of UPF1 on HIV-1 RNA metabolism. siRNA-mediated depletion of UPF1 led to a dramatic negative effect on steady-state HIV-1 RNA levels (Figs. 8–11; Fig. 15), indicating that UPF1 is a principal player in maintaining the stability of HIV-1 RNA. The striking regulation of UPF1 on HIV-1 RNA is novel. Nevertheless, a role for UPF1 in the control of RNA stability of cellular mRNAs is not without precedence. For example, UPF1 was shown to affect the stability of both PTC- and non-PTC-containing mRNAs in earlier work [32, 33]. Recent studies

identified a role for UPF1 in the stability of the intronless histone H2a mRNA in a cell-cycle-dependent manner in mammalian cells [34] and an effect on *Xist* RNA, but in this latter case, this RNA is entirely restricted to the nucleus [35]. While there is at least one similarity between NMD and the effects of UPF1 on H2A and HIV-1 RNA, including the dependence on UPF1 expression and ongoing translation (Fig. 10), HIV-1 RNA degradation does not appear to be due to UPF1-mediated NMD. First, there was no increase in HIV-1 RNA when cells were treated with siUPF1. Second, current data support the notion that NMD is dependent on splicing and the deposition of an EJC [36, 37], suggesting that genomic RNA would not be a substrate, which is consistent with our findings. Third, despite evidence that extended 3'-UTRs may be recognized for EJC-dependent NMD [38, 39], our data demonstrate that UPF1 expression protects unspliced RNA from degradation, even with its almost 7-kb 3'-UTR. Fourth, recent work shows that the order of cotranscriptional intron removal from HIV-1 genomic RNA does not generate substrates for NMD [40]. And finally, introducing a PTC in the HIV-1 *gag* ORF does not lead to its degradation (Fig. 10). We cannot completely rule out the possibility, however, that the effects on HIV-1 RNA and Gag expression are an indirect consequence of inhibiting NMD, for example, especially since UPF1 depletion can alter the expression levels of many genes [41].

Our data reveal that the effects of UPF1 on HIV-1 Gag expression are not dependent on its association to UPF2, an interaction that is appreciated to be important, but not critical, for NMD (Fig. 13; Table 3;[31]). While NMD is still

active in HIV-1-expressing cells (Fig. 8), the regulation of HIV-1 expression by UPF1, which is independent of UPF2, may be achieved in the context of a distinct RNP. The association of UPF1 with the HIV-1 RNP, although potentially mediated via Staufen1 [20], does not require UPF2 to enhance expression. In fact, our Western and mass spectrometry analyses of the composition of Staufen1 complexes also confirmed that UPF2 is not detectable in Staufen1-TAP eluates (M. Milev and A.J. Mouland, unpubl.). The absence of UPF2 may favor the ability of UPF1 to bind RNA and perhaps that of HIV-1 [42], but this will require further analysis.

Most of the UPF1 NMD mutants, including the helicase transdominants and the UPF2-binding mutants (Table 3), up-regulated HIV-1 RNA (Figs. 13,14). While $pr55^{Gag}$ expression levels increased when TDN and $UPF1^{RRAA}$ were overexpressed, the ATPase-/NMD-deficient mutant $UPF1^{DE}$ did not (Fig. 14). Expression of this mutant increases the size and number of P-bodies, increases the level of normal transcripts in P-bodies, promotes the recycling of P-body components and compromises RNA binding capacity of UPF1 [32, 40, 43]. Because P-bodies are translationally silent and are sites of mRNA storage and degradation, we speculate that HIV-1 genomic RNA could be directed to PBs for storage explaining why $pr55^{Gag}$ levels do not change when we express this ATPase mutant. This idea is echoed by a recent study showing that PB-sequestered ARE-mRNAs prevent translation on polysomes, thereby silencing ARE-mRNA function even when mRNA decay is delayed [44].

The lack of correspondence between HIV-1 RNA and $pr55^{Gag}$ expression levels in our rescue experiments support an enhancing role for UPF1 on HIV-1 *gag* mRNA translation (Figs. 8, 9; Fig. 16). The incomplete rescue of RNA levels is indicative that UPF1 could function at a specific time in the late expression stages, and the RNA defect, due to UPF1 depletion, cannot be rescued. Nevertheless, UPF1 still maintains quite a strong effect on gag mRNA translation. The role of UPF1 in HIV-1 RNA translation is supported by its association to polysomes[3, 45], its regulation of ribosomal frameshifting [46], and its possible roles in translation termination [38]. Nevertheless, the mechanism by which UPF1 enhances $pr55^{Gag}$ expression levels is not well understood, but could be due to its association to sequences within the *gag* ORF since the tethering of many NMD factors including UPF1 was shown to mediate translational enhancement [47]. Translational enhancement could also be caused by a UPF1-mediated recruitment of residual HIV-1 RNA (from a pool that might not be translated) into polyribosomes or even by enhanced ribosome recycling following translation termination, a proposed role for UPF1 in earlier work [47]. The near complete rescue of $pr55^{Gag}$ expression indicates that UPF1 is a principal regulator of viral structural protein synthesis. Moreover, these results support the notion that separable roles for UPF1 in HIV-1 RNA degradation and translation exist.

Ongoing translation of the *gag* ORF was necessary for HIV-1 RNA degradation that ensues when UPF1 is depleted from cells (Fig. 10). The translational enhancing effects mediated by UPF1 may, in fact, have two functions: (1) to enhance $pr55^{Gag}$ synthesis; and (2) to prevent RNA degradation due to stalling

ribosomes. Furthermore, at first sight, the results showing that the *gag* RNA expressed from a Rev- provirus succumbs to the effects of UPF1 depletion might seem contradictory. While negligible levels of genomic RNA do indeed enter the cytoplasm and are translated even in a Rev- background [21], these results could be explained by separable processes that are regulated by UPF1 in nuclear and cytosolic compartments.

A nuclear function for UPF1 is strongly supported by our data because of the striking effects of UPF1 knockdown on nuclear-retained HIV-1 genomic RNA. The ability of UPF1 to shuttle between the nucleus and cytoplasm [30] and its role in nonsense-mediated alternative exon skipping [30], in exon skipping [48], in regulating nuclear *Xist* RNA [35], and in DNA repair [49], all support nuclear roles. Our results suggest that UPF1 is needed for late gene expression stages of HIV-1, early following transcription to protect the viral mRNA from degradation. When we expressed the Rev- provirus when UPF1 was depleted, the Rev-dependent, 9-kb genomic RNA was selectively targeted, but the 2-kb RNAs that are generated by complete splicing and rapidly exported to the cytoplasm were not (Fig. 11). This selectivity for the genomic RNA strongly suggests that UPF1 exerts its protective effects on the genomic RNA when the decision for splicing is made, concomitant or upstream of the activity of Rev. CRM1-mediated nuclear export of both of these cargoes may also functionally link this host protein with the genomic RNA.

A *cis*-acting RNA sequence is likely necessary for the elicited effects of UPF1 depletion. The insensitivity of the 2-kb RNAs, observed most notably in the context of a Rev- provirus, could also be explained by the absence of CRS/INS in these RNAs upon complete splicing of the genomic RNA. Moreover, it has been speculated that the zinc fingers of UPF1 are implicated in binding INSs [50] and the lack of effect of $UPF1^{\Delta 20–150}$ on HIV-1 expression is likely due to the deletion of a zinc-finger motif in this mutant (Fig. 12). The C-terminal mutant $UPF1^{I–1074}$ also did not have any effect on HIV-1 RNA. The deleted region in this mutant is speculated to be important for protein–protein or protein–RNA interactions, and these events might be important for the effects on HIV-1 [50]. These observations would be consistent with the results obtained with $UPF1^{DE}$, which also has compromised RNA-binding capacity.

Based on our data, we speculate that UPF1 acts to prevent the recruitment of nuclear exosome degradative machinery [51] to intron-containing HIV-1 RNAs (in both Rev+ and Rev- backgrounds). On the one hand, when UPF1 is depleted from cells, intron-containing HIV-1 RNAs would be detected and rapidly cleared. On the other hand, UPF1 overexpression leads to enhanced protection of genomic RNA following transcription, and the RNA would efficiently access the cytoplasm, increasing its availability to the translation apparatus. UPF1 interacts directly with several components of the nuclear and cytoplasmic exosome [52], providing support for this model. The potential to correct genetic disease by targeting NMD [53] highlights the importance of studying UPF1 function in order

to potentially home in on HIV-1 RNAs before, during, and after their export to the cytoplasm.

ACKNOWLEDGMENTS

We thank Miles Wilkinson and Melissa Moore for reagents and constructive discussions; Juan Ortin, Jens Lykke-Andersen, Larry Kleiman, Marv Wickens, Damian Purcell, David Ott, Anne Gatignol, Éric Cohen, Resa Sadaie, Bruce Chesebro, Hardy Chen, and Michael Phelan for antisera, reagents, and genetic clones; and the NIH AIDS Reference and Reagent Program for reagents. We thank Martin Lehmann for supplying additional images for analysis. L.A. (the first author) is a recipient of a Canadian Institutes of Health Research (CIHR) doctoral research fellowship. P.V.I. is supported by a FEBS long-term fellowship. A.J.M. is a recipient of a CIHR New Investigator Award. This work was supported by grants from the Deutsche Forschungsgemeinschaft to N.H.G. and A.E.K. (KU 563/7, KU 563/8, KU563/11) and the CIHR to A.J.M. (MOP-38111) and to A.J.M., A.E.K., and N.H.G. (OPC-83178).

MATERIALS AND METHODS

siRNAs

siRNA duplexes were synthesized by QIAGEN-Xeragon. siNS is commercially available nonsilencing control duplex (QIAGEN-Xeragon). The sequence for siUPF1 (5'-AAGATGCAGTTCCGCTCCATT-3') was reported earlier [5]. An additional siUPF1 was employed (On-Target Plus, catalog #J-011763-07, Dharmacon: 5'-GCAGCCACAUUGUAAAUCAUU-3') having reduced off-target effects with similar efficiencies of UPF1 knockdown. The corresponding On-Target Plus siNS was also used.

Cell lines and transfections

Stable TAP- and Staufen1-TAP-expressing cell lines were created following transfection of 293T cells with the Staufen1-TAP vector generously provided by Juan Ortin (Centro Nacional de Biotecnologia, Spain) as described [24]. Single clones were expanded in the presence of 600 μg/mL G418. Expression was verified by Western blot analyses and was stable for more than 22 passages. Stable control TAP-expressing cells were verified by resistance to neomycin and by PCR of genomic DNA to identify integral TAP DNA sequences. These experiments were performed in the absence and presence of transfected HIV-1 HxBRU DNA.

All other experiments employed HeLa cells. Transfection of proviruses and siRNAs was performed essentially as described [25] except HeLa cells were plated in six-well plates at 300,000/well for 24 h before transfection. A total of 2 μg of DNA was added per well (1 μg of proviral DNA plus 1 μg of carrier pKSII with or without siRNA RNA duplexes). siUPF1 and siNS were used at 100 nM as described [54, 55]. Proviral DNAs HxBRU and pNL4-3 were described earlier [56]. 3C1 (PTC-) and 7C3 (PTC+) HeLa cell lines were provided by Miles Wilkinson (University of Texas) [26], and NMD was assessed by Northern blotting for TCR-β transcripts. In control experiments, 100 nM cycloheximide was added 6 h before cell harvesting in order to abrogate NMD in TCR-β mRNA-expressing cell lines or to examine the dependence on translation of siUPF1-mediated effects on HIV-1 RNAs.

Total cellular RNA was isolated from cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The RNA was re-precipitated with ethanol before loading onto denaturing agarose/formaldehyde gels. Unspliced and spliced HIV-1 and *gapdh* mRNAs were identified by Northern blot analyses as described previously [57]. For the Rev- experiments, the genomic RNA was identified using a radiolabeled *pol*-specific cDNA probe that corresponded exactly to the riboprobe used in the FISH analyses [55]. TCR-β mRNA was identified using a cDNA probe to the second VDJ exon generated by PCR using the following primers (sense: 59-ACACATGGAGGCTGCAGTCA; antisense: 59-CGAAACAGT CAGTCTGGTTC) and the β433 TCR-β minigene DNA construct (generously provided by Miles Wilkinson, University of Texas). RSV mRNA was

identified by Northern blot analysis. Eric A. Cohen (IRCM, Canada) provided the HxBRUΔp6 in which a stop codon was introduced following the p6 ORF [58]. David Ott (NIH/NCI, Frederick, MD) provided the pNL4-XX and pNL4-X proviral constructs as described [29]; and the NIH AIDS Reference and Reagent Program provided the pMRev(-) contruct [59]. Anne Gatignol (McGill University) provided the pMAL and pADA M-tropic proviral constructs. The HIV-2 ROD27 is described by Mouland et al. (2000). pC1-Flag/UPF1-Rescue ($UPF1^{Rescue}$, harboring silent mutations that resists siUPF1), wild-type pC1-Flag-UPF1 ($UPF1^{WT}$), and pC1-λN-UPF3 (λN/UPF3) expression constructs were described previously [54]. pC1-Flag-UPF1(VV204DI) ($UPF1^{DI}$), pC1-Flag-UPF1 (LECY181VRVD) ($UPF1^{VRVD}$), and pC1-Flag-UPF1(TLH169RVD) ($UPF1^{RVD}$) were generated in $UPF1^{Rescue}$, while the ATPase mutant pC1-Flag/UPF1(DE637AA) ($UPF1^{DE}$), the helicase mutants pC1-Flag/UPF1(RR857AA) ($UPF1^{RRAA}$), and pC1-Flag-UPF1(R844C) (TDN), pC1-Flag/UPF1(1–1074) ($UPF1^{1–1074}$), and pC1-Flag/UPF1Δ20–150 ($UPF1^{\Delta 20–150}$) were generated in $UPF1^{WT}$ by site-directed mutagenesis. For $UPF1^{1–1074}$, a stop codon was introduced at position 1075. For $UPF1^{\Delta 20–150}$, primers were designed to delete the region in the context of wild-type UPF1. $UPF1^{\Delta N40}$ was generated by PCR of UPF1 with a 59-primer starting at position 41. $UPF1^{1–1074}$ lacks all C-terminal phosphorylation sites that are speculated to be important for protein–protein or protein–RNA interactions; $UPF1^{\Delta 20–150}$ is deficient for interaction with UPF2 and is missing one of the zinc fingers important for its binding to INSs on RNAs; and $UPF1^{\Delta N40}$ lacks part of the putative SMG5 interaction site.

Antibodies and reagents

Antisera to UPF proteins were generously supplied by Jens Lykke-Andersen (University of Colorado). Anti-p24 (CA) and anti-GAPDH antisera were purchased from Intracell and Techni-Science and were described previously[55]. Anti-Flag antisera and cycloheximide were purchased from Sigma-Aldrich.

IF/FISH analyses

Immunofluorescence and fluorescence in situ hybridization were performed exactly as previously described [55].

Colocalization analyses

For colocalization studies, laser scanning confocal microscopy (LSCM) was performed using a Zeiss Pascal LSM5 confocal microscope. Cells were mock transfected with Flag DNA or transfected with proviral HIV-1 DNA, pNL4-3, fixed at 30–36 h post-transfection, and processed for immunofluorescence as previously described [55]. Fixed cells were costained with anti-Gag p17 antisera (1:400, sheep anti-p17; from Michael Phelan, NIH AIDS Reference and Reagent Program) and rabbit anti-UPF1 antisera (1:200; from Jens Lykke-Anderson) as previously described [55]. The amount of colocalization was calculated using Manders' overlap coefficient (for entire cells) or colocalization coefficients *m1* and *m2* using Colocalizer Pro Software (Colocalization Research Software) as

described previously [55]. The latter quantitates the contributions of each channel [(red) UPF1; (blue) Gag] to a region of interest providing the percentage of UPF1 that colocalizes with the Gag signals in the depicted region of cell is shown in the figure inset. The results shown are representative of two independently performed experiments and were calculated from more than 50 cells per condition.

Immunoprecipitation analyses

HeLa cells were transfected as described above. Cells were lysed for 30 min on ice by NP-40 lysis buffer, and Gag was immunoprecipitated as described previously using 2 mg of protein and affinity-purified mouse anti-p24 antisera (hybridoma 183-H12- 5C) [60] from the NIH AIDS Reference and Reagent Program [25]. Signals obtained in Western blotting of UPF1 and Gag (input and output levels) were quantitated by ImageJ software (NIH, Bethesda, MD) in order to estimate the proportion of UPF1 and Gag found in Staufen1-TAP eluates.

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FIGURES AND FIGURE LEGENDS

FIGURE 7: *Long-term expression of Staufen1-TAP and identification of UPF1 in the HIV-1 RNP.*

(A) Stable Staufen1-TAP (STF1- TAP)- (#11 and #12) and TAP-expressing 293T cell lines were created. Expression was verified by Western blot analysis for Staufen1 identifying Staufen1-TAP fusion (MW = 75 kDa) and endogenous Staufen1 (55 kDa and 63 kDa) proteins. Staufen1-TAP expression for clones #11 and #12 is shown. The identity of TAP-expressing cell lines was verified by PCR of harvested genomic DNA (data not shown). (B) Cells were mock transfected or transfected with HIV-1 provirus, and TAP affinity purification was performed. Full-length fusion protein (Staufen1-TAP), truncated, TEV protease cleaved Staufen1 containing the calmodulin binding domain (Staufen1-CBD), and viral proteins were verified by Western blotting of cell extracts before (left) and after (right) tandem affinity purification. The identification of $pr55^{Gag}$ as a Staufen1-binding partner and not mature Gag proteins in the eluates was used for validation of the assay. UPF1 was identified in Staufen1-TAP, but not in TAP eluates (bottom). (C) $pr55^{Gag}$ was immunoprecipitated from mock (+siNS), pNL4-3+siNS-, or pNL4-3+siUPF1-transfected cells using a monoclonal anti-p24 antisera as described in the Materials and Methods. UPF1, $pr55^{Gag}$, and GAPDH [as loading control in cell lysates and negative control in immunoprecipitation (IP) lanes] were identified by Western blotting analyses in input cell lysates and

Gag immunoprecipitates. (D) To determine the cellular localization of Gag and UPF1, cells were mock transfected (*top* panels) or transfected with pNL4-3 (*bottom* panels), followed by LSCM analyses for UPF1 and Gag. The number in the inset in HIV-1- expressing cells represents the colocalization coefficient (%) of the UPF1 signal (red fluorescence) found to colocalize with Gag (blue fluorescence signal) at the cell periphery indicated by magenta colored regions and calculated as described in the Materials and Methods.

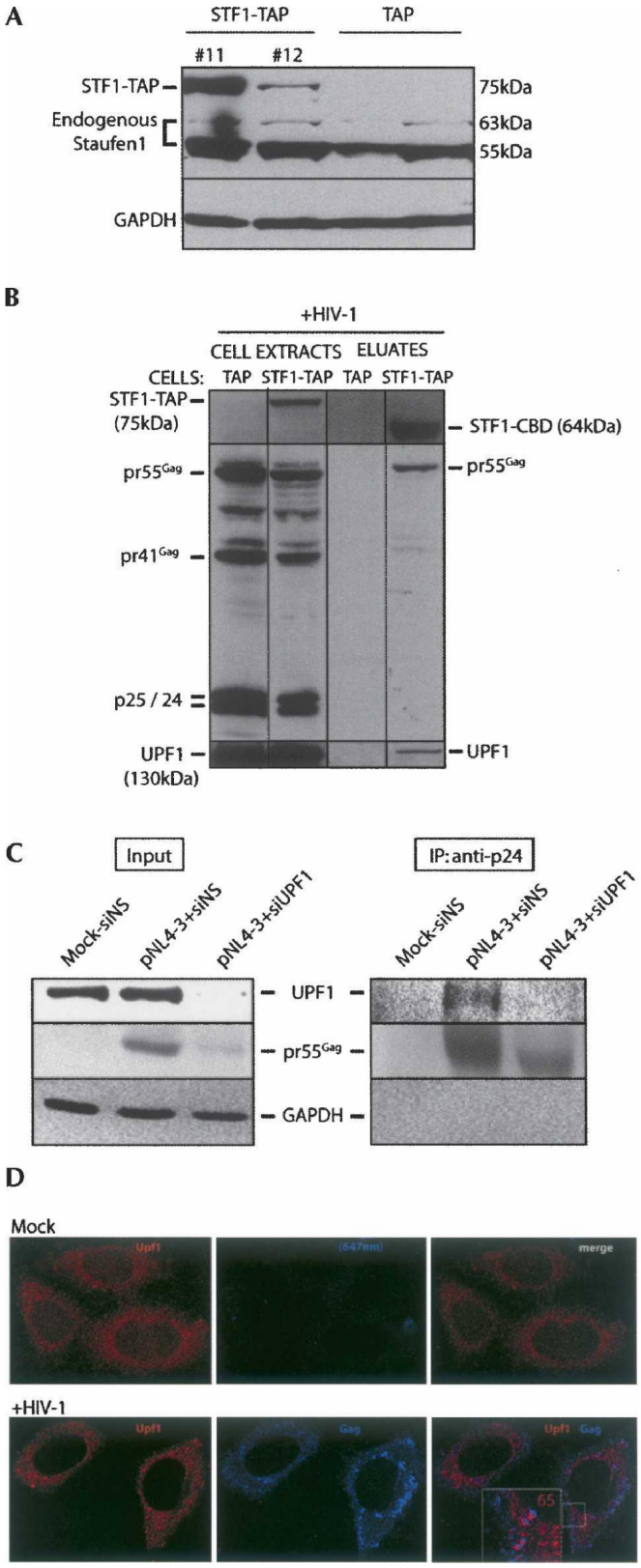

FIGURE 7: *Long-term expression of Staufen1-TAP and identification of UPF1 in the HIV-1 RNP.*

FIGURE 8: *UPF1 expression is essential for NMD and HIV-1 RNA stability.*

HeLa cells expressing a TCR-β transgene harboring either a wild-type codon (PTC-, 3C1 cells) or an introduced nonsense codon (PTC+, 7C3 cells) as depicted in A (adapted from [61]) were tested for their NMD responsiveness by inhibiting translation by a brief treatment with cycloheximide (CHX). Both cell lines were mock transfected, treated with CHX, or transfected with siNS as described in the Materials and Methods. Expression of UPF1, GAPDH (as loading control), TCR-β, and *gapdh* mRNA levels were examined at 30 h post-transfection by Western and Northern analyses. Two exposures for TCR-β mRNA are shown. (B,C) 7C3 cells were transfected with siNS (odd-numbered lanes) or siUPF1 (even numbered lanes) for 24 h. They were then mock transfected (lanes 1,2), or transfected with pNL4-3 (wild-type HIV-1, lanes 3–8), with Flag (lanes 3,4), $UPF1^{WT}$ (lanes 5,6), or a $UPF1^{Rescue}$ (1 mg, lanes 7,8). UPF1, UPF2 (as loading control), $pr55^{Gag}$, and GAPDH (as loading control) levels were monitored by Western blot analysis. (C) TCR-β, *gapdh* (as loading control), and HIV-1 RNAs were assessed by Northern blot analysis. All three HIV-1 RNA species (unspliced genomic, 9 kb; singly spliced, 4 kb; and multiply spliced, 2 kb) are shown here.

