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Novel antiviral mechanism of IFN-stimulated gene 20(ISG20) via translational suppression

Nannan Wu

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Nannan Wu. Novel antiviral mechanism of IFN-stimulated gene 20(ISG20) via translational suppression. Microbiology and Parasitology. Université de Lyon; East China normal university (Shanghai), 2016. English. NNT: 2016LYSEN005 . tel-01424182

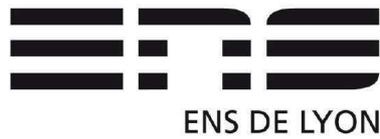
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Numéro National de Thèse : 2016LYSEN005

THESE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée par
l'Ecole Normale Supérieure de Lyon

Ecole Doctorale N° 340
Biologie Moléculaire, Intégrative et Cellulaire (BMIC)

Spécialité de doctorat : Sciences de la Vie

Discipline : Virologie et Immunologie

Soutenue publiquement le 20/05/2016, par :

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**Novel antiviral mechanism of
IFN-stimulated gene 20 (ISG20)
via translational suppression**

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Acknowledgement

Among the entire thesis, this is the most difficult part for me because I am bad at expressing my gratitude and I want to thank too many people. So even this first simple sentence I took one month to type it on this page.

Four years pass by since I first arrived to Lyon in 2012. I would like first to express my sincere gratitude to my supervisors, Dr Andrea Cimarelli, Prof Mingyao Liu and Dr Bing Du. I spend most of the time in Andrea's team in France. I want to tell him that thanks for your complete and systematic training, for your encouragement and patience. I adore your rich knowledge base and scientific literacy, which promote me to try to become a real scientist like you. I would like also thank Prof Mingyao Liu and Dr Bing Du, for accepting me to be your student, I am really proud of that. Dr Du really help me a lot, not only in scientific training, but also in coping with the tedious affairs from the school in China.

My thanks also goes to the rest members of committee, Dr Jianhua Wang, Prof Yuping Lai and Prof Biao Zheng. Dr Wang and Prof Lai also reviewed my manuscript, with accompany of Dr Frederick Arnaud, Dr Lanrent Chavatte and Prof Wenzheng Jiang. Thank you all for your precious time and useful suggestions for optimizing my thesis.

I'll not forget that it's my master supervisors, Dr Yongjin Wang and Prof Tianhou Wang who helped me to get through the examination of ECNU-ENS cotraining PhD project. It's them who showed me this route that otherwise I'll never experience.

The results I present in this thesis is achieved with participation of Li, Kevin, Romain, Xuan-Nhi and Baptiste who have made an indispensable contribution to this project. I would like also to thank Fabrice, Qing and Wen Yue for your generous offering of some protocols and materials.

I would like to thank everybody of our team here in France, the two Steph, Kevin, Fanny, Cindy who left already. And Xuan-Nhi, Mathilde, Vero, Claire, Lucie, Eloise, Louise, Stepania, Megane, Nina and Romain. It's so nice to meet you and I'll always remember your enthusiasm and keep this friendship in mind. Chengfei, Yinglei and Jie who work in Dr Du's team, although I never meet you yet, you already help me a lot, soon I'll have the opportunity to say thanks to you face to face.

Thanks to my family, my girlfriend Jingyun, thank you for your accompany. Thanks to all my friends here, it's you make me feel not far from home. Thanks to our neighbors from team OR, EBV, TEV, AAV and team COSSET. Thanks to the secretariat of CIRI, BMIC and ENS-Lyon in France, and also the secretariat of Life science school and ECNU in China, as well as Halima, Huajun, Madame Qian, Xiaoling, Xiaoyan from international department of ENS-lyon and ECNU.

In the end, I want to give my big thanks to my country, where I get the foundation for this four years PhD study. Thanks also to people who work in the Embassy and CSC.

Abstract

Interferons specify a complex antiviral response that upon the detection of pathogens through various cellular pattern-recognition receptors (PRRs) lead to the induction of hundreds of genes named interferon-stimulated genes (ISGs). Several ISGs have been reported to restrict viral infection, however the antiviral role/s of many of them remains either unknown or poorly characterized. During my thesis I have focused on the characterization of ISG20 during the replication of two viruses, VSV and HIV-1. ISG20 had been previously identified as an antiviral 3'-5' exonuclease and was thought to act by directly degrading viral genomes. However, the decrease in viral RNAs specified by ISG20 was controversial.

To gather further insights into the mechanism with which ISG20 interfered with viral replication, I constructed several mutants of ISG20. The results we have obtained indicated that the antiviral activity of ISG20 does not solely rely on its ability of ISG20 to degrade RNA, as several mutants were identified that lost their antiviral properties despite a robust RNase capacity *in vitro*.

We have found here that ISG20 could block viral replication through a block in translation. This block occurred both during viral infection as well as during the ectopic expression of reporter genes in ISG20-expressing cells. The results we have obtained indicate that ISG20 affects both cap- and IRES-mediated translation in a manner that is very likely independent from translation initiation.

To substantiate the antiviral role of ISG20 during viral infection, knock-out *isg20* ^{-/-} mice were generated and then analyzed for their ability to support VSV infection *in vivo*. The results obtained, clearly implicate ISG20 in the natural control of viral spread *in vivo*, strongly supporting our data *ex vivo*.

Overall, the data obtained during my thesis indicate that ISG20 is an important antiviral factor and shed light on a novel mechanism of viral inhibition whereby ISG20 interferes with viral mRNA translation.

Key words

ISG20; RNase; VSV; HIV-1; Translation

Résumé

La réponse interféron est une réponse antivirale complexe qui, après la détection de pathogènes par des PRR (récepteurs de motifs associés aux pathogènes), conduit à l'induction de centaines de gènes appelés ISG (gènes stimulés par l'interféron). Dans la littérature, il existe plusieurs ISG capables de s'opposer à l'infection virale ; cependant le rôle antiviral précis d'un grand nombre d'entre eux reste inconnu ou mal caractérisé. Pendant ma thèse, je me suis concentré sur la caractérisation d'ISG20 pendant la réplication de deux virus, VSV et le VIH-1. La protéine ISG20 a été décrite au préalable comme une exonucléase 3'-5' antivirale en agissant sur la dégradation directe du génome viral. Cependant, la diminution de la quantité d'ARN viraux liée à ISG20 était controversée.

Afin de mieux comprendre le mécanisme par lequel ISG20 interfère avec la réplication virale, j'ai construit plusieurs mutants d'ISG20. Les résultats obtenus indiquent que l'activité antivirale d'ISG20 ne repose pas uniquement sur sa capacité à dégrader l'ARN, puisque plusieurs mutants ont perdu leurs propriétés antivirales malgré une robuste activité RNase *in vitro*.

Mes résultats montrent qu'ISG20 peut bloquer la réplication virale en bloquant la traduction. Dans les cellules exprimant ISG20, ce blocage intervient à la fois pendant l'infection virale et lors de l'expression ectopique de gènes rapporteurs. Les résultats que nous avons obtenus indiquent que la protéine ISG20 affecte la traduction qu'elle soit cap- ou IRES-dépendant. Cette inhibition de la traduction est très probablement indépendante de l'initiation.

Afin d'étayer le rôle antiviral d'ISG20 pendant l'infection virale, des souris invalidées pour *isg20* (-/-) ont été générées et leur capacité à supporter l'infection par VSV *in vivo* a été analysée. Les résultats obtenus impliquent clairement ISG20 dans le contrôle naturel de la propagation virale *in vivo*, confirmant nos données *ex vivo*.

Dans l'ensemble, les données obtenues pendant ma thèse indiquent qu'ISG20 est un important facteur antiviral et mettent en évidence un nouveau mécanisme d'inhibition virale où ISG20 interfère avec la traduction d'ARNm viral.

Mots clés:

ISG20, RNase, VSV, VIH-1, Traduction

Abbreviations

A:

AEN (ISG20L1): apoptosis enhancing nuclease;
AIM2: absent in melanoma 2;
ALR: AIM-2-like receptors;

APOBEC3: apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3;
ART: antiretroviral treatment;

B:

BVDV: Bovine viral diarrhoea virus;

C:

CARDs: caspase activation and recruitment domains;
CBs: Cajal Bodies;
c-di-AMP: cyclic (3'-5') diadenylate;
c-di-GMP: cyclic (3'-5') diguanylate;

CDNs: cyclic dinucleotides;
cGAS: cyclic GMP-AMP synthase;
CpG: cytosine-guanosine;
CrPV: Cricket paralysis virus;

D:

DAI (ZBP1): DNA-dependent activator of IRFs;
DAMPs: damage-associated molecular patterns;
DDX41: DEAD box polypeptide 41;

DENV: Dengue virus;
DNA-PK: DNA-dependent protein kinase;

E:

eIF: eukaryotic initiation factor;
EMCV: Encephalomyocarditis virus;

ESCRT-I: endosomal sorting complex required for transport I;

F:

FMD: Foot-and-mouth disease;

H:

HAV: Hepatitis A virus;
HBV: Hepatitis B virus;
HCV: Hepatitis C virus;

HEM45 (ISG20): human estrogen regulated transcript 45;
HIV-1: human immunodeficiency virus 1;
HSV-1: Herpes simplex virus 1;

I:

IAV: Influenza A virus;
IFI16: gamma-interferon-inducible protein 16;
IFIT1: interferon-induced protein with tetratricopeptide repeats 1;
IFIX: interferon-inducible protein X;
IFN-I: type I interferons;

IFNAR: IFN- α receptors;
IKK: I κ B kinase;
IRFs: IFN regulatory factors;
IRES: internal ribosome entry site;
IRF: interferon regulatory factor;
ITAF: IRES trans-activating factors;

L:

LPG2: laboratory of genetics and physiology 2

M:

MAP: Mitogen-activated protein;
MEFs: mouse embryonic fibroblasts;
MLV: murine leukemia virus;

MRE11: meiotic recombination 11;
MYD88: myeloid differentiation primary-response protein 88;

N:

NEMO: NF- κ B essential modulator;
NNS: nonsegmented negative strand;

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells;

O:

OAS: 2'-5'-oligoadenylate synthetase;

P:

PABP: poly(A)-binding protein;
PAMPs: pathogen-associated molecular patterns;

PKR: protein kinase R;
PRR: pattern-recognition receptors;
PV: Poliovirus

R:

RdRp: RNA-dependent RNA polymerase;
RIG-I: retinoic-acid-inducible gene-I;

RLR: RIG-I like receptors;
RNP: ribonucleoprotein;

S:

SARS-CoV: Severe acute respiratory syndrome coronavirus;
SG: stress granules;

SINV: Sindbis Virus;
SMN: survival motor neuron;

T:

TIR: Toll-IL-1 receptor
TLRs: Toll-like receptors;
TBK1: TRAF family member-associated NF-kappa-B activator (TANK)-binding kinase 1;

TREX1: Three prime repair exonuclease 1;
TRIF: Toll-IL-1 receptor (TIR) domain-containing adaptor protein inducing IFN β ;

V:

VPg: viral protein genome-linked protein;

VSV: Vesicular stomatitis virus;

W:

WNV: West Nile virus

Y:

YFV: Yellow fever virus

Z:

ZAP: zinc finger antiviral protein;

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Introduction

I. Interferon responses and the multiple functions of interferon-stimulated genes in anti-viral responses

Pathogen infection awakes host innate immune responses through the action of pattern-recognition receptors (PRRs) that specifically recognize pathogen-associated molecular patterns (PAMPs), as well as cellular damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004). Toll-like receptors (TLRs), retinoic-acid-inducible gene-I (RIG-I)-like receptors (RLRs), and a series of cytoplasmic DNA sensors such as cyclic GMP-AMP synthase (cGAS) are known PRRs, which sense viral nucleic acids and initiate signaling pathways that activate transcriptional regulators including NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), MAP (Mitogen-activated protein) kinases and IFN regulatory factors (IRFs), for regulating the transcription of genes encoding type I interferon and other inflammatory cytokines (Kawai and Akira, 2011; Ablasser et al, 2013a).

IFNs α/β , collectively called type I interferons (IFN-I) are then secreted from the cell where they associate in a paracrine or autocrine manner to specific cell surface receptor (IFNAR). Upon ligand binding, these receptors convey a signal to the nucleus through the so-called JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway that activates the transcription of hundreds of IFN-stimulated response elements (ISRE)-driven or gamma-activated sequence (GAS, in the case of type II interferon responses)-driven ISGs (Stark et al, 2012; Schoggins et al, 2011). The IFN signaling plays anti-pathogen activities in various ways by stimulating both innate, as well as adaptive immune responses (Schoenborn and Wilson, 2007).

I.A Pathogens recognition and innate immune responses

I.A.1 PAMPs recognition and type I IFN production

PAMPs, DAMPs and PRRs

PAMPs (pathogen-associated molecular patterns) are small molecular motifs conserved within a certain group of microbes essential for their life cycle. PAMPs consist of a vast array of molecule types. In bacteria they mainly comprise molecules derived from extracellular structures such as Flagellin, the cell wall component lipopolysaccharides (LPS), lipoteichoic acid (LTA) and peptidoglycan. However, also nucleic acids normally associated with viral pathogens either in their RNA, DNA or DNA:RNA hybrid form which are most well-known PAMPs. In contrast to nucleic acids that are conserved between host and pathogen, many PAMPs such as LPS and LTA are typical microbial molecules that are not produced by the host, allowing the host to clearly distinguish them as nonself. In contrast, nucleic acids are genetic information features present in all known forms of life, issue that opens up the important question as to how the cell discriminates between self and non-self. This issue will be discussed below.

Lastly, DAMPs (damage-associated molecular patterns) are generally cellular proteins, represented by intracellular high-mobility group box 1 (HMGB1) and heat-shock proteins, extracellular glycans and

glycoconjugates response to tissue injury (Scaffidi et al, 2002; Panayi et al, 2004; Scheibner et al, 2006). Non-protein DAMPs such as uric acid, ATP nucleotides and adenosine, and nucleic acids themselves can also serve as danger signals and trigger innate immune responses (Shi et al, 2003; Bours et al, 2006; Farkas et al, 2007; Bernard et al, 2012).

Nucleic acids are detected by PRRs (pattern-recognition receptors). PRR can be mainly divided in three groups: TLRs (Toll-like receptors), RLRs (RIG-I-like receptors) and cytoplasmic DNA sensors. TLRs are membrane-localized proteins that are mostly expressed in immune cells such as dendritic cells (DCs), macrophages, and B cells. Cell surface TLRs mainly respond to bacterial associated ligands such as Flagellin and LPS, while various forms of nucleic acids derived from bacteria and viruses are detected by endosome-localized TLRs (Kawai and Akira, 2011).

RLRs are most well-known receptors for cytosolic nucleic acids sensing, including RIG-I, MDA5 (melanoma differentiation gene 5) and LPG2 (laboratory of genetics and physiology 2) (Yoneyama et al, 2004&2005). Finally, cytoplasmic DNA are detected by many cellular sensors such as DNA-dependent activator of IRFs (DAI, also known as ZBP1), Gamma-interferon-inducible protein 16 (IFI16), and cyclic GMP-AMP synthase (cGAS) (Takaoka et al, 2007; Unterholzner et al, 2010; Sun et al, 2013).

Viral nucleic acids recognized by intracellular TLRs in endosome

TLRs are transmembrane proteins expressed on the cell surface or in intracellular vesicles. Each single TLR protein has an ectodomain for the recognition of PAMPs, a transmembrane domain, and a cytosolic Toll-IL-1 receptor (TIR) domain for adaptors binding. Upon recognition of respective PAMPs, TLRs form into dimers and recruit a specific set of adaptor molecules that recognize the TIR domain. Known adaptors of this domain include: myeloid differentiation primary-response protein 88 (MYD88), TIR domain-containing adaptor protein inducing IFN β (TRIF), MYD88-adaptor-like protein (MAL) and TRIF-related adaptor molecule (TRAM).

Ten members of TLRs (TLR1-10) have been characterized in humans. Apart from TLR10 which only performs sensing, but not signaling (Guan et al, 2010), each other TLR detects distinct PAMPs derived from a large heterogeneity of pathogens. Among TLRs, four are involved in nucleic acid sensing in endosomes: TLR3 (also localized on the plasma membrane of human fibroblasts) recognizes viral dsRNA (Alexopoulou et al, 2001); TLR7 and TLR8 share a common ligand in single-strand RNA (ssRNA) derived from RNA viruses (Heil et al, 2004); TLR9 recognizes bacterial and viral DNA containing the cytosine-phosphate-guanine (CpG) motif, a rare motif in mammalian genomes (Hemmi et al, 2000; Ohto et al, 2015).

Upon recognition, TLR7, TLR8 and TLR9 recognition leads to MyD88-dependent pathway activate, while TLR3 activates a TRIF-dependent pathway. Both pathways converge in the activation of transcription factors such as NF- κ B, IRFs, to induce production of type I IFN and inflammatory cytokines (O'Neill et al, 2013). (Figure I-1)

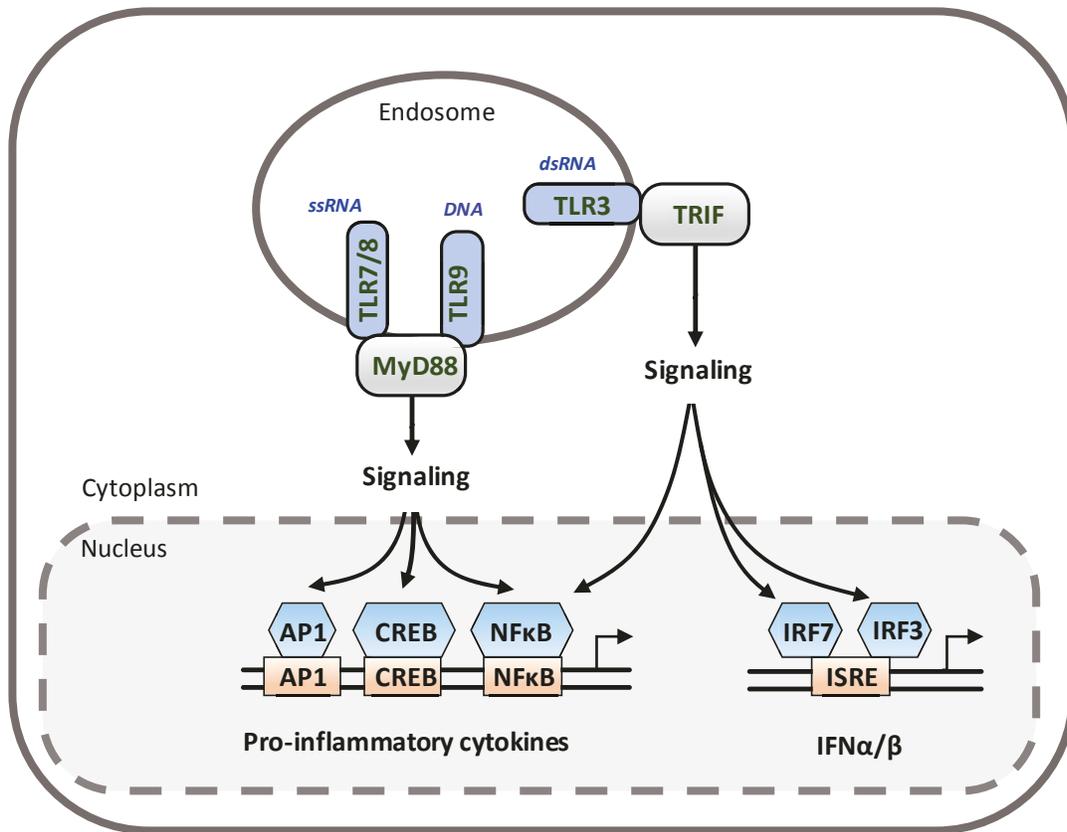


Figure I-1, Nucleic acids detection by toll-like receptors in endosome.