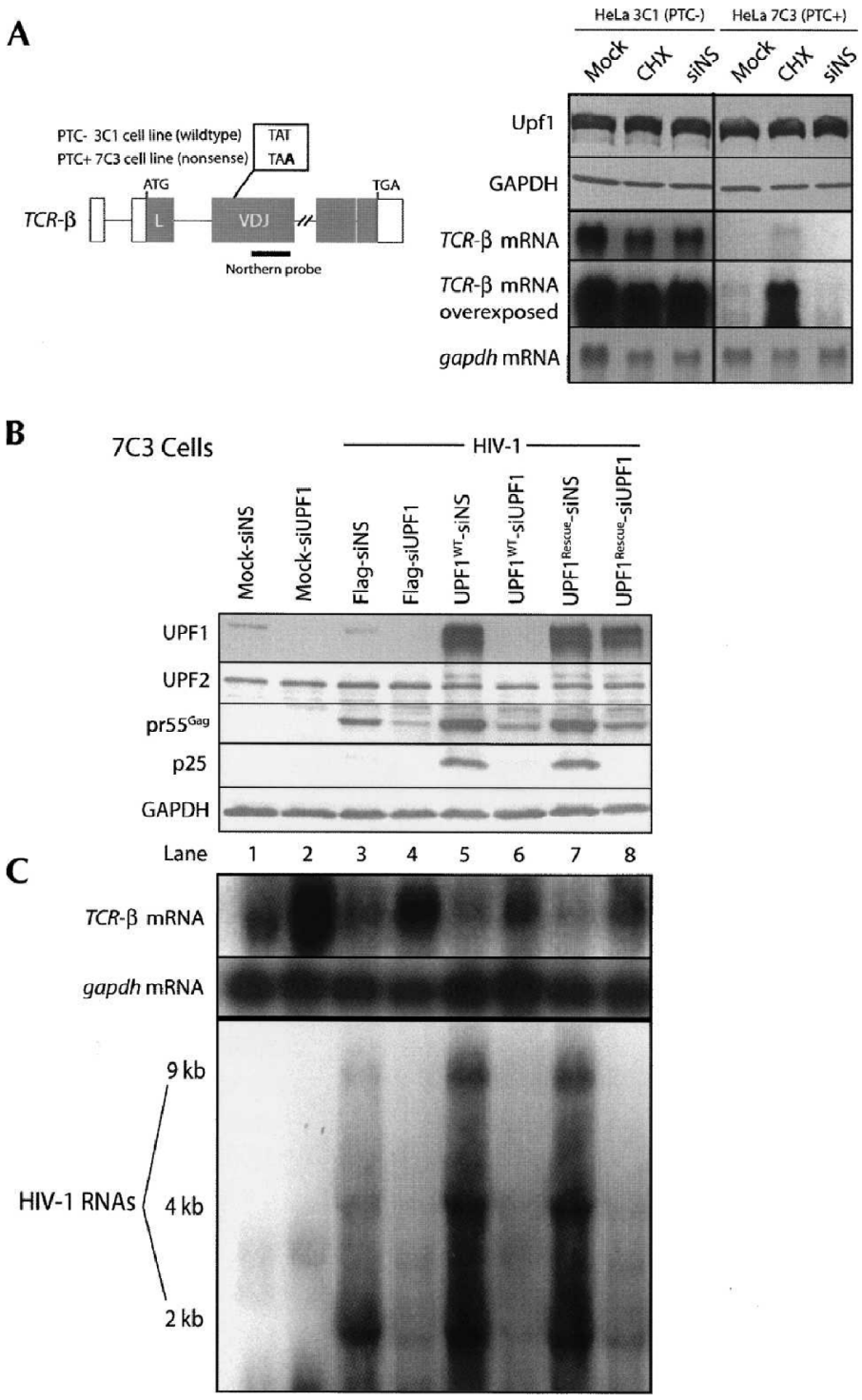

FIGURE 8: *UPF1 expression is essential for NMD and HIV-1 RNA stability.*

FIGURE 9: *Time-course effects of siUPF1 and UPF1 overexpression on HIV-1 pr55^{Gag} and RNA levels.*

HeLa cells were transfected with siNS or siUPF1 for 24 h. They were then transfected with pNL4-3 (HIV-1) and Flag or UPF1^{Rescue} (2 μg) and again with siNS or siUPF1 (Time 0). At 6, 12, 24, and 30 h post-transfection (hr PT), cells were harvested for Western and Northern analyses. (A) A set of mock-transfected cells (no pNL4-3) with siNS or siUPF1 is shown on the left at 30 h post-transfection. Expression levels of UPF1, UPF2, pr55^{Gag}, and GAPDH (as loading control) were monitored by Western analyses. Expression levels of HIV-1 genomic RNA and *gapdh* mRNA (as loading control) were assayed by Northern blotting. Two exposures of the results for HIV-1 genomic RNA are shown. Flag and (lanes 1–4) siNS- or (lanes 5–8) siUPF1-transfected cells; UPF1^{Rescue} and (lanes 9–12) siNS- or (lanes 13–16) siUPF1-transfected cells. (B) Genomic RNA levels based on densitometric scanning of the lower exposure shown in A were expressed relative to those of *gapdh* mRNA at each time point post-transfection. (C) The levels of (gray bars) HIV-1 pr55^{Gag} and (black bars) RNA were expressed relative to the corresponding expression levels in the UPF1^{Rescue}-siNS treatment group that were considered maximal.

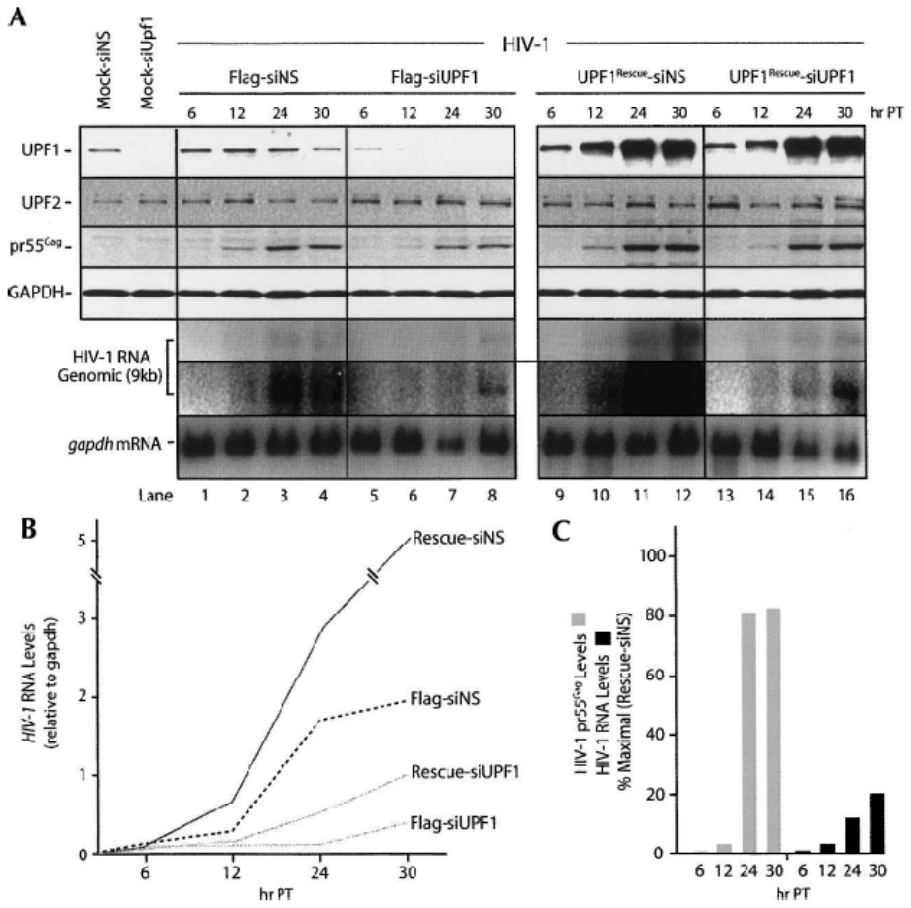

FIGURE 9: *Time-course effects of siUPF1 and UPF1 overexpression on HIV-1 pr55^{Gag} and RNA levels.*

FIGURE 10: *UPF1 depletion affects actively translating HIV-1 genomic (gag) RNA.*

HeLa cells were transfected with siNS or siUPF1 for 24 h. HeLa cells were then mock transfected or transfected with pNL4-3, pNL4-XX, or HxBRUΔp6 proviral constructs depicted in the top panel and again with siNS or siUPF1 for each condition. In pNL4-3, the ATG initiation codon (ATG) is indicated, and translation of the RNA generates $pr55^{Gag}$ protein. Translation from this codon in pNL4-XX is mutated, preventing translation initiation at this site, but translation can initiate at a downstream initiation codon. A stop codon was introduced downstream from this internal site to prevent Gag synthesis [29]. HxBRUΔp6 possesses a stop codon preventing completion of p6 synthesis and results in the synthesis of a truncated Gag protein (prGagΔp6). Virus produced from HxBRUΔp6-expressing cells show demonstrable budding and maturation defects [58]. Cells were harvested at 30 h. UPF1, UPF2, $pr55^{Gag}$, truncated prGagΔp6, and GAPDH were assessed by Western blot analyses. Genomic RNA and *gapdh* mRNAs were quantitated by Northern blotting as described in the Materials and Methods. The ribosomal frameshift site (FS) is indicated on the RNAs. The abundance of the genomic RNA was related to *gapdh* mRNA level (loading control), and levels were related to signal intensities found in siNS-treated cells expressed as a percentage of the amount remaining (±SD) calculated from 10 experiments. The asterisk (*) identifies a processed form of Gag that is found only in the HxBRUΔp6-expressing cells.

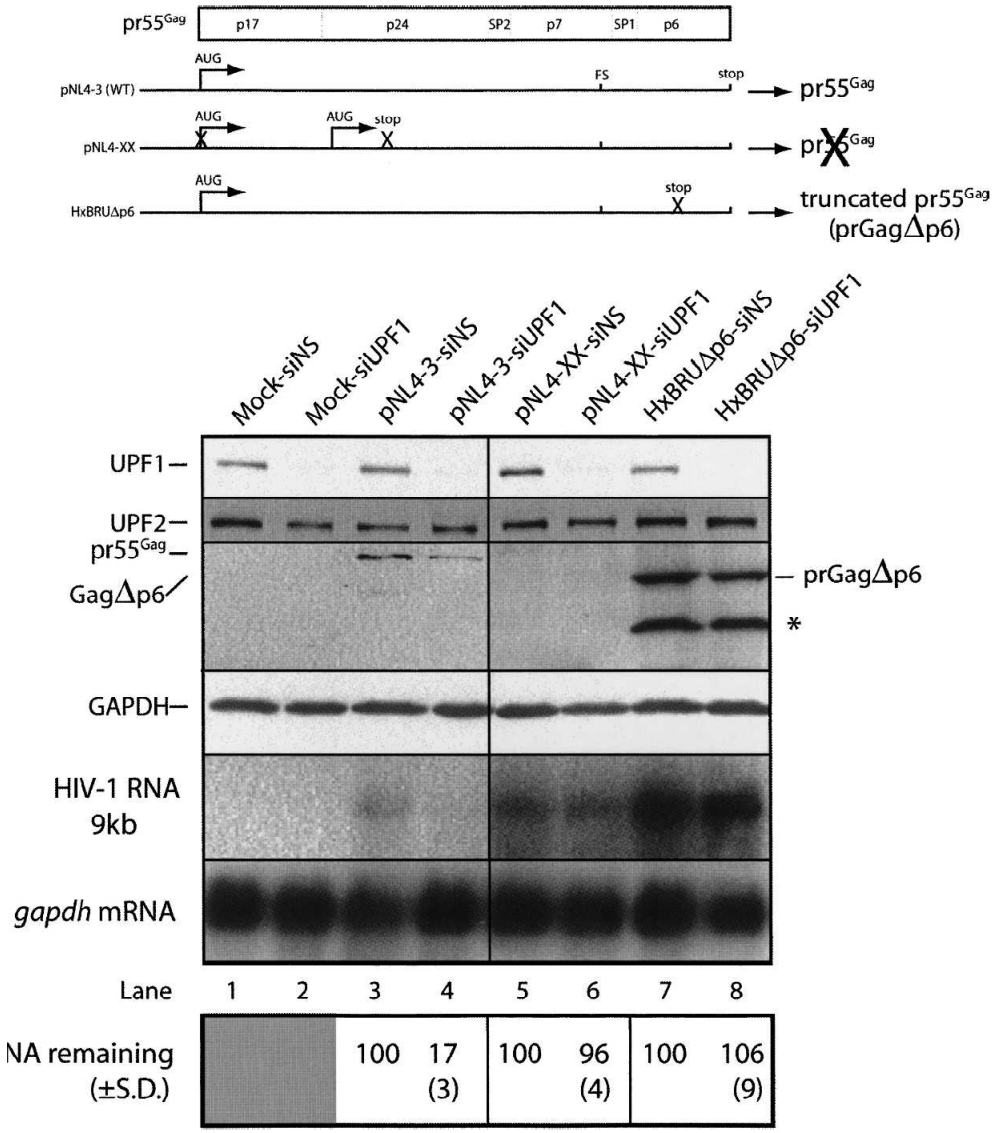

FIGURE 10: *UPF1 depletion affects actively translating HIV-1 genomic (gag) RNA.*

FIGURE 11: *Unspliced HIV-1 RNA is sensitive to UPF1 depletion in the nucleus.*

HeLa cells were transfected with siNS or siUPF1 for 24 h. HeLa cells were then mock transfected or transfected with HxBRU or the Rev-defective provirus, pcMRev(-), with either siNS or siUPF1 for each condition. (A) Western blot analysis of UPF1, $pr55^{Gag}$, and GAPDH. (B) Northern blot analysis of HIV-1 unspliced 9-kb and 2-kb RNAs identified using a *pol*-specific cDNA probe or a 5' TAR-specific probe, respectively. (C) FISH analyses of HIV-1 genomic RNA (green fluorescence in all panels) in cells transfected with HxBRU or pcMRev(-) in siNS or siUPF1 knockdown conditions as indicated *above* each panel. Cells were counterstained with DAPI to identify nuclei (blue). Merged images are shown including transmitted light/phase contrast to highlight cell periphery. Red arrows indicate nontransfected cells. These results are representative of three independently performed experiments.

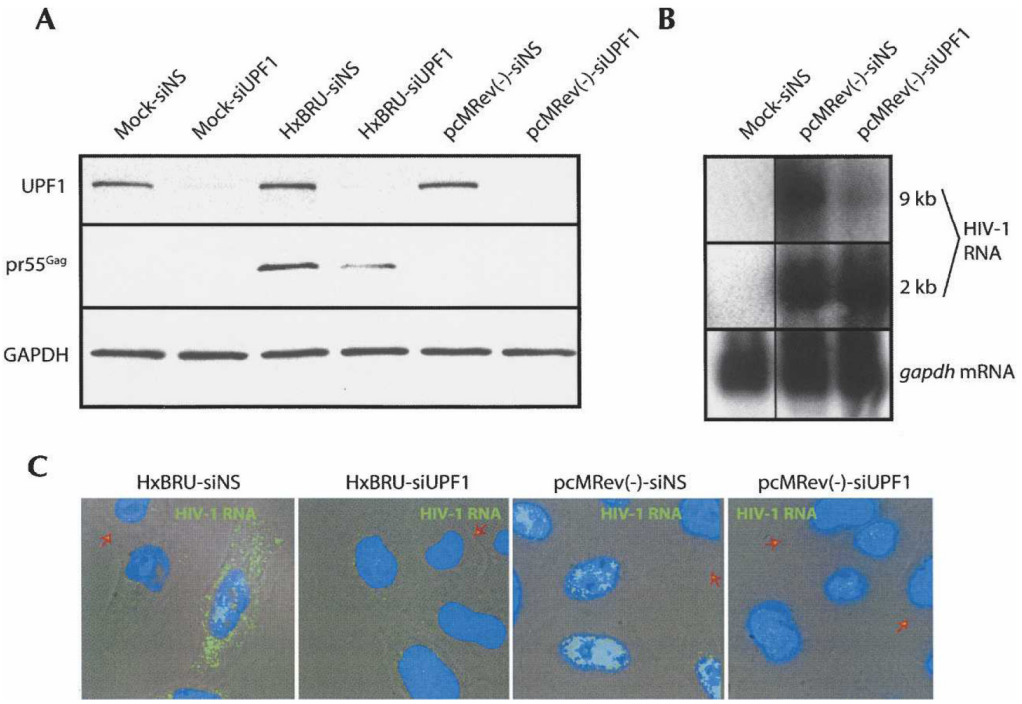

FIGURE 11: *Unspliced HIV-1 RNA is sensitive to UPF1 depletion in the nucleus.*

FIGURE 12: *Selective up-regulation of HIV-1 RNA levels by an intact UPF1 protein.*

Cells were (lane 1) mock transfected or co-transfected with (lane 2) HIV-1 proviral DNA (pNL4-3, HIV-1) and $UPF1^{WT}$, (lane 3) an expression construct for UPF3b with a λ-N-terminal tag (λN/UPF3b), (lane 4) TDN (R844C), or Flag-tagged expression constructs coding for deletion mutants of UPF1 including (lane 5) the C-terminal deletion mutant, $UPF1^{I–1074}$, (lane 6) the internally deleted $UPF1^{\Delta 20–150}$, and (lane 7) the N-terminal deleted $UPF1^{\Delta N40}$. At 30 h post-transfection, cells were harvested for Western blot analysis for UPF1, UPF3b, and GAPDH (loading control). RNA was also isolated from cell extracts, and Northern blotting was performed to measure steady-state levels of HIV-1 and *gapdh* mRNA. Because the primary anti-UPF1 antibody did not efficiently recognize the truncation and deletion mutants, Western blotting using an anti-Flag antibody was employed to identify the Flag-tagged UPF1 proteins expressed *in trans* (bottom panel). The percentages represent genomic RNA levels related to the signal obtained in $UPF1^{WT}$ and HIV-1-expressing cells. The results shown are representative of three independently performed experiments.

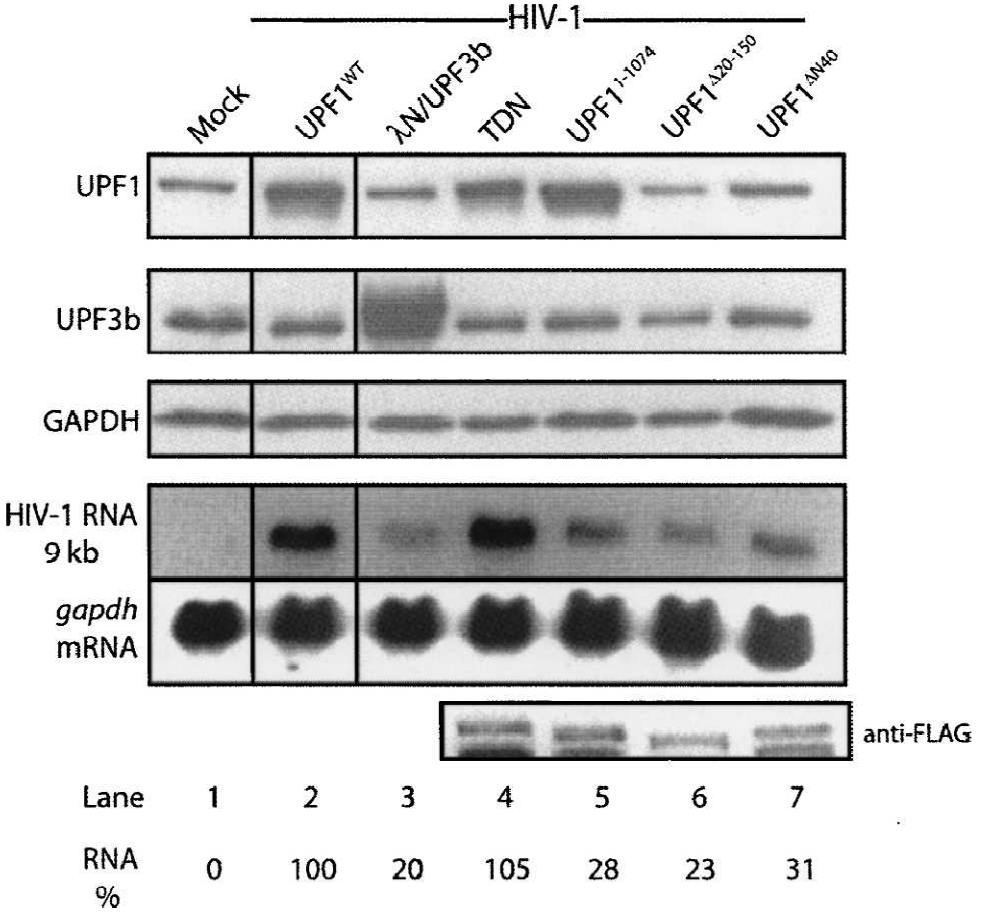

FIGURE 12: *Selective up-regulation of HIV-1 RNA levels by an intact UPF1 protein.*

FIGURE 13: *UPF2 binding is not necessary for UPF1-mediated upregulation of HIV-1.*

Cells were (lane 1) mock transfected or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) with Flag, TDN (R844C), $UPF1^{RVD}$, or UPF2-binding-deficient mutants $UPF1^{VRVD}$ or $UPF1^{DI}$. At 30 h post-transfection, cells were harvested for Western blot analysis for UPF1, UPF3b (as loading control), $pr55^{Gag}$, and GAPDH (as loading control). Cell-associated pr25, a product of $pr55^{Gag}$ maturation, is shown to demonstrate up-regulation more clearly than the signals obtained for $pr55^{Gag}$. The percentages represent the amount of $pr55^{Gag}$ related to the signal obtained in Flag and HIV-1-expressing cells. The values from three independently performed experiments did not vary by more than 17%.

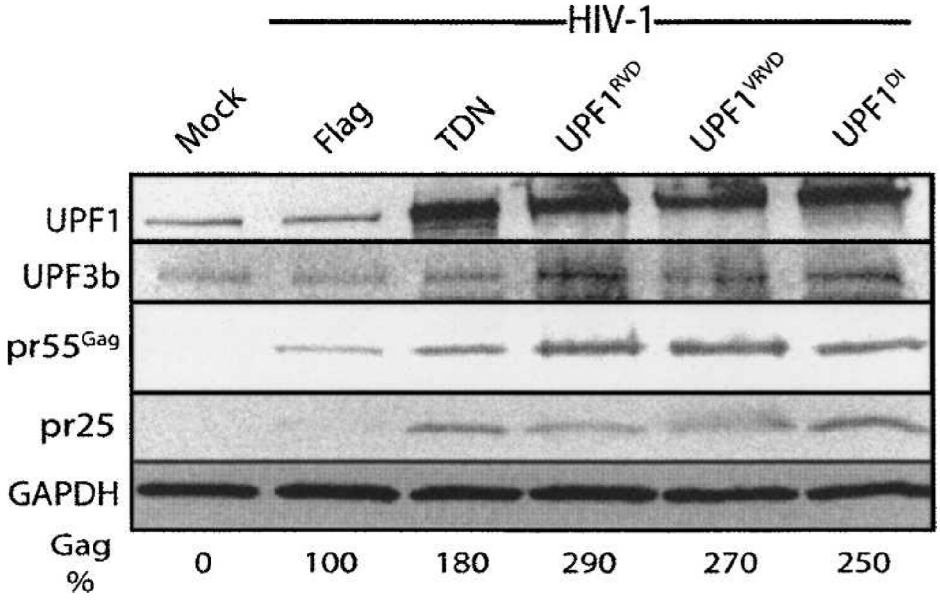

FIGURE 13: *UPF2 binding is not necessary for UPF1-mediated upregulation of HIV-1.*

FIGURE 14: *ATPase activity of UPF1 is required for HIV-1 upregulation.*

Cells were (lane 1) mock transfected or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) with Flag, $UPF1^{WT}$, TDN (R844C), $UPF1^{RRAA}$, and $UPF1^{DE}$. At 30 h post-transfection, cells were harvested for Western blot analysis for UPF1 expressed *in trans* using an anti-Flag epitope tag, UPF2 (as loading control), Gag, and GAPDH (as loading control). The relative levels of $pr55^{Gag}$ are related to the signal obtained in Flag and HIV-1-expressing cells, set to 1. The histogram shows the averages (±SD).

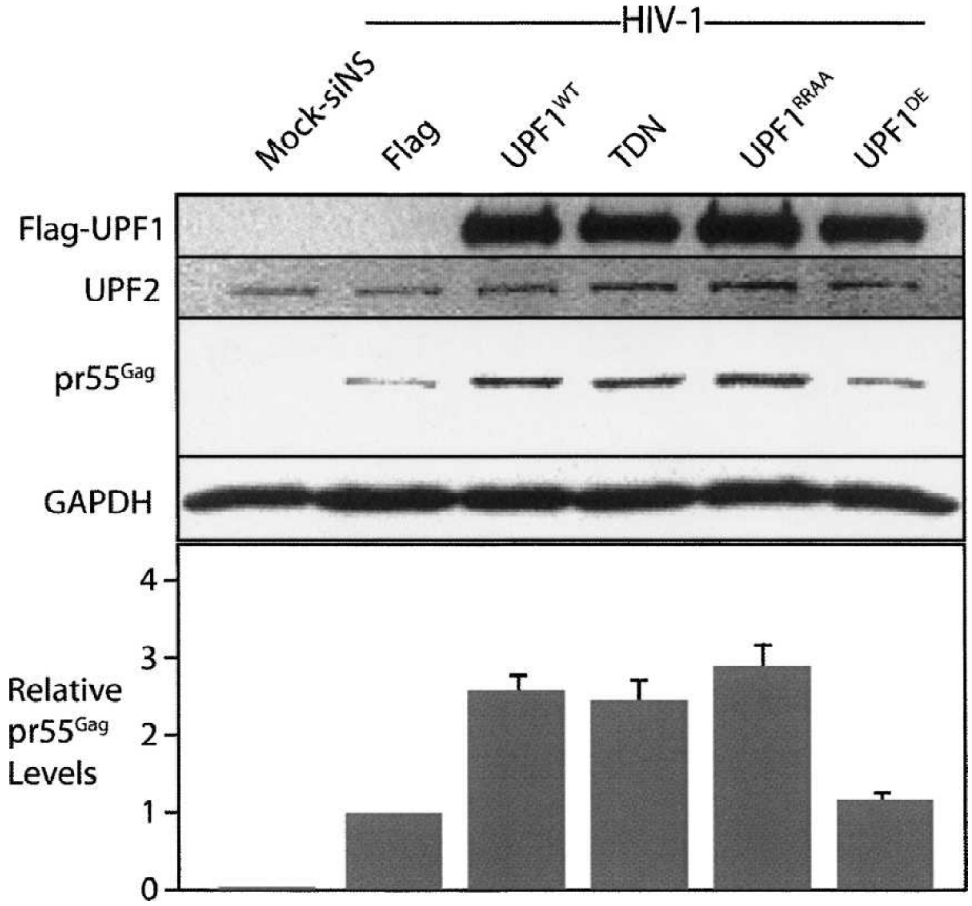

FIGURE 14: *ATPase activity of UPF1 is required for HIV-1 upregulation.*

TABLE 1. UPF1 mutants used in this study

UPF1 mutant	NMD	UPF2 interaction
Deletions (C-, N-, and internal)		
$UPF1^{\Delta N40}$	ND	Yes
$UPF1^{\Delta 20-150}$	ND	No
$UPF1^{1-1074}$	ND	Yes
Helicase		
TDN (R844C)	No[a]	Yes
$UPF1^{RRAA}$ (RR857AA)	No[b]	Yes
ATPase		
$UPF1^{DE}$	No[b]	Yes
UPF2 binding		
$UPF1^{RVD}$	Yes[c]	Yes
$UPF1^{VRVD}$	No[c]	No
$UPF1^{DI}$	Yes[c]	No

(ND) Not determined.
[a]Sun et al. (1998).
[b]Mendell et al. (2002).
[c]Ivanov et al. (2008).

TABLE 3: *UPF1 mutants used in the study.*

RESULTS FOR SUPPLEMENTAL FIGURES

Effects of UPF1 depletion on T- and M- tropic viruses and HIV-2

In order to determine if the knockdown effects of UPF1 are observed not only on T-cell-tropic viruses (pNL4.3), we expressed two macrophage or M-tropic viruses, pMAL and pADA, and HIV-2 with siNS or siUPF1. As observed with pNL4-3, UPF1 depletion resulted in a dramatic decrease of pMAL, pADA and HIV-2 genomic RNAs (Supplementary Figure 15).

UPF1 expression rescues pr55^{Gag} but not mRNA levels

Our lack of ability to rescue HIV-1 RNA levels using the Rescue plasmid provided the impetus to study this phenomenon further. An experiment was designed to assess to what extent we could rescue pr55^{Gag} expression and mRNA levels using increasing doses of the UPF1^{Rescue}. HeLa cells were mock transfected or transfected with HIV-1 proviral DNA, pNL4-3, siNS, siUPF1 and increasing doses of the Rescue DNA. As shown in Supplementary Figure 16 we were able to rescue pr55^{Gag} expression levels to 74% compared to levels achieved in siNS-treated cells expressing 1μg of the UPF1^{Rescue} in siNS conditions (set to 100%; Supplementary Figure 16A, lane 2). However, when HIV-1 RNA levels were assessed, while abundant expression of HIV-1 mRNA was achieved by overexpressing the UPF1^{Rescue} as shown in Supplementary Figure 16 (lanes 5 &

7), there was little evidence of an efficient rescue of the HIV-1 RNAs at any dose of the UPF1^{Rescue} used (Supplementary Figure 16B; the 9kb RNA is shown). At the highest dose of UPF1^{Rescue}, HIV-1 RNA was only enhanced to 8% of that found in UPF1^{Rescue}/siNS-expressing cells levels.

FACS Analyses following siNS and siUPF1 treatment at 30 hr and 4 days post-transfection

We performed cell cycle analysis as a control in light of recent report that demonstrated that UPF1 knockdown leads to a S-phase accumulation [1]. At 30 hr post-transfection, cells were harvested and processed for cell cycle analysis exactly as described before [2,3]. The results of cell cycle analyses of cells treated with siNS or siUPF1 ± HIV-1 DNA showed similar cell cycle distributions in all conditions (Supplementary Figure 17). When cells were harvested for analyses at 4 days post-transfection, a marked S-phase accumulation was observed in siUPF1-treated, but not siNS-treated cells at 4 days post-transfection as shown previously [1].

REFERENCES FOR SUPPLEMENTAL MATERIAL

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SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

FIGURE 15: *UPF1 depletion results in decreased HIV-1 genomic RNA in T- and M- tropic viruses, as well as in HIV-2.*

Cells were mock transfected or transfected with pNL4.3, pMAL, pADA or HIV-2 with siNS or siUPF1. At 30 hr post-transfection, cells were harvested for Western blot analysis for $pr55^{Gag}$, UPF1 and GAPDH. RNA was isolated using TRIzol and Northern blotting was performed to measure genomic RNA levels and *gapdh* mRNA. A *pol*-specific cDNA probe was used to identify genomic RNA of pNL4-3, pMAL and pADA. To identify HIV-2 genomic RNA, a specific radiolabelled HIV-2 cDNA probe was generated by digesting HIV-2 proviral DNA ROD27 with *Hinc*II and *Sca*I.

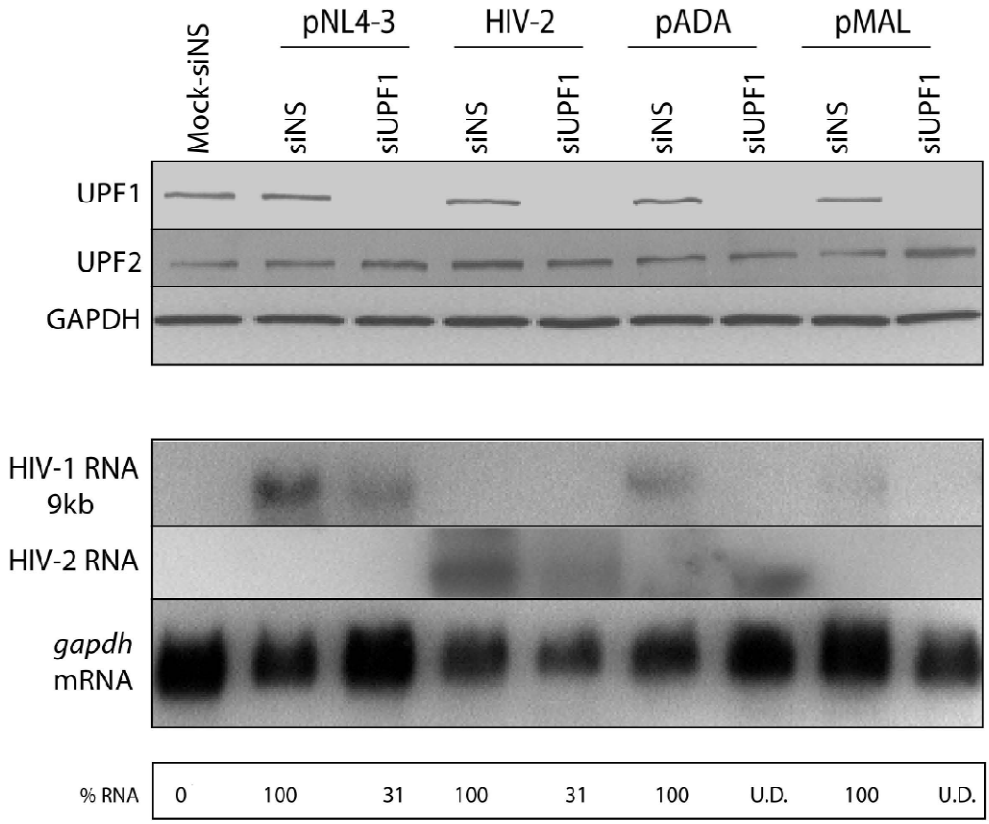

FIGURE 15: *UPF1 depletion results in decreased HIV-1 genomic RNA in T- and M- tropic viruses, as well as in HIV-2.*

FIGURE 16: *Expression of siRNA-insensitive UPF1 rescues pr55^{Gag} synthesis but not gag mRNA levels.*

HeLa cells were mock transfected (lane 1) or transfected with pNL4-3 proviral DNA, siNS and 1µg UPF1^{Rescue} (lane 2), or siUPF1 and increasing concentrations (1, 2 or 3µg) of UPF1^{Rescue}; lanes 3-5). Cells were harvested at 30hr and monitored for UPF1, pr55^{Gag} and GAPDH (as loading control) expression levels by Western blotting. HIV-1 RNA levels were measured by Northern blot analyses (bottom panel) using a pol-specific probe for the genomic RNA. *gapdh* mRNA served as a loading control.

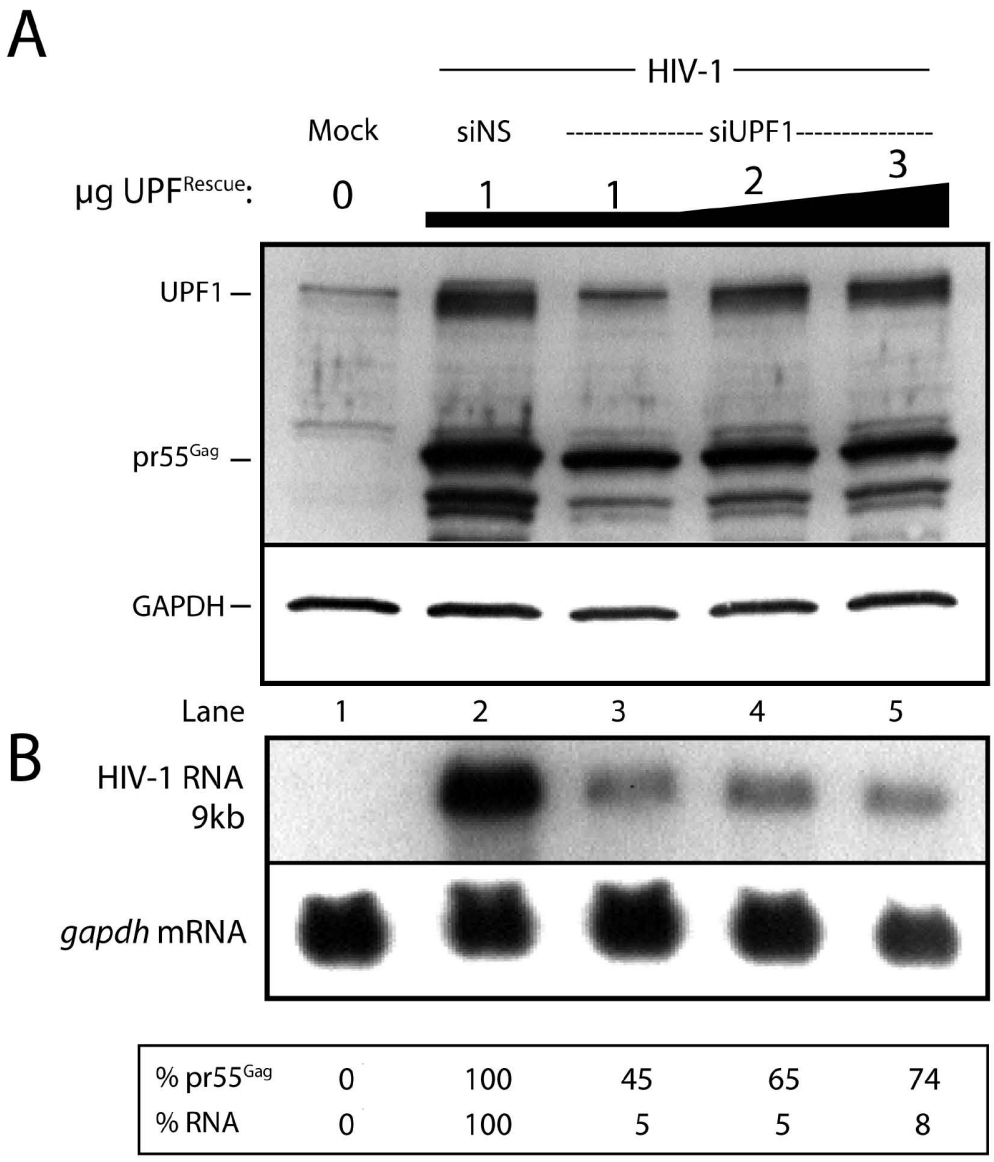

FIGURE 16: *Expression of siRNA-insensitive UPF1 rescues pr55^{Gag} synthesis but not gag mRNA levels.*

FIGURE 17**:** *FACS analysis of siNS- and siUPF1-treated cells.*

HeLa cells were mock transfected or transfected with pNL4-3 and siNS or siUPF1 and cultured for 30 hr or 4 days. At each time point, cells were harvested and cell cycle was assessed by FACS analysis [55]. Upper panels show UPF1 knockdown at 30 hr for mock and pNL4-3-transfected (HIV-1) cells and middle panels show cell cycle distribution with gated channels for Go/G1, S and G2/M phases indicated with bars. The bottom stacked histograms show 30 hr FACS cell cycle results as indicated in colour representing averages calculated from 4 independent experiments. 4-day results are representative of 2 independent experiments.

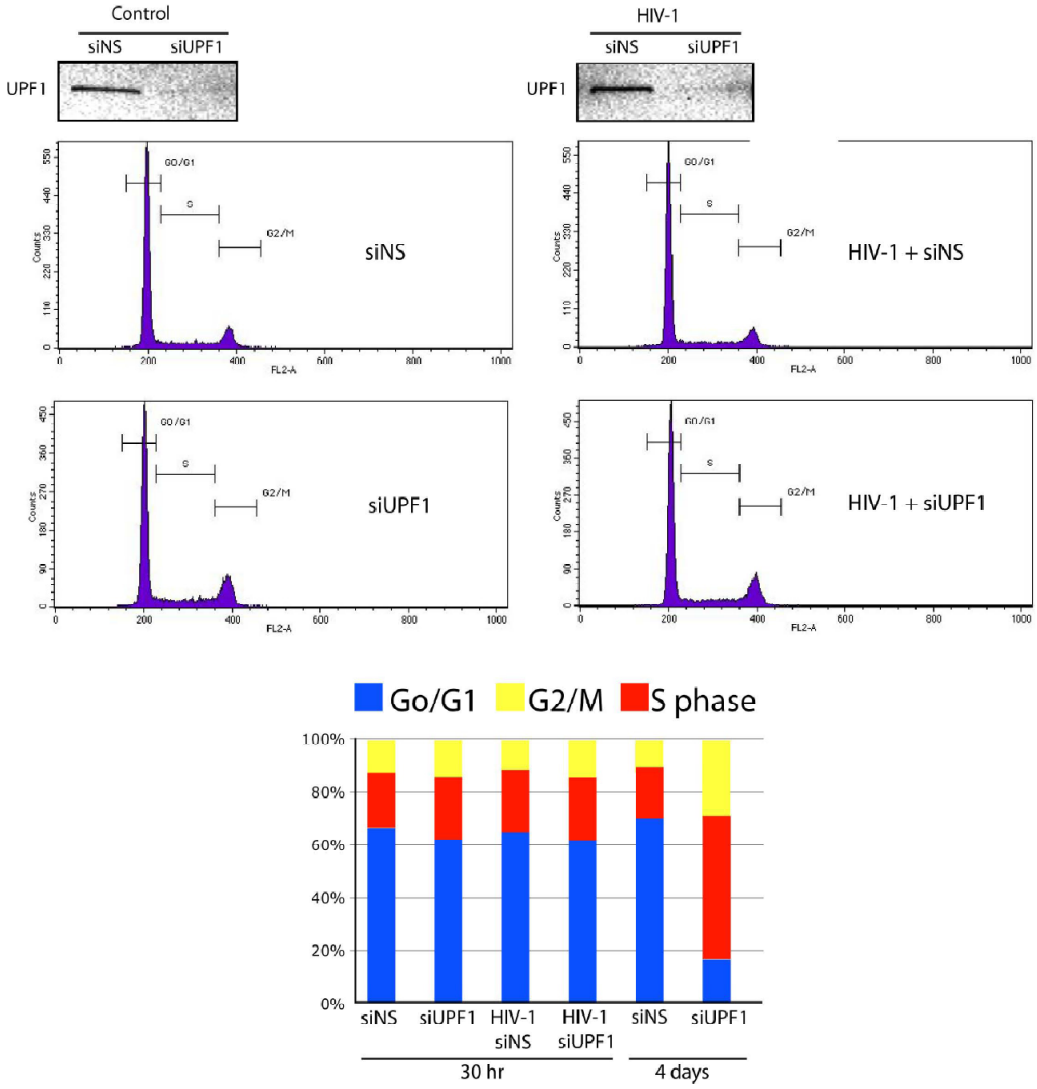

FIGURE 17: *FACS analysis of siNS- and siUPF1-treated cells.*

Chapter 3

Chapter 3: Research hypothesis and specific aims

In Chapter 2, we showed that UPF1 is involved in HIV-1 genomic RNA stability and translation. These effects were independent of its well characterized role in NMD, which is to degrade PTC containing mRNA. Furthermore, UPF1's role in the stabilization of the HIV-1 genomic RNA was observed in both nuclear and cytoplasmic cellular compartments.

A subset of cellular proteins co-opted by HIV-1 increases HIV-1 genomic RNA export via an interaction in the nucleus. Because UPF1 is associated with the newly transcribed nuclear HIV-1 genomic RNA and our studies in Chapter 2 point out to a nuclear role of UPF1, this led us to our second hypothesis: the nucleocytoplasmic shuttling function of UPF1 is required for HIV-1 genomic RNA export.

Chapter 3

UPF1 shuttling is required for nucleocytoplasmic trafficking

of the HIV-1 genomic RNA

UPF1 shuttling is required for nucleocytoplasmic trafficking of the HIV-1 genomic RNA

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Manuscript in preparation

ABSTRACT

The HIV-1 ribonucleoprotein complex includes Gag, the genomic RNA (vRNA) and several cellular proteins including upframeshift protein 1 (UPF1), a key factor in nonsense-mediated mRNA decay (NMD). In earlier work, we demonstrated that UPF1 controlled vRNA stability and translation and these effects were independent of its functions in NMD. Here we reveal that UPF1 promotes nucleocytoplasmic vRNA export that was dependent on UPF1 nucleocytoplasmic shuttling mediated by its nuclear export and localization sequences (NES, NLS). UPF1 relieved the nuclear retention of vRNA that was imposed by either a mutation in rev or pharmacologically. Importantly, we established that the shuttling function of UPF1 is required for proper export. UPF1 coimmuno-precipitated with Rev, CRM1 and DDX3 as well as the nucleoporin p62 supporting a dominant role for UPF1 in HIV-1 vRNA trafficking during the late expression phase of HIV-1.

INTRODUCTION

The nucleocytoplasmic export of macromolecules (RNA and protein) and RNA-protein (RNP) complexes are critical steps in ensuring proper cellular function. A great deal of information on this highly regulated process has been derived from studies on retroviral export [1]. For instance, the nucleocytoplasmic export of intron-containing viral RNAs is achieved by the recruitment of factors that help overcome nuclear retention signals inherent in viral genomic RNAs [2]. Several retroviruses use the chromosome region maintenance 1 (CRM1)-mediated export [e.g., human T-lymphotropic virus type I (HTLV-1); human immunodeficiency virus type I (HIV-1)], while simple retroviruses such as Mason-Pfizer monkey virus (MPMV) possess constitutive transport elements (CTE) to program nucleocytoplasmic transport of their RNAs via the nuclear RNA export factor 1 (NXF1) pathway [3].

HIV-1 utilizes two different export processes to transport its more than 30 mRNAs from the nucleus [4, 5]. The full-length, unspliced, 9kb viral genomic RNA (vRNA) and the singly-spliced, 4kb RNAs bypass nuclear splicing events and are exported via the CRM1 export pathway [6]. HIV-1 encodes the accessory protein, Rev which binds to the Rev-responsive element (RRE) within the envelope RNA coding sequence in HIV-1 to initiate nucleocytoplasmic export of these intron-containing viral RNAs [6]. The multiply-spliced, 2kb RNAs that are

generated early, are trafficked to the cytoplasm in a Rev-independent manner via the NXF1 export pathway [7].

Studies have highlighted the requirement for various host proteins in these processes. For example, the cellular protein eukaryotic translation initiation factor 5A (eIF5A) acts as an adaptor for Rev and CRM1 [8, 9]. RNA helicases such as DEAD box protein 1 and 3 (DDX1, 3) also bind CRM1 and Rev and promote Rev-mediated export [10]. Src-associated protein in mitosis, 68 kDa (Sam68) is not only required for Rev-dependent export, but is also involved in exporting RRE-containing RNAs in a Rev- and CRM1-independent manner [11, 12]. Nuclear pore complex proteins such as nucleoporin p62 (Nup62) may also gate HIV-1 vRNA as it exits the nucleus [13]. Following nuclear export, additional host factors play roles at the nuclear periphery or in the cytoplasm. For example, human Rev-interacting protein (hRIP) is required for the release of the HIV-1 vRNA from the nuclear periphery [14, 15]; heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) mediates cytoplasmic trafficking [16, 17]; and Sam68 appears to be responsible for shuttling HIV-1 vRNA to the translation apparatus [18, 19]. Whereas these examples identify positive activators of nucleocytoplasmic export, to date at least two have been identified to act in a negative fashion and these include insulin-like growth factor II mRNA binding protein 1 (IMP1) and HS1-associated protein X-1 (Hax-1) [20, 21]. Thus, the roles of auxiliary host factors in nuclear export are intensely studied because they represent suitable targets to block HIV-1 gene expression and assembly.

UPF1 is an RNA-binding protein that possesses Zn-finger motifs, RNA helicase and ATPase domains as well as acidic and basic amino acid clusters [22] and shuttles between the nucleus and cytoplasm via CRM1 [23]. UPF1's roles in mammalian cells are quite diverse and these include roles in RNA stability [24, 25], DNA repair and cell cycle progression [26] and completion of DNA replication and telomere metabolism [27]. Its best understood function is in nonsense-mediated mRNA decay (NMD), a RNA surveillance mechanism in which UPF1 mediates the decay of aberrant mRNAs that harbor premature terminations [28, 29]. UPF1 is also integral in Staufen1-mediated RNA decay (SMD) whereby Staufen1 recruits UPF1 to mRNAs for decay. Overall, UPF1 serves many diverse functional roles in cell physiology [22]. UPF1 also has functional importance during HIV-1 replication in HIV-1 RNA metabolism and in the control of Gag synthesis that are independent of its function in NMD [[30] & this work]. UPF1 has also been characterized in the context of other retroviruses such Rous Sarcoma Virus (RSV) where the RSV RNA is subject to NMD when a pre-termination codon (PTC) is introduced in the gag open reading frame [31].

We have shown that UPF1 expression levels modulate steady-state vRNA levels of both laboratory and clinical strains of HIV-1 and of other retroviruses [30]. Importantly, UPF1 selectively stabilizes the vRNA and our earlier work also strongly supports a nuclear role for UPF1 in stabilizing de novo synthesized HIV-1 vRNA. In the cytoplasm, UPF1 is an integral component of the HIV-1 RNP [30] such that HIV-1 induces UPF1 co-localization with Gag and vRNA at viral assembly sites, likely via an interaction with Gag [30] and/or with the HIV-1

vRNA [32]. In this report a new role for UPF1 in HIV-1 vRNA fate is described. We demonstrate that UPF1 shuttling promotes the nucleocytoplasmic export of vRNA and identify UPF1 binding domains required for this activity. UPF1 also overcomes vRNA nuclear export constraints imposed by genetic and pharmacologic approaches. This new role for UPF1 reveals an unsuspected link between nuclear and cytoplasmic roles of UPF1 during the late expression phase of HIV-1 replication.

RESULTS

Mapping the domains of UPF1 required for Gag and vRNA upregulation and binding

Our earlier work showed that both wild-type UPF1 ($UPF1^{WT}$) and the trans-dominant negative (TDN) and NMD null RNA helicase mutant, $UPF1^{R844C}$ increased HIV-1 $pr55^{Gag}$ (herein called Gag) and vRNA levels, indicating that UPF1's function in HIV-1 vRNA metabolism is independent of its role in NMD. Also, while NMD can require the interaction between UPF1 and UPF2 [33, 34], this association was not required for the resulting effects on HIV-1 gene expression [30]. Therefore, we decided to identify domains of UPF1 that are important in conferring these effects. We co-expressed GFP-tagged wildtype $UPF1^{WT}$ and several UPF1 mutants (Figure 18A & Figure 25) with the proviral DNA pNL4-3 and examined levels of Gag and vRNA by western blot and RT-PCR analysis. As shown before, $UPF1^{WT}$ overexpression increased Gag expression and vRNA levels (Figure 18B). The co-expression of a helicase UPF1 mutant (HelΔI, Ia) also resulted in an increase in both Gag and vRNA levels. The UPF1 mutant lacking a functional nuclear export signal, ΔNES, increased vRNA levels when overexpressed but had little effect on Gag protein levels. UPF1 mutants ΔNLS, HelΔIV-VI, ΔPS with deleted nuclear localization signal, helicase domains IV-VI and a C-terminus, respectively, did not elicit changes either in vRNA or Gag expression levels (Figure 18B). We further investigated vRNA

binding to each of these UPF1 proteins (Figure 18C). GFP-tagged mutants were immunoprecipitated and the bound vRNA was extracted, purified and RT-PCR analysis as described in Materials and Methods. vRNA co-immunoprecipitated with $UPF1^{WT}$, consistent with its presence in the HIV-1 RNP [30]. Likewise, both ΔNES and HelΔI,Ia co-immunoprecipitated with the vRNA whereas vRNA was not detected when the mutants ΔNLS, HelΔIV-VI and ΔPS were expressed (Figure 18C). These results show that ΔNES, although it does not lead to enhanced Gag synthesis, markedly co-immunoprecipitated with the vRNA providing evidence for a functional interaction with nuclear vRNA that could occur between the NLS, Helicase IV-VI and/or C-terminal domain. Moreover, Gag remains constant upon expression of these mutants, indicating that these domains of UPF1 also contribute to the regulation of Gag synthesis and vRNA levels.