Host cells recognize pathogens-derived nucleic acids from endosomes and cytoplasm. TLR3, TLR7, TLR8 and TLR9 localize to the endosomes, where they sense microbial derived DNAs or RNAs and recruit adaptor proteins MyD88 and TRIF to trigger the downstream pathway. Several transcriptional factors were activated and translocated to the nucleus for inducing the transcription of pro-inflammatory cytokines and type I IFNs genes which contain the related binding sites.

Abbreviations: ss/ dsRNA (single/ double-strand RNA); TLR (toll-like receptor); MyD88 (myeloid differentiation primary-response protein 88), TRIF (TIR domain-containing adaptor protein inducing IFN β); AP1 (activator protein 1); CREB (cyclic AMP-responsive element-binding protein); NF- κ B (nuclear factor- κ B); IRF (IFN regulatory factor); ISRE (IFN-stimulated response element).

Viral RNAs recognized by RLRs in the cytosol

TLRs are expressed in selected immune type cells, most other non-immune cells like epithelial cells and fibroblasts do not express TLRs, but retain the ability to mount effective innate immune responses against pathogen infections (Diebold et al, 2003), indicating that other sensors are existed in these cells. In 2004 and 2005, three RIG-I like receptors, RIG-I, MDA5 and LPG2 were identified as general sensors of cytosolic viral RNA (Yoneyama et al, 2004&2005).

As shown in Figure I-2 (Wu and Chen, 2014), the RLRs have highly conserved secondary structures. The C-terminal domain (CTD) contributes to specific recognition and binding of the RNA ligand, and the interaction of CARD domains connecting RIG-I and MDA5 with their adaptor MAVS and trigger downstream signaling (Kawai et al, 2005; Seth et al, 2005). LPG2 has the helicase domain and CTD for dsRNA-binding, but is devoid of a CARD domain, making it deficient for activating downstream antiviral signal and likely suggesting it as a regulatory molecule for RIG-I and MDA5 signaling (Yoneyama et al, 2005; Satoh et al, 2010).

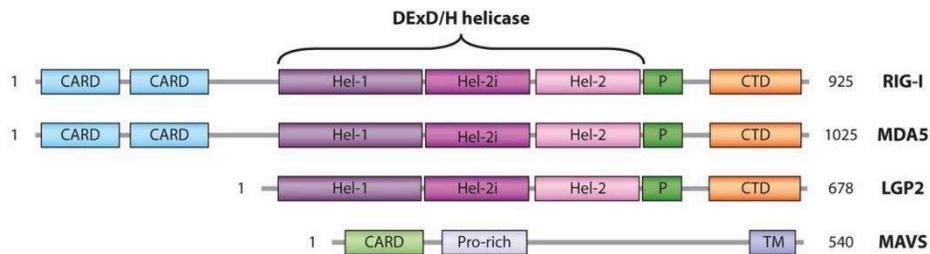


Figure I-2, Domain Alignment of RLRs and their adaptor MAVS (Wu and Chen, 2014)

The three RLRs share highly conserved domain organizations. From 5' to 3', there are two CARDS in the N-terminal, a DExD/H-box helicase domain in the centre which contain two helicase domains (Hel-1 and Hel-2) and an insertion termed as Hel-2i, a helical extension pincer domain and a C-terminal domain (CTD). The adaptor of RLR, MAVS also comprises a N-terminal CARD domain, followed by a proline-rich domain, and a transmembrane domain to anchor its C terminus into the mitochondrial membrane. LPG2 lacks the CARD domain, thus does not signal through MAVS-dependent pathway.

Abbreviations: RIG-I (retinoic-acid-inducible gene-I); MDA5 (melanoma differentiation gene 5); LPG2 (laboratory of genetics and physiology 2); MAVS (mitochondrial antiviral signaling); CARD (caspase activation and recruitment domains); Hel (helicase); P (pincer); CTD (C-terminal domain); Pro (proline); TM (transmembrane).

Among the three RLRs, ligands for RIG-I are well characterized, including 5'-triphosphate (5'ppp)-bearing panhandle structures synthesized by polymerases of some negative-strand ssRNA viruses, as well as 5'-diphosphate (5'pp) dsRNAs of some viral genomes, represent by influenza A virus and reovirus, respectively (Pichlmair et al, 2006; Schlee et al, 2009; Schmidt et al, 2009; Goubau et al, 2014). These RNAs are typically viral products that lack in the host and therefore allow the host to discriminate them from self material. Cellular or viral 5'-hydroxyl (5'-OH) and 3'-monophosphoryl short RNA molecules generated by OAS/RNase L system are also recognized by RIG-I (Malathi et al, 2007&2010). The ligand for MDA5 and LPG2 is less well characterized. In comparison with RIG-I, MDA5 prefer longer, high molecular weight double-strand RNA complex, such as synthesized poly I:C and the long replicative form (RF) dsRNA of picornaviruses (Kato et al, 2008; Pichlmair et al, 2009; Feng et al, 2012). However, there is no evidence that specific terminal groups are required for MDA5 ligands. While MDA5 possesses a nonspecific but weak RNA binding activity, LPG2 displays the highest RNA binding affinity among RLRs,

but it lacks downstream signaling. Increasing evidences suggest that LPG2 may function to modulate MDA5 sensing, as reviewed by Bruns and Horvath in detail (Bruns and Horvath, 2015).

As shown in Figure I-3, upon association with their ligand, the CARD domains of RIG-I and MDA5 recruit K63-linked ubiquitin chains and form oligomers. Oligomerized CARD domains then bind to the CARD of MAVS and trigger the polymerization of MAVS, which in turn activates several E3 ligases tumor necrosis factor receptor associated factors (TRAFs). NF- κ B essential modulator (NEMO) is the regulatory subunit of cytosolic kinases IKK (I κ B kinase) and TBK1 complexes. NEMO recruits polyubiquitin chains synthesized by TRAFs to the complexes and the two kinases in the complexes then phosphorylate I κ B α and IRF3, respectively. The activated transcriptional factors transport into the nuclear and trigger the induction of type I interferons and numerous pro-inflammatory cytokines (reviewed by Wu and Chen, 2014).

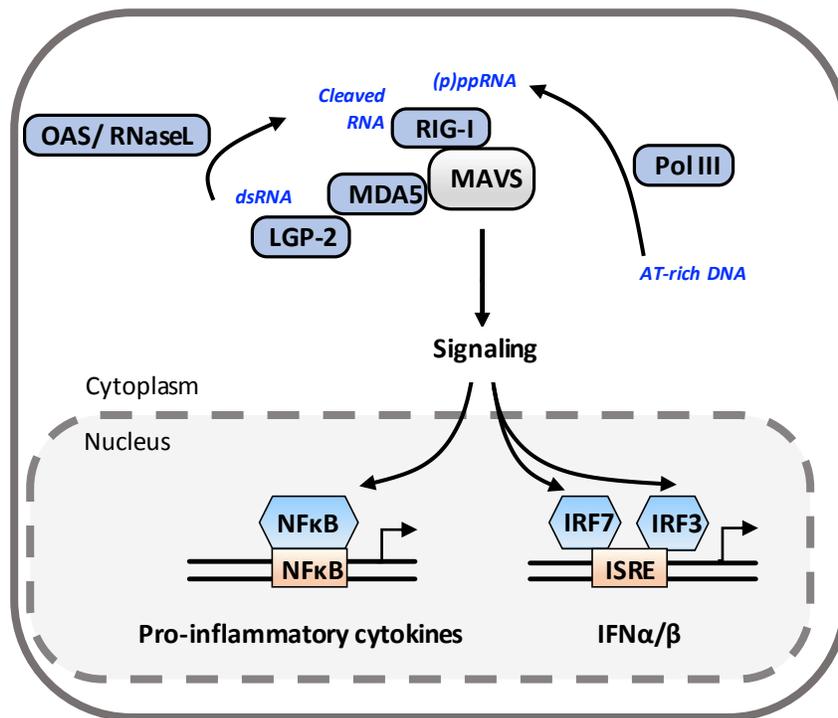


Figure I-3, Nucleic acids detection by cytosolic RIG-I like receptors.

Cytosolic RNAs are mainly detected by RIG-I like receptors including RIG-I, MDA5 and LGP2. RIG-I targets viral RNA containing 5'ppp and panhandle-like secondary structures, while MDA5 detects viral long dsRNA. LGP2 may function as a regulator to modulate the activity of MDA5. RIG-I and MDA5 recruit adaptor proteins MAVS to trigger the downstream signaling and activate transcriptional factors such as NF- κ B, IRF3 and IRF7. These activated factors import to the nucleus and stimulate the transcription of pro-inflammatory cytokines and type I IFNs. RNA polymerase III (Pol III) detects cytosolic AT-rich DNAs from bacteria and DNA viruses and transcribes them into 5'ppp-RNAs that are ligands of RIG-I (Chiu et al, 2009). dsRNA also activates the 2'-5' Oligoadenylate Synthetase (OAS)/RNase L pathway. RNase L digests the dsRNA into small fragments that are also detected by RIG-I (Malathi et al, 2007&2010).

Abbreviations: (p)ppRNA (5' di-/ tri-phosphate RNA); RIG-I (retinoic-acid-inducible gene-I); MDA5 (melanoma differentiation gene 5); LGP2 (laboratory of genetics and physiology 2); MAVS (mitochondrial antiviral signaling); OAS (2'-5'-oligoadenylate synthetase); RNaseL (Ribonuclease L); Pol III (RNA

polymerase III); *NF-κB* (nuclear factor-κB); *IRF* (*IFN* regulatory factor); *ISRE* (*IFN*-stimulated response element).

DNA sensors in the cytosol

DNA sensors in the cytosol are less characterized as compared to TLRs and RLRs, although earlier studies had already indicated that the downstream molecules TBK1, IRF3 and IRF7 were a part of an important cytosolic DNA sensing pathway (Stetson et al, 2006) (Figure I-4). DAI (DNA-dependent activator of *IFN*-regulatory factors) was the first identified cytosolic DNA sensor involved in type I *IFN* induction (Takaoka et al, 2007). In the following year, the most important adaptor, named STING (stimulator of interferon genes, also termed MPYS, MITA, ERIS by several groups independently) was identified to act upstream of TBK1 kinase (Ishikawa and Barber, 2008; Jin et al, 2008; Zhong et al, 2008; Sun et al, 2009). Following the discovery of STING, multiple DNA sensors were proposed to initiate the STING-TBK1-IRF signaling, including IFI16 (Unterholzner et al, 2010), the DExD/H helicase DDX41 (Zhang et al, 2011), DNA-PKcs (catalytic subunit of DNA-dependent protein kinase) (Ferguson et al, 2012), Mre11 (Meiotic recombination 11) (Kondo et al, 2013), and cGAS (Sun et al, 2013; Wu et al, 2013). Other STING-independent sensors were also suggested to respond to cytosolic DNA recognition, such as Pol III, LRRFIP1 and LSm14A (Chiu et al, 2009; Yang et al, 2010; Li et al, 2012c). However, it is still unclear why and how the cell devotes such a large number of DNA sensors and what their true importance in DNA pathogen recognition may be. It is possible that a certain number of these sensors are expressed in a tissue and cell type specific manner, thereby restricting *de facto*, the number of molecules that in a given cell is available for DNA sensing.

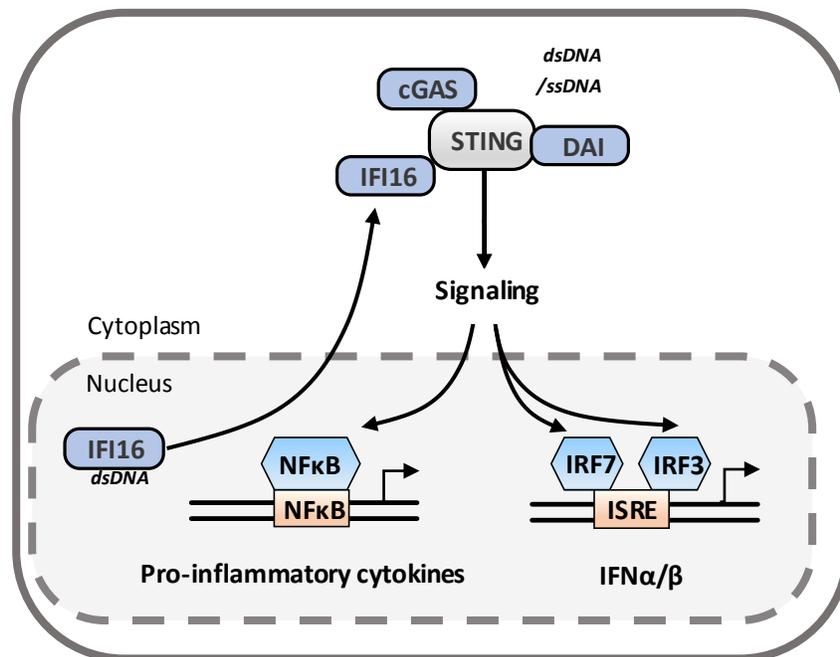


Figure I-4, Nucleic acids detection by DNA receptors.

There are several identified DNA sensors response to cytosolic DNA detection, such as cGAS, DAI and IFI16 (also detects DNA in nucleus). These sensors have a common adaptor protein, STING (stimulator of interferon genes) to transmit the signals for type I *IFN* induction.

Abbreviations: ss/ dsRNA (single-/ double-strand RNA); cGAS (cGAMP synthase); IFI16 (Interferon- γ -inducible protein 16); DAI (DNA-dependent activator of IRFs); STING (stimulator of interferon genes); NF- κ B (nuclear factor- κ B); IRF (IFN regulatory factor); ISRE (IFN-stimulated response element).

DAI also named Z-DNA binding protein 1 (ZBP1), as it contains N-terminal Z-DNA-binding domains (Schwartz et al, 2001). This protein was shown to play an important role on Herpes simplex virus 1 (HSV-1)-mediated induction of type I IFNs in murine L929 cells. However, in DAI-deficient mice or many human cell lines it mounts a normal type I IFN response to cytosolic DNA stimulation (Ishii et al, 2008; Lippmann et al, 2008), suggesting that this protein may not be an indispensable DNA sensor.

IFI16 together with AIM2 and the newly identified IFIX (interferon-inducible protein X), are members of PYHIN family member that contains pyrin domain and HIN200 domain in the N-terminal and C-terminal, respectively. They are also classified as AIM-2-like receptors (ALR). IFI16 and IFIX senses DNA and triggers IFN responses in both the cytoplasm and the nucleus (Unterholzner et al, 2010; Kerur et al, 2011; Li et al, 2012b; Orzalli et al, 2012; Diner et al, 2015). In addition to the role in IFN induction via STING-TBK1 signaling, IFI16 also participates in the inflammasome pathway that leads to caspase-1 activation. In the case of IFI16 however, the outcome of infection seems also to depend on the specific nature of the virus. Indeed, while IFI16 sensing leads to caspase-1 activation during herpesviruses infection, but rarely to cell death (Kerur et al, 2011; Ansari et al, 2015), activation of this pathway seem to lead to massive cell death in the case of human immunodeficiency virus 1 (HIV-1) infection. This massive cell death by IFI16-mediated pyroptosis has been hypothesized to be a leading cause in the depletion of CD4 T cells and in the progress towards acquired immune deficiency syndrome (AIDS) (Monroe et al, 2014).

DDX41 belongs to the DExD/H-box (DDX) protein family, the other renowned members in this family are the three RIG-I like receptors. DDX41 was identified to sense cytosolic DNA and trigger type I IFN induction in dendritic cells (Zhang et al, 2011). Further studies determined that DDX41 can also directly interact with cyclic dinucleotides (CDNs) and stimulate CDN-induced type I IFN (Parvatiyar et al, 2012; Zhang et al, 2013). A recent study characterized DDX41, cGAS and IFI202 to be required for MLV (murine leukemia virus) nucleic acid recognition (Stavrou et al, 2015). Thus, these results suggest that DDX41 may play an important in STING-dependent DNA and CDN signaling.

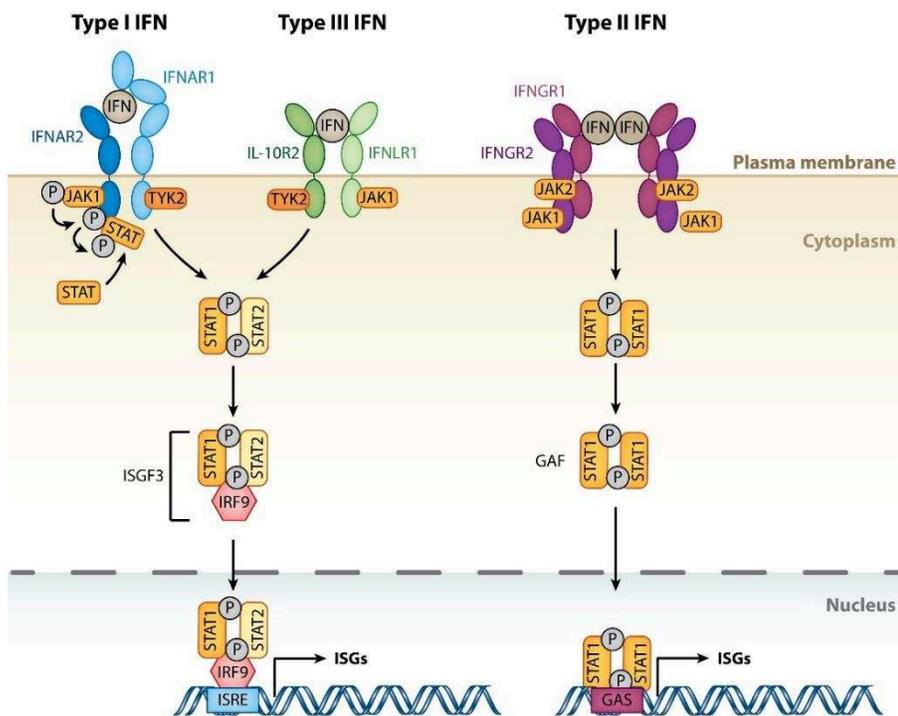
Cyclic diguanylate (c-di-GMP) and cyclic diadenylate (c-di-AMP) are members of CDNs and much emphasis has been recently given to them. In bacteria, they serve as second messengers with multi-functions (Tamayo et al, 2007). These CDNs have been shown to directly bind STING for its downstream signaling (Burdette et al, 2011). The identification of cGAS, the nucleotidyltransferase (NTase) that produces cGAMP from ATP and GTP upon DNA binding (Ablasser et al, 2013b; Sun et al, 2013; Wu et al, 2013; Gao et al, 2013) led to the explanation of an apparent puzzle in the field as to how STING could function as a sensor of bacterial signaling molecules, as well as an adaptor in the DNA-induced IFN response. Many viruses' infection activates the cGAS-STING pathway, including Kaposi's sarcoma-associated herpesvirus (KSHV), HSV-1, HIV-1, Adenovirus, *Retroviruses*, as well as Venezuelan equine encephalitis virus (VEEV) (Gao et al, 2013; Lahaye et al, 2013; Rasaiyaah et al, 2013; Lam et al, 2014; Dai et al, 2014; Schoggins et al, 2014&2015; Ma et al, 2015). A recent study demonstrates that cGAMP can be incorporated into viral particles of *lentiviruses* and *herpesviruses* and trigger a STING-dependent antiviral response in new infected

cells (Bridgeman et al, 2015; Gentili et al, 2015). Various cell types from cGAS-deficient mice failed to produce innate cytokines in response to DNA stimulation from transfection or virus infection, cGAS-deficient mice were more susceptible to several viral infections (Ablasser et al, 2013b; Li et al, 2013; Schoggins et al, 2014&2015), indicating that cGAS functions as a nonredundant cytosolic DNA sensor in response to virus infection.