The shuttling function of UPF1 is important for HIV-1 vRNA export

Since the vRNA co-precipitated with ΔNES UPF1 to the highest degree (Figure 18C), we next determined the localization of UPF1 proteins in relation to the vRNA using fluorescence in situ hybridization/immunofluorescence (FISH/IF) co-analyses (Figure 19). $UPF1^{WT}$ exhibited a mainly cytoplasmic distribution, like endogenous and in these cells, we observed a noticeable increase in vRNA signal intensity in cells transfected with $UPF1^{WT}$ and HIV-1 (Figure 19A) consistent with our earlier work [30]. ΔNES accumulated in the nucleus as demonstrated earlier [23] but surprisingly, vRNA staining localized principally to the nucleus

(Figure 19B). The shuttling mutant that lacks a functional nuclear localization signal, ΔNLS, was entirely cytoplasmic and vRNA intensity did not exhibit any apparent increase (Figure 19B & Figure 18C). Because the overexpression of ΔNES confines the vRNA to the nucleus even in the presence of an active Rev protein (Figure 19B), we examined Gag and vRNA expression levels under $UPF1^{WT}$ overexpression and vRNA localization upon overexpression of $UPF1^{WT}$, ΔNES and ΔNLS during the expression of a Rev- proviral DNA. Under these conditions, the overexpression of FLAG-$UPF1^{WT}$ resulted in a slight increase in Gag expression but a more marked increase in vRNA levels was observed similar to that found in Rev+ conditions (Figure 20A, right lanes). Under UPF1 depletion conditions we also showed that the vRNA exhibited exquisite sensitivity to UPF1 levels in both Rev- and Rev+ conditions [30]. Since a basal increase in Gag expression was observed, we decided to investigate whether this could be explained by a translocation of the vRNA to the cytoplasm. We overexpressed GFP-tagged $UPF1^{WT}$, ΔNES and ΔNLS under Rev- conditions and determined the localization of the GFP-tagged UPF1 proteins in relation to the HIV-1 vRNA by FISH/IF co-analyses (Figure 20B-20D). Laser scanning confocal microscopy (LSCM) analysis revealed that under Rev- conditions, vRNA was confined to the nucleus as expected (Figure 20B white arrows). Surprisingly, $UPF1^{WT}$ overexpression resulted in the translocation of the vRNA to the cytoplasm (Figure 20B & 20E) while the shuttling ΔNES and ΔNLS mutants did not further affect vRNA localization (Figure 20C-20E). We did not observe any major changes in total mRNA distribution as determined by staining with the dye SYTO14 (Figure

26A [16]) indicating that the effects of UPF1 on HIV-1 vRNA localization were specific.

These results support a new role for UPF1^{WT} in the promotion of HIV-1 vRNA nucleocytoplasmic export that requires its ability to shuttle between the nucleus and cytoplasm.

CRM1 export pathway is favored by UPF1 for efficient HIV-1 vRNA export

The two essential nucleocytoplasmic export pathways in cells are those mediated by CRM1 and NXF1. HIV-1 uses both export pathways in the export of its different RNA transcripts such that multi-spliced RNAs are exported via NXF1 while singly-spliced and the unspliced (vRNA) transcripts that require Rev, are exported to the cytoplasm via CRM1 [6, 35]. To differentiate between these two export pathways, we first co-transfected FLAG-UPF1^{WT} with or without Rev-proviral DNA and with or without LMB treatment to block CRM1-mediated export at 6 h post transfection. LMB treatment resulted in a decrease in Gag expression under both pNL4-3 and pMRev(-), as expected [36] (Figure 21A). FLAG-UPF1^{WT} overexpression also increased Gag expression under Rev-conditions. To validate these results, FISH/IF co-analyses were employed to visualize the localization of the HIV-1 vRNA under the same conditions; a negative transdominant form of NXF1 (HA-TapA17) DNA was also included to inhibit NXF1-mediated export [37]. LMB treatment resulted in the nuclear accumulation of the HIV-1 vRNA as well as a partial but significant nuclear

localization of UPF1 as previously shown by others (Figure 21B, top panel) [23]. In pNL4.3 and Rev- conditions with LMB, prominent cytoplasmic staining for the HIV-1 vRNA was observed only in cells in which FLAG-UPF1^{WT} was expressed (78%, n=55 cells in the experiment shown; Figure 21C, top and bottom panels). Thus, UPF1 is able to overcome the block to vRNA nuclear export imposed by LMB treatment. In contrast, a normal cytoplasmic and punctate distribution of the vRNA was observed when NXF1-mediated export was blocked using HA-TapA17, under Rev+ conditions (Figure 21D). Moreover, under conditions in which UPF1 promotes HIV-1 vRNA export (i.e., Rev+ and Rev- with UPF1 overexpression), there was no detectable block to HIV-1 vRNA nuclear export in the presence of the HA-TapA17 (Figure 21D). LSCM analysis revealed that LMB treatment caused a partial but significant nuclear accumulation of UPF1 [23] while there was no significant change in UPF1 localization with HA-TapA17 (Figure 21). We therefore conclude that UPF1 can promote HIV-1 vRNA nucleocytoplasmic export in normal conditions and in the face of both CRM1 and NXF1 blockades.

Since NXF1 blockade has been shown not to affect HIV-1 RRE vRNA export [37] and since UPF1 shuttles via CRM1, our results indicate that UPF1 uses CRM1 rather than NXF1 for HIV-1 vRNA export. To validate specificity, we therefore decided to directly determine if this newly characterized UPF1 export function, which is CRM1-dependent in the context of bona fide HIV-1 expression, is also required for CTE-directed export of the *gag* mRNA. Therefore, we co-expressed Gag-CTE with either FLAG-UPF1^{WT} or HA-TapA17 and assessed Gag

expression levels by western blotting (Figure 22). The overexpression of HA-TapA17 blocked NXF1-mediated export and resulted in a decrease in Gag-CTE, while, importantly; there was no change in Gag-CTE when FLAG-UPF1^{WT} was expressed (Figure 22). This result shows that while UPF1 can promote the export of *Gag* mRNAs amenable for CRM1 export, but cannot enhance CTE/NXF1–mediated export, at least in the context of Gag.

UPF1 is present in a complex with Rev, CRM1, DDX3 and Nup62

To understand how UPF1 promotes Rev-independent as well as Rev-dependent RNA nuclear export, we determined if CRM1 and UPF1 interacted. We transfected cells with FLAG-UPF1^{WT} followed by immunoprecipitation analysis. The immunoprecipitates were then subjected to western analysis for CRM1 and DDX3. UPF1 immunoprecipitated with CRM1 in all three conditions (mock, Rev+ and Rev- conditions) (Figure 23A). DDX3 also immunoprecipitated with UPF1 and its presence in the UPF1 RNP was increased only in the Rev+ condition, indicating that UPF1 is found in an export competent complex containing DDX3 and CRM1 [10].

HIV-1 encodes the regulatory viral protein Rev to export singly-spliced and unspliced vRNAs to the cytoplasm [6]. Rev binds to the HIV-1 vRNA by interacting with its NLS and the RRE element on the vRNA and exports the vRNA through the CRM1-mediated pathway through the nuclear pore via an interaction with various nucleoporins such as Nup98 and Nup214 [8, 38, 39]. Our

results highlight the importance of the shuttling function of UPF1 in HIV-1 vRNA export in both a Rev-dependent and -independent manner. Therefore, we decided to identify if UPF1 was present in the RNP complex required for HIV-1 vRNA nucleocytoplasmic export. To do this, we overexpressed FLAG-UPF1^{WT} with or without a Rev expressor (Rev-R-YC) and immunoprecipitated FLAG-UPF1^{WT}. Rev, DDX3 and CRM1 all co-immunoprecipitated with UPF1^{WT} in the presence and absence of Rev (Figures 23A & 23B). In work published from our lab, we showed that the depletion of Nup62 resulted in a block to vRNA nuclear export and Nup62 was also found to be part of the HIV-1 Rev RNP complex [8, 13]. We therefore sought to determine if UPF1 could also co-precipitate with Nup62. Indeed, Nup62 was also found to be part of the UPF1 RNP strengthening the role of UPF1 in HIV-1 vRNA export.

Since our previous results have shown that UPF1 co-immunoprecipitated with Gag [30] and here with Rev, DDX3, CRM1 and Nup62 (Figures 23A-C), we determined if UPF1 was part of two separate complexes in cells by immunoprecipitating Gag, which is predominantly cytoplasmic. We therefore immunoprecipitated Gag in mock- and in pNL4-3-transfected cells. Flag-UPF1^{WT} co-immunoprecipitated with Gag as shown earlier, while DDX3, Nup62 and CRM1 were not detectable in this Gag-UPF1 complex (Figure 23D). These results therefore suggest that UPF1 can be found in two different HIV-1 RNPs: the first being composed of RNA nucleocytoplasmic export factors such as CRM1, Rev, Nup62 and DDX3 and a second UPF1 complex that includes Gag and a variety of

other cytoplasmic proteins likely to be important for viral assembly as proposed [30].

DISCUSSION

In this study, we identify a novel function for UPF1 in the regulation of HIV-1 vRNA nucleocytoplasmic export. The overexpression of $UPF1^{WT}$ led to the nuclear export of vRNA, under both Rev+ and Rev- conditions and even when vRNA export was blocked pharmacologically. Nevertheless, Gag expression did not increase substantially in Rev- conditions since Rev is required for its translation (Figure 20A) [40-44]. UPF1 ΔNES blocked vRNA nucleocytoplasmic export in Rev+ conditions (Figure 19D) providing evidence for a transdominant effect by this UPF1 shuttling mutant. These results correlate with our previous data such that by increasing UPF1 levels by overexpression (Figures 18 & 19), Gag expression levels are enhanced; an effect that is likely due to enhanced HIV-1 vRNA nucleocytoplasmic export [30].

Based on our current findings, UPF1 requires its shuttling function (NES and NLS domains) for proper HIV-1 vRNA export. The modular binding of UPF1 reflects the current model on how HIV-1 vRNA nucleocytoplasmic export is achieved in that Rev binds to the HIV-1 vRNA via the NLS [38]. The binding patterns are quite similar, it is yet left to be determined if both Rev and UPF1 require similar binding domains. Studies have also shown that Rev and UPF1, upon actinomycin D treatment results in the loss of their nuclear localization thus hinting that they are components of transport between the nucleus and cytoplasm [45, 46].

A role for UPF1 in RNA export is not without precedence, however. Yeast-two hybrid studies have shown that UPF1 interacts with nucleoporins Nup100/116 (mammalian homolog is Nup98), suggesting that this interaction ensures the association of UPF1 with the newly synthesized mRNAs [47, 48]. Interestingly, Nup98 is also a cofactor of CRM1/Rev as well as an important nucleoporin for HIV-1 vRNA export [49]. Further studies revealed Nup62 is in the Rev RNP and recent work from our laboratory demonstrated that Nup62 is a valuable player in nucleocytoplasmic export. Nup62's association with UPF1 strengthens the importance of UPF1 in HIV-1 vRNA export. It was shown that ataxia-telangiectasia-mutated kinase (ATM), while not only involved in HIV-1 DNA integration and DNA damage, it also promotes HIV-1 RNA nucleocytoplasmic export by activating Rev [50]. Interestingly, ATM phosphorylates UPF1 [50, 51]. Future work will be needed to determine if this post-translational modification of UPF1 is required for its activity in nucleocytoplasmic export.

Our previous studies showed that UPF1 knockdown negatively affected steady-state levels of both cytoplasmic and nuclear HIV-1 vRNA [30]. Since it was shown that UPF1 depletion increased Hax-1 mRNA levels [52], it is possible that the similarity between what we observe when UPF1 is depleted [30] and when Hax-1 is overexpressed [52] is due to the reformation of the nuclear vRNA RNP to negatively regulate HIV-1 vRNA metabolism prior to nuclear export. This could also explain why the nuclear export mutant of UPF1 does not affect negatively vRNA levels because it is still tightly associated with the vRNA and inhibitors such as Hax-1 are not able to bind the viral. Other studies have also

shown that Sam68 knockdown affects vRNA levels because inhibitors, like Hax-1, are recruited to the HIV-1 vRNA RNP to negatively affect steady-state levels and vRNA export [11, 20].

Both NXF1 and CRM1, in most cases, do not directly bind RNAs and therefore require adaptor proteins to recruit mRNAs for nuclear export [53, 54]. Likewise, HIV-1 must generate an environment so that its own RNAs are efficiently exported. Since our results indicate that UPF1's role is RRE-specific and is not involved in CTE-mediated export (Figures 21-22) and is able to overcome LMB and Rev- phenotypes, UPF1 thus represents a novel adaptor protein that binds the HIV-1 vRNA and promotes its export through its interaction with CRM1, Nup62, DDX3 among other proteins. Because UPF1 shuttles via CRM1 [23], HIV-1 may have evolved a means to efficiently traffic its mRNAs by co-opting UPF1 function. We cannot rule out the possibility that under LMB treatment UPF1 efficiently exports the HIV-1 vRNA through the NXF1 export pathway since NXF1 and UPF1 have been shown to be in complex together with other export factors [55]. Further studies are needed to test the possibility that UPF1 might promote selectively Rev-dependent export under LMB conditions.

Likewise, recent proteomic studies now reveal that UPF1 binds to HIV-1 vRNA in the nucleus thus making it an attractive target for therapy [56] and in this proteomic study they identified DDX3, whose roles in RRE-dependent export have been characterized. Since bulk mRNA export depends on NXF1 and UPF1 overexpression does not affect total cellular RNA distribution (Figure 26A), it is

likely that UPF1 does not affect this export machinery. These results are similar to what was found for the host protein DDX3 in HIV-1 vRNA export, where DDX3 only affects RRE- but not CTE-mediated export [10]. Moreover, UPF1 binds CRM1, Rev, Nup62 and DDX3 (Figures 23) which are key components required for efficient HIV-1 vRNA export, thus strengthening notion that UPF1 plays a regulatory role in HIV-1 vRNA nucleocytoplasmic export. Data revealing that UPF1 is found in two different HIV-1 RNP complexes (Figure 23), one in which DDX3, CRM1, Nup62 and Rev are found, and another in which Gag is present provide support for both nuclear and cytoplasmic functions of UPF1 in the HIV-1 replication cycle. In the nucleus, UPF1 most likely enhances the association of the HIV-1 vRNA with export factors by remodeling of the HIV-1 RNP and then accompanies the vRNA into the cytoplasm where it enhances stability and translation independently of its nuclear remodeling function.

We propose the following model for the role of UPF1 in HIV-1 replication. First, UPF1 is recruited to the HIV-1 vRNA via its NLS domain as well as the helicase IV-VI and/or C-terminal domains and this may steer this intron-containing mRNA from the nuclear RNA quality control surveillance machinery as well serving as an adaptor protein for Rev/CRM1-mediated export. Next, Rev NLS binds the RRE and other critical interactions will occur between UPF1 NES and export factors as well as between Rev-NES and CRM1. This large nuclear vRNA RNP acquires additional nuclear proteins including UPF1, DDX3, DDX1, eIF5A amongst others and is poised for nuclear export through the nuclear pore. The RNP engages several nuclear pore proteins, such as Nup98, Nup214 and/or

Nup62 [8, 49]& Figure 23C & 23D) resulting in the efficient export and cytoplasmic localization of the HIV-1 vRNA (Figure 24).

In summary, UPF1 is identified here as a novel cellular factor required for efficient HIV-1 vRNA nucleocytoplasmic transport providing new insight into the complex interplay between HIV-1 vRNA and host proteins and machineries. The results shown here also highlight how UPF1 mutants can act in a transdominant negative manner to block nucleocytoplasmic export even when all viral regulatory proteins are expressed. Current strategies using Rev-transdominant RevM10 to block vRNA export have failed because HIV-1 develops resistance so targeting of Rev alone will likely not be sufficient [57]. Therefore, cofactors or cellular proteins that aid in Rev function remain viable targets. Since we have shown that UPF1 is required for HIV-1 vRNA stability, translation and now nucleocytoplasmic export, targeting UPF1 might represent a suitable multi-pronged approach that will contribute to the arsenal that we use to block HIV-1 replication.

ACKNOWLEDGEMENTS

We thank Jens Lykke-Andersen, Harry Dietz, William Rigby, Alan Cochrane, Ruth Brack-Werner and Bryan Cullen for antisera and clones and Reza Sadaie, Bruce Chesebro and the NIH AIDS Reference and Reagent Program for reagents. We thank Kishanda Vyboh for technical support, Fernando Valiente-Eccheveria and Marie-Joelle Miron for critical reading of the manuscript and other members of the lab for helpful discussions. L.A. is a recipient of a Canadian Institutes of Health Research (CIHR) Doctoral Research Award; A.J.M. is supported by a McGill University Fraser, Monat and MacPherson Scholarship. This work was supported by grants from the CIHR (#MOP-38111) and the Canadian Foundation for AIDS Research to A.J.M.

MATERIALS AND METHODS

Cell culture, plasmids and transfections

Cell culture of HeLa cells and transfections of proviral DNAs, pNL4-3 and pMRev(-) were performed as described before [30]. Total cellular RNA was isolated by TriZol Reagent or TriZol LS (Invitrogen) according to the manufacturer's instructions. pMRev(-) construct was provided by the NIH AIDS Reference and Reagent Program (generously provided by Reza Sadaie). pC1-FLAG-UPF1 ($UPF1^{WT}$) and pC1-FLAG were described earlier [30]. Bryan Cullen (Duke University) provided the HA-TapA17 plasmid [37] and Alan Cochrane (University of Toronto) provided the CTE-Gag plasmid. The following GFP-tagged UPF1 wildtype ($UPF1^{WT}$) and UPF1 mutants were described earlier [23]. ΔNES lacks the nuclear export signal (NES) as well as both zinc fingers, HelΔI,Ia lacks the RNA helicase domains I and Ia, ΔNLS lacks the RNA helicase domains II and III as well as the nuclear localization signal (NLS), HelΔIV-VI lacks the RNA helicase domains IV and V, and ΔPS lacks phosphorylation sites at the carboxy terminus. Rev-R-YC was provided by Ruth Brack-Werner (GSF-National Research Center for Environment and Health, Neuherberg, Germany) [58]. LMB was purchased from Sigma-Aldrich. For the LMB experiments, cells were transfected and 20 h prior to collection, cells were treated with 2nM of LMB as described [59]. ImageJ was used to quantify the relative expression levels of

mRNA and proteins normalized using *gapdh* mRNA reverse transcription-PCR and western analysis.

Antisera and reagents

Antisera to UPF1 was generously supplied by Jens Lykke-Andersen (University of California, San Diego). Antisera to DDX3 was generously supplied by Luc DesGroseillers (University of Montreal). Mouse anti-p24 and mouse anti-GAPDH antisera were purchased from Intracell and Techni-Science, respectively. Mouse anti-Digoxin was purchased from Sigma-Aldrich and sheep anti-Digoxin was purchased from Roche. Mouse and Rabbit anti-FLAG antibodies were purchased from Sigma-Aldrich and mouse anti-GFP was purchased from Roche. Rabbit anti-CRM1 was purchased from Santa Cruz. Rabbit and mouse anti-HA was purchased from Santa Cruz and Roche, respectively. Nup62 antibody was purchased from Sigma-Aldrich. For western analysis, horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Rockland Immunochemicals (PA, USA). For indirect immunofluorescence and FISH, secondary fluorophore-conjugated antisera AlexaFluor donkey anti-mouse 488, 594 and 647, donkey anti-sheep 488 and 647, and donkey anti-rabbit 594 and 647, were purchased from Invitrogen [59].

FISH/IF co-analyses

Immunofluorescence and fluorescence in situ hybridization co-analyses were performed as described before. Microscopy was performed on a Zeiss LSM5 Pascal laser-scanning confocal microscope or on a WaveFX spinning disk confocal microscope system (Quorum Technologies, Inc.). Filter sets and laser wavelengths were described earlier [59, 60]. Intensity plots and imaging analyses were performed by Imaris software v. 7.0 (Bitplane, Inc.) in the measurement panel through the center focal plane, as described previously [60, 61]. All imaging experiments were performed at least 4-5 times with similar results. Representative phenotypes that are shown were observed in 78 to 85% cells (n>50 cells per condition) in each experiment, except when otherwise noted.

Immunoprecipitation analyses

HeLa cells were transfected as described above and were described earlier [30]. Briefly, 500µg of protein was immunoprecipitated with agarose-conjugated anti-GFP beads for 1 h and the bound vRNA was extracted and purified with TriZol LS followed by RT-PCR. RT-PCR was performed for vRNA and gapdh mRNA as described previously by using SuperScript One-Step RT-PCR (Invitrogen) [61]. For the FLAG immunoprecipitation, 500µg of total lysate was incubated 1 h with agarose conjugated anti-FLAG beads and the bound complex was analyzed via SDS-PAGE analysis as described earlier [61]. HeLa cells were transfected as described above. Cells were lysed in NP40 lysis buffer and Gag was immunoprecipitated as described previously using 1.5mg of protein and affinity-

purified mouse anti-p24 antisera (hybridroma 183-H12-5C) [62] from the NIH AIDS Reference and Reagent Program. [30].

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FIGURE AND FIGURE LEGENDS

FIGURE 18: *Mapping the functional domains of UPF1*.

(A) Schematic representation of UPF1. Zinc fingers are shown in blue bars. The seven helicase domains are shown in green bars. The ATPase activity resides in helicase domains Ia and II. The nuclear export signal (NES) corresponds to amino acids 55-416 and the nuclear localization signal (NLS) corresponds to amino acids 596-697. (B) HeLa cells were mock transfected with pCMV-GFP or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) with pCMV-GFP, $UPF1^{WT}$, ΔNES, HelΔI,Ia, ΔNLS, HelΔIV-VI and ΔPS. At 30h post-transfection, cells were harvested for Western blot analysis for GFP, UPF1, Gag and GAPDH (loading control). RNA was also isolated from cell extracts and RT-PCR was performed to measure steady state levels of HIV-1 and *gapdh* mRNA. The relative levels of Gag and vRNA are related to the signal obtained in pCMV-GFP and HIV-1 expressing cells, set to 1. Average fold changes are calculated from three separate experiments. (C) HeLa cells were transfected as in B and GFP was immunoprecipitated mock+pCMV-GFP, pNL4-3+pCMV-GFP, pNL4-3+$UPF1^{WT}$, pNL4-3+ΔNES, pNL4-3+HelΔI,Ia, pNL4-3+ΔNLS, pNL4-3+HelΔIV-VI and pNL4-3+ΔPS transfected cells by using agarose conjugated anti-GFP beads and the immunoprecipitated vRNA was identified by RT-PCR as described in Materials and Methods. GFP-tagged mutants were identified by anti-GFP antibody. The relative levels of vRNA are related to the signal obtained in

UPF1^{WT} and HIV-1 expressing cells, set to 1. ImageJ was used to quantify the relative expression levels of vRNA and Gag that were normalized using GAPDH protein and mRNA for western and RT-PCR analyses. (N.D.: not determined).