Apart from the cytosolic RNA and DNA sensors I mentioned before, there are also several dual-sensors that have been shown to sense both RNA and DNA, such as the high mobility group box (HMGB) proteins that bind DNA and RNA and promote the activation of their sensors such as RIG-I and TLRs (Yanai et al, 2009). The binding of RNA and DNA by LSm14A triggers RIG-I-MAVS and STING-TBK-1 signaling, respectively (Li et al, 2012c). LRRFIP1 also interacts with both DNA and RNA upon infection of bacteria and virus, but induces IFN- β expression by activating the transcriptional co-activator β -catenin (Yang et al, 2010).

Figure I-5. IFN signaling via the JAK-STAT pathway (Schneider et al, 2014).

Type I, II, III IFNs bind to heterodimer of IFNAR1/2, IFNGR1/2, IL-10R2: IFNLR1. Despite using distinct



membrane receptors, type I and type III IFNs use the same pathway to successively activate the JAK1 and TYK2, then heterodimer of STAT1 and STAT 2. The dimer recruits the IRF9 to form the ISGF3. The phosphorylated complex translocates to the nucleus to induce transcription of genes regulated by ISRE promoter elements. Type II IFN recruits JAK1 and JAK 2 to activate STAT1 and lead to the formation of a homodimer called the gamma-activated factor (GAF), which translocates to the nucleus and induces genes regulated by their gamma-activated sequence (GAS) promoter elements.

Abbreviations: IFNAR (IFN- α receptor); IFNLR (IFN- λ receptor); IFNGR (IFN- γ receptors); IL-10R (Interleukin-10 receptor); JAK (Janus kinase); TYK2 (tyrosine kinase 2); STAT (signal transducer and activator of transcription); ISGF3 (Interferon-stimulated gene factor 3); GAF (IFN- γ -activated factor); GAS (IFN- γ -activated sequence); IRF (IFN regulatory factor); ISRE (IFN-stimulated response element); ISG (IFN-stimulated genes).

I.A.2 IFNs activate JAK-STAT pathway and induce the production of hundreds ISGs

The interferons (IFNs) were discovered in the 1950s and ever since a number of studies have revealed the complex effects that IFNs played on virus restriction, immune modulation, as well as cell proliferation.

According to their receptors at the cell surface, IFNs are grouped into types I, II and III. Type I IFNs comprises of more than a dozen of IFN- α s and the single IFN- β , binding to heterodimer receptors IFN- α receptors 1:2 (IFNAR1:IFNAR2). Type II IFN has a single member IFN- γ , binding to IFN- γ receptors 1:2 (IFNGR1:IFNGR2). Type III IFNs (also known as IFN- λ s) including IFNL1, 2, 3 and 4 are recognized by Interleukin-10 receptor 2 (IL-10R2) and IFN- λ receptor 1 (IFNLR1) heterodimers. Binding of IFNs to their receptors activates the JAK-STAT pathway, leading to the transcriptional regulation of a large number of IFN-regulated, or -sensitive genes (IRGs, or ISGs). (Reviewed by Schneider et al, 2014; Figure I-5).

I.B ISGs: proteins with multiple functions in anti-viral responses

I.B.1 ISGs involved in IFN signaling

Hundreds of proteins are stimulated by IFNs. These proteins play multiple roles in IFN signaling and in IFN-mediated immune responses, which are notoriously antiviral. After sensing of the pathogen, the transcription of a large antiviral program is initiated (see Figure I-6). Hundreds of ISGs are therefore transcribed that play numerous and diverse roles in the protection of the cell against the infection. Here I will focus on those that pertain to the regulation of translation, after a brief introduction on the subject.

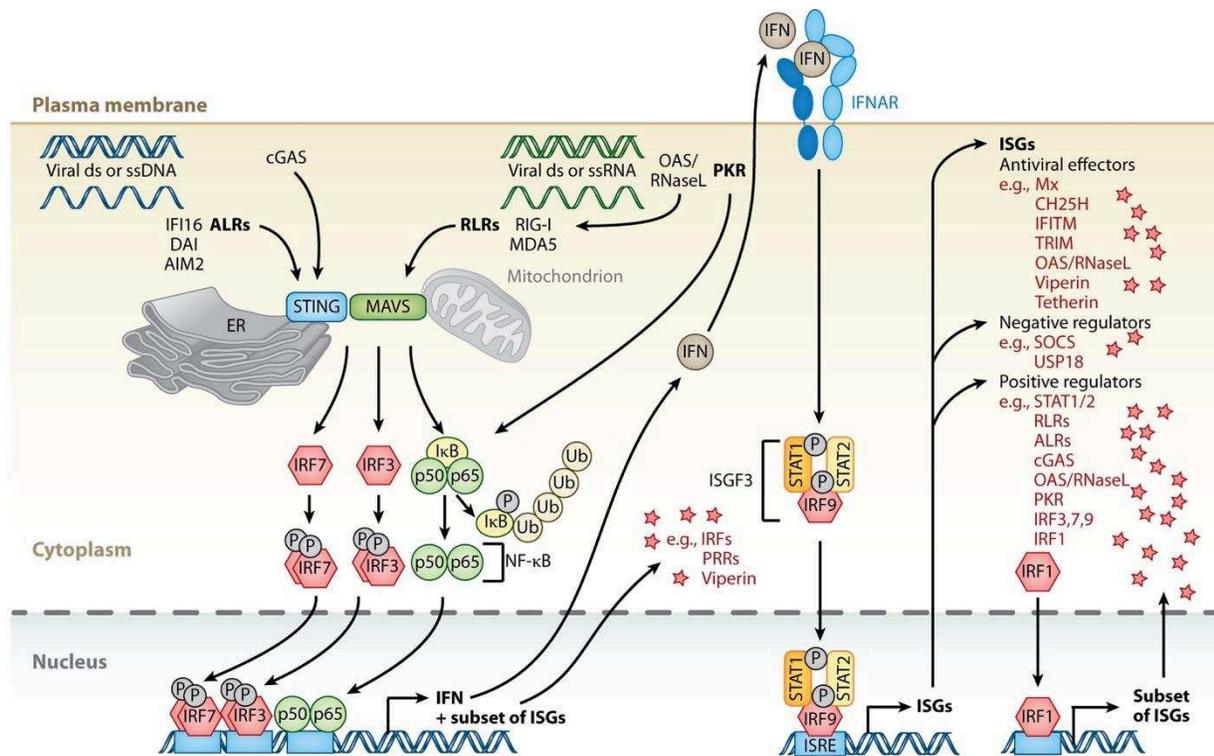


Figure I-4, Pathways leading to ISGs induction and the multiple functions of ISGs (Schneider et al, 2014).

PAMPs' recognition by cellular PRRs was briefly introduced above. ISGs are induced by IFN through JAK-STAT pathway. Some of ISGs are signal proteins such as PRRs and IRFs that act as positive regulators to amplify the IFN signaling, while some others act as negative regulators to suppress the IFN responses. Another group of ISGs act as antiviral effectors that directly inhibit viral replication, such as IFITMs and tetherin (Bailey et al, 2014; Neil et al, 2008).

Abbreviations: AIM2 (absent in melanoma 2); ALRs (AIM2-like receptors); PKR (Protein kinase R); I κ B (inhibitor of κ B); P (phosphorylation); Ub (Ubiquitin); Mx (myxovirus resistance); CH25H (Cholesterol-25-hydroxylase); IFITM (IFN-inducible transmembrane); TRIM (tripartite motif); SOCS (suppressor of cytokine signaling); USP18 (ubiquitin-specific peptidase 18).

I.B.2 Canonical cap-dependent and IRES-dependent translation initiation

Protein synthesis, or translation, can be schematically divided into three processes: initiation, elongation and termination. Interference with translational abilities of the pathogen is a well known inhibitory pathway of IFNs. Most reported regulation of translation by ISGs has been shown to occur at the initiation stage. Translation initiation can be initiated through several routes. In eukaryotic cells, the most commonly used mode of translation is through canonical cap-dependent initiation, which accounts for 95-97% of all translation initiation events within the cell. A second mechanism of translation occurs through internal ribosome entry site (IRES) mediated initiation (Komar and Hatzoglou, 2015). In this mechanism, ribosomes

associate to specific sequences present at the internal of the mRNA thereby bypassing the classical cap-dependent mechanism of translation.

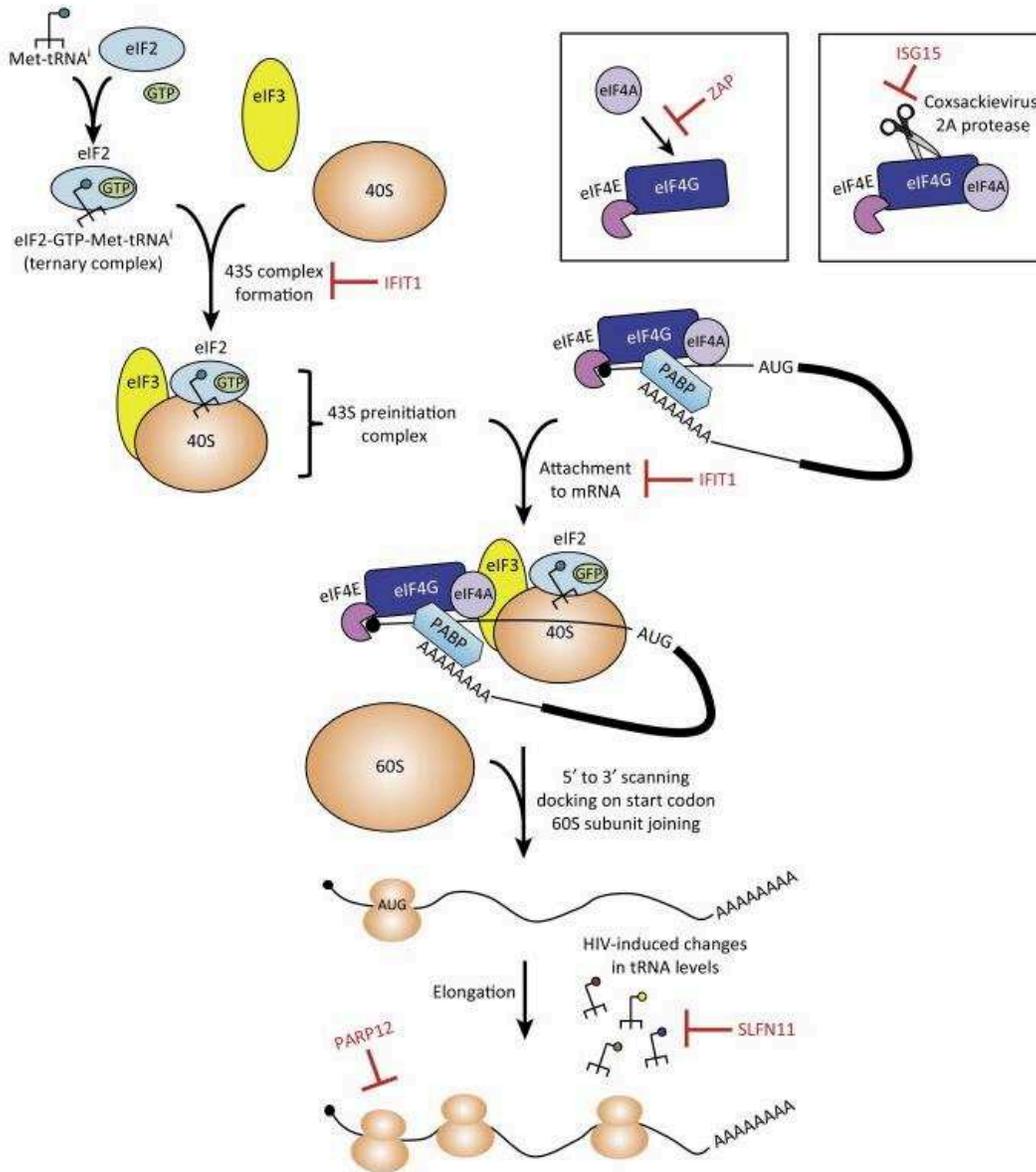


Figure I-7, Canonical cap-dependent translation initiation pathway and the regulators target different steps (Li et al, 2015).

The initiation of mRNA translation needs participation of tRNA, ribosomes and groups of initiation factors. Several important events occur during the translation process, such as (i) 43S initiation complex formation, (ii) eIF4F-mRNP complex activation, (iii) interaction of (i) and (ii), (iv) initiation and elongation. Numerous ISGs were identified to target these steps and regulate viral or host translation events.

Abbreviations: Met-tRNA (Methionine-transfer RNA); eIF (eukaryotic translation initiation factor); GTP (Guanosine-5'-triphosphate); IFIT1 (IFN induced protein with tetratricopeptide repeats 1); ZAP (zinc-

finger antiviral protein); *ISG15* (*IFN-stimulated gene 15*); *PABP* (*Poly(A)-binding protein*); *PARP12* (*Poly (ADP-Ribose) Polymerase 12*); *SLFN11* (*schlafen family member 11*).

Canonical cap-dependent translation initiation

As shown in figure I-7, canonical cap-dependent translation initiation can be divided into several steps (Jackson et al, 2011; Hinnebusch, 2014; Li et al, 2015). (i), Formation of 43S preinitiation complex (PIC) composed of a 40S ribosome subunit, the eIF2-GTP-Met-tRNA^{iMet} ternary complex (TC), eukaryotic initiation factor (eIF) 3, 1 and 1A (For 43S preinitiation complex, this picture only shows the minimal constituents without eIF1 and eIF5). (ii), Activation of the mRNP (messenger ribonucleoprotein), 5' m7G(5')ppp(5')N cap and 3' poly(A) structure of mRNA are recognized by eIF4E and poly(A)-binding protein (PABP), respectively. eIF4B and the ATP-dependent helicase eIF4A are also recruited at the cap-proximal region, where they unwind the region in an ATP-dependent manner. The scaffold protein eIF4G can binds to eIF4E, PABP, and eIF3, leading to mRNA circularization and 43S PIC interaction. (iii), scanning of mRNA and recognition of the start codon by 43S PIC, where the 48S PIC is formed and 60S ribosomal subunits is joined, accompanied with release of several initiation factors. (iv), Formation of the elongation-competent 80S initiation complex (IC) by the two ribosomal subunits. Upon termination of translation, the 80S complex disassembles and is recycled to start a new translation.

IRES-dependent translation initiation

Many viruses translate their own mRNA simply mimicking cellular mRNAs and therefore use cap-dependent translation. This is the case of viruses as diverse as HIV, influenza viruses and VSV. Other viruses lacking the cap structure at the 5'-end of their vRNA, and some cellular mRNAs such as cMyc, IGF1 involved in stress survival processes, have evolved an alternative IRES-dependent translation (Jackson, 2013). Unspliced HIV-1 gRNA was also shown to process an IRES-dependent translation during the G2/M cell cycle when cap-dependent translation is inhibited in this phase (Brasey et al, 2003). According to the RNA length, structure, initiation factors requirement and 40S ribosome assembly position, IRES-dependent initiations can be divided into four typical groups (see Figure I-8) (Picard-Jean et al, 2013).

(1) Viruses such as Cricket paralysis virus of the *Dicistroviridae* have the simplest IRES that directly recruits the 40S ribosomal subunit to the translation initiation site and initiate the translation with no need of initiation factors and methionyl-tRNA (Pisarev et al, 2005).

(2) The second group of IRES can also directly bind the 40S ribosomal subunit, but is longer than the first group. IRESes of this group require eIF2, eIF3 and methionyl-tRNA to initiate the translation, represented by viruses of *Flaviviridae* family such as Hepatitis C virus from the genus *Hepacivirus* (Niepmann, 2013).

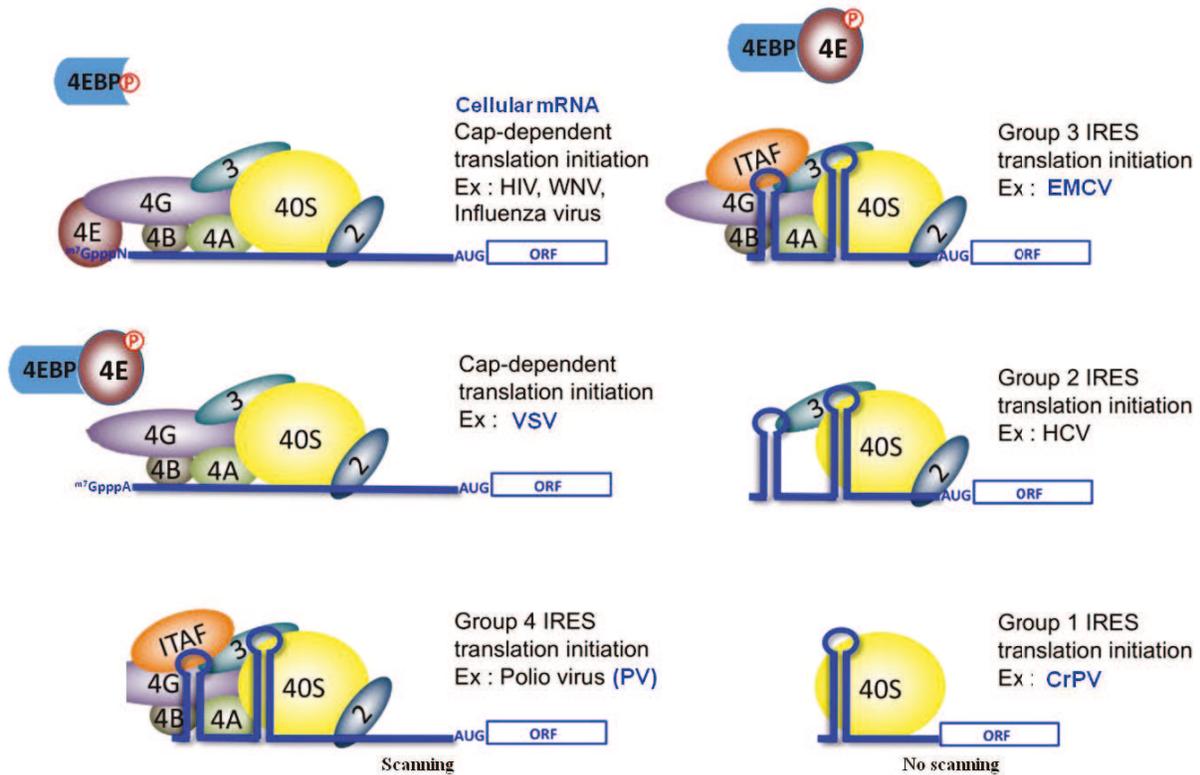


Figure I-8, Modes of translational initiation (Adapt from Picard-Jean et al, 2013).

eIF4E specifically recognizes cellular and viral mRNAs contain the cap structure and initiates the cap-dependent translation, while the conserved viral IRESes in 5' untranslated region (UTR) directly recruit ribosomes for translation initiation. According to the requirement of initiation factors and mRNA scanning, IRES-dependent initiations are divided into four types. eIF4E binding protein (4EBP) is normally phosphorylated and inactivated, some viruses such as EMCV and VSV can activate 4EBP and transfer the Pi to eIF4E, leading to its inactivation and dissociation from eIF4G binding. There is another well-known mode called VPg (viral protein genome-linked)-dependent translation initiation, which is quite similar to the cap-dependent initiation with the exception that it uses the viral-encoding VPg instead of cap structure for eIF4E binding.

Abbreviation: 4EBP (eIF4E binding protein); P (phosphorylation); HIV (human immunodeficiency virus); WNV (West Nile virus); VSV (Vesicular stomatitis virus); EMCV (Encephalomyocarditis virus); HCV (Hepatitis C virus) and CrPV (Cricket paralysis virus); IRES (internal ribosome entry site); ITAF (IRES trans-activating factors); ORF (Open reading frame).

(3) and (4) Both group 3 and group 4 IRESes are found in the *Picornaviridae* family viruses and display the highest length and complexity. These IRESes don't normally need eIF4E (except IRES of Hepatitis A virus), but require all the remaining initiation factors as well as additional proteins called IRES trans-activating factors (ITAF) to recruit the ribosome and initiate the translation (Ali et al, 2001; Costa-Mattioli et al, 2004). The assembly position of the 40S ribosome distinguishes the viruses of this family into group 3 (represented by *Aphthovirus* and *Cardiovirus*, e.g. Foot-and-mouth disease virus and EMCV) and group 4 (*Enterovirus* and *Hepatovirus*, e.g. poliovirus and Hepatitis A virus), which recruits the ribosome at the initiating codon or upstream from the ORF, respectively. The latter group requires scanning of the ribosomes on the mRNA to reach the initiating codon for translation initiation (Asnani et al, 2016).

Some viruses of the *Caliciviridae* (e.g. Norwalk virus, Sapporo virus) and *Potyviridae* (plant viruses) lack of the 5' cap structure, but still take over the entire cap-dependent translation initiation machinery, benefiting from their viral protein genome-linked (VPg) protein. These VPg specifically binds to 5'-end vRNA and serves as cap structure for eIF4E targeting and translation initiation (Goodfellow, 2011; Jiang and Laliberté, 2011). Members of the *Picornaviridae* family also encode VPg, however, their VPg-RNAs switch to use IRES-dependent translation initiation. Actually, the binding of VPg is important for viruses to prevent the degradation of vRNA by cellular exonucleases, as well as evade the detection of 5'-triphosphate vRNA by RIG-I (Goodfellow, 2011).

I.B.3 Translation regulation mediated by ISGs

PKR (protein kinase R) is the most well characterized ISG in the inhibition of viral translation by inactivating eIF2. PKR can sense dsRNA and lead to the phosphorylation of the α -subunit of initiation factor eIF2 (Balachandran et al, 2000). The eIF2 α phosphorylation-mediated translation suppression has been classically reported during infection with several viruses and may also linked to the formation of stress granules (SG) (Beckham and Parker, 2008; Ruggieri et al, 2012; Okonski and Samuel, 2013). PKR is also involved in enhanced induction of IFN β in some cell types (Diebold et al, 2003; McAllister et al, 2010).

However, many RNA viruses are known to selectively block host translation and hijack the translation machinery for their own purposes, as for instance VSV, EMCV, Poliovirus (PV) and Coxsackievirus (CV), circumventing the PKR-mediated block in translation. In the case of VSV infection, the eIF4E binding protein 1(4EBP1) was found to be activated and to lead to the recruitment of eIF4E and to its dissociation from the eIF4F complex, therefore inhibiting cap-dependent translation (Connor and Lyles, 2002; Richter and Sonenberg, 2005). Translation initiation of EMCV, PV and CV also bypasses eIF4E, while EMCV applies the similar pathway to lead the dephosphorylation of eIF4E by 4EBP1, PV and CV were shown to cleave eIF4G from the eIF4E binding site by viral protease. Bypass of eIF4E facilitates viral translation over the hosts (Balvay et al, 2009; Foeger et al, 2002).

Other ISGs were reported to regulate the translation initiation pathways or to counteract the viral-mediated shutoff of host translation. Here I will present the case of three proteins that seem to exert interesting roles in this mechanism: the zinc finger antiviral protein (ZAP), the interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and schlafen 11 (SLFN11).

ZAP disrupts the interaction between eIF4A and eIF4G

ZAP (also named PARP13) is an IFN induced protein that belongs to the poly (ADP-ribose) polymerase (PARP) family. Two isoforms of ZAP have been identified, termed ZAPL and ZAPS. Both isoforms display N-terminal zinc fingers that mediate RNA binding, and diverge at the C terminus that has been suggested to regulate the functions of ZAP isoforms (Kerns et al, 2008; Hayakawa et al, 2011). ZAP has been shown to restrict a range of viruses such as *Retroviruses*, *Alphaviruses*, *Filoviruses* and Hepatitis B virus (HBV) (Gao et al, 2002; Bick et al, 2003; Müller et al, 2007; Mao et al, 2013). The most described antiviral mechanism seems to involve both direct degradation of viral mRNA and/or a block in viral genome RNA translation (Zhu et al, 2011; Guo et al, 2007; Todorova et al, 2014). The translation suppression activity of ZAP was first demonstrated in Sindbis virus (Bick et al, 2003) and was later on determined to depend on the ability of ZAP to bind to eIF4A and prevent it from eIF4G binding, lead to the inhibition of translation initiation (Zhu et al, 2012) (Figure I-5).

Another ISG from the same PARP family, PARP12 (mPARP12L in mouse) was also shown to restrict *Alphaviruses* by translation suppression (Atasheva et al, 2014; Welsby et al, 2014). The mechanism is still understudied, however, mass spectrometry analysis on mPARP12L co-immunoprecipitated complex revealed the interaction of several ribosomal and translation-associated proteins with mPARP12L, suggesting that its translational suppression may be achieved by directly binding to ribosomes (Atasheva et al, 2014; Welsby et al, 2014).

ISG15 prevents the cleavage of eIF4G through binding to CVB3 2A protease and limiting its proteolysis activity

As I just mentioned, 2A protease of Coxsackievirus B3 (CVB3) was the first viral-encode protease that disassociate eIF4E from the initiation complex by cleaving its binding protein, eIF4G, thus selectively allowing its IRES-driven translation, while blocking host translation (Gradi et al, 1998). ISG15 is a 15kDa ubiquitin family protein which can modify virus-encoded proteins through the ISG15 conjugation system, with participation of some E1, E2 and E3 ligases (Morales and Lenschow, 2013). ISG15 was also reported to interfere with CVB3 2A protease and prevent the cleavage of eIF4G during viral infection (Figure I-5). The interference was also depending on ISG15 conjugates while the mutants deficient in conjugation site lost the interference activity (Rahnefeld et al, 2014).

IFIT1 specifically binds mRNA with cap-0 structure and prevents its interaction with initiation factors

5' structures of mRNA such as cap, 5'ppp, 5'pp and ribose-2'-O methylated cap are essential for the host to discriminate between self and non-self material. Cellular mRNAs bear a N7 methylated guanosine cap 0 structure linked to the 5' terminal. In higher eukaryotes and some viruses (ex. VSV), mRNA is further methylated at the 2'-O position of the first ribose to generate the cap 1 structure (graphical explanation in the VSV introduction part, Figure II-3) (Hyde and Diamond, 2015). IFIT1 (also named ISG56 or p56) can

outcompete eIF4E for binding of viral capped mRNAs lacking a 2'-O methylation and thereby selectively inhibiting virus translation (Züst et al, 2011; Daffis et al, 2010; Habjan et al, 2013). IFIT1 also process a lower binding affinity to the viral 5'ppp mRNA and inhibits translation of these viruses (Pichlmair et al, 2011; Kimura et al, 2013). Interestingly, tRNAs also competitively bind to IFIT1, suggesting that rather than sequestering viral mRNA, IFIT1 may also reduce viral protein production by sequestering tRNAs from the translating pool (Kumar et al, 2014). This hypothesis must be taken with precaution however, because the stoichiometry of IFIT1 and tRNAs within the cell would clearly disfavor a competitive model in which IFIT1 sequesters all tRNA available. Prior to these studies, IFIT1 was known as an eIF3-associated protein that inhibited both cap- and HCV IRES-dependent translation through destabilization of the 43S complex and eIF3 itself (Guo et al, 2000; Hui et al, 2003; Wang et al, 2003). However, this mechanism has not been fully confirmed, since the IRES of EMCV also need eIF3 to initiate the translation, but does not appear to be targeted by IFIT1 (Hui et al, 2003). (Figure I-7).

Viruses have also evolved strategies to evade or counteract the antiviral mechanisms mediated by IFIT1 (reviewed by Hyde and Diamond, 2015). Introduction of mutations on the 2'-O-methyltransferase (2'-O-MTases) leads to viruses such as severe acute respiratory syndrome coronavirus (SARS-CoV) that becomes susceptible to IFIT1, suggesting that generating the cap 1 structure is essential for viruses to evade IFIT1-mediated restriction (Menachery et al, 2014). *Alphaviruses* using secondary structural motifs in their 5' UTRs to prevent its 2'-O methylation therefore lacking the cap structure required for IFIT1 recognition (Hyde et al, 2014). Some other viruses employ additional strategies such as snatch cap structure from cellular mRNA, or process a cap-independent translation to escape this inhibition.

SLFN11 selectively inhibits viral mRNA translation in HIV-infected cells in a codon-usage-dependent manner

The mechanism of translation inhibition is in this case peculiar and so far controversial (Li et al, 2012a; van Weringh, 2011; Finer-Moore et al, 2015). SLFN11 binds tRNA in a codon-usage-dependent manner and is thought to inhibit HIV-1 by modulating the pool of tRNAs within the cell (see Figure I-7). This mechanism of differential inhibition between virus and cell must therefore rely on a distinct codon usage between virus and cell mRNAs (Li et al, 2012a). Codon usage bias has been reported in the case of HIV-1, but its significance has not been truly examined.

II. Vesicular Stomatitis and Vesicular Stomatitis Virus

In 1901 Theiler reported a stomatitis disease in horses and mules in Transvaal, South Africa, and referred to a prior outbreak in 1884. In affected animals, the first signs were increased temperature and decreased appetite, followed by marked salivation, formation and then rupture of vesicles on the gums, tongue and lips, leaving broad ulcerations. This disease was later on characterized by Oltsky (1926) and Cotton (1927) as vesicular stomatitis (VS). Outbreaks in cattle of South Africa have been described since 1897 (Bekker et al, 1934; Mason et al, 1940; Wheeler et al, 1945). In cattle and pigs the symptoms are clinically similar to foot and mouth disease (FMD) (Hanson, 1952; Rodriguez, 2002), first described in 1904 by Mohler JR. However, despite the fact that the lesions of the mouth, muzzle, udder, and feet are indistinguishable between VS and FMD, the former disease is followed by a rapid recovery and rarely leads to the death of the animals (Hanson, 1952). The etiologic agent of VS, VSV was finally isolated from the tongue epithelium of a cattle in Richmond, Indiana in 1925 (Cotton, 1926). VSV belongs to the family of *Rhabdoviridae*, genus *Vesiculovirus*, is an insects-borne virus that primarily affects rodents, cattle, mule, pig and horses. Natural infection in sheep and goats is rare, although both species can be infected experimentally. Many other species of laboratory animals are also susceptible as the virus is zoonotic. In humans, VSV infection leads to light influenza-like symptoms, normally in the absence of vesicles (Tesh et al, 1987, Krauss et al, 2003). The disease is limited to the Americas; however, it was sporadically reported in France, South Africa and in Asia (Hanson, 1952; Krauss et al, 2003).

II.A Vesicular stomatitis (VS)

II.A.1 Agents

Vesicular stomatitis is mainly caused by the Vesicular Stomatitis virus (VSV), a member of the genus *Vesiculovirus*. At least 28 Vesiculoviruses infect vertebrates and invertebrates (Wunner et al, 1995). The two serotypes of VSV: New Jersey (VSNJV) and Indiana (VSIV), contribute the main clinical cases (Hanson, 1968). Another two serotypes of VSV, Cocal and Alagoas, as well as some other vesiculoviruses include Piry, Isfahan and Chandipura viruses, have also been identified to infect humans and domestic animals. The summary of them is shown as following in table 1 (Letchworth et al, 1999).

II.A.2 Host range and clinical signs

VSV is an insect-borne virus that has an extremely broad host range and that is naturally transmitted by insects to swine, rodents, cattle, mule and horse. Natural disease in other species is rare, although many of them can be infected experimentally, such as sheep, goat, guinea pig, hamster, ferret, mouse, and chicken (Hanson, 1981; Carbrey, 1982; Fultz and Holland, 1985; Huneycutt et al, 1994).

Common name	Serotype	Host	Isolate place, year	Reference
Indiana	VSIV-1	Bovine	Indiana, USA, 1925	Cotton, 1926
New Jersey	VSNJV-1	Bovine, Equine	New Jersey, USA, 1926	Cotton, 1927
Cocal	VSIV-2	Insect, Rodents	Trinidad, Brazil, 1964	Jonkers et al, 1964
Alagoas	VSIV-3	Equine, Bovine, Human	Brazil, 1964	Federer et al, 1967
Chandipura	Chandipura	Human	India, 1965	Bhatt and Rodrigues, 1967
Piry	Piry	Opossum	Brazil, 1973	Theiler and Downs, 1973
Isfahan	Isfahan	Human	Iran, 1975	Tesh et al, 1977

Table I-1, First isolation of vesiculoviruses from infected human and animals (adapted from Letchworth et al, 1999).

Infection in livestock represents a major economic concern. Although the disease does not generally cause animals death, the clinical signs of vesicular stomatitis are very similar to those of FMD in cattle and pigs and those of swine vesicular disease in swine and the only manner to distinguish them is through laboratory tests.

In affected livestock, generally, after a 2 to 8 days' incubation period, excessive salivation comes as the first sign, with raised body temperature and lower appetite. This is followed by an expansion of vesicles or blister-like lesions on the lips, gums, palate and tongue, as well as other organs such as coronary band, nostrils, prepuce, vulva, and udder. The vesicles later on undergo rupture, leaving broad ulcerations that can interfere with normal feeding and/or milk production. While this occurs in adult animals, infection of young animals in endemic areas commonly occurs in the absence of clinical symptoms, perhaps because of protection from maternal antibodies (Vanleeuwen et al, 1995).

In contrast to the protection seemingly provided by maternal antibodies to newborn animals, young and adult horses appear equally susceptible to infection with VSV (Burton, 1917; Webb et al, 1987). Natural lesions in horse normally appear 1-3 days after viral exposure, on the tongue, lips, palate, gums and around nostrils, the blanched and macules lesions are first developed and then quickly formed into vesicles (Burton, 1917; Mohler, 1940; Knight and Messer, 1983; Thorne al, 1983; Carver, 1984). Vesicles also found on the coronary band, mammary gland and prepuce (Acree, 1964; Carver, 1984), sometimes on the ears and under surface of the belly as well (Heiny, 1945). Affected horses are debilitated and present a transient fever until vesicles rupture (Webb et al, 1987). Oral erosions can be healed in 3 to 21 days, whereas udder and foot lesions require more time (Knight and Messer, 1983). Experimental infection of Cocal, Alagoas or Piry viruses lead to more moderate lesions than VSIV or VSNJV, that fail to yield spreading of the infection between horses, in contrast to Isfahan and Chandipura viruses (Yedloutsching and Dardiri, 1977; Wilks and House, 1986).

Infected pigs usually first show signs of lameness caused by foot lesions (USDA-APHIS, 2012) and high mortality rates in pigs affected by the NJ virus have been observed. Swine infection leads to the development of vesicles on the animal lips, snout, coronary bands and interdigital space of the foot (Chow and McNutt, 1953; Patterson et al, 1955) and animals recover from infection in about 2 weeks.

Human can also be infected by VSV-IN, VSNJV, Alagoas, Chandipura and Piry viruses. Infection leads to conjunctivitis followed by influenza-like symptoms characterized by fever, chills, nausea, vomiting, headache, muscle aches, pharyngitis and lymphadenitis (Hanson et al, 1950; Patterson et al, 1958; Johnson et al, 1966; Fields and Hawkins, 1967; Reif et al, 1987). Encephalitis is rarely found in infected human adults, but is more common in children (Rodrigues et al, 1983; Jones, 1987). However, as reported for cattle some serotypes display higher pathogenicity, the Chandipura serotype has been associated to epidemic acute encephalitis in India and a high fatality rate during the outbreaks of 2003 and 2004 (Rao et al, 2004; Chadha et al, 2005). More than 75% fatality rate was reported during the outbreak of 2004 in the eastern state of Gujarat of India. This evidence indicated possible emergence of Chandipura virus as a deadly human pathogen in Indian Subcontinent (Basak, 2007). Inoculation of young laboratory or wild mice with VSV usually also results in viremia that precedes death from encephalitis (Bruno-Lobo et al, 1968a&b; Cornish et al, 2001).