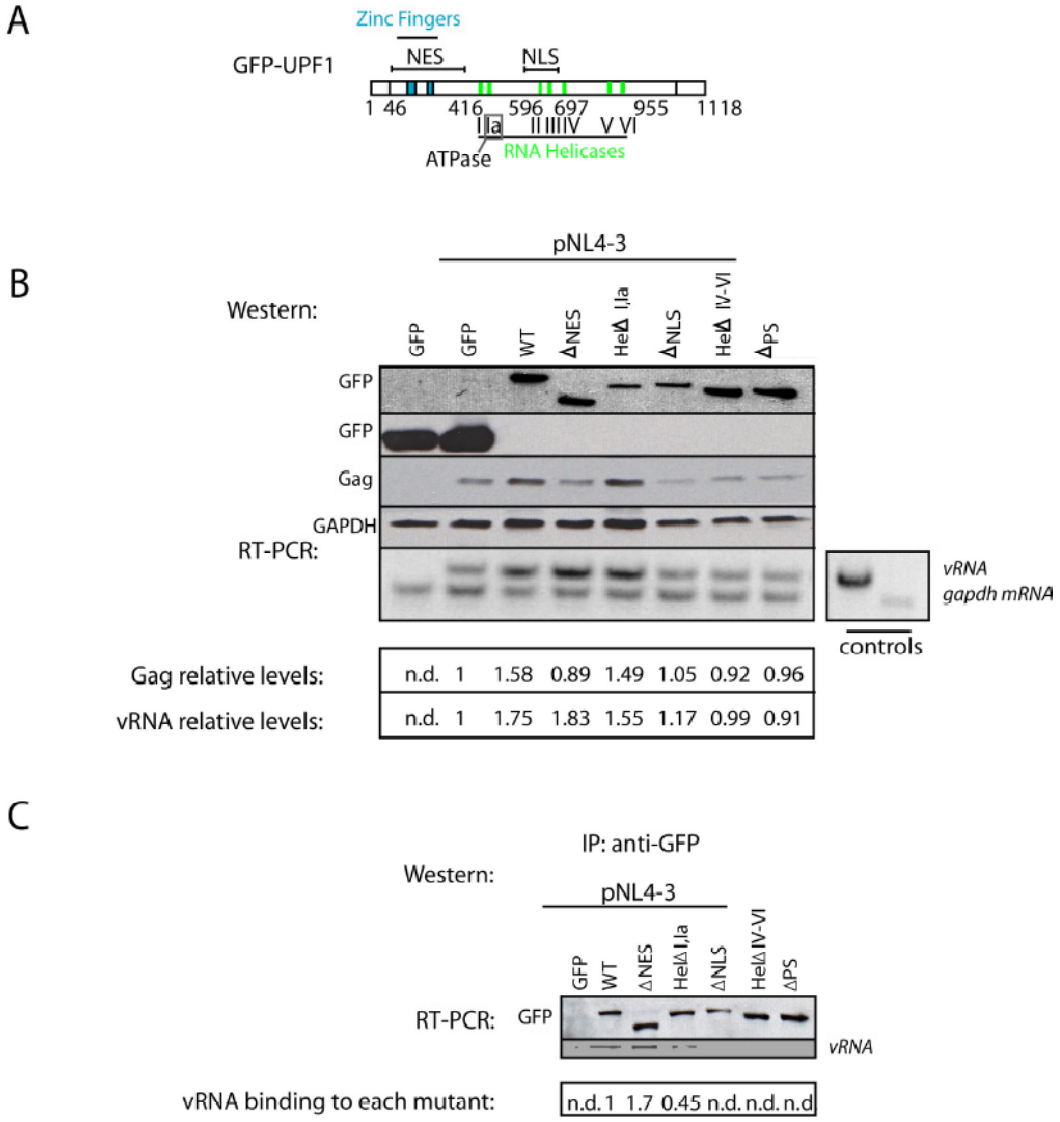

FIGURE 18: *Mapping the functional domains of UPF1.*

FIGURE 19: *Nuclear Export Mutant of UPF1 traps the vRNA in the nucleus.*

To determine the cellular localization of $UPF1^{WT}$, ΔNES and ΔNLS with HIV-1 vRNA, HeLa cells were seeded onto coverslips at $1.5x10^5$ cells per mL, and were transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and $UPF1^{WT}$ (A), ΔNES (B) and ΔNLS (C). At 30h post-transfection, cells were fixed for FISH/IF co-analyses for the vRNA (red) and $UPF1^{WT}$ (A), ΔNES (B) and ΔNLS (C) (green). (D) Intensity plots were created using Imaris 7.0 software. HIV-1 vRNA (red) and GFP tagged UPF1 protein staining from point A to B are shown in Figure 19A-C and Figure 19D shows the dotted lines which distinguish the nucleus and cytoplasm. White arrows indicate HIV-1 transfected cells. Size bars are 10μm. Imaging results shown are representative of the phenotypes observed in >85% cells in each condition, in 3-4 experiments.

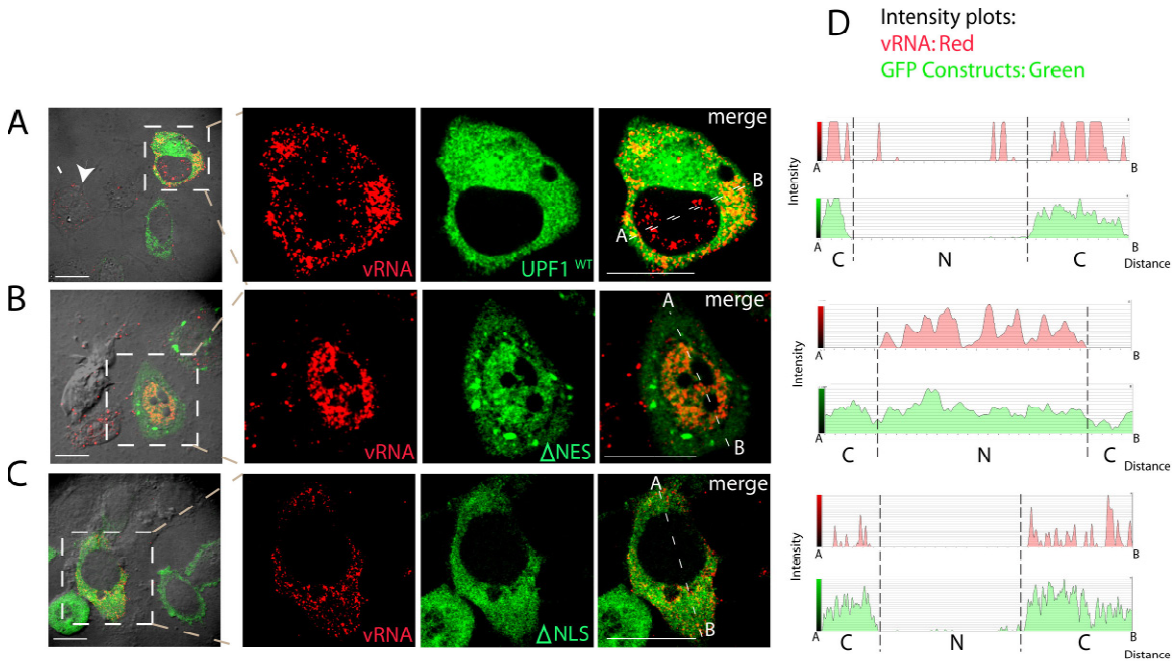

FIGURE 19: *Nuclear Export Mutant of UPF1 traps the vRNA in the nucleus.*

FIGURE 20: *Shuttling function of UPF1 is required for efficient HIV-1 vRNA export independently of Rev.*

(A) HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and pCI-FLAG or FLAG-UPF1^{WT}, or with a Rev-defective provirus pMRev(-) and pCI-FLAG or FLAG-UPF1^{WT}. At 30h post-transfection, cells were harvested for Western blot analysis for UPF1, Gag and GAPDH (loading control). RNA was also isolated from cell extracts and RT-PCR was performed to measure steady state levels of HIV-1 and *gapdh* mRNA. (B) To determine the cellular localization of UPF1 ^{WT}, ΔNES and ΔNLS with HIV-1 vRNA, HeLa cells were seeded onto coverslips at 1.5x10^5 cells per mL and were co-transfected with a Rev-defective provirus pMRev(-) with UPF1^{WT} (B), ΔNES (C) and ΔNLS (D). At 30h post-transfection, cells were fixed for FISH/IF co-analyses for the vRNA (red) and UPF1^{WT} (B), ΔNES (C) and ΔNLS (D) (green). (E) Intensity plots were created using Imaris 7.0 software. HIV-1 vRNA (red) and GFP tagged UPF1 staining from points A to B (panels 20B-20D). Dotted lines distinguish between nuclear and cytoplasmic compartments. White arrows indicate HIV-1 transfected cells. Size bars are 10μm. Imaging results are representative of the phenotype observed in >85% cells in each condition, in 5 experiments.

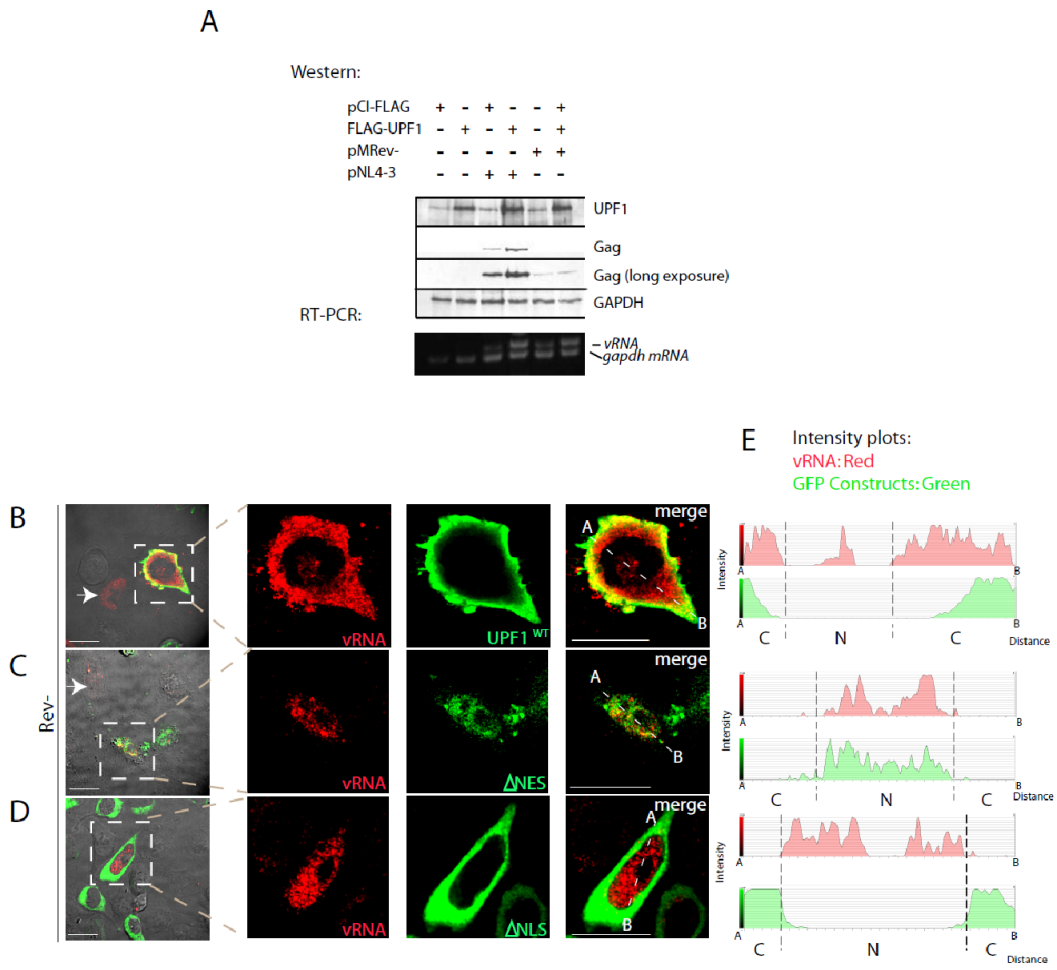

FIGURE 20: *Shuttling function of UPF1 is required for efficient HIV-1 vRNA export independently of Rev.*

FIGURE 21: *UPF1 exports HIV-1 vRNA via the CRM1 export pathway and not the NXF1 export pathway.*

(A) HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and pCI-FLAG or with a Rev-defective provirus pMRev(-) and pCI-FLAG or FLAG-UPF1^{WT}. At 20 h prior to harvesting, samples were treated with 2 nM of LMB. At 26 h post-transfection, cells were harvested for Western blot analysis for UPF1 (anti-Flag), Gag and GAPDH (loading control). B) To determine if LMB treatment or HA-TapA17 affected HIV-1 vRNA localization, HeLa cells were seeded onto coverslips and were transfected with pNL4-3 and treated with LMB at described above or co-transfected with pNL4-3 and HA-TapA17. At 26 h post-transfection, cells were fixed for FISH/IF co-analyses for endogenous UPF1 (red), HIV-1 vRNA (green) and HA-TapA17 (blue). (C) HeLa cells were seeded onto coverslips at 1.5x10^5 cells per mL and were transfected with pNL4-3 or pMRev- and co-expressed with FLAG-UPF1. 20 h prior to harvesting, cells were treated with 2 nM of LMB. At 26h post-transfection, cells were fixed for FISH/IF co-analyses for HIV-1 vRNA (green) and FLAG-UPF1 (red). (D) To determine if HA-TapA17 inhibited HIV-1 vRNA export via UPF1, HeLa cells were seeded onto coverslips at 1.5x10^5 cells per mL, and were transfected with pNL4-3 and co-expressed with FLAG-UPF1 and HA-TapA17. At 26 h post-transfection, cells were fixed for FISH/IF co-analyses for HIV-1 vRNA (green), FLAG-UPF1 (red) and HA-TapA17 (blue). Size bars are 10μm. White arrows indicated nuclear accumulation of HIV-1

vRNA under LMB treatment in B-D. Imaging results shown are representative of the phenotypes observed in 78-85% cells in each condition, in 3 experiments.

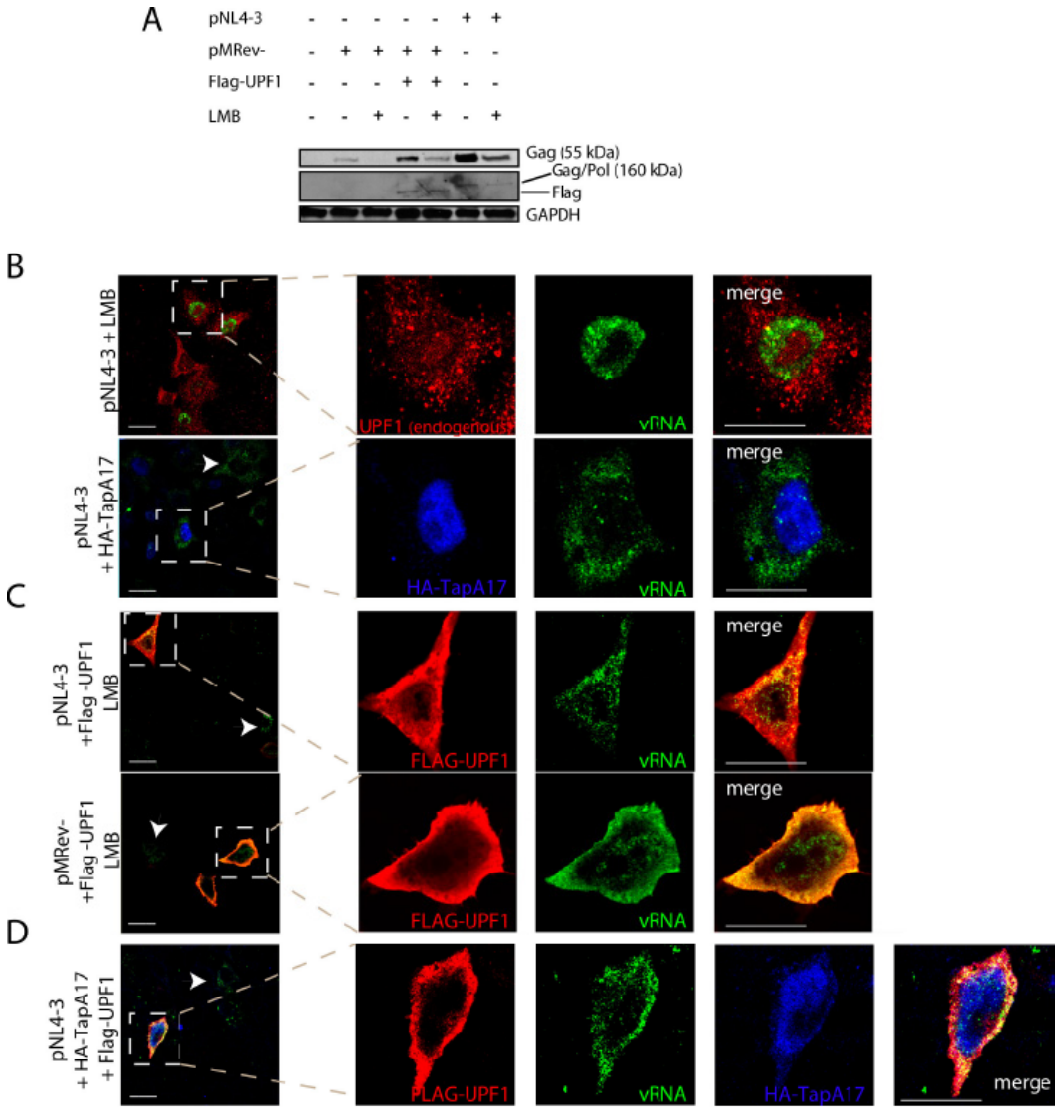

FIGURE 21: *UPF1 exports HIV-1 vRNA via the CRM1 export pathway and not the NXF1 export pathway.*

FIGURE 22: *UPF1 is not involved in CTE-mediated export.*

HeLa cells were mock transfected with pCI-FLAG or GAG-CTE without or with FLAG-UPF1 or HA-TapA17. At 30h post-transfection, cells were harvested for Western blot analysis for UPF1, HA, Gag and GAPDH (loading control). *Indicates Gag-CTE band (lower). ImageJ was used to quantify the relative expression levels of Gag normalized using GAPDH protein Western analysis. Results shown represent averages from 2 independent experiments with <10% deviation between experiments.(N.D.: not determined).

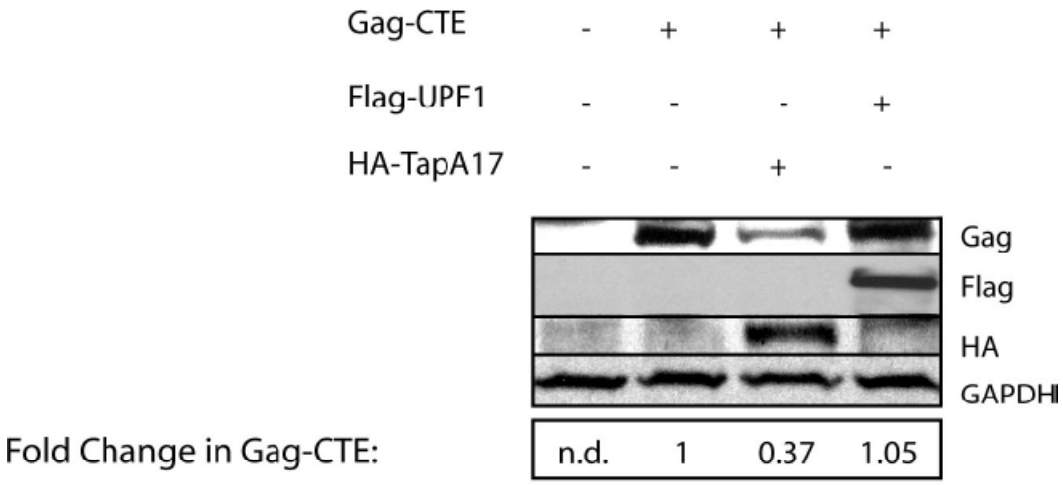

FIGURE 22: *UPF1 is not involved in CTE-mediated export.*

FIGURE 23: *UPF1 is found in two different HIV-1 RNPs: one with Rev-CRM1-DDX3-Nup62 and another with Gag.*

(A) HeLa cells were mock transfected with pCI-FLAG or FLAG-UPF1 or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) or pMRev-. At 30h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Material and Methods. Bound complexes were analyzed by Western blot analysis for UPF1, Gag, DDX3, CRM1 and GAPDH (loading control). HC: Heavy Chain IgG. Results shown are representative of three independent experiments. (B) HeLa cells were mock transfected with pCI-FLAG or FLAG-UPF1 or co-transfected with FLAG-UPF1 and Rev-R-YC. At 24h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Material and Methods. Western blot analysis for UPF1, CRM1, DDX3 and Rev. HC: Heavy Chain IgG. Asterisk (*) indicates band corresponding to CRM1. (C) HeLa cells were mock transfected with pCI-FLAG or FLAG-UPF1. At 24h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Materials and Methods. Western blot analysis is shown for FLAG and Nup62. HC: Heavy Chain IgG. (D) HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and pCI-FLAG or FLAG-UPF1^{WT}. At 30h post-transfection, cells were harvested and Gag was immunoprecipitated using a monoclonal anti-p24 antiserum as described in Material and Methods [30]. Western blot analysis for UPF1 (anti-FLAG), Gag, Nup62, CRM1 and DDX3 was performed. LC: Light Chain IgG.

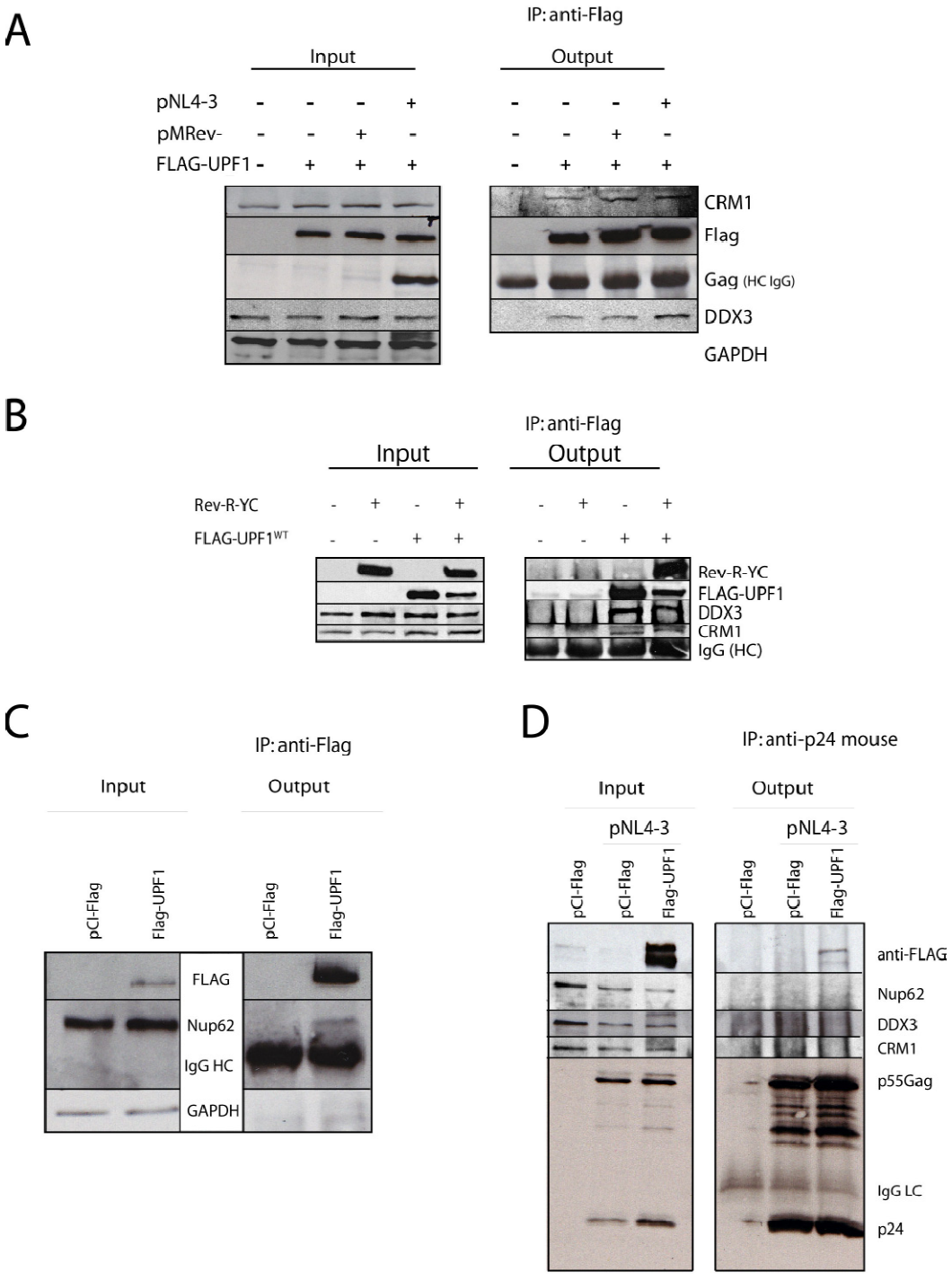

FIGURE 23: *UPF1 is found in two different HIV-1 RNPs: one with Rev-CRM1-DDX3-Nup62 and another with Gag.*

FIGURE 24: *Model of UPF1 mediated HIV-1 vRNA export.*

During HIV-1 expression, UPF1 binds the HIV-1 vRNA via its NLS or domains IV-VI as well as the C-Terminal and acts as an adaptor protein bridging the nuclear HIV-1 RNP with export factors through its NES region. The nuclear export complex on the HIV-1 vRNA comprised of UPF1 with Rev, CRM1, DDX3 and DDX1 then associates with the nuclear pore complex, Nup98 and/or Nup214, as well as Nup62, thus resulting in the efficient export of the unspliced HIV-1 vRNA.