II.A.3 Transmission

How the vesicular stomatitis virus spreads is not fully known, insect vectors, mechanical transmission, and movement of animals are all possible factors (USDA-APHIS, 2012). The viruses have been isolated from hematophagous insects such as sandflies (*Diptera: Psychodidae*), black flies (*Diptera: Simuliidae*), mosquitoes (*Diptera: Culicidae*) and culicoides (*Diptera: Ceratopogonidae*) (Tesh et al, 1969; 1987; Sudia et al, 1967), as well as from non-hematophagous insects, such as house flies and eye gnats (Walton et al, 1987). There was a long-standing hypothesis that VSV could be transmitted by biting insects to livestock (Heiny, 1945). However, scientific data to support this hypothesis is currently lacking, although experimental transmission of VSNJV has been reported from black flies (*Simulium vittatum*) to domestic swine and cattle (Mead et al, 2004a, b; 2009), or from biting midges (*Culicoides sonorensis*) to cattle (Perez de Leon and Tabachnick, 2006).

Once the disease takes place in a herd, infection may then spread from animal to animal or to human by contact or exposure to saliva or fluid from ruptured vesicles (Sellers and Maarouf, 1990; Patterson et al, 1955; Hanson, 1981).

II.B Vesicular stomatitis virus (VSV)

II.B.1 Classification and serotypes

Vesicular stomatitis virus (VSV) belonging to the family *Rhabdoviridae*, genus *Vesiculovirus*. The *Rhabdoviridae* family includes a wide variety of bullet-shaped nonsegmented negative strand (NNS) RNA viruses with a large host tropism (vertebrates, invertebrates, and plants) (Lyles et al, 2007). In addition to VSV, another well-known representative virus of this family is the rabies virus (genus *lyssavirus*) that cause

an acute and lethal infection in humans in the absence of prompt treatment. The *Rhabdoviridae* family belongs to the *Mononegavirales* order consisting of a large and diverse group of NNS RNA viruses like Measles and Nipah virus (*Paramyxoviridae*), Ebola virus (*Filoviridae*) and Borna disease virus (*Bornaviridae*). Despite the various morphological and biological characteristics, these NNS RNA viruses share similar genome structures and multifunctional RNA-dependent RNA polymerase (RdRp) large (L) proteins (Baltimore, 1970; Poch, 1990; Briese, 1994; Volchkov, 1999) and therefore share common strategies for genome transcription and replication. As VSV possesses highest *in vitro* RdRp activity among known NNS RNA viruses and mildly pathogenic to humans, extensive studies on NNS viral mRNA biogenesis have used VSV as model.

There are two distinct serological serotypes of VSV: New Jersey (NJ, VSNJV) and Indiana (IND, VSIV). Based on serological relationships, two additional subtypes of the Indiana serotype (Cocal-like VSIV-2 and Alagoas-like VSIV-3) were added (Federer, 1967, Allende and Germano, 1993). VSNJV and classical VSIV (VSIV-1) are endemic in livestock in areas of northern South America, Central America and southern Mexico, where VSNJV contributes over 80% of the clinical cases and the VSIVs for the remaining (Hanson, 1968). Sporadic outbreaks have been reported in Bolivia, northern Mexico and western United States. In the rest of South America, VSV has only been reported in Brazil and Argentina, where VSIV-2 has only been isolated from horses (Rancharia-Brazil/66, Riberao-Brazil/79, Salto-Argentina/63, Maipú-Argentina/86) (Alonso et al. 1985; 1991). While the VSIV-3 subtype has been identified only in Brazil from horse (Alagoas-Brazil/64) and cattle (Espinosa-Brazil/77 strain) (Alonso et al, 1985; 1991).

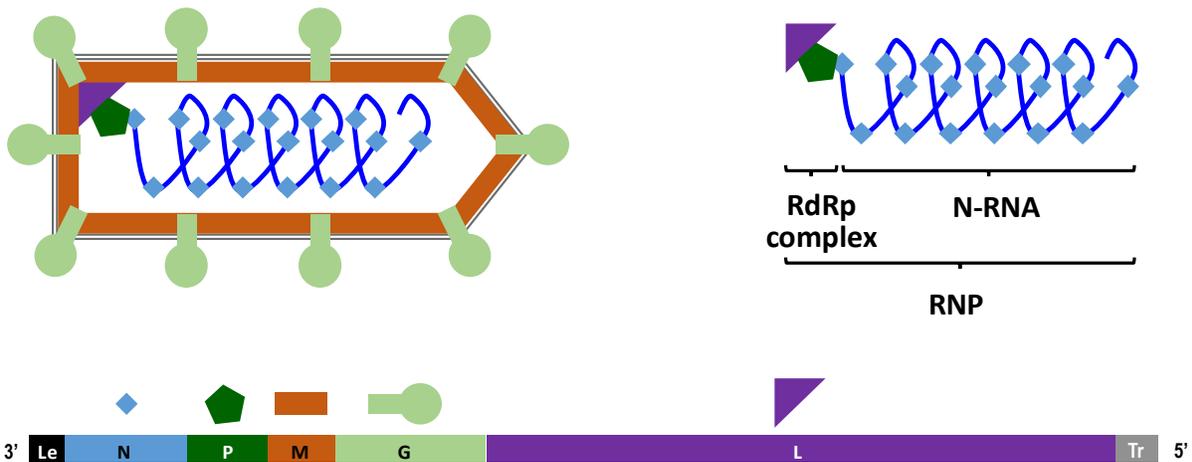


Figure II-1. Genome composition and virion structure of VSV.

VSV is a bullet-shaped, enveloped RNA virus with a linear, negative-sense ssRNA. The genomic RNA consisting of a leader (Le) sequence followed by genes encoding structural proteins N, P, M, G, L, and ended with a trailer (Tr) sequence. The viral RNA is completely coated by the N protein to form a helical nucleocapsid (N-RNA). Phosphoprotein (P) bridges N-RNA and the RdRp large L proteins to form the ribonucleoprotein (RNP). The RNP is enveloped in a phospholipid bilayer, coated with the membrane-associated M and G protein to assemble into a bullet shaped viral particle.

Abbreviation: Le (leader); Tr (trailer); N (nucleocapsid protein); P (Phosphoprotein); M (matrix protein); G (glycoprotein); L (polymerase); RdRp (RNA-dependent RNA polymerase); RNP (ribonucleoprotein).

II.B.2 Genome and virion structure

As shown in Figure II-1, VSV is a bullet-shaped, enveloped virus, approximately 70 nm in diameter and 170 nm in length (Acha and Szyfres, 2003; Cureton, 2010). Among VSV subtypes, VSIV-1 is widely used as a laboratory model of RNA virus because it easily replicates to high titers in various culture cells and laboratory animals (Rodriguez et al, 2002). The genome of the virus is a linear, single stranded, negative-sense RNA that consists of a 3' 47 nucleotide leader (Le) promoter sequence followed by the 5 structural genes and a 59 nucleotide trailer (Tr) at the 5' terminus. The molecule size of genome RNA is approximately 11 kb, within 70 nucleotides are not transcribed. Including 2-nt at each of the four gene junctions, 3-nt at the Le-N junction and the 59-nt trailer (Tr) sequence (Gallione et al, 1981).

The five structural genes code the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L). Two additional small proteins (C and C') are encoded in a second open reading frame within the P gene (Spiropoulou and Nichol, 1993). The five viral proteins are assembled into a bullet shaped virus particle that is enveloped in a phospholipid bilayer and coated with the protruding transmembrane glycoprotein (Lichty, 2004).

The viral RNA is completely coated by the viral nucleoprotein (N) to form a helical nucleocapsid (N-RNA). Phosphoprotein (P) bridges interactions between N-RNA and the RdRp large L proteins (Green and Luo, 2009) to form the ribonucleoprotein (RNP). The RNP is enveloped in a phospholipid bilayer, coated with the membrane-associated matrix protein (M) and glycoprotein (G).

II.B.3 Life cycle in cells

VSV can replicate in a wide variety of culture cell types, including nearly all mammalian cells as well as insect cells (Mudd et al, 1973), yeast (Makarow et al, 1986) and nematodes (Wilkins et al, 2005; Schott et al, 2005). The remarkably large host range is due to the virus surface glycoprotein, VSV-G, which for this reason is widely used for pseudotyping other viruses and viral vectors (Lichty, 2004; Naldini et al, 1996).

The life cycle of VSV is entirely cytoplasmic. VSV entry begins with the attachment of the virion to the cell surface. Early studies excluded membrane proteins as VSV receptors, because proteolytic digestion of the cell surface proteins did not affect VSV binding (Schloemer and Wagner, 1975). The receptor was thought to be the lipid component of plasma membrane such as phosphatidylserine and phosphatidylinositol (Schlegel et al, 1982&1983; Mastromarino et al, 1987). However, recent work refutes those results but suggests that it is in fact initiated by recognition of protein receptors (Coil and Miller, 2004; Bloor et al, 2010; Regan and Whittaker, 2013). Finkelshtein et al proved that VSIV G protein mediates viral attachment through interaction with the low-density lipoprotein receptor and its family members (Finkelshtein et al, 2013).

Viral attachment to the cell leads to viral particles internalization through clathrin-dependent endocytosis (Sun et al, 2005; Johannsdottir et al, 2009; Cureton et al, 2009&2010). The endocytic pathway transfers the particles to the early endosomes (EEs). The acidic pH (≤ 6.3) triggers conformational rearrangements in G proteins, which in turn drive fusion between the viral and cellular membranes, leading to the cytoplasmic release of the viral RNP (White et al, 1981; Baquero et al, 2015). In the cell cytoplasm, M proteins are released from the RNP to facilitate mRNA synthesis (Rigaut et al, 1991; Mire et al, 2010). The site(s) within

the cytoplasm for VSV RNA synthesis remain uncertain. The transcription and replication of Rabies virus take place within the inclusion-like structures termed Negri bodies (Lahaye et al, 2009; Menager et al, 2009). Similar cytoplasmic inclusions have also been reported in cells infected with VSV. Indeed, Heinrich et al showed that although VSV RNA synthesis occurs throughout the cytoplasm, the viral RNA synthesis machinery is redirected to inclusions following protein synthesis (Heinrich et al, 2010).

In infected cells the polymerase complex, consisting of L and P, drives the formation of two types of RNA products synthesized from the genome RNA. One is a full-length positive copy that serves as an intermediate for genome replication, while the other serves for viral protein production through the generation of five unique viral mRNAs in addition to a short uncapped, non-polyadenylated 47-nucleotide leader RNA (Le+) (Ball and Wertz, 1976; Colonno and Banerjee, 1976&1978; Testa et al, 1980; Iverson and Rose, 1981; Emerson, 1982; Morin et al, 2013).

Transcription of viral protein coding mRNAs is regulated from a single promoter at the 3' end of the genome, in a pause and reinitiation mode that is regulated by specific 23 nucleotide sequences located at each gene junction. These conserved sequences contain the transcription initiation and polyadenylation signals (Rodriguez et al, 2002). This mode of production results in a gradient of protein expression from N towards L in which approximately 30% of each transcript is lost in the order: 3' Le > N > P > M > G > L 5' (Ball and Wertz, 1976; Iverson and Rose, 1981).

Translation of the viral mRNA is of course essential to support viral genome replication. At a later stage of infection, the polymerase switches translation to replication, which together with translated products, yield nucleocapsids containing the full-length sense antigenome RNA. The antigenome RNAs provide templates for synthesis of the antisense genome RNA, both the nascent antigenome and the genome RNAs are bound by the N protein and the newly synthesized genomic RNPs then serve either as templates for secondary transcription and/or replication. The viral genome and proteins are then recruited at the plasma membrane where new viral particles are assembled and released.

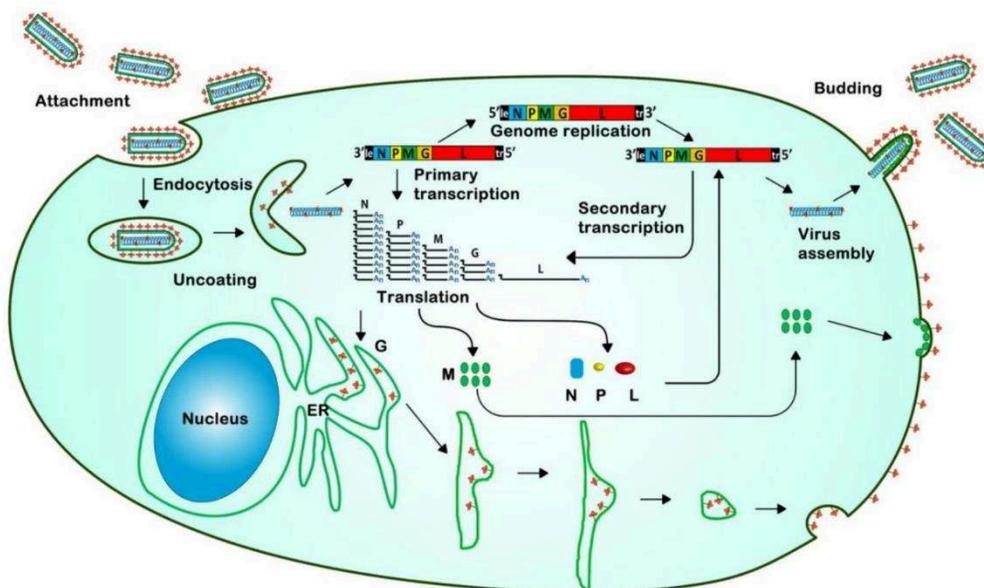


Figure II-2, VSV life cycle in cell (Li and Zhang, 2012).

The life cycle of VSV is entirely cytoplasmic. VSV entry cells through endocytosis, leading to the release of the viral RNP (ribonucleoprotein) into cytoplasm. In infected cells the RdRp (RNA-dependent RNA polymerase) complex drives the formation of two types of RNA products synthesized from the genome RNA. One is a full-length positive copy that serves as an intermediate for genome replication, while the other serves for viral protein production by transcribing to five unique viral mRNAs. The newly synthesized genomic RNPs then serve either as templates for secondary transcription and/or replication. Transcription of viral protein coding mRNAs results in a gradient of protein expression from N towards L. The synthesised viral genomes and proteins are then recruited at the plasma membrane where new viral particles are assembled and released.

Abbreviation: ER (endoplasmic reticulum); N (nucleocapsid protein); P (Phosphoprotein); M (matrix protein); G (glycoprotein); L (polymerase).

II.B. 5 Strategies of VSV mRNA synthesis and translation

The multifunctional viral RdRp L protein, which binds the N-RNA template indirectly via the bridging cofactor P protein (Green and Luo, 2009), catalyzes all enzymatic reactions required for viral mRNA synthesis and processing by its RdRp and AdoMet-dependent MTase domains (Sleat and Banerjee, 1993; Li et al, 2005a; Ogino and Banerjee, 2007; Galloway and Wertz, 2008; Ogino et al, 2010).

The formation of the 5'-terminal cap structure is one of the major RNA processing events that occurs during mRNA biogenesis and is essential for mRNA stability and translation (Furuichi and Shatkin, 2000; Cougot et al, 2004). VSV process an unconventional mRNA capping reactions different from eukaryote (Ogino and Banerjee, 2011). As shown in Figure II-3, the multifunctional L protein catalyzes a series of enzymatic reactions to form the cap structure of the VSV mRNAs. First, the γ -phosphate of GTP is hydrolyzed by the guanosine 5'-triphosphatase (GTPase) activity of the L protein (Ogino and Banerjee, 2007, 2008). Then, the 5'-monophosphorylated RNA (pRNA) is transferred from 5'-triphosphorylated RNA (pppRNA) to GDP by the RNA:GDP polyribonucleotidyltransferase (PRNTase) activity to form the GpppRNA structure through the (L)-pRNA intermediate (Ogino and Banerjee, 2007; Ogino et al, 2010). VSV-associated MTases methylate the cap core structure at the ribose-2'-OH position followed by the guanine-N7 position to generate GpppAm- and finally m7GpppAm- (Testa and Banerjee, 1977).

The L polymerase initiates mRNA synthesis at the first gene start and the cap structure is added co-transcriptionally (Whelan and Wertz, 2002; Whelan et al, 2004). After transcription of a viral gene, the *cis*-acting signal at the junction of two genes helps the polymerase to terminate synthesis and generate the 3' polyadenylate tail by reiterative copying of a U tract (Barr and Wertz, 2001). The termination of an upstream gene is critical for reinitiation of the following one. The sequential order of transcription resulting in a decreasing amount of each transcript in the order: 3' Le > N > P > M > G > L 5' (Ball and Wertz, 1976; Iverson and Rose, 1981).

While transcription requires only virally coded components, how VSV diverts most of the host translation machinery towards viral protein synthesis remains unclear. Many RNA viruses can selectively block host translation and hijacking the translation machinery for their own purposes and similar documentation exists for VSV (Lyles, 2000; Gale et al, 2000; Bushell and Sarnow, 2002; Schneider and Mohr, 2003; Walsh and Mohr, 2011). This mechanism is interesting as it supports the rapid production of viral proteins, while

suppressing potential host antiviral factors and host immune responses. However, since most mRNA viral transcripts undergo through the same modification and similar structural formation as cellular mRNAs, the underlying mechanism of translation inhibition must be selective.

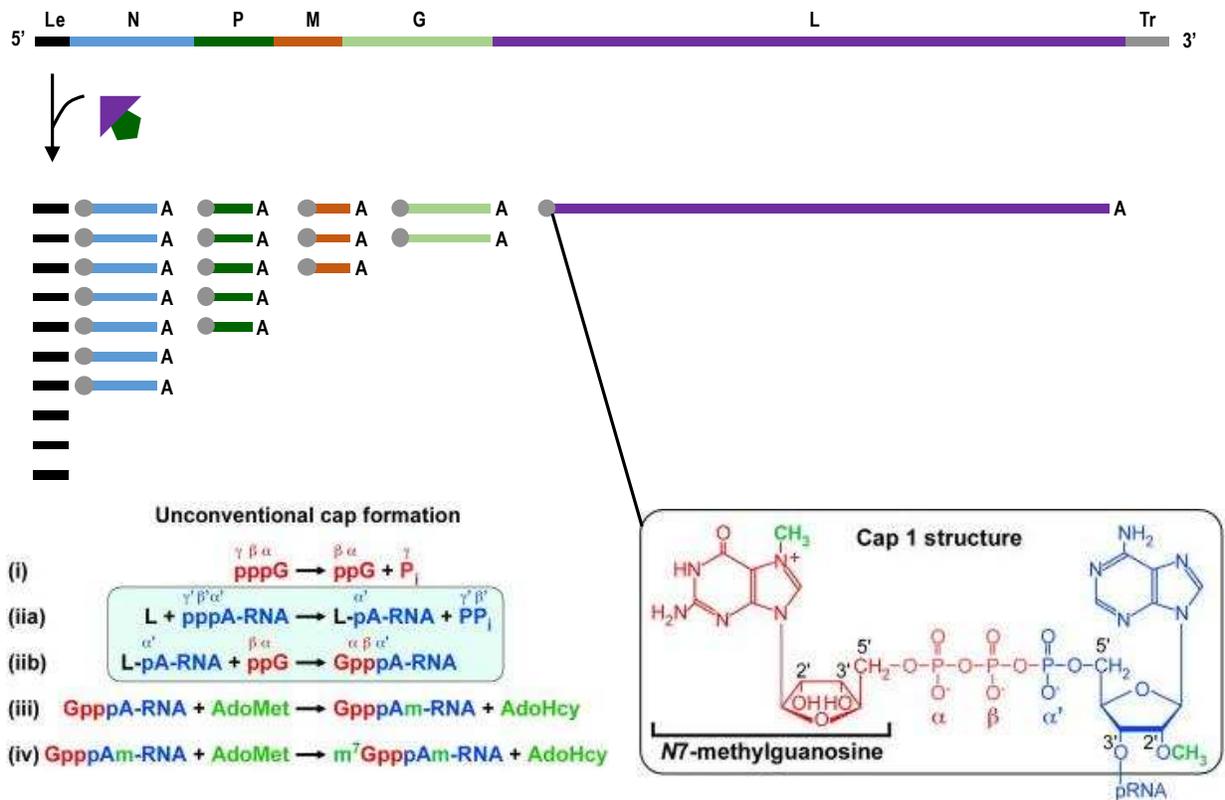


Figure II-3. VSV mRNAs synthesis and 5' cap structure formation (Adopt from Ogino, 2014).

The RdRp (RNA-dependent RNA polymerase) complex catalyzes transcription of viral protein encoding mRNAs and results in a gradient of mRNA production. The formation of cap 1 structure of VSV is detailed in the text.

Abbreviation: m^7G (N^7 -methylguanosine); pi (phosphate); pRNA (monophosphorylated RNA); AdoMet (S -Adenosyl methionine); AdoHcy (S -Adenosyl- L -homocysteine).

In fact, during VSV infection, both host and viral translation are apparently inhibited, as the global inhibition of translation also blocks viral protein synthesis (Williams, 1999). There are two phase of translation inhibition, the first is a rapid inhibition of host translation early after infection, followed by a later inhibition of viral translation (Lyles, 2000; Connor and Lyles, 2005).

Early studies showed that in VSV-infected cells eIF2 was inactivated, as protein translation was rescued *in vitro* by adding eIF2 (Centrella and Lucas-Lenard, 1982; Dratewka-Kos et al, 1984). Later studies suggested

that eIF2 α phosphorylation by the double-stranded RNA-activated kinase PKR was responsible for the selective inhibition of host translation (Williams, 1999; Balachandran et al, 2000). eIF2 α is an initiation factor within the multiprotein complex eIF2. The complex functions in the recruitment of the initiator tRNA_{MET} to the 40S ribosome subunit in a GTP-dependent manner. Phosphorylation of eIF2 α inhibits this activity therefore blocking the overall function of the complex (Hinnebusch, 2014). However, Connor and Lyles showed that phosphorylation of eIF2 α occurs after and not prior to host protein synthesis inhibition. In addition, induction of eIF2 α phosphorylation has been shown to block viral protein synthesis suggesting that eIF2 α phosphorylation is not a viable mechanism with which specific translation inhibition can be achieved in the case of VSV (Connor and Lyles, 2005).

Another initiation factor complex is eIF4F found be altered during VSV infection (Connor and Lyles, 2002). The eIF4F complex contains the cap-binding protein eIF4E, the scaffolding protein eIF4G and the eIF4A helicase. This complex is responsible for binding to the cap structure of mRNAs and delivery them to the 40S ribosome (Jackson et al, 2010). The alteration of the eIF4F complex driven by the dephosphorylation of the eIF4E subunit, has been shown to be responsible for the inhibition of host translation (Connor and Lyles, 2002; Richter and Sonenberg, 2005). eIF4E dephosphorylation has been associated to the activation of the eIF4E binding protein 1 (eIF4E-BP1), which dissociates eIF4E from the eIF4F complex, thereby inhibiting translation.

VSV infection also diminishes the pool of ribosomal RNA by inhibiting the cleavage of the 45S precursor rRNA to 28S and 18S ribosomal RNA (Zan et al, 1990). The VSV matrix protein (M) is the main viral determinant of this effect and seems to do this according to two pathways. The first one occurs by directly inhibiting ribosomal RNA transcription, while the second occurs by blocking the Rae1 mRNP export pathway, thus trapping the ribosomal RNA into the nucleus (Ahmed and Lyles, 1998; Faria et al, 2005).

However, despite the existence of these studies, it remains unclear how VSV impairs host translation. A recent study suggests that ribosomal proteins may facilitate VSV mRNAs translation during host translational shutoff. Indeed, Lee et al found that VSV translation requires specifically a 60S ribosomal protein, rpL40. Depletion of this protein didn't abolish processing of mature ribosomal RNAs or cellular translation, but specially decreased replication of VSV and other nonsegmented negative strand (NNS) viruses including rabies virus, measles virus, and Newcastle disease virus. This suggests that the cells may have an alternative rpL40-dependent translation mechanism which is employed by negative-sense RNA viruses for transcript-specific initiation (Lee et al, 2013).

II.C Cellular antiviral responses and VSV counteracting measures

II.C.1 Cellular innate immunity and antiviral factors against VSV

Pathogen infection awakes host innate immune responses by PRRs that specifically recognize PAMPs. The detection of pathogen ligands is followed by the engagement of signaling adaptor molecules and leads to the activation of transcription factors such as NF- κ B, MAP kinases and the interferon-regulatory factors (IRFs). These regulators control the transcription of genes encoding type I interferon and other inflammatory

cytokines. IFN-alpha and the IFN-beta subspecies activate a common type I IFN receptor (IFNAR) that sends a signal to the nucleus through the JAK-STAT pathway, which in turn activates the transcription of hundred ISRE-driven ISGs, which play antiviral activities in various ways.

During infection, VSV RNA is mainly sensed by RIG-I, although TLR7 was also found to recognize the VSV RNA in plasmacytoid dendritic cells (pDCs) and mouse B cells (Lund et al, 2004). Another receptor, TLR3 that is localized in endosomes has also been suggested to sense VSV infection, although its contribution is less clear since *TLR3*^{-/-} mice can fully control VSV infection (Edelmann et al, 2004).

IFN production is tightly regulated by host immune system, as the excessive production of IFN is also harmful to the host. Modifications of the transcription factors such as IRF3 and IRF7 are crucial mechanisms to balance the Type I IFN production. VSV infection has been reported to impair Type I IFN gene expression by triggering small ubiquitin modifier (SUMO)-ylation of IRF3 and IRF7. Accordingly, viral infection triggers higher level of IFN mRNA induction in cells express SUMOylation deficient IRF3 and IRF7 (Kubota et al, 2008).

Many ISGs serves as antiviral restriction factors and a number of them is implicated in both intrinsic and innate immune activities, which act cooperatively to inhibit particular stages of viral replication (Figure II-4). During the life cycle of VSV, the cellular factors IFITM3 and Tetherin proteins have been shown to prevent viral entry and release, respectively (Weidner et al, 2010). Additionally, Cholesterol 25-hydroxylase (CH25H) that converts cholesterol to a soluble antiviral factor, 25-hydroxycholesterol (25HC) has also been shown to inhibit VSV entry by blocking the fusion between viral and cellular membranes (Liu et al, 2013). Additional factors have been described to play an antiviral role against VSV infection among which: MxA (Pavlovic et al, 1990; Staeheli and Pavlovic, 1991) and PML (Chelbi-Alix et al, 1998; El Asmi et al, 2014) that inhibit primary and secondary transcription, respectively; PKR (Stojdl et al, 2000) and IFITs (Schmeisser et al, 2010; Fensterl et al, 2012) that inhibit viral translation, although the overexpression of PKR and the 2'-5' oligoadenylate synthetases have not been reported to alter VSV infection (Krishnamoorthy et al, 2008).

Some other ISGs act indirectly on anti-VSV. Such as ADAR1 was suggested to suppress activation of PKR and inhibition of VSV growth in response to IFN treatment (Li et al, 2010). Some TRIM proteins include PML (TRIM19) also recognized as important immune signaling mediators (El Asmi et al, 2014; Panda et al, 2011). ISG20 was reported to impair the VSV mRNA and protein accumulation by degrade viral RNA (Espert et al, 2003), I will detail and fully introduce this study in the following ISG20 introduction part.

II.C.2 Simple but efficient contract activity of VSV: shutdown of host gene expression by Matrix protein

As mentioned above, VSV is able to drive a fast shutdown of host gene expression that leads to a rapid inhibition of IFN responses. In particular cell types, the capacity of VSV to shutdown host gene expression is correlated with the capacity to induce IFN induction (Wertz and Youngner, 1970). As we already discussed before, Matrix protein plays a key role in this process. Apart from the structural function of M protein for viral assembly and budding, it is also responsible for the cytopathic effects associated with VSV infection. M protein inhibits both synthesis and nuclear export of cellular mRNA. (Ahmed and Lyles, 1998; Faria, et al, 2005). Overexpression of M inhibits Polymerase II-mediated cellular transcription by

inactivating the basal transcription factor TFIID (Black et al, 1993; Yuan et al, 1998), while the single mutations in VSV M have been shown losing the ability to inhibit host RNA synthesis and IFN production (Ahmed and Lyles, 1998). M protein located in the nucleus blocks nuclear export of cellular mRNAs by interacting with the nucleoporin Nup98 and the export factor Rae (Von Kobbe et al, 2000; Petersen et al, 2000; Enninga et al, 2002; Faria, et al, 2005). At present, the manner through which VSV affects host cell translation remains unclear.

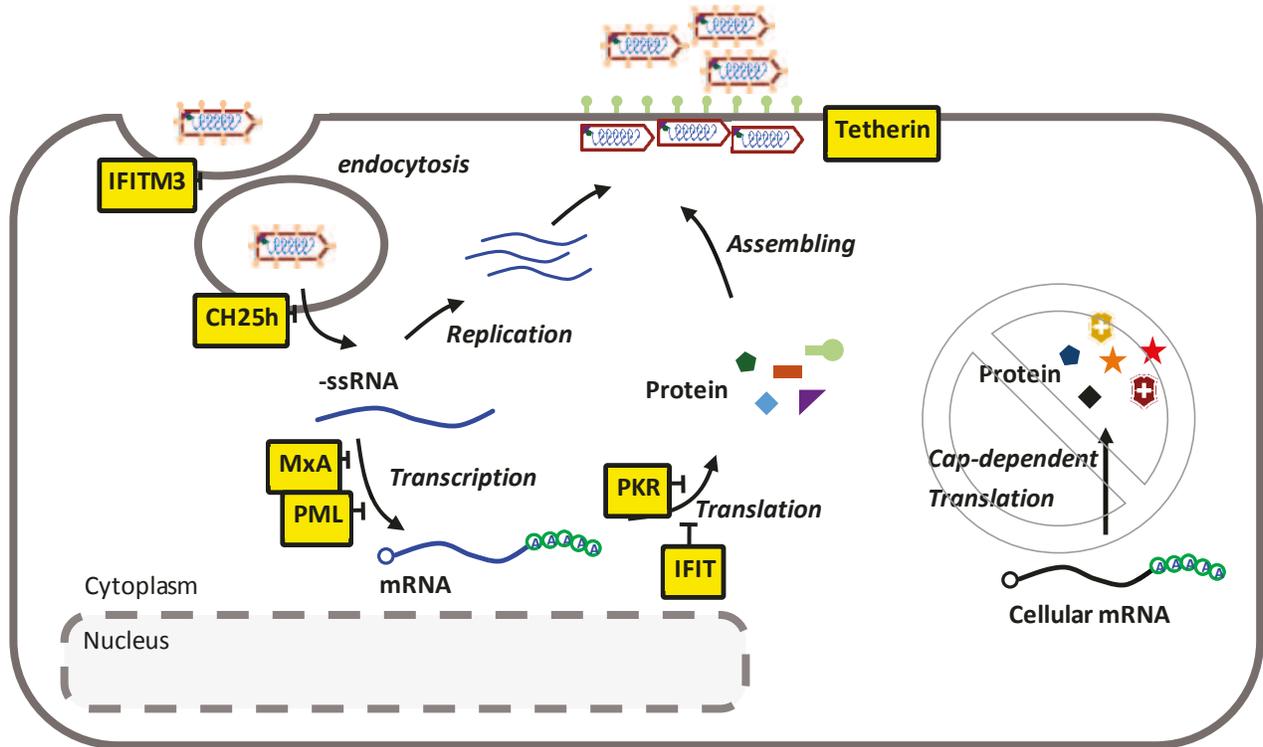


Figure II-4. interaction between VSV and host antiviral factors.

Many ISGs serves as antiviral restriction factors and restricts VSV replication in every step of viral life cycle. VSV also evolves counteracting mechanism by fast shutoff of host translation.

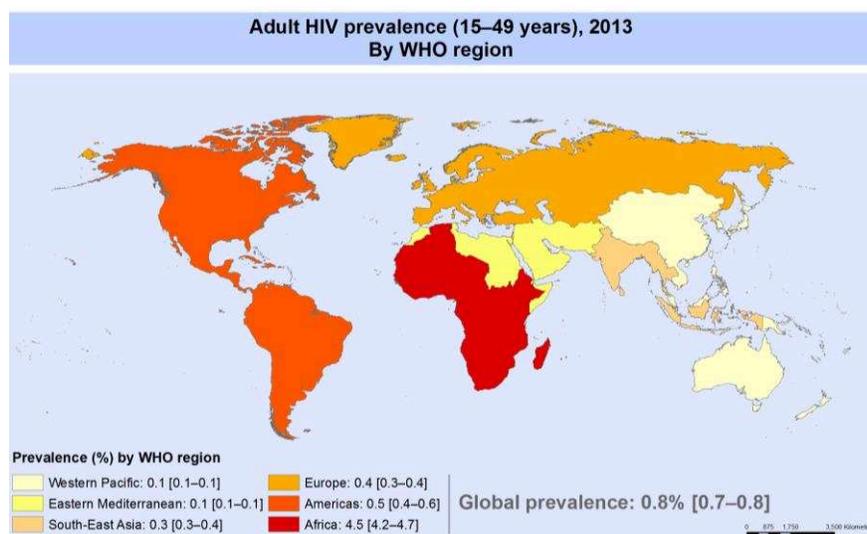
Abbreviations: IFITM3 (IFN-inducible transmembrane 3); CH25H (Cholesterol-25-hydroxylase); MxA (myxovirus resistance A); PML (Promyelocytic leukemia protein); IFIT (IFN induced protein with tetratricopeptide repeats 1).

III. Acquired Immune Deficiency Syndrome and Human Immunodeficiency Virus (AIDS and HIV)

AIDS was first clinically observed in 1981 in Los Angeles in homosexual men who showed a rare opportunistic infection symptoms of *Pneumocystis carinii* pneumonia (PCP) (CDC, 1981). Soon afterwards, more and more PCP patients were identified, as well as patients presenting other opportunistic diseases, Kaposi's sarcoma and lymphadenopathy (CDC, 1982a&b), which all commonly occurred in people with compromised immune systems. In 1983 two separate laboratories from France and United states independently isolated the Human Immunodeficiency Virus type 1 (HIV-1) (Gallo et al, 1983; Barre-Sinoussi et al, 1983).

According to data from the World Health Organization (WHO) HIV/AIDS Department, about 78 million people have been infected with the HIV virus and half of them were died of AIDS, since the beginning of the epidemic. The improvement of the preventive awareness as well as the expanding access to antiretroviral treatment (ART), the global epidemiology of HIV infection has remarkably changed. Since 2000, new HIV infections and AIDS-related deaths have fallen by 35% and 25%, respectively (Fact sheet 2015, UNAIDS); 41% of all adults and 32% of all children living with HIV had access to antiretroviral treatments in 2014, which is an overall worldwide increase given that in 2010 the percentage of treatment for these populations was only 23% and 14%. In low-income and middle-income countries, especially in Africa, with comparatively low penetration of ART, HIV-related tuberculosis continues to be a major cause of mortality (Cohen et al, 2010; Glaziou et al, 2013), although the global mortality caused by tuberculosis is decreasing. In high-income countries, only half deaths in people on ART are caused of AIDS, non-AIDS-defining cancers (23.5%), cardiovascular disease (15.7%) and liver disease (14.1%) are major contributors to non-AIDS-related deaths (Antiretroviral Therapy Cohort Collaboration, 2010).

Figure III-1, HIV prevalence in young-adult (15-49 years) in 2013 (Data from WHO).



Globally, a total of 35 million people are living with HIV at the end of 2013. About 0.8% of adults aged 15-49 years worldwide are HIV carriers. Sub-Saharan Africa remains the hardest hit region, and contributes nearly 71% of people living with HIV worldwide.

III.A Acquired immunodeficiency syndrome (AIDS)

III.A.1 Agent

In 1983, two separate labs led by Luc Montagnier in France and Robert Gallo in United states independently isolated a novel retrovirus from patient of AIDS (Gallo et al, 1983; Barre-Sinoussi et al, 1983; Shaw et al, 1984). They named the virus ‘lymphadenopathy-associated virus (LAV)’ and ‘human T-lymphotropic viruses III (HTLV-III)’, respectively. Later reports indicated LAV and HTLV-III to be the same etiological agent of AIDS. In 1986, the International Committee on Taxonomy of Viruses renamed the agent to HIV (Human Immunodeficiency Virus) instead of HTLV-III and LAV (Coffin et al, 1986).

HIV belongs to the genus *Lentivirus*, part of the family *Retroviridae*. The name ‘Lenti’ means ‘slow’ in Latin, indicating that those group of viruses are characteristically responsible for the long-time elapsing between the initial infection and the onset of the disease. HIV has two genetically distinct subtype: HIV-1 and HIV-2, both of which cause AIDS. However, when compared to HIV-1, HIV-2 is less virulent, less infectious and because of its relatively poor capacity for transmission, its distribution is restricted to West Africa (Barin et al, 1985; Clavel et al, 1986a, b&1987; Kanki et al, 1986&1994).

III.A.2 Clinical signs

Acute infection stage

HIV infection can be divided into three stages (Figure III-2), the first of which is an acute infection that leads to an influenza-like illness. Within 2 to 4 weeks post infection, most people develop an influenza-like illness with symptoms like: fever, swollen lymph nodes, sore throat, headache, as well as rash, muscle and joint pains. It’s the body’s natural response to the HIV infection and its intense replication. Large amounts of virus are produced during this phase in CD4+ T cells and destroyed in the process.

During this stage, the diseases are not often recognized as HIV leading signs, as the symptoms are nonspecific and usually overlapping with many common infectious diseases. At this stage, the levels of virus circulating in body fluid are very high and patients are at high risk of transmitting the disease.