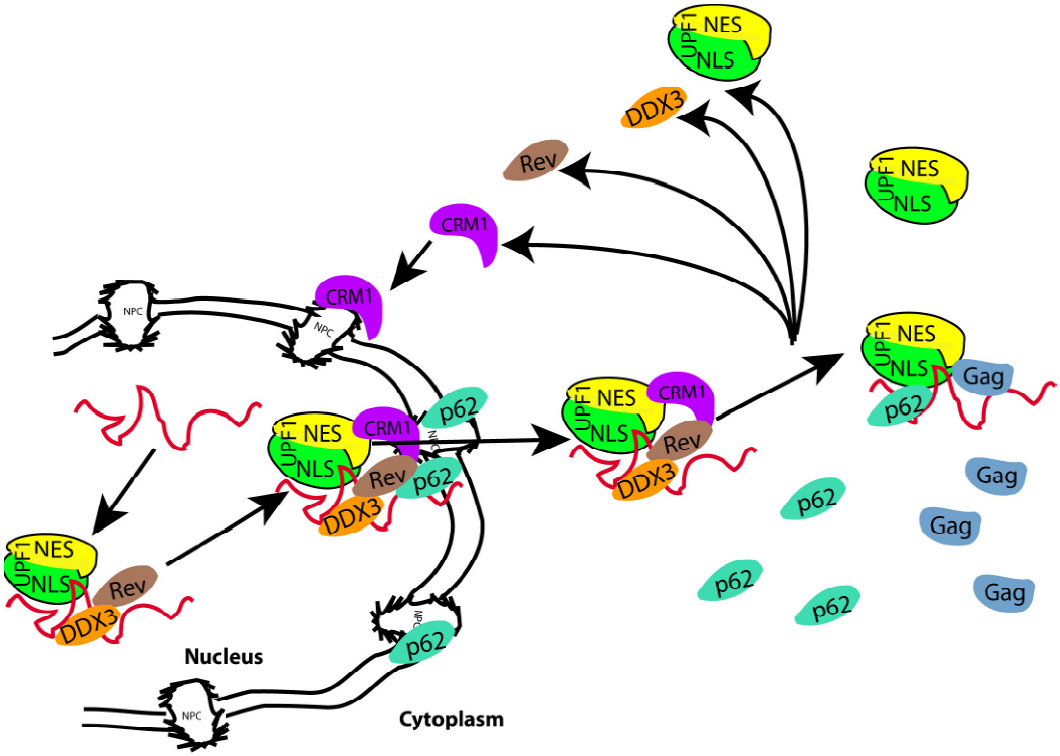

FIGURE 24: *Model of UPF1 mediated HIV-1 vRNA export.*

RESULTS FOR SUPPLEMENTAL FIGURES

UPF1 mutants

The figure shows the schematic representation of the different mutants of UPF1 (Figure 25).

Effects of UPF1 on HIV-1 vRNA export are specific to the RRE-Rev dependent export

In order to determine if UPF1 overexpression affected total cellular RNA distribution, we overexpressed FLAG-UPF1 and cells were fixed for IF analysis for UPF1 (FLAG) and total cellular mRNA was stained with the dye, SYTO14 [1]. Our results show that UPF1 overexpression does not effect total cellular RNA distribution (Figure 26).

REFERENCES SUPPLEMENTAL RESULTS

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SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

FIGURE 25: *Schematic representation of the different mutants of UPF1.*

(A) Wild-type UPF1. *(B)* ΔNES lacks the nuclear export signal (NES) as well as both zinc fingers (Δ55-416), *(C)* HelΔI,Ia lacks helicase domains I and Ia, (Δ419-593) *(D)* ΔNLS lacks helicase domains II and III as well as the nuclear localization signal (NLS) (Δ596-697), *(E)* HelΔIV-VI lacks helicase domains IV and V (Δ700-952), and *(F)* ΔPS is a C-terminal truncation mutant that lacks phosphorylation sites (Δ955-1118).

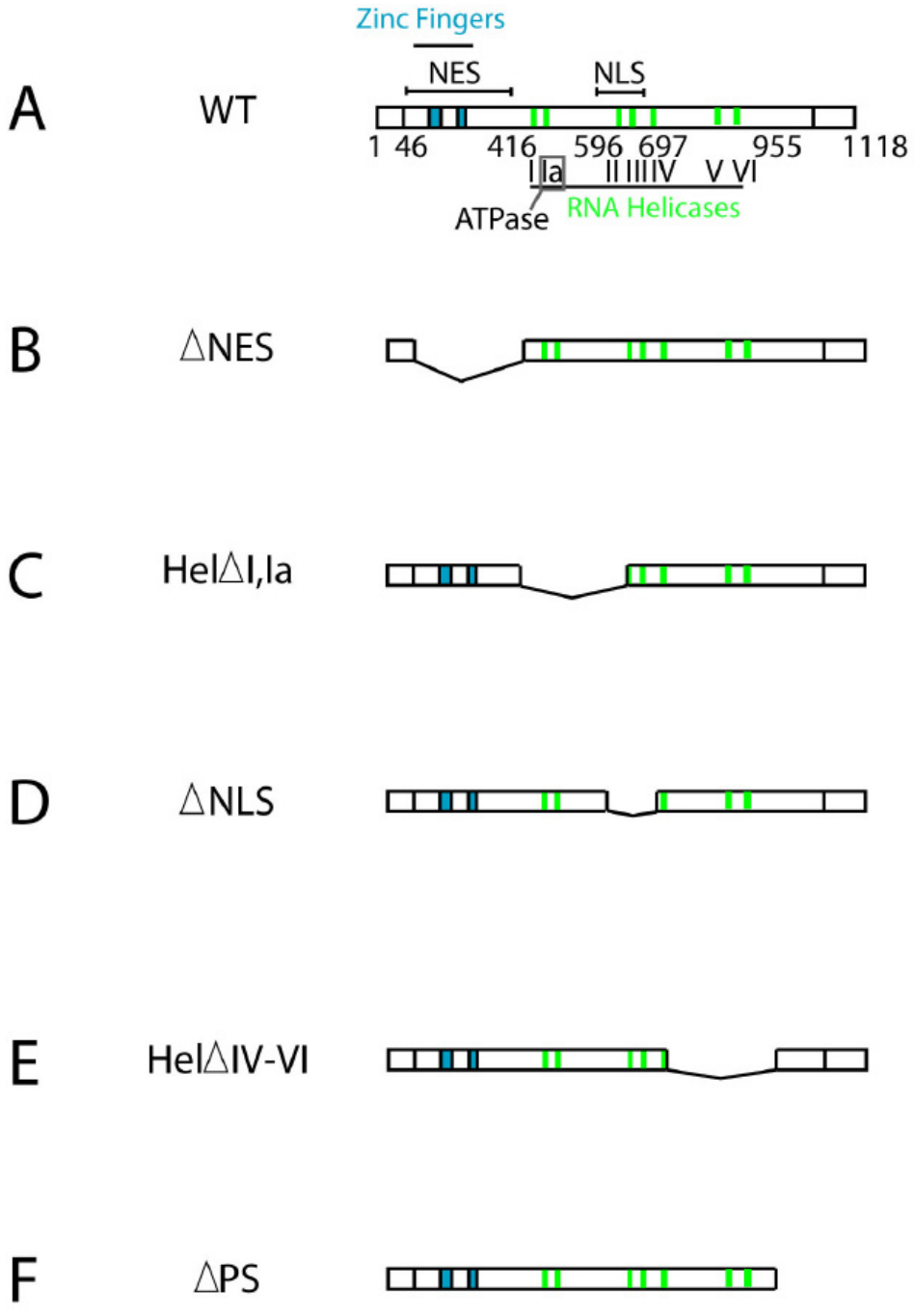

FIGURE 25: *Schematic representation of the different mutants of UPF1.*

FIGURE 26: *UPF1 overexpression does not affect total cellular RNA* distribution but only HIV-1 genomic RNA expression and localization.

HeLa cells were seeded onto coverslips at of $1.5x10^5$ cells per mL and transfected with $FLAGUPF1^{WT}$. At 30 h post-transfection, cells were fixed for IF analysis for FLAG (blue) and total cellular mRNA was stained with the dye SYTO14 (red). Size bars are 10 μm. Image results shown are representative of the phenotypes observed in >90% cells (n=60), in 2 experiments.

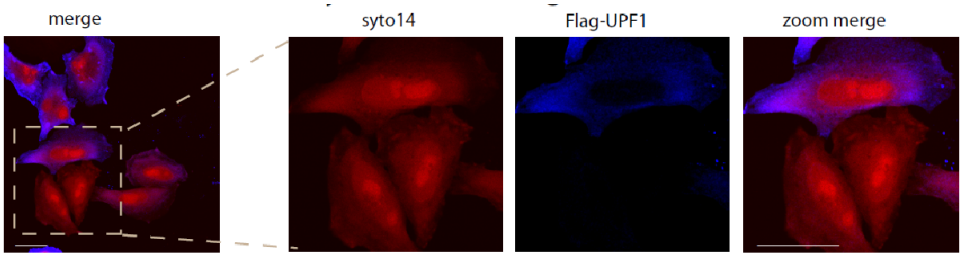

FIGURE 26: *UPF1 overexpression does not affect total cellular RNA distribution but only HIV-1 genomic RNA expression and localization.*

Chapter 4

Chapter 4: Research hypothesis and specific aims

In Chapter 2, we showed that UPF2 was excluded from the Staufen1-HIV-1 RNP complex where UPF1 was upregulated and UPF3b remained constant [1]. We also demonstrated that the role of UPF1 was distinct from its NMD function and that UPF2 binding to UPF1 wasn't an important interaction for HIV-1 RNA metabolism and translation [1]. In addition, UPF1 is encapsidated in virus particles and UPF2 is excluded (Abrahamyan L, Mouland AJ, data not shown; [2]). It was recently shown that UPF2 binding to UPF1 decreased its affinity to single stranded RNAs [3] and UPF2.

The presence and/or absence of the different UPF proteins in the Staufen1-HIV-1 RNP complex led us to our third hypothesis: UPF2 and UPF3a/3b may be involved in HIV-1 genomic RNA export and translation.

Chapter 4

UPF2 negatively regulates HIV-1 genomic RNA export

UPF2 negatively regulates HIV-1 genomic RNA export

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Manuscript in preparation

ABSTRACT

HIV-1 genomic RNA requires the activities of a variety of cellular proteins for efficient nucleocytoplasmic export as well as the viral protein Rev. UPF1 is enriched in Staufen1-HIV-1 RNP complexes and more recently, we showed that UPF1's shuttling function was required for HIV-1 genomic RNA export by binding to CRM1, Rev, DDX3 and Nup62. The interaction between UPF3b, UPF2 and UPF1 is required for the classical nonsense-mediated mRNA decay pathway. Since, we have already shown that UPF3b overexpression does not affect HIV-1 RNA metabolism, we therefore decided to investigate the role of UPF2 in HIV-1 RNA export and translation. We have identified UPF2 as being a negative regulator of HIV-1 genomic RNA export. UPF1 in complex with UPF2, results in the nuclear sequestration of the HIV-1 genomic RNA. Our results confirm that the exclusion of UPF2 from the HIV-1 RNP complex is necessary for UPF1 to efficiently export the HIV-1 genomic RNA.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1), encodes three structural, four accessory and two regulatory genes. *Rev,* one of the regulatory genes, is translated from the multiply-spliced 2kb RNAs and is required for proper export of the full-length HIV-1 unspliced 9kb viral genomic RNA (vRNA) and the singly-spliced 4kb RNAs by binding to the Rev-response element (RRE) [4].

At each step of the HIV-1 replication cycle, numerous cellular proteins are required for HIV-1 pathogenesis and HIV-1 Rev-mediated vRNA export is no different. Many studies have shown a number of cellular proteins required for proper HIV-1 vRNA export. Dead box protein 3 (DDX3) was shown to bind chromosome region maintenance 1 (CRM1) and Rev and to be involved in the Rev-mediated export [5]. In addition, eukaryotic translation initiation factor 5A (eIF5A) was shown to be an adaptor for Rev and CRM1 [6, 7] and sam68 (Src-associated protein in mitosis, 68 kDa) was demonstrated to be involved in Rev-independent and dependent HIV-1 vRNA export [8, 9]. Moreover, UPF1 was shown to be implicated in HIV-1 vRNA export in the presence or absence of Rev and was not involved in CTE-mediated export (Chapter 3). Additionally, UPF1 immunoprecipitated with Rev, CRM1, DDX3 and the nucleoporin Nup62 (Chapter 3). A recent study conducted by Kula et al. identified UPF1 associated with the nuclear HIV-1 genomic RNA immediately following transcription indicating that UPF1's role in HIV-1 is an early nuclear event [10].

While these represent positive activators of nucleocytoplasmic export, other host proteins act in a negative fashion. Insulin-like growth factor II mRNA binding protein 1 (IMP1) and HS1-associated protein X-1 (Hax-1) have been shown to alter the subcellular localization of Rev thus inhibiting its function [11, 12]. Therefore, studying the roles of various cellular proteins involved in HIV-1 vRNA export can lead to the identification of suitable targets to block HIV-1 infection.

Nonsense-mediated decay (NMD) is an RNA surveillance pathway that selectively identifies and degrades mRNAs containing premature termination codons (PTCs) thus eliminating the accumulation of truncated proteins [13, 14]. NMD also regulates many physiological mRNAs which do not contain PTCs. The NMD core proteins are the *trans*-acting factors called up-frameshift (UPF) proteins, initially discovered in *Saccharomyces cerevisiae.*

Up-frameshift protein 1 (UPF1) is an RNA binding protein and a nucleic acid dependent ATPase. It contains two zinc-fingers, seven helicase motifs and a nuclear export and localization signal. UPF1 has been found in many different ribonucleoprotein (RNP) complexes besides NMD such as with Staufen1 called Staufen1-mediated decay where UPF1 is recruited to the 3'UTR of certain mRNAs via Staufen1 thus eliciting its decay [15]. It is a component of the HIV-1 Staufen1 RNP complex in which UPF3b is present and UPF2 is excluded [1]. Up-frameshift protein 2 (UPF2), is a phosphoprotein that interacts with UPF1 and UPF3b to trigger NMD. Studies have shown that its interaction with UPF1

decreases UPF1's ability to bind RNA by altering its conformation [3]. In addition, UPF2 and Staufen1 compete for the same binding site on UPF1, making both NMD and SMD competing pathways [16]. Up-frameshift protein 3 (UPF3) has two paralogs UPF3aL and UPF3b. They both trigger NMD differently [17]. UPF3b regulates UPF3aL such that depletion of UPF3b increases UPF3aL levels and this regulation is unidirectional. Moreover, they both compete for UPF2 binding such that UPF2 binding to UPF3aL stabilizes UPF3aL levels [18]. UPF3a has two isoforms: UPF3aL and UPF3aS, the latter lacking exon 4 and does not bind UPF2 [19]. Moreover, it was shown that UPF1 was found in two different complexes one with UPF3aS, PP2A, SMG5, SMG7 and phosphorylated UPF1 (P-UPF1) (called 'pre-dephosphorylation) and a complex lacking SMG5/7 but containing UPF2, UPF3aL and P-UPF1 (called 'post-phosphorylation') [20]. This indicates that UPF3b and UPF3a as well as UPF3aL/S have differential roles in distinct RNPs.

In this work, we extended our previous findings by examining the roles for other UPF proteins in HIV-1 replication. We identified a new role for UPF3aL in HIV-1 vRNA nucleocytoplasmic export. UPF3aL recruits UPF1 in complex with UPF2 causing a block in HIV-1 RNA export. A mutant of UPF3aL (UPF3aS) that binds UPF1 but does not bind to UPF2 did not block HIV-1 vRNA export. In addition, we have characterized a new role for UPF2, previously shown to be excluded from the HIV-1 RNP, as a negative regulator of HIV-1 RNA export. Mutants of UPF2 that do not bind UPF1 did not sequester HIV-1 vRNA in the nucleus. These results strengthen our previous results where UPF1 mutants which do not bind

UPF2 still upregulated HIV-1 vRNA levels and translation and that UPF2 is excluded from the Staufen1-HIV-1 RNP complex [1]. Therefore, UPF2 in complex with UPF1 leads to the nuclear sequestration of the HIV-1 genomic RNA by blocking UPF1's role in HIV-1 genomic RNA export.

RESULTS

HIV-1 increases the interaction between UPF1 and UPF3b by bypassing the requirement of UPF2

Our earlier work showed that UPF2 was excluded from the HIV-1-Staufen1 RNP complex and that the association between UPF1 and UPF2 wasn't essential [1]. We also demonstrated that UPF3b was present in the Staufen1-HIV-1 RNP complex even though its overexpression did not increase HIV-1 genomic RNA levels [1]. We therefore decided to see if UPF2 was part of the Gag-HIV-1 RNP complex. HeLa cells were transfected with or without proviral DNA pNL4-3 and wildtype UPF1 (FLAG-UPF1^{WT}). Gag was immunoprecipitated and the presence of UPF1, UPF2 and UPF3b was assessed (Figure 27). Our results show that UPF1, as previously reported [1], immunoprecipitated with Gag while UPF2 was absent from this complex; thus confirming the absence of UPF2. UPF3b was also present (weakly) in the Gag RNP complex. We then decided to test if there was a change between the interactions of the UPF proteins in HIV-1. To address this, we isolated UPF1 complexes in the absence and presence of HIV-1 and we checked to see if UPF2 and UPF3b association was enriched or not (Figure 28). HeLa cells were transfected with FLAG DNA (empty vector pCI-FLAG) or wildtype UPF1 (FLAG-UPF1^{WT}) with or without proviral pNL4-3. FLAG complexes were then immunoprecipitated with FLAG-conjugated agarose beads and the eluted complexes were assessed for FLAG (UPF1), UPF2, UPF3b, Gag and GAPDH by

Western analysis (Figure 28). We observed that UPF3b and UPF2 co-immunoprecipitated with $UPF1^{WT}$ and UPF3b was immunoprecipitated even more with UPF1 in HIV-1 conditions where no change in UPF2 was observed. These observations indicate that the interaction between UPF3b and UPF1 is increased due to the absence of UPF2.

UPF3aL overexpression blocks HIV-1 vRNA nucleocytoplasmic export by binding to UPF1 and UPF2

The major binding partners of UPF2 are UPF1, UPF3aL and UPF3b. Our previous results demonstrate that UPF3b overexpression does not increase HIV-1 genomic RNA levels or Gag translation. It was recently shown that UPF3aL and UPF3b compete for binding to UPF2. In addition, UPF2 binding to UPF3b destabilizes UPF3aL, while UPF2 binding to UPF3aL stabilizes its levels. Moreover, this unidirectional regulation depends on the availability of UPF2 [18]. We therefore overexpressed UPF3aL to observe if its role was similar to its paralog UPF3b. We first decided to investigate the localization of HIV-1 vRNA under UPF3aL and UPF3b overexpression conditions by using fluorescence *in situ* hybridization/immunofluorescence (FISH/IF) co-analyses (Figure 29a-b). As expected, overexpression of FLAG-UPF3b did not affect HIV-1 vRNA localization (Figure 29b), consistent with our earlier work where UPF3b overexpression did not increase HIV-1 vRNA levels [1]. To our surprise, overexpression of UPF3aL blocked vRNA export resulting in a phenotype similar to Rev- (Figure 29a). Since we have shown that UPF1 is involved in HIV-1

vRNA export, we validated to see if both proteins bound UPF1 differently thus resulting in the sequestration of the HIV-1 genomic RNA (Figure 29c). HeLa cells were transfected with FLAG DNA (empty vector pCI-FLAG) with or without proviral pNL4.3 and UPF3aL (FLAG-UPF3aL) or with UPF3b (FLAG-UPF3b) with proviral pNL4-3. FLAG complexes were then immunoprecipitated with FLAG-conjugated agarose beads and the eluted complexes were assessed for FLAG, UPF1 UPF2 and GAPDH by Western analysis (the top of Figure 29c). We observed UPF2 with UPF3b and UPF3aL and, UPF1 immunoprecipitated with both UPF3aL and UPF3b. No change in HIV-1 genomic RNA levels was observed under UPF3b overexpression (the bottom of Figure 29c). A slight increase in HIV-1 genomic RNA levels were observed under UPF3aL overexpression conditions (the bottom of Figure 29c). These results indicate that UPF3aL in complex with UPF1 blocks HIV-1 nucleocytoplasmic export.

Since UPF2 was present in both UPF3aL and UPF3b complexes, but only UPF3aL specifically blocked HIV-1 genomic RNA export (Figure 29), we overexpressed a UPF3aL mutant (UPF3aΔe4 or UPF3aS) which does not bind UPF2 (FLAG-UPF3aS) [20] and looked at the HIV-1 vRNA localization by using fluorescence *in situ* hybridization/immunofluorescence (FISH/IF) co-analyses (Figure 30a-b). As shown in Figure 29a, overexpression of FLAG-UPF3aL results in a block in HIV-1 vRNA export (Figure 30a). Interestingly, overexpression of FLAG-UPF3aS did not sequester the HIV-1 vRNA (Figure 30b).

To confirm UPF3aS binding to UPF1 but not UPF2, we overexpressed FLAG-UPF3aL and FLAG-UPF3aS with proviral pNL4-3, FLAG immunoprecipitated these complexes and assessed for the presence of FLAG, UPF1, UPF2 and GAPDH (the top of Figure 30c). Our results show that UPF3aS does not bind to UPF2 while UPF1 is present in the immunoprecipitate. UPF3aL overexpression resulted in an increase in vRNA levels (the bottom of Figure 29c), however, a greater increase in HIV-1 genomic RNA was observed with UPF3aS (the bottom of Figure 30c). This could be due to less binding of UPF1 with UPF2 thus increasing the availability of UPF1 to bind the HIV-1 genomic RNA and export it. Since UPF3aS did not block HIV-1 genomic RNA export, our data shows that UPF1 in complex with UPF2 (as well as with UPF3aL) blocks nucleocytoplasmic export of HIV-1 vRNA.

UPF2 blocks HIV-1 genomic RNA export by binding to UPF1

Since our results point to UPF2 negatively affecting HIV-1 genomic RNA export, we decided to overexpress wildtype UPF2 (FLAG-UPF2) and mutants of UPF2 which do not bind UPF1. FLAG-UPF2 1173 harbors a point mutation disrupting its interaction with UPF1 and FLAG-UPF2 1-1096 is deleted at its C-terminus which also does not bind UPF1 [21]. We overexpressed these UPF2 constructs with proviral DNA and looked at the HIV-1 vRNA localization by using fluorescence *in situ* hybridization/immunofluorescence (FISH/IF) co-analyses (Figure 31a-d). Wildtype UPF2 blocked HIV-1 genomic RNA export (Figure 31b). This is similar to what was observed with the overexpression of UPF3aL

and a Rev- phenotype. The point mutation 1173 in UPF2 resulted in about a 50% nuclear sequestration (Figure 31c) and the C-terminal deletion mutation (1-1096) did not block HIV-1 genomic RNA export (Figure 31d) (see Figure 32 for statistics). These results show that UPF2 wildtype blocks HIV-1 nucleocytoplasmic export.

In view of the fact that the point mutation 1173 still blocked HIV-1 genomic RNA export by about 50% and this mutant is not suppose to bind UPF1, we decided to overexpress UPF2 wildtype, FLAG-UPF2 1173 and FLAG-UPF2 1-1096 with proviral DNA pNL4-3 and immunoprecipitate the FLAG complexes and check for the presence of UPF1 and UPF3b (the top of Figure 31e). UPF1 immunoprecipitated with wildtype UPF2 and UPF2 1173 immunopreciptated weakly with UPF1, indicating why we saw about a 50% nuclear sequestration of the HIV-1 genomic RNA. On the other hand, the C-terminal UPF2 mutant (1-1096) barely immunoprecipitated with UPF1. All these immunoprecipitated with UPF3b (the top of Figure 31e). The UPF2 regions that bind UPF1 are C and N terminal. It is therefore possible that this point mutation still binds UPF1 via the remaining C-terminal and its N-terminal [19, 21]. Nevertheless, the C-terminal is the main binding site for UPF1 and that is why the deletion mutant 1-1096 barely immunoprecipitates with UPF1. No significant change in HIV-1 genomic RNA levels was observed with the overexpression of the UPF2 constructs (the bottom of Figure 31e). Importantly, it was shown that UPF2 Δ1095-1272 was still able to bind UPF3aL and UPF3b [19]. These results demonstrate that UPF2 binding to UPF1 blocks HIV-1 genomic RNA export.

DISCUSSION

In this study we identified UPF2 as being a negative regulator of HIV-1 genomic RNA export. Since we have shown that UPF1 stimulates HIV-1 genomic RNA export (Chapter 3), it is possible that UPF2 binding to UPF1 blocks UPF1 from binding and exporting the HIV-1 RNA, thus resulting in the sequestration of the HIV-1 genomic RNA in the nucleus. Our results are supported by the conformational change that is induced on UPF1 by UPF2 resulting in decreased RNA binding of UPF1 [3]. Studies have shown that both isoforms of UPF3a, UPF3aS and UPF3aL, are found in different complexes with UPF1 [20]. The presence of both these complexes in the cell can explain why both UPF3a isoforms, which differ in their ability to bind UPF2, have different effects on HIV-1 genomic RNA export. These two complexes are UPF3aL-UPF2 with phosphorylated UPF1 (P-UPF1) and UPF3aS-SMG5/7-PP2A-PUPF1 called post-phosphorylation and pre-dephosphorylation complexes respectively [20]. It is yet to be determined if the phosphorylation state of UPF1 is affected leading to a block in HIV-1 genomic RNA nucleocytoplasmic export. Interestingly, studies have pointed out that the cycle of phosphorylation and dephosphorylation of UPF1 is important in nonsense-mediated mRNA decay as well as maintaining RNA and DNA stability [22, 23].

Moreover, our results are also supported by the data that UPF2 is not part of the HIV-1 Gag (Figure 27) or Staufen1 RNP complex and that mutants of UPF1 that

do not bind UPF2 do not effect UPF1 function in maintaining HIV-1 genomic RNA stability and increased translation [1]. In addition, UPF2 is absent in HIV-1 particles (Abrahamyan L., Mouland AJ., data not shown) while UPF1 is encapsidated [2].

HIV-1 excludes UPF2 from its RNP complex for numerous reasons. First and foremost, HIV-1 bypasses the RNA quality control mechanism NMD by recruiting UPF1 immediately following transcription to ensure efficient HIV-1 RNA export and translation [1, 10]. Secondly, since the classical NMD pathway is UPF2 dependent, UPF2 is not coopted by HIV-1 to evade NMD. Lastly, UPF2 in complex with UPF1 blocks HIV-1 genomic RNA export.

The proposed model is that immediately following transcription, UPF1 is recruited to the HIV-1 genomic RNA and efficiently exports it through the CRM1 export pathway. In the cytoplasm, UPF2 is not recruited to HIV-1 genomic RNA since UPF1's presence protects it from binding to UPF2. Moreover, since UPF2 and Staufen1 compete for the same binding site on UPF1 [16], it is possible that Staufen1 binding to UPF1 blocks the interaction between UPF1 and UPF2. [1, 2, 15].

In conclusion, the significance of the selective nature of HIV-1 in coopting cellular factors demonstrates that the interaction of two proteins, whose association is essential for an RNA quality control mechanism, can be detrimental in HIV-1 replication. Thus, it is interesting how HIV-1 coopts UPF1 for HIV-1

genomic RNA export and translation and excludes UPF2 due to its inhibitory function.

ACKNOWLEDGMENTS

We thank Jens Lykke-Andersen for antisera and Bruce Chesebro and the NIH AIDS Reference and Reagent Program for reagents. We thank the other members of the lab for helpful discussions. L.A. is a recipient of a Canadian Institutes of Health Research (CIHR) Doctoral Research Award; A.J.M. is supported by a McGill University Fraser, Monat and McPherson Scholarship. This work was supported by grants from the CIHR (#MOP-38111) and the Canadian Foundation for AIDS Research to A.J.M.

MATERIAL AND METHODS

Cell culture, plasmids and transfections

Cell culture of HeLa cells and transfections of the proviral DNA, pNL4-3 were performed as described before [1]. Total cellular RNA was isolated by TriZol Reagent or TriZol LS (Invitrogen) according to the manufacturer's instructions. pC1-FLAG-UPF1 (UPF1^{WT}) and pC1-FLAG were described earlier [1]. FLAG-UPF3aS, FLAG-UPF3aL, FLAG-UPF3b, FLAG-UPF2^{WT}, FLAG-UPF2 ^{1173} and FLAG-UPF2 $^{1-1096}$ were provided by Niels Gehring [18, 19, 21, 24, 25]. FLAG-UPF3aS lacks exon4 from UPF3aL, FLAG-UPF2 ^{1173} has a point mutation which abolishes its interaction with UPF1 and FLAG-UPF2 $^{1-1096}$ has a deletion at the C-terminal of the protein and does not bind UPF1 [17, 19, 21, 24, 26].

Antisera and reagents

Antisera to UPF1, UPF2 and UPF3b were generously supplied by Jens Lykke-Andersen (University of California, San Diego). Mouse anti-p24 and mouse anti-GAPDH antisera were purchased from Intracell and Techni-Science, respectively. Mouse anti-Digoxin was purchased from Sigma-Aldrich and sheep anti-Digoxin was purchased from Roche. Mouse and Rabbit anti-FLAG antibodies were purchased from Sigma-Aldrich. For western analysis, horseradish peroxidase-

conjugated anti-rabbit and anti-mouse antibodies were purchased from Rockland Immunochemicals (PA, USA). For indirect immunofluorescence and FISH, secondary fluorophore-conjugated antisera AlexaFluor donkey anti-mouse 488 and 594, donkey anti-sheep 488, and donkey anti-rabbit 488 and 594, were purchased from Invitrogen.

FISH/IF co-analyses

Immunofluorescence and fluorescence *in situ* hybridization co-analyses were performed as described before. Microscopy was performed on a Zeiss LSM5 Pascal laser-scanning confocal microscope. Filter sets and laser wavelengths were described earlier [1, 2, 27]. Statistical significance was calculated with the program Prism (GraphPad version 5.0a) (Figure 32).

Immunoprecipitation analyses

HeLa cells were transfected as described above and were described earlier [1]. For the FLAG immunoprecipitation, 1mg of total lysate was incubated overnight with agarose conjugated anti-FLAG beads and the bound complex was analyzed via SDS-PAGE analysis as described earlier. Cells were lysed in NP40 lysis buffer and Gag was immunoprecipitated as described previously using 1mg of protein and affinity-purified mouse anti-p24 antisera (hybridroma 183-H12-5C) [28] from Bruce Chesebro and the NIH AIDS Reference and Reagent Program [1].

First Strand cDNA synthesis followed by PCR

HeLa cells were transfected as described above and were described earlier [1]. For PCRs in Figure 29c, 30c and 31e, RNA was purified using TriZol. 1ug of total RNA was treated with DNAse I Amplification Grade (Invitrogen) and first strand cDNA synthesis was made using Superscript II RT (Invitrogen). 2ul of total cDNA was taken and PCR amplified for *gapdh* mRNA and HIV-1 vRNA using the same primers as described in Abrahamyan et al., 2010 [2].

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FIGURES AND FIGURE LEGENDS

FIGURE 27: *UPF2 is absent in the HIV-1 Gag RNP complex.*

HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and FLAG-UPF1^{WT}. At 30h post-transfection, cells were harvested and Gag was immunoprecipitated using a monoclonal anti-p24 antiserum as described in Material and Methods. Western blot analysis for UPF1 (anti-FLAG), Gag, UPF2, UPF3b and eF1α (positive control) were performed. HC: Heavy Chain IgG. LC: Light Chain IgG. Results are representative of two experiments.

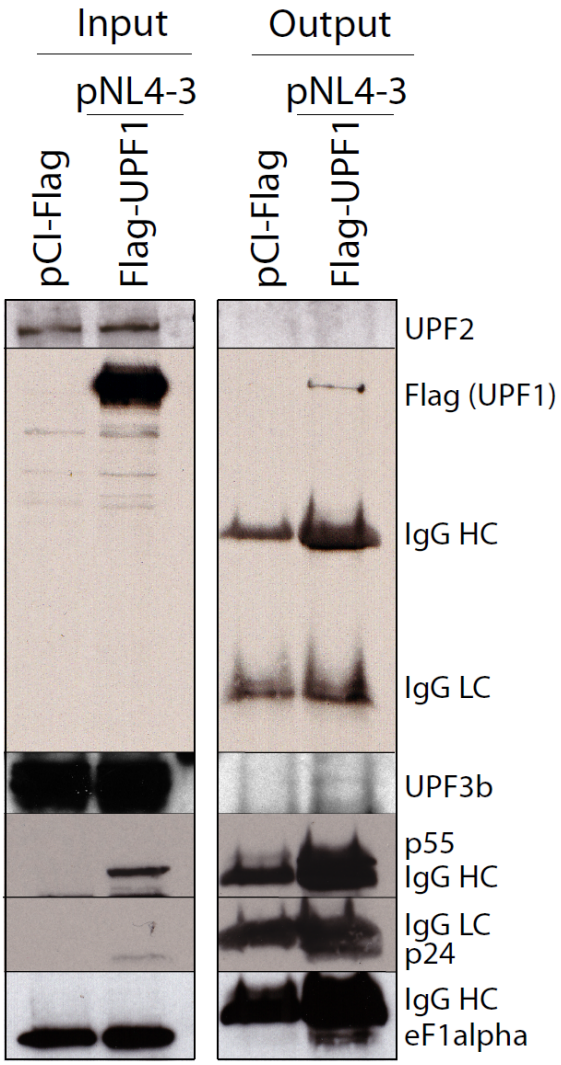


FIGURE 27: *UPF2 is absent in the HIV-1 Gag RNP complex.*

FIGURE 28: *UPF3b immunoprecipitates more with UPF1 under HIV-1 conditions while no change in UPF2 is observed.*

HeLa cells were mock transfected with pCI-FLAG or FLAG-UPF1 or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1). At 30h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Material and Methods. Bound complexes were analyzed by Western blot analysis for UPF1 (anti-FLAG), UPF2, UPF3b, Gag and GAPDH (loading control). Results shown are representative of three independent experiments. HC: IgG Heavy Chain, LC: IgG Light Chain.

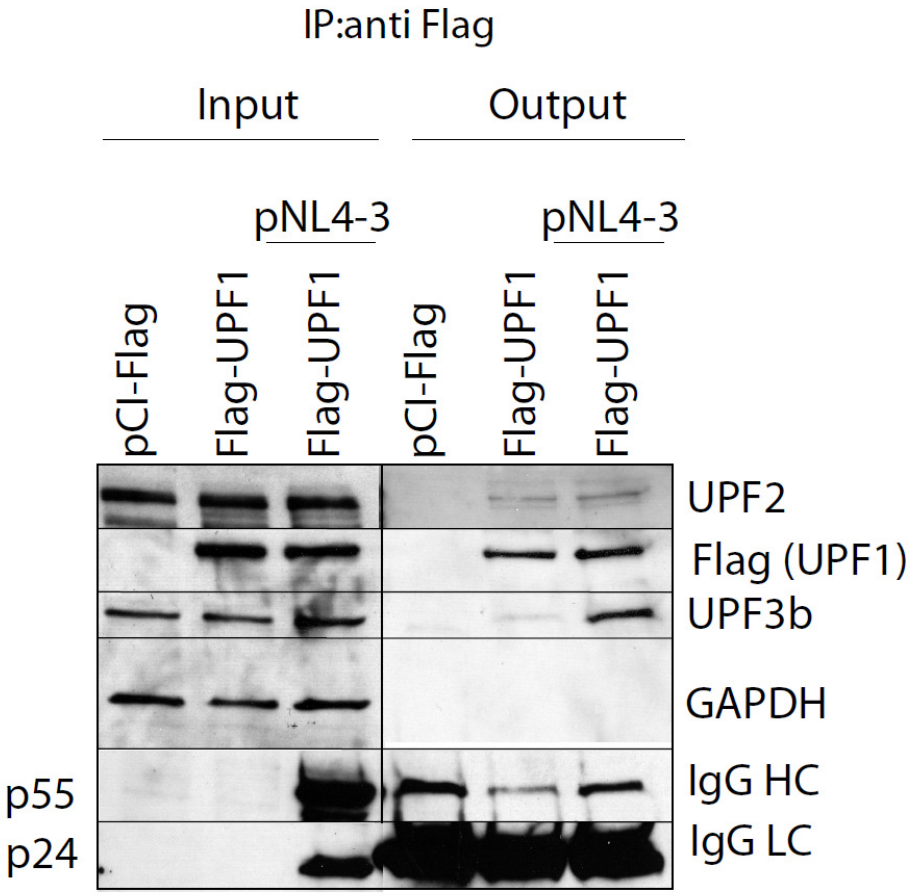


FIGURE 28: *UPF3b immunoprecipitates more with UPF1 under HIV-1 conditions while no change in UPF2 is observed.*

FIGURE 29: *UPF3aL overexpression blocks HIV-1 genomic RNA export while UPF3b has no effect.*

To determine the cellular localization of the HIV-1 genomic RNA with UPF3aL and UPF3b, HeLa cells were seeded onto coverslips at $1.5x10^5$ cells per mL, and were co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) with UPF3aL *(A)* and UPF3b *(B)*. At 48 h post-transfection, cells were fixed for FISH/IF co-analyses for the vRNA (green) and UPF3aL *(A) and* UPF3b *(B)* (red). The statistical significance of phenotypes observed is shown in Figure 32. White arrows indicate cells only transfected with pNL4-3. White box in A is the pNL4-3 transfected cell shown on top. *(C) (Top)* HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and pCI-FLAG or FLAG-UPF3aL or FLAG-UPF3b. At 48 h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Material and Methods. Bound complexes were analyzed by Western blot analysis FLAG, UPF1, UPF2 and GAPDH (loading control). *(Bottom)* HIV-1 genomic RNA and *gapdh* RNA were assessed by RT-PCR in the input. Results shown are representative of 2 independent experiments.

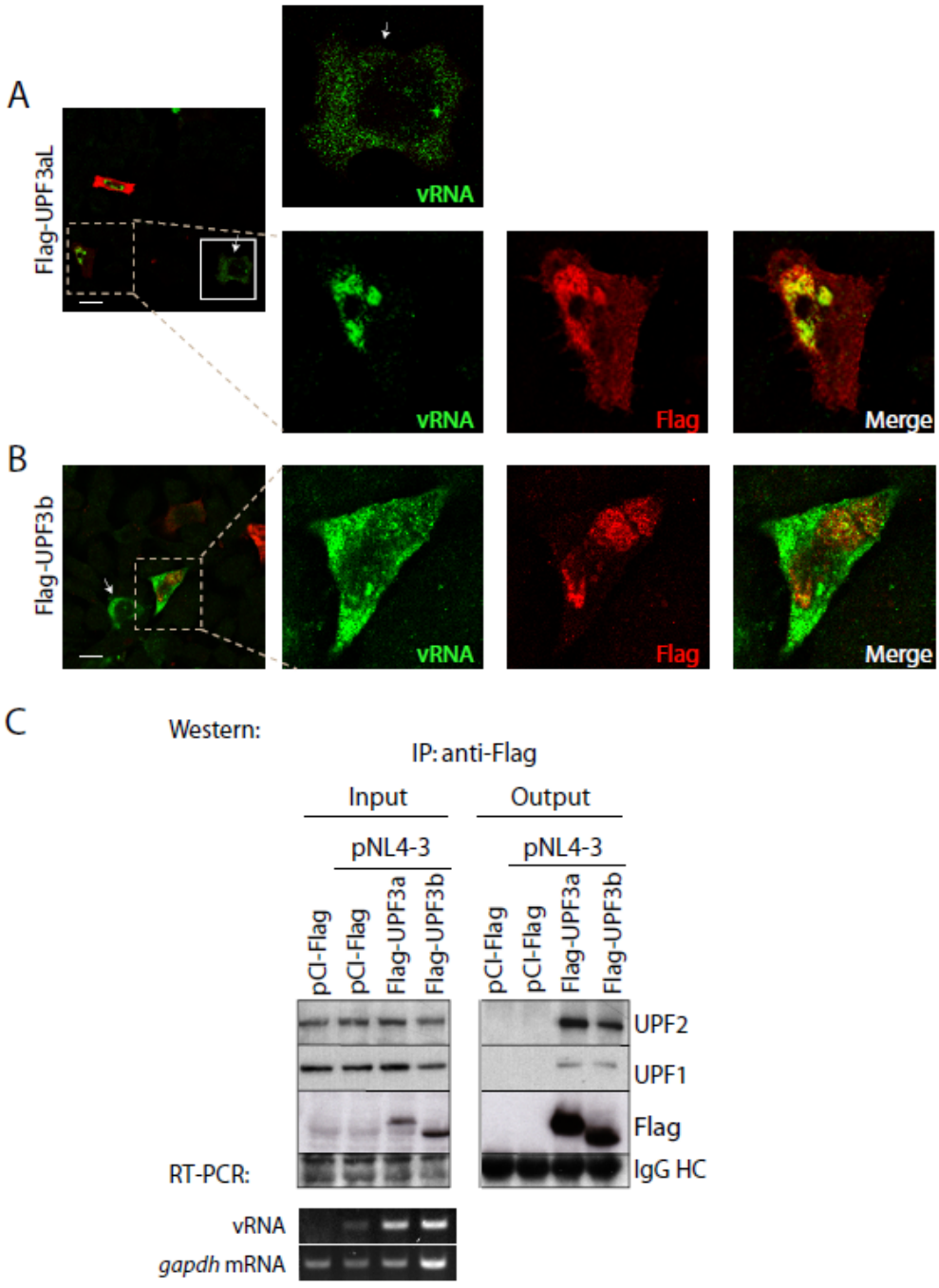


FIGURE 29: *UPF3aL overexpression blocks HIV-1 genomic RNA export while UPF3b has no effect.*

FIGURE 30: *UPF3aL overexpression blocks HIV-1 genomic RNA export by binding to UPF2, while UPF3aS has no effect.*

To determine the cellular localization of the HIV-1 genomic RNA with UPF3aL and UPF3aS, HeLa cells were seeded onto coverslips at $1.5x10^5$ cells per mL, and were co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) with UPF3aL *(A)* and UPF3aS *(B)*. At 48h post-transfection, cells were fixed for FISH/IF co-analyses for the vRNA (green) and UPF3aL *(A) and* UPF3aS *(B)* (red). The statistical significance of phenotypes observed is shown in Figure 32. White arrows indicate cells only transfected with pNL4-3. White box in A is the pNL4-3 transfected cell shown on top. *(C) (Top)* HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and pCI-FLAG or FLAG-UPF3aL or FLAG-UPF3aS. At 48h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Material and Methods. Bound complexes were analyzed by Western blot analysis FLAG, UPF1, UPF2 and GAPDH (loading control). *(Bottom)* HIV-1 genomic RNA and *gapdh* RNA were assessed by RT-PCR in the input. Results shown are representative of two independent experiments.

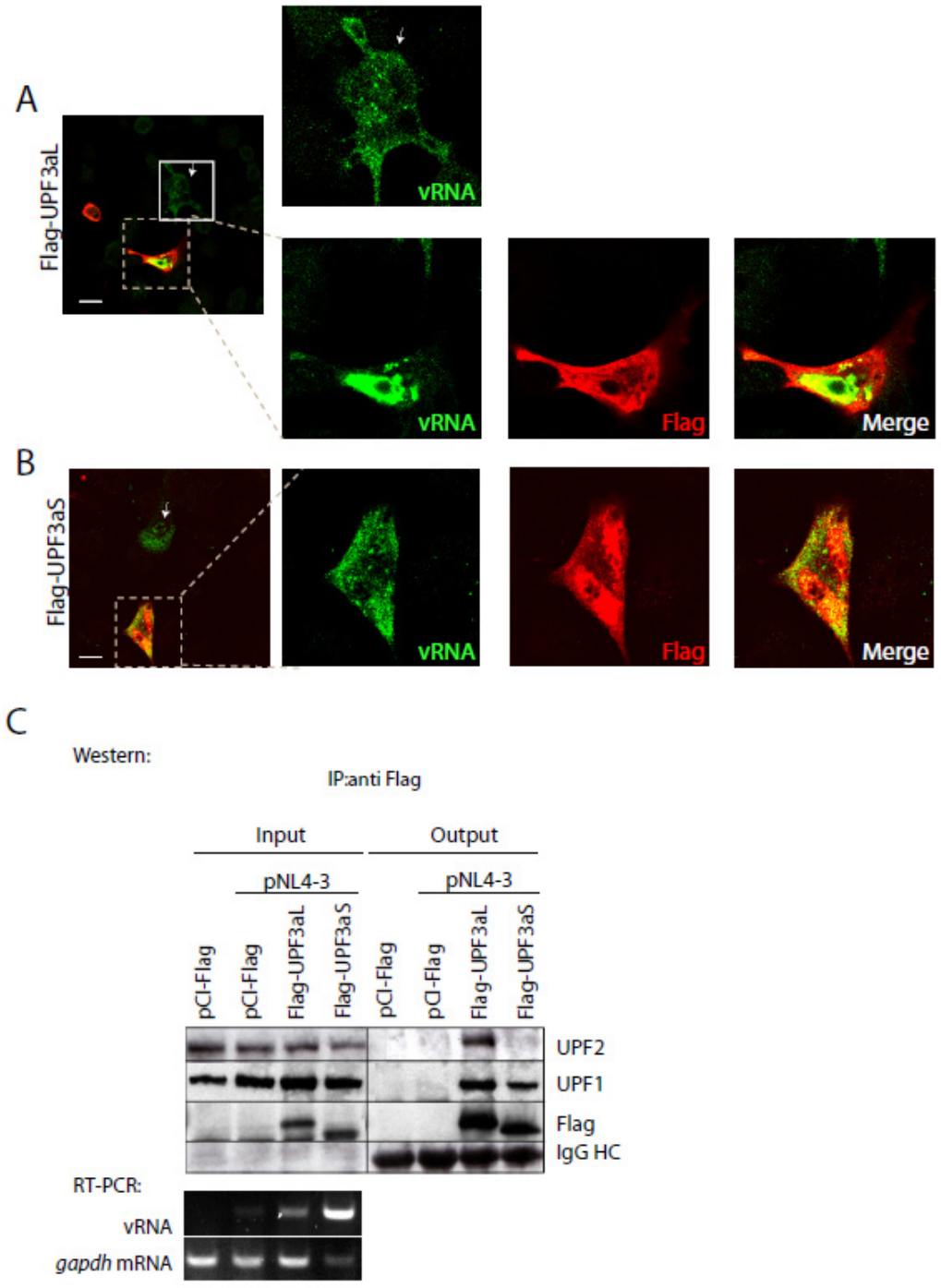


FIGURE 30: *UPF3aL overexpression blocks HIV-1 genomic RNA export by binding to UPF2, while UPF3aS has no effect.*

FIGURE 31: *UPF2 binding to UPF1 blocks HIV-1 vRNA export.*

To determine the cellular localization of the HIV-1 genomic RNA with UPF2^{WT}, UPF2 ^{1173} and UPF2 $^{1-1096}$, HeLa cells were seeded onto coverslips at 1.5x10^5 cells per mL, and were co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) with pCI-FLAG *(A),* UPF2^{WT} *(B),* UPF2 ^{1173} *(C)* and UPF2 $^{1-1096}$ *(D).* At 48h post-transfection, cells were fixed for FISH/IF co-analyses for the vRNA (green) and pCI-FLAG *(A),* UPF2^{WT} *(B),* UPF2 ^{1173} *(C)* and UPF2 $^{1-1096}$ *(D)* (red). The statistical significance of phenotypes observed is shown in Figure 32. White arrows indicate cells only transfected with pNL4-3. (*E) (Top)* HeLa cells were mock transfected with pCI-FLAG or co-transfected with HIV-1 proviral DNA (pNL4-3, HIV-1) and UPF2^{WT} or UPF2 ^{1173} or UPF2 $^{1-1096}$. At 48h post-transfection, cells were harvested and FLAG was immunoprecipitated as described in Material and Methods. Bound complexes were analyzed by Western blot analysis FLAG, UPF1, UPF3b and GAPDH (loading control). *(Bottom)* HIV-1 genomic RNA and *gapdh* RNA were assessed by RT-PCR in the input. Results shown are representative of two independent experiments.