Clinical latency stage

The acute phase is followed by a phase that is called of clinical latency. During this phase that can last months to years, viral replication continues but is in an equilibrium with the immune system and its responses, that, to a certain extent, control the virus. During this phase the patient may present no symptom, so this stage is also named “asymptomatic HIV infection”. This nomenclature is however misleading as the virus continues to burden the immune responses leading to a decrease in the number of helper T CD4+ cell which constitute a central component in the orchestration of proper immune responses. As the disease progresses, symptoms such as fever, weight loss, lymph nodes enlargement occurs eventually leading to the *overt* phase of the disease, AIDS.

Acquired immunodeficiency syndrome (AIDS)

The third and final phase of HIV infection occurs when the blood CD4⁺ T cells drop to less than 200 cells per μL , an amount at which their number becomes limiting for the proper functioning of anti-pathogen immune responses. It is at this point that patients develop HIV associated opportunistic diseases such as pneumonia, wasting syndrome or esophageal candidiasis, Kaposi sarcoma etc.

The decrease of helper T cells below a certain threshold is functionally nefast to the overall immune system, as this type of T cells are essential for both humoral and cellular immunity. The damaged immunity fails to protect the body from opportunistic infections caused by otherwise unharmed organisms such as viruses, bacteria, fungi or parasites.

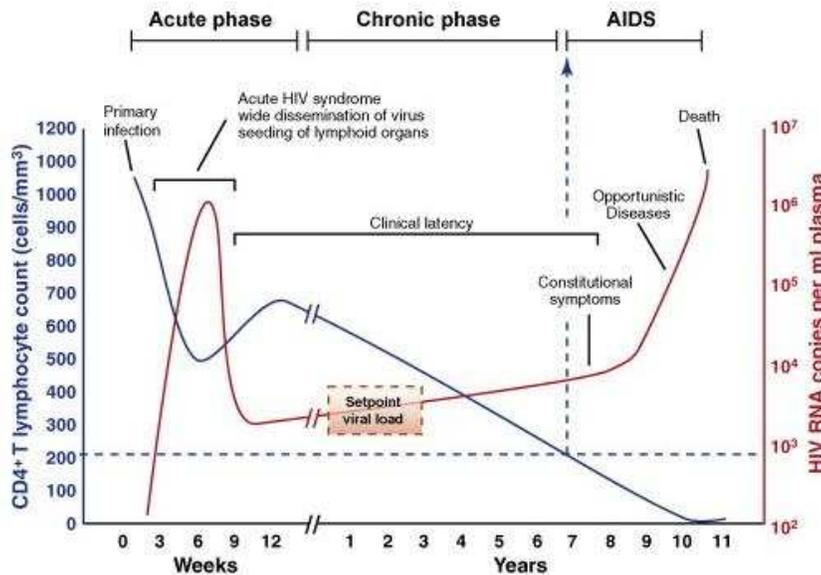


Figure III-2, A typical course of HIV-1 infection (An and Winkler, 2010).

After infected with HIV, infection can be divided schematically into three phases: an acute phase lasting 6-12 weeks in which patients present flu-like symptoms, high viral loads and a significant drop in CD4⁺ T cells; an asymptomatic phase that can last few years in which viral replication continues but to more moderate levels than the previous one, due to the involvement of immune responses. This phase is characterized by a decline in CD4 T cells; then the final phase called of overt AIDS associated to increasing viral replication and sharp decline in CD4⁺ cell counts to $<200/\text{mm}^3$.

Without treatment, most of infected individuals develop AIDS within ten years. People who progress to AIDS typically survive about 3 years in absence of ART. However, a small proportion of exceptional individuals have been reported, including Exposed uninfected (EU) individuals who show resistance to HIV under high risk exposures (O'Brien and Nelson, 2004; Shacklett, 2006); Elite controllers (EC) who are infected but restrain HIV to a low level (less than 50 copies/ml) (Walker, 2007); Long-term non-progressors (LTNP) who maintain stable CD4 levels and low virus level for decades (Poropatich K and Sullivan). On the contrary, few infected individuals develop AIDS with much faster rates than others. These are called fast progressors (O'Brien and Nelson, 2004). All this suggests that the individual responses to HIV may depend on different host genetic backgrounds.

III.A.3 Transmission

It is widely accepted that both HIV-1 and HIV-2 have emerged by cross-species transmission from non-human primates to humans in the early 20th Century (Holmes, 2001).

In humans, there are four main ways for HIV transmission, sexual intercourses, sharing of contaminated needles, transfusion of contaminated blood and vertical transmission from mother to child. Contact with snot, saliva, sputum, sweat, tears, urine, feces or vomit normally brings no risk, unless they are contaminated with blood (Kripke, 2007). Plasma viral load is the most important factor for HIV-1 transmission. People in the stage of acute HIV infection have very high plasma viral loads and are the major drivers of HIV epidemics (Cohen et al, 2011). For heterosexual HIV-1 transmission, a 0.7 log₁₀ reduction of plasma viral load leads to 50% reduction of transmission (Lingappa et al, 2010).

Heterosexual contacts remain the most frequent route of HIV transmission worldwide. The risk of HIV transmission per sexual act in low-income countries appears to be four to ten times higher than in high-income countries (Boily et al, 2009). Many factors associated with increased risk of sexual transmission. Sexually transmitted infections such as genital ulcers, bacterial vaginitis and herpes simplex type-2 infection, increase the risk of transmission (Glynn et al, 2009; Dosekun and Fox, 2010). Anal intercourse strongly increases the risk of transmission in both heterosexual and homosexual contacts, especially for the receptive person (Beyrer et al, 2012).

Most blood-borne transmission of HIV, as well as other blood-borne viruses such as HBV and HCV, occurs among people who inject drugs (PWID) using contaminated equipment. The high risk of this transmission route is mainly due to contaminated injecting equipment, frequency of drug injecting, and drug use behavior such as unprotected coitus (Crawford and Vlahov, 2010; WHO, 2014). Apart from drug injecting, health care settings are the major places where HIV to be transmitted via contaminated injection, surgical and other piercing operation, blood transfusion, tissue transplantation, as well as accidental occupational exposure like needle-stick injuries. Benefit from the implementation of safety measures on donor selection, blood screening, disinfected and single used equipment, most high-income countries have significantly low risk of HIV transmission during blood transfusion. While in low-income countries, however, with lacking routine testing or of low quality, transfusion of blood continues to be a risk for HIV transmission (WHO, 2013&2014).

The mother-to-child transmission (MTCT) of HIV is another most common route which accounts for the most cases of HIV in children. HIV can be transmitted in three major periods during pregnancy, delivery, or breastfeeding (Newell, 1998).

New HIV infections among children had declined by 58% between 2001 and 2013. In 2014, 73% [68%-79%] of pregnant women living with HIV had access to ART to prevent transmitting HIV to their babies. The risk of MTCT can be reduced to 5% or even less if the mother takes effective antiretroviral therapy during pregnancy, delivery and breastfeeding. Despite the significant progress of prevention of MTCT (PMTCT), there were still estimated 220 000 [190 000 -260 000] children worldwide became newly infected in 2014 (UNAIDS, 2014&2015).

III.B Human Immunodeficiency Virus

III.B.1 Classification and genetic variability

HIV belongs to the genus *Lentivirus*, part of the family *Retroviridae*. Retroviruses are single-stranded, enveloped viruses. Upon entry into the cell, the viral positive-sense genomic RNA is reverse transcribed into a double-stranded DNA intermediate by the viral encoded reverse transcriptase.

Many species are infected by *lentiviruses*, including primates, horse, cattle, sheep, goat and cat (Campbell and Robinson, 1998; Leroux et al, 2004). Primates are the natural host for several lineages of closely related simian and human immunodeficiency viruses (SIV and HIV, respectively). SIVs have been identified in at least 45 species of African non-human primates (Peeters et al, 2014). SIVs are named according to the host species, such as chimpanzee, cpz; sooty mangabey, sm; western gorilla, gor; Mona monkey, mon; Red-capped mangabey, rcm and so on. SIVcpz and SIVsm are believed to have crossed the species barrier into humans, resulting in HIV-1 and HIV-2, respectively (Gao et al, 1999; Chen et al, 1997). Both HIV-1 and HIV-2 cause AIDS. Unlike HIV, SIV infection is non-pathogenic in the natural host, although the infected natural host also displays high viral replication and an acute immunologic response similar to pathogenic SIV/HIV infections (Pandrea et al, 2008; Mir et al, 2010). However, the infection becomes pathogenic when transmitted to a different species, as is the case in humans, and as also confirmed by experimental infections of monkeys (Kestler et al, 1990; Silvestri, 2008; Brenchley et al, 2010).

HIV displays high genetic variability thanks to the high mutation rate and low fidelity of the reverse transcriptase enzyme as well as due to the recombination that occur during reverse transcription (Rambaut et al, 2004). The two major types, HIV-1 and HIV-2 can be further divided into groups. HIV-1 is divided into Group M (Major), Group O (Outlier) and Group N (non-M, non-O), as well as newly emerged Group P (pending the identification of further human cases). The origins are shown in figure III-3 (Sharp and Hahn, 2011).

Group M is by far the most common and pathogenic strain of HIV, leading to more than 90% of HIV/AIDS cases. The other three groups are quite uncommon and only occur in certain region in Africa. Group M can be further subdivided into at least nine subtypes based on genetic sequence data. These are subtypes A, B, C, D, F, G, H, J, K, as well as unique recombinant forms (URFs) and circulating recombinant forms (CRFs) derived from recombination between different subtypes (Aldrich and Hemelaar, 2012; Kouri et al, 2015).

Nine HIV-2 groups were identified and designated A to I (Ayoubia et al, 2013), of which only groups A and B are epidemic and spread mainly in West Africa (Santiago et al, 2005).

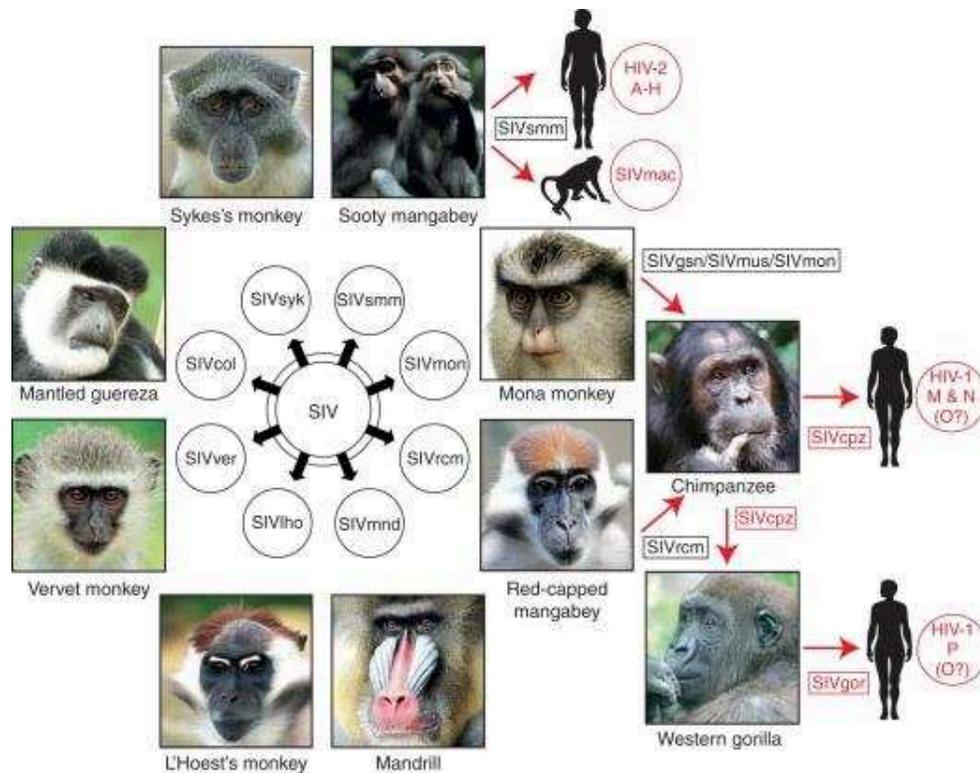


Figure III-3, Origins of human AIDS viruses (Sharp and Hahn, 2011).

Old World monkeys are naturally host of more than 40 different Simian immunodeficiency viruses (SIVs). Occasionally, new pathogens generated while some of these SIVs transmitted to great apes and humans. Known examples of cross-species transmissions are highlighted in red.

III.B.2 Structure of HIV genome and viral particle

HIV-1 genome RNA is approximately 9.8 kb long, 5' capped and 3' polyadenylated (Figure III-4). In this mRNA the viral open reading frames (ORFs) are flanked by two long terminal repeats (LTRs). HIV-1 employs an exquisite splicing mechanism to generate its different gene products: a 9.2kb unspliced transcript encoding the Gag and Pol (polymerase) precursors; a 4.5kb single spliced transcript encoding Env, Vif, Vpr and Vpu; and a 2 kb multiply spliced transcript encoding for Tat, Rev and Nef. The polyprotein precursors are further processed by the viral protease. Specifically, Gag (p55) is processed to p17 (MA_{matrix}), p24 (CA_{capsid}), p7 (NC_{nucleo-capsid}) and p6 proteins; Pol is processed to p10 (PR_{reverse transcriptase}), p66/p51 (RT_{reverse transcriptase}), and p31 (IN_{integrase}). The Envelope glycoproteins are also translated as a single precursor (gp160) that is processed by a cellular protease called furin in the ER/Golgi to yield the external gp120 and the transmembrane gp41 (Hallenberger et al, 1992). While Gag-Pol and Env are common to all retroviruses, the remaining proteins are specific for lentiviruses and play an important part in either expression regulation (Tat, Rev) or in pathogenesis (Vif, Vpr, Vpu and Nef). Given that this is not the specific topic of this thesis the role of these proteins will not be detailed further. (Debaisieux et al, 2012; Pollard and Malim, 1998).

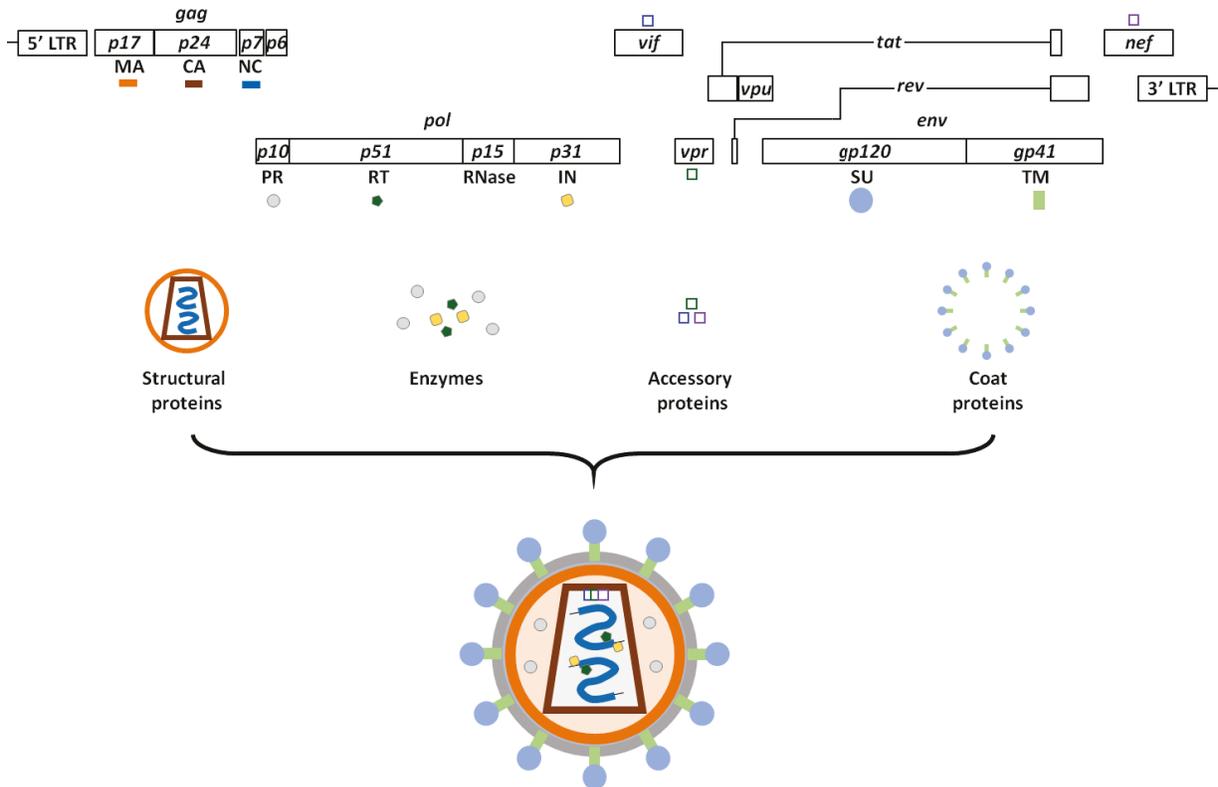


Figure III-4, Structure of HIV-1 genome RNA and virion.

The RNA genome of HIV-1 consists of nine genes, encoding in the 19 final proteins. The three major genes, gag, pol and env, encoding major structural proteins and essential enzymes. Apart from them, there are six accessory genes, tat, rev, nef, vpr, vif and vpu, encoding regulatory proteins that control the enter ability, virulence and reproduction of HIV.

Abbreviation: LTR (Long terminal repeats); MA (Matrix); CA (Capcid); NC (Nucleocapsid); PR (Protease); RT (Reverse-transcriptase); IN (Integrase); Vif (Viral infectivity factor); Vpr (Viral protein R); Vpu (Viral protein unique); Tat (trans-activating protein); Rev (Regulator of Virion); Env (Envelope); Nef (Negative factor); SU (surface); TM (Transmembrane); gp (glycoprotein).

HIV has a roughly spherical virion structure with a diameter of about 100-120 nm. From inside to outside, two copies of positive-sense single-stranded RNA are tightly bound to p7 nucleocapsid proteins and essential enzymes such as reverse transcriptase and integrase. The RNA-NC-Enzymes complex is then enclosed by capsid protein p24 forming the higher molecular order structure called core. The core is then packaged and surrounded by p17 matrix proteins that bind to the plasma membrane that envelopes the viral particle. On this membrane stands the gp41 and gp120 that constitutes the viral envelope proteins (Figure III-4).

III.B.3 HIV life cycle in cells

HIV-1 infects various immune cells including macrophages, CD4+ T cells, microglial cells and dendritic cells (DCs). According to its coreceptor binding specificity, the virus can be divided into R5 tropic and X4 tropic strains that use cellular coreceptors CCR5 and CXCR4, respectively. Some strains called dual-tropic or R5/X4 are able to use both CCR5 and CXCR4 (Berger et al, 1998). The Env consists of two noncovalently associated subunits, the external gp120 subunit with the receptor binding site, and the membrane-spanning gp41 subunit with a hydrophobic fusion peptide, which is directly involved in membrane fusion. HIV-1 life cycle in cells can be divided into two phases, before integration called early phases and after called late phases (Figure III-5).