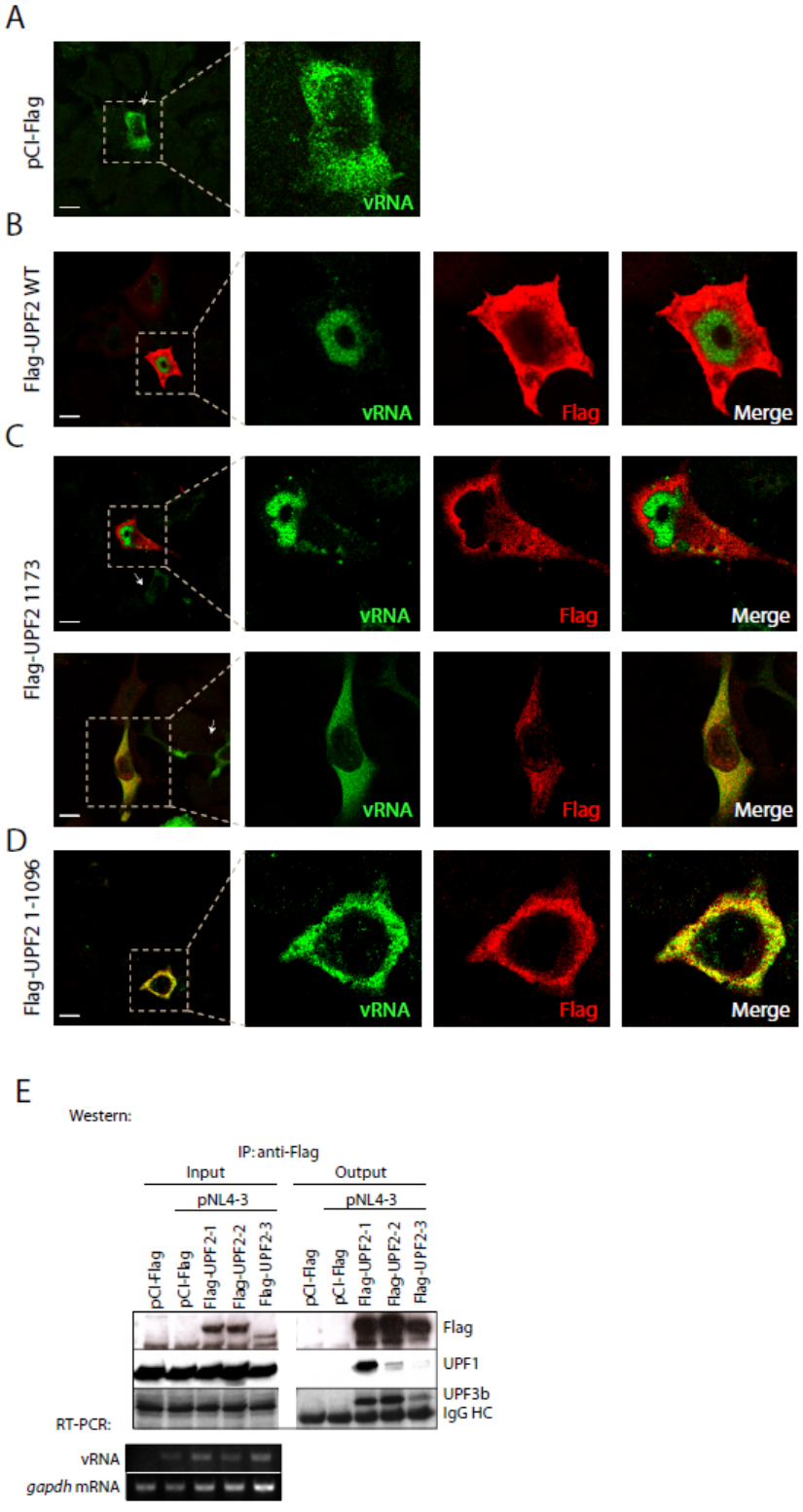


FIGURE 31: *UPF2 binding to UPF1 blocks HIV-1 vRNA export.*

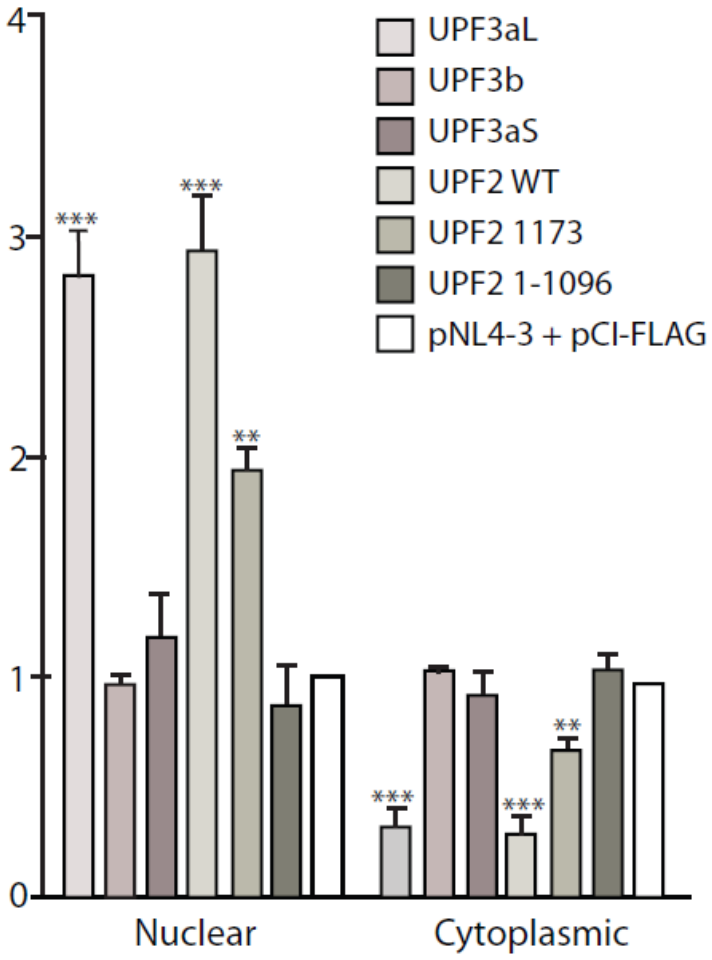


FIGURE 32: *Statistic for figures 29-31.*

Chapter 5

Chapter 5

Discussion, Future Directions and Claims to Original Research

DISCUSSION

Summary

This work highlights the importance of UPF1 at different stages of the HIV-1 replication cycle. We first demonstrated that UPF1 is involved in maintaining HIV-1 genomic RNA stability and is required for Gag genomic RNA translation (Chapter 2). In addition, our results hinted towards a nuclear role for UPF1 in HIV-1 genomic RNA metabolism [1], which was confirmed and elaborated in Chapter 3. We also showed that the role of UPF1 in HIV-1 RNA metabolism was independent of its role in NMD and did not require its association to UPF2 [1]; two aspects important in nonsense-mediated mRNA decay (NMD) (Chapter 2).

Since our data hinted at a nuclear role for UPF1 (Chapter 2), we studied how the shuttling function of UPF1 influenced HIV-1 RNA nucleocytoplasmic export and if UPF1 was associated with the already characterized HIV-1 nuclear export protein complex. We found that this shuttling function (NES, NLS) was required for proper trafficking of the genomic RNA into the cytoplasm. To our surprise, the NES mutant of UPF1 sequestered the HIV-1 genomic RNA in the nucleus. In addition, the nuclear localization mutant had no effect. Our data demonstrate that the nuclear export UPF1 mutant immunoprecipitated with the HIV-1 genomic RNA to the highest extent while the nuclear localization UPF1 mutant did not immunoprecipitate with the RNA. This strengthens the importance of UPF1's role

in binding to the HIV-1 genomic RNA in the nucleus. In addition, UPF1 binds to the genomic RNA in the nucleus (via its NLS and/or C-terminal) and once it is exported to the cytoplasm, translation occurs and UPF1 shuttles back to export more HIV-1 genomic RNA or stays on the RNA for translation, packaging and viral production (Chapter 3). These data are supported by recent work that identified UPF1 associated to *de novo* transcribed nuclear HIV-1 genomic RNA [2], and the discovery that UPF1 was found in HIV-1 virus particles [3]. Moreover, we also demonstrated that, UPF1 was able to export Rev-dependent RNAs in the presence or absence of Rev (Chapter 3). In addition, this nuclear export function was specific to HIV-1 since UPF1 did not affect CTE-mediated export through the NXF1 export pathway (Chapter 3).

HIV-1 Rev-mediated export requires a number of cellular proteins such as CRM1 and DDX3/1 and various nucleoporins [4] [5-8]. Our results show that UPF1 co-immunoprecipitates with Rev, DDX3 and CRM1, as well as the nucleoporin Nup62 during HIV-1 nucleocytoplasmic RNA export (Chapter 3). Since these export factors, besides UPF1, do not interact with Gag, our results indicate that UPF1 is found in two different HIV-1 RNP complexes: one for nucleocytoplasmic export and stability and one for translation. The export/stability complex is derived from factors that directly bind the newly transcribed HIV-1 genomic RNA before this nuclear HIV-1 RNP complex reaches the cytoplasm. In constrast, the translation complex is cytoplasmic and is involved in Gag synthesis. It has yet to be determined if the second complex is derived from the first one following the recycling of these factors.

We also demonstrated that UPF2 was excluded from the Staufen1-HIV-1-RNP and the HIV-1 RNP complex. In addition, we show that UPF2 is absent from HIV-1 particles containing UPF1 (Abrahamyan L, Mouland AJ, data not shown). This exclusion of UPF2 may be the result of a competition between Staufen1 and UPF2 for the same binding site on UPF1. Unexpectedly, we found that UPF1 in complex with UPF2 results in the nuclear sequestration of the HIV-1 genomic RNA, most likely by blocking UPF1 function [9-12] (Chapter 2,4). These results are strengthened with other published data showing that UPF2 binding to UPF1 decreases UPF1's ability to bind single-stranded RNAs [9]. This is expected to block in UPF1-mediated HIV-1 genomic RNA export.

All of our results strongly suggest that the recruitment of UPF1 to HIV-1 prevents HIV-1 genomic RNA to be degraded through NMD. Moreover, UPF1 is recruited to HIV-1 to stabilize and export its genomic RNA and enhance its translation (Chapters 2 & 3). These post-transcriptional processes involving UPF1 depends on UPF1's shuttling function, its binding to the genomic RNA in the nucleus, its interaction with the cellular proteins DDX3, CRM1, Nup62 and the viral protein Rev and, importantly, bypassing of the requirement for UPF2 (Chapters 2-4).

Our proposed model for UPF1's role in the HIV-1 replication cycle is shown in Figure 24. UPF1 binds to the HIV-1 genomic RNA immediately following transcription to stabilize it and inhibit the binding of negative regulators before exporting the HIV-1 genomic RNA into the cytoplasm. This interaction is most likely mediated by the C-terminal of UPF1. Through multiple interactions

between UPF1 (NES domain) and the cellular and viral proteins DDX3, Rev and other yet unknown factors, the nuclear HIV-1 RNP complex translocates to the nuclear pore. At the nuclear pore, interactions between UPF1 and Nup62/ CRM1/Rev as well as between Rev/DDX3 and other unknown interactions, allows this nuclear HIV-1 RNP complex to translocate through the nuclear pore into the cytoplasm. Once in the cytoplasm, some of these factors recycle back to their original subcellular locations but other proteins, such as UPF1, remain on the HIV-1 genomic RNA. The genomic RNA is then translated and the viral life cycle is completed with trafficking, assembly and budding of viral particles. In the nucleus and cytoplasm, UPF1 remains bound to the HIV-1 genomic RNA (Chapter 3), and as a consequence it is protected from negative regulators such as Hax-1 and does not associate with UPF2.

FUTURE DIRECTIONS

Changes in the nuclear and cytoplasmic subcellular compartments in the presence of HIV-1.

Every step of the HIV-1 replication cycle necessitates numerous cellular proteins contributing to the production of infectious virus particles. In addition, retroviruses need to escape host RNA quality control mechanisms such as NMD to ensure spread of infection. One important aspect to address is to look at how HIV-1 influences the composition of UPF1, UPF2 and UPF3a/b RNPs. Moreover, the specific differences in both subcellular compartments (nuclear and cytoplasmic) would give additional insight since these proteins harbour NES and NLS signals. One way to do this is to fractionate (nucleus, cytoplasm) the HIV-1 negative (as comparison) and positive cells, immunoprecipitate the complexes of UPF1, UPF2, UPF3aL and UPF3b and analyze by mass spectrometry the different cellular proteins present in each complex in both subcellular compartments. The changes observed would indicate how HIV-1 remodels these complexes to efficiently replicate and produce viral particles.

It is clear that HIV-1 causes differential expression of numerous cellular proteins [13-17]. It would be interesting to subfractionate HIV-1 infected cells and examine the changes in the abundance of cellular proteins (by mass spectrometry, for example) in both the nuclear and cytoplasmic compartments by comparing

them to the non-infected conditions. Identifying the compositional changes and how they are modulated in various subcellular compartments will hint to HIV-1's capacity changes to displace inhibitors or coopt factors required at different steps of its replication cycle. In addition, these findings would explain in part how nuclear proteins are identified in the cytoplasm or in HIV-1 particles. This would therefore open new avenues of research and may identify new candidates for therapy.

Does UPF1 have a role in the early stages of the HIV-1 replication cycle?

Abrahamyan et al. showed that UPF1 was encapsidated in virus particles [3]. Moreover, under Staufen1 knock down conditions, which result in an increase in HIV-1 genomic RNA encapsidation, more UPF1 is incorporated in virus particles [3]. This could most likely be due to its association with the HIV-1 genomic RNA. In addition, the experiments that we have used only look at the late stages of the HIV-1 replication cycle. Since UPF1 is found in virus particles, it would be interesting to study if UPF1, as well as the other UPF proteins, has a role during the early stages of HIV-1 replication. Azzalin et al. showed that UPF1 binds to chromatin and that it is involved in the cell cycle progression [5, 6]. Therefore, there is the possibility that UPF1 could be involved in the integration of the HIV-1 genome into the host. It is known that HIV-1 preferentially integrates into active transcription sites. Thus, one way to approach this would be to knock down or overexpress UPF1 and look at the transcriptional activation resulting from integration into active or silent transcription sites. In addition, during these

conditions, the presence of unintegrated provirus can be looked at and this would indicate if a block in integration occurs.

Does HIV-1 alter the post-translational modification of the UPF proteins?

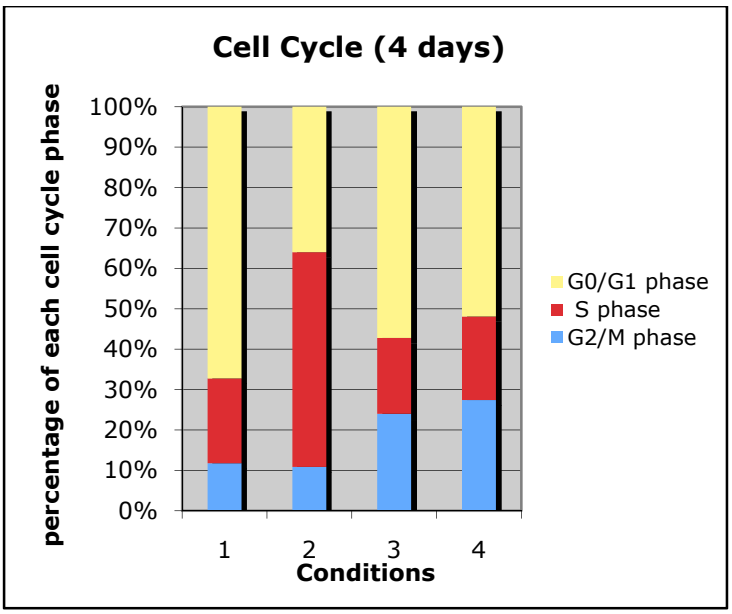
Post-translational modifications of proteins affect numerous cellular processes as well as their localization and interactions. UPF1 is phosphorylated more than 20 times and yeast UPF1 has been shown to auto-ubiquitinate [18]. UPF2, also has phosphorylation sites, serine residues at its N-terminal [11, 19]. In addition, the phosphorylation and dephosphorylation of UPF1 has been shown to be involved in RNA/DNA stability and is important for NMD [11, 20, 21]. UPF3 proteins and UPF2 were also shown to be involved in UPF1 phosphorylation. It would therefore be interesting to know if UPF1 and/or UPF2 phosphorylation states are altered in HIV-1 positive conditions. Moreover, since UPF3b and UPF1 interaction is increased in HIV-1 conditions (Chapter 3), it would be appealing to determine if this increase in interaction does affect the phosphorylation status of UPF1. Expression of UPF1 mutants or UPF3b mutants unable to bind each other could shed light if UPF1 phosphorylation, if changed in HIV-1, is dependent on UPF3b binding.

Recently, a link was made between protein ubiquitination and RNA export [22, 23]. Since yeast UPF1 has been identified as an E3 ligase and the self-ubiquitination of UPF1 required an interaction with UPF3b over UPF2 [24]; and our results show that the shuttling function of UPF1 is important for efficient

HIV-1 genomic RNA export (Chapter 3), it would be interesting to see if UPF1 is indeed ubiquitinated in HIV-1 conditions and if this ubiquitination is required for HIV-1 genomic RNA export. This could be achieved by overexpressing HA-ubiquitin in HIV-1 negative and positive conditions, immunoprecipitating HA (ubiquitin) and blotting for UPF1 to check its presence. This would indicate if any changes in UPF1 ubiquitination are present between conditions. Mutant UPF1 proteins lacking the ubiquitination sites identified by Takahashi et al. [24] can be used to validate the requirement for ubiquitination of UPF1 and the promotion of HIV-1 genomic RNA export.

HIV-1, UPF1 and cell cycle progression.

HIV-1 has been shown to affect cell cycle progression. Moreover, UPF1 has also been involved in cell cycle progression such that knock down of UPF1 results in an S phase arrest after 4 days [20, 21] [1] (Chapter 2). In addition, this observed cell cycle arrest was specific to UPF1 since knock down of UPF2 did not alter the cell cycle progression [20, 21]. We have preliminary data showing us that this S-phase arrest resulting from UPF1 knock down is abrogated in HIV-1 positive cells (Figure 33). There is a possibility that a viral protein can overcome this S phase arrest such as Vif, Vpr and Tat who have roles in cell cycle. Moreover, Vpr arrest cells in G2 phase of the cell cycle and this has been linked as being important in viral replication and apoptosis [25]. Thus, there is a possibility that Vpr pushes the cells into G2 by overcoming the block in S phase resulting from UPF1 knock down [26]. Using deletion strains of HIV-1 lacking viral



	1	2	3	4
	Mock + siNS	Mock + siUPF1	pNL4-3 + siNS	pNL4-3 + siUPF1
G2/M Phase	11.78	10.87	24.01	27.42
S Phase	20.97	53.12	18.8	20.67
G0/G1 Phase	67.25	36.01	57.28	51.91

FIGURE 33: *Cell cycle arrest 4 days following transfection.*

HeLa cells were transfected with pNL4-3 with or without siNS or siUPF1. Cells were collected, fixed and treated with RNAse A and stained with propidium iodide. Cell cycle was analyzed by FACS analysis. Results shown are representative of one experiment. (similar results were obtained in 2 independent experiments).

proteins and assessing S phase arrest under UPF1 knock down can identify which viral protein is altering the cell cycle. This would illustrate another reason as to why HIV-1 commandeers UPF1 function.

UPF1 and the microRNA pathway.

MicroRNAs (miRNAs) are short RNAs (19-25 nucleotides) whose main function is to block protein synthesis. HIV-1 infection and reactivation from latency have identified numerous miRNAs that were upregulated [27]. HIV-1 Tat has the unique property of being secreted from HIV-1 infected cells and can enter the uninfected cells. This property was observed in neuronal cells. In neurons, Tat increased the expression of cellular miRNAs: mir-374, -128a/b, -25 ,-100, -99a, among others [28]. Interestingly, it was shown that mir-128, this brain-specific miRNA, downregulated UPF1 (as well as MLN51/BTZ) by targeting its 3'UTR, thus resulting in the repression of NMD [29]. It would be interesting to see how this pathway is affected since we have not observed a decrease of UPF1 in HIV-1 positive cells.

Where and how does UPF1 bind to the HIV-1 genomic RNA?

Kula et al. [2] identified UPF1 as one of the proteins binding to nuclear HIV-1 genomic RNA immediately following transcription [2]. It would be interesting to identify the binding site of UPF1 on the HIV-1 genomic RNA. UPF1's zinc fingers have been proposed to bind to instability (INS) sequences [30] and HIV-1

genomic RNA contains numerous INS elements to which UPF1 can possibly bind [31-33]. Since our UPF1 NES mutant co-immunoprecipitates with the HIV-1 genomic RNA, we therefore can rule out the involvement of the zinc fingers (Figure 18). We can therefore mutate other functional domains and study the binding of UPF1. Moreover, we can study the binding pattern of UPF1 on HIV-1 genomic RNA by doing PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation analysis). This technique is used to isolate RNA binding proteins bound to their RNA sequence targets and identify the bound RNA sequence by using the tool PARalyzer [34]. Identifying the binding pattern of UPF1 on HIV-1 can shed light if the binding sites differ in regards to UPF1's presence in two different HIV-1 RNP complexes: export/stability and translation.

Live cell visualization is a novel technique that can bring new insight on many dynamic cellular processes (i.e. RNA export, translation, trafficking). We can use this technique by studying the dynamic binding pattern of UPF1 in the nucleus to the HIV-1 genomic RNA and the export of the HIV-1 RNP complex. Since we identified UPF1 in two different HIV-1 RNP complexes, one for stability/export and one for stability/translation, we actually do not have any evidence if these two complexes stem from one complex (where a variety of cellular factors are released and UPF1 stays on) or are there two different UPF1 complexes. This live cell technique is a newly optimized technique in our laboratory. We can therefore conduct these experiments by using $UPF1^{WT}$ as well as the NES and NLS mutants (GFP tagged) and an HIV-1 plasmid containing numerous MS2 sites to which a

MS2-tagged fluorescent protein (different from GFP: RFP) can bind. Then time lapse could be captured to identify the changes in the different association/binding patterns between $UPF1^{WT}$, the NES and the NLS mutants with the HIV-1 genomic RNA. Live cell imaging can also assist in identifying if UPF1 always stays bound to the HIV-1 genomic RNA in the nucleus or if its interaction in the nucleus and cytoplasm are distinct. The combination of deep sequencing analysis (PAR-CLIP) and live cell imaging can help clarify if there is one UPF1 RNP complex which gets remodelled in the cytoplasm, or if there is the presence of two distinct RNP complexes.

Possible roles of the UPF proteins in HIV-1 latency.

Latency still remains one of the main obstacles to eradicate HIV-1. Thus, identifying new cellular proteins aiding during this replication stage can identify new therapeutic strategies. A genome-wide siRNA screen identified a number of RNA helicases altered in expression following reactivation from latency [35]. Interestingly, DDX3 and DDX1, where among those identified [35]. Both of these RNA helicases are involved in HIV-1 genomic RNA export. Moreover, cellular proteins binding the newly transcribed HIV-1 genomic RNA immediately following transcription are more likely to be involved in latency. The study conducted by Kula et al. [2] identified 32 cellular proteins binding to the newly transcribed nuclear HIV-1 genomic RNA [2]. From this list, UPF1, as well as other proteins involved in HIV-1 genomic RNA export were identified. Among this list were DDX3 and PTB. DDX3 and PTB have been implicated in HIV-1

nucleocytoplasmic RNA export. It was shown that the HIV-1 multiply spliced RNAs are present in the nucleus of latently infected patient cells [36]. Furthermore, overexpression of PTB reverted the cells from latency [36]. Since UPF1 is required for HIV-1 replication, more specifically for HIV-1 genomic RNA export, and UPF2 blocked HIV-1 genomic RNA export, we speculate that they may differentially regulate HIV-1 latency. We can perform knockdown and overexpression studies of the UPF proteins in latently infected cells and observe if there is a reactivation of latency by looking at HIV-1 genomic RNA levels and Rev and Gag translation [36]. Studying their roles in latency can represent future therapeutic therapies. Decreasing the pool of viral latency reservoirs, by identifying cellular proteins that can revert cells out of latency is a must in winning this war against HIV-1.

CLAIMS TO ORIGINAL RESEARCH

The work presented in this thesis highlights new findings that are of importance not only in the HIV field but as well as in RNA export and metabolism research areas. The research conducted and presented here shows:

- That HIV-1 escapes the RNA quality control mechanisms, such as nonsense-mediated mRNA decay (NMD), by coopting UPF1 function.
- The role of UPF1 in HIV-1 RNA metabolism is distinct from its role in NMD, since the transdominant negative form of UPF1 (R844C) still upregulated HIV-1 genomic RNA.
- UPF1 activities are present in both the nucleus and the cytoplasm.
- UPF1 is involved in HIV-1 genomic RNA export, stability and translation.
- The shuttling function of UPF1 is required to efficiently export HIV-1 genomic RNA.
- We have also uncovered new and distinct functions of UPF1 and UPF2.
- The binding between UPF1 and UPF2 is not required in HIV-1 RNA stability and export.
- While UPF1 is a positive regulator for HIV-1 genomic RNA export, UPF2 seems to be a negative regulator. UPF2 binding to UPF1 results in a block in HIV-1 genomic RNA export.

This work highlights novel roles for UPF1 and UPF2 in HIV-1 RNA metabolism. Furthermore, the future directions described in the previous section can also lead to numerous new projects.

Numerous host proteins are required at each step of the HIV-1 replication cycle. Identifying and characterizing the specific roles of each of these host factors, in this case UPF1 and UPF2, can lead to new antiretroviral therapies.

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