Early phases

Entry of HIV-1 in the cell is initiated by primary binding of the viral gp120 with CD4 receptor, followed by secondary binding with the chemokine coreceptors CCR5 or CXCR4. This stable binding allows gp41 to change its conformation and to fuse the viral to the cellular membrane leading to the subsequent entry of the viral capsid into the cell cytoplasm (Chan and Kim, 1998; Berger et al, 1999). Fusion occurs mainly at the plasma membrane although HIV has also been shown to enter cells by clathrin-dependent as well as clathrin-independent endocytosis (Permanyer et al, 2010).

Once the viral core is released into the cytosol, the process uncoating occurs, leading to the disassembly of the conical shells that contain the viral genome and its associated proteins (Ambrose and Aiken, 2014). Prior or concomitantly to uncoating, the HIV reverse transcriptase converts the viral genomic RNA into the final double-stranded viral DNA. Once completed, viral DNA is then transported to the nucleus for chromosomal integration, which is catalyzed by viral Integrase. Only a few copies of HIV DNA are imported into nucleus and integrated into host genome DNA, while the rest is either not completed or directly degraded by cytoplasmic nucleases.

Late phases

Once the viral DNA is integrated into a host chromosome, the integrated DNA provirus is transcribed mostly using the host transcription machinery with two exceptions. The beginning of transcription leads to the production of small transcripts from the viral LTR promoter. To extend them fully, the viral protein Tat must act positively on viral transcription. Indeed, the HIV Tat protein which stand for "Trans-Activator of Transcription", can drastically enhances the efficiency of viral transcription (Debaisieux et al, 2012). After mRNA transcription, another viral protein, the Rev protein acts on splicing by binding to full-length, unspliced viral RNAs and delivering them to the cytoplasm (Pollard and Malim, 1998). These unspliced RNAs function as mRNAs for translating the structural proteins Gag and Pol, as well as new copies of the virus genome.

The final steps of the HIV-1 viral cycle were particularly well reviewed by Eric in 2015 (Eric, 2015). For the viral particle to be produced, the Gag and Gag-Pol polyproteins, are recruited to the plasma membrane where they begin to bud from the membrane with the help of cellular proteins such as the endosomal sorting complex required for transport I (ESCRT-I) (Jouvenet et al, 2011). While Gag is the only protein absolutely

required for the formation and release of virion particles, the formation of infectious particles requires Env, which reaches the site of assembly of virion particles via the endoplasmic reticulum and then the Golgi, as well as the inclusion, or packaging, of the viral genomic RNA. This incorporation is mediated by a specific association between the packaging sequence (Psi)-present on the viral genome and the NC portion of Gag. After formation, virion particles bud out from the cell and are released in the cell supernatant where they can begin the next cycle of infection.

III.C Regulation of HIV infection

Host factors and cellular machineries are extensively used by HIV-1 during viral replication. HIV-1 only produces 9 viral genes, however, over 1000 candidate host factors have been revealed to participate in HIV-1 infection throughout the viral life cycle (Brass et al, 2008; Börner et al, 2010; Pache et al, 2011; Landi et al, 2014; Li, 2015). As of today only a few have been truly characterized mechanistically.

III.C.1 Cellular cofactors

These are generally referred to as cellular proteins that play a pro-viral role and that therefore are recruited by the virus to complete its life cycle. Despite the fact that large genome-wide screens have identified many potential candidates, only few have been characterized in detail and some of them will be mentioned below.

Attachment, entry and uncoating

The cellular receptors CD4, CXCR4 and CCR5 are essential for binding and fusion of virus with target cell (Moore et al, 2004) and are therefore key essential co-factors in HIV replication. However, additional cellular factors have also been shown to mediate viral attachment to the cell, such as DC-SIGN and Galectin-1 (Geijtenbeek et al, 2000; St-Pierre, 2010). Cyclophilin A (Cyp A) and peptidyl-prolyl cis/trans isomerase Pin1 have been shown to play a role, albeit controversial in regulating the equilibrium between uncoating and reverse transcription (Li et al, 2009; Misumi et al, 2010).

Reverse transcription, nuclear import and integration

Distinct pathogen recognition receptors such as cyclic GMP-AMP (cGAMP) synthase (cGAS) and IFN γ -inducible protein 16 (IFI16) target viral DNA in cytoplasm. However, HIV-1 seems to avoid triggering an IFN response in many cultured cells (Manel and Littman, 2011). This has been ascribed to the beneficial role played by the host cytoplasmic DNase called Three prime repair exonuclease 1 (TREX1). This protein has been shown to remove the excess of viral DNA from the cell cytoplasm, leading to a drastic decrease in the ability of the cell to detect viral DNA during the process of infection. In this respect, TREX1 plays a pro-viral role that ultimately silences the ability of the cell to detect viral infection (Yan et al, 2010).

After reverse transcription, formation of the pre-integration complex (PIC) is essential for nuclear import. The PIC consisting of viral dsDNA associated with both viral and cellular proteins and a key important co-

factor that associates to PICs through its association to the viral Integrase is extenso (LEDGF/p75). This protein is absolutely required to tether the viral PIC to the host chromatin that allowing integration (Vandegraaff et al, 2006; Schrijvers et al, 2012). A recently study revealed that the presence of the cellular integration cofactors LEDGF/p75 and Nup153 are indispensable for the peripheral integration site selection of the virus (Marini et al, 2015).

Transcription and nuclear export

Cyclin-dependent kinase (CDK9) and cyclin T1 constitute the Positive transcription elongation factor b (P-TEFb), which is a crucial transcription factor for HIV provirus transcription. HIV-1 Tat protein recruits the P-TEFb to the transactivation response (TAR) RNA, where P-TEFb triggers transcriptional elongation (Zhou et al, 1998).

While completely spliced transcripts are exported by the canonical nuclear export pathway, export of the unspliced and the partially spliced viral RNAs require formation of the nuclear export complex that consisting of CRM1 (chromosome region maintenance 1) and Ran-GTP (GTP-binding nuclear protein Ran). These intron-containing transcripts contain the Rev responsive element (RRE) for binding of Rev, allowing the recruitment of human CRM1 and Ran-GTP and the formation of the transport complex (Köhler and Hurt, 2007; Rojas-Araya et al, 2015).

Translation and viral protein trafficking

HIV-1 transcripts are synthesized by cellular RNA polymerase II, thus are capped and polyadenylated by the host machinery (Karn J and Stoltzfus, 2012). HIV-1 has been shown to use both cap-dependent and the internal ribosome entry sites (IRES)-dependent translation mechanisms to synthesis viral proteins, dependent on the physiological conditions of the cell and the status of the ongoing infection (Brasey et al, 2003; De Breyne et al, 2013). Both translation routes require the cellular translation machinery including the essential initial factors.

Rab GTP proteases is a large family of small GTPases that play an important role in vesicular trafficking, control membrane identity and vesicle budding, uncoating, motility and fusion through the recruitment of effector proteins, such as sorting adaptors, tethering factors, kinases, phosphatases and motors (Silvestri, 2008). Several Rab GTPases, as well as other vesicular trafficking components including the clathrin adaptor protein (AP) complexes, the Golgi-localized γ -ear containing Arf-binding (GGA) and ADP ribosylation factor (ARF) proteins have also been implicated in viral protein trafficking and virus release (Bonifacino and Traub, 2003; Azevedo et al, 2009; Joshi et al, 2008).

Budding

The endosomal sorting complexes required for transport (ESCRTs) is an ancient and conserved system for membrane fission, consist of ESCRT-I, ESCRT-II and ALIX, which recognize cargo, the ESCRT-III complex and the VPS4 ATPase work together to catalyze membrane fission (Carlton and Martin-Serrano,

2007; McCullough et al, 2013). The release of nascent particle from the cell surface is mediated by the cellular ESCRT machinery, which is hijacked by Gag (Freed, 2015).

III.C.2 Cellular restriction factors

In addition of anti-HIV-1 infection mediated directly via IFN production, the IFNs encore induce multiple restriction factors. Most of the known anti-HIV-1 restriction factors are IFN-stimulated genes (ISGs) induced by IFN α . The known restriction factors were briefly reviewed by Doyle et al (Figure III-5, Doyle et al, 2015). Here I will detail only a few of them to provide examples of how the cell may try to impair HIV replication and how on the other hand, the virus may try to circumvent their negative effects.

The cellular cytidine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) is the first identified HIV-1 restriction factor. APOBEC3G inhibits infection of vif-deficient HIV-1 by deaminating cysteines in single-stranded HIV DNA to uracils (Sheehy et al, 2002; Wang et al, 2014). This deamination that is carried out in single-stranded viral DNA specifies a C to T mutation in that is then copied into a G to A mutation in the positive strand DNA, during the process of reverse transcription. The editing, or deamination activity of APOBEC3G can be particularly dense, resulting on what has been called death by mutagenesis, a process whereby the accumulation of G to A mutations is so heavy that most of the viral ORFs are mutated. APOBEC3G belongs to a protein of similarly related proteins and most members of the APOBEC3 family have been shown to exert similar antiviral effects on HIV.

A second strong and more recent antiviral effector against HIV is Tetherin/Bst2. Tetherin is an interferon-regulated protein with a strong antiviral effect. This protein retains virion particles at the cell surface and lead to the degradation of virions by subsequent endocytosis (Neil et al, 2008).

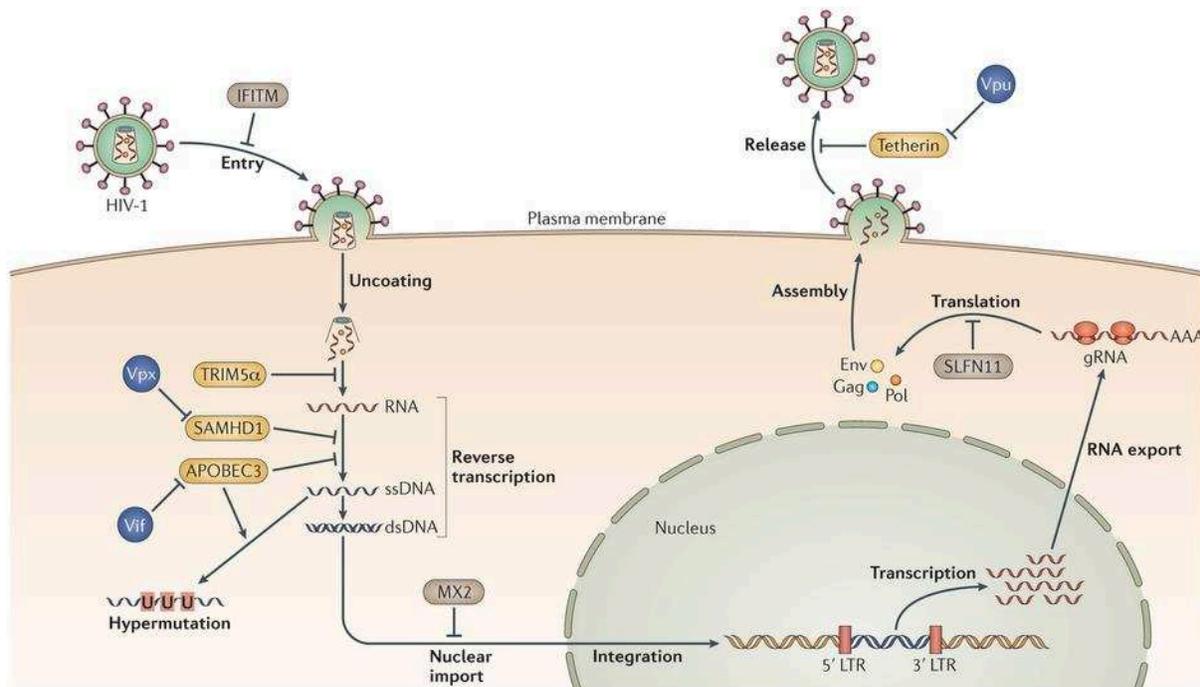


Figure III-5, Interaction between cellular restriction factors and viral antagonists during HIV-1 life cycle in cells (Doyle et al, 2015).

The life cycle of HIV-1 as well as other retroviruses begins with entry into the cell, followed by uncoating, reverse transcription, integration, transcription, translation, assembly release and maturation. The life cycle can be divided into early and late phases according to integration of the viral genome into the host cell genome. Interactions between viral and host cell factors occur at every stage of the viral life cycle: antiviral factors such as IFITMs, TRIM5, SAMHD1, APOBEC3s, MX2 and Tetherin and viral proteins such as Vif, Vpu and Vpx (of HIV-2 and SIV) that can rescue virus by counteracting some restriction factors.

Abbreviation: IFITM (Interferon-induced transmembrane protein); TRIM5 α (Tripartite motif-containing protein 5 α); SAMHD1 (SAM domain and HD domain-containing protein 1); APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3); Vpx (Viral protein X); Vif (Viral infectivity factor); Vpu (Viral protein unique); LTR (Long terminal repeats); gag (group-specific antigen); pol (polymerase); env (envelope); SLFN11 (Schlafen Family Member 11).

II.C.3 Counter-restriction mechanisms performed by HIV-1 accessory proteins

The HIV accessory proteins Vif, Vpr, Vpu (or Vpx of HIV-2) and Nef may seem not necessary for viral replication in tissue culture. However, they play important roles in primary cells as well as of course *in vivo*, as they are strictly conserved. One typical example is the protein Vif (Viral Infectivity), which was dispensable for replication in some, albeit not all, established cell lines, but important for HIV replication in primary cells.

Indeed cells can be divided into permissive and non-permissive based on their ability to support the replication of vif-deficient (Δ vif) HIV-1. Vif is required for HIV-1 replication in non-permissive cells, as HIV-1 Δ vif viruses produced from these cells are much less infectious than wild-type viruses. While Δ vif viruses produced from permissive cells have indistinguishably infectious from wild-type viruses. (Gabuzda et al, 1992; Sakai et al, 1993). By comparing correlative RNA expression in non-permissive (CEM) and permissive (CEM-SS) cell lines, and subsequent functional screening, Sheehy et al identified APOBEC3G as the restriction factor against Δ vif HIV-1 (Sheehy et al, 2002). In absence of Vif, APOBEC3G is packaged into the virion and inhibits viral replication in target cells through cytidine deamination of the viral genome (Mariani et al, 2003). Vif prevent APOBEC3G to be packaged into virions by binding it in the producer cell and recruits an E3 ubiquitin ligase complex for its degradation (Sheehy et al, 2003; Mariani et al, 2003).

A second example of the same kind is Vpu. Δ Vpu HIV-1 accumulate in massive clusters on the cell surface (Klimkait et al, 1990). A few years later bone marrow stromal cell antigen BST2 (Tetherin) was identified as the agent that blocking Δ vpu virion release (Neil et al, 2007). Vpu interacts with tetherin and causing its endocytosis and proteasomal degradation (Neil et al, 2008). In absence of Vpu, HIV-2 use viral Env to antagonize tetherin (Bour and Strebel, 1996), while many SIVs use Nef protein instead of Vpu (Jia et al, 2009; Zhang et al, 2009).

It is well accepted that only a few antiviral factors have been identified, cells express some others that remain to be discovered and the characterization of one potential candidate was one of the goals of my thesis work.

IV. IFN-stimulated gene 20kDa (ISG20)

ISG20/ HEM45 (human estrogen regulated transcript 45) was identified independently by two teams as a protein inducible by interferon as well as by the estrogen hormone (Gongora et al, 1997; Pentecost et al, 1998). Further studies demonstrated that the induction by IFN requires the binding of the IFN regulatory transcription factor 1 (IRF-1) to the IFN stimulated response Element (ISRE) localized on the promoter of ISG20 (Gongora et al, 2000; Espert et al, 2004). ISG20 belongs to the DEDDh subgroup of the DEDD exonuclease superfamily. Members of this family may possess both RNase and DNase activities (Zuo and Deutscher, 1999&2001; Yuan et al, 2015), and share a conserved domain required for the binding of two divalent metal ions, which are essential for catalysis (Steitz and Steitz, 1993; Derbyshire et al, 1995; Horio et al, 2004). Among the members of this family, ISG20 is perhaps the major interferon-regulated protein (Gongora et al, 2000; Espert et al, 2004; Zahoor et al, 2015; Zhao et al, 2013) and since its discovery, it has been linked to antiviral innate immunity. Up to this day, few studies have truly examined the antiviral potential of ISG20. However, in those that did it, the antiviral property of ISG20 has been ascribed to its ability to directly degrade viral RNA, given its strong 3'-5' exonuclease activity (Espert et al, 2003; Jiang et al, 2008; Nguyen et al, 2001). This activity has been however contradicted by results shown in other studies (Zhang et al, 2007; Zhou et al, 2011). While the results I have obtained during my PhD do not exclude this possibility, they also indicate that an additional function of ISG20 may be to inhibit the pathogen translation.

IV.A Classification of ISG20

Nucleases are enzymes able to digest a polynucleotide chain by hydrolyzing its phosphodiester bonds. Depending on the location of the cleavage, nucleases can be divided into exonucleases or endonucleases (from 3' or 5' nucleic acid ends, or from the middle of the polynucleotide chain, respectively). There are six exonuclease superfamilies, five of them (DEDD, RNR, RBN, PDX and RRP4) catalyze 3'-5' cleaving, while the sixth superfamily, 5PX, catalyze 5'-3' cleaving (Zuo and Deutscher, 2001).

All members of DEDD superfamily have a structurally related exonuclease domain that contains three separated catalytic motifs termed Exo I, Exo II and Exo III (Moser et al, 1997). These domains are characterized by the repetition of four conserved amino acids (three aspartate (D) and one glutamate (E, see figure IV-1). Members of the DEDD family can be further subdivided into DEDDh and DEDDy subgroups, depending on the presence of a conserved histidine (h) or tyrosine (y) residues at Exo III. ISG20 belongs to the DEDDh subgroup.

The DEDDh subgroup is composed of more than seven thousand members of RNases (among them RNase D and T, Oligoribonuclease, 3'hExo, Pan2, PARN, Rex4, ISG20L1&L2) and DNases (ex. ϵ subunit of bacterial DNA polymerase III, TREX1) involved in various aspects of RNA and DNA processing (Moser et al, 1997; Zuo and Deutscher, 2001; Zhang et al, 1998; Dominski et al, 2003; Boeck et al, 1996; Couté et al, 2008; Körner et al, 1997; Mazur and Perrino, 1999).

The crystal structure of ISG20 exonuclease domain has been determined and shown to be very similar to the Escherichia coli (*E. coli*) DNA polymerase III ϵ subunit and exonuclease I (Horio et al, 2004). In humans, two closely related homologs of ISG20 have been described: ISG20L1 (originally named apoptosis enhancing nuclease, AEN) and ISG20L2, whose C-terminal ExoIII exonuclease domain is highly matched to that of ISG20 (57% and 51%, respectively) and also display a notable 3'-5' exonuclease activity (Lee et al, 2005; Couté et al, 2008). The antiviral properties of these two genes are currently unknown although both of them have been described to be devoid of antiviral activity (Zhou et al, 2011).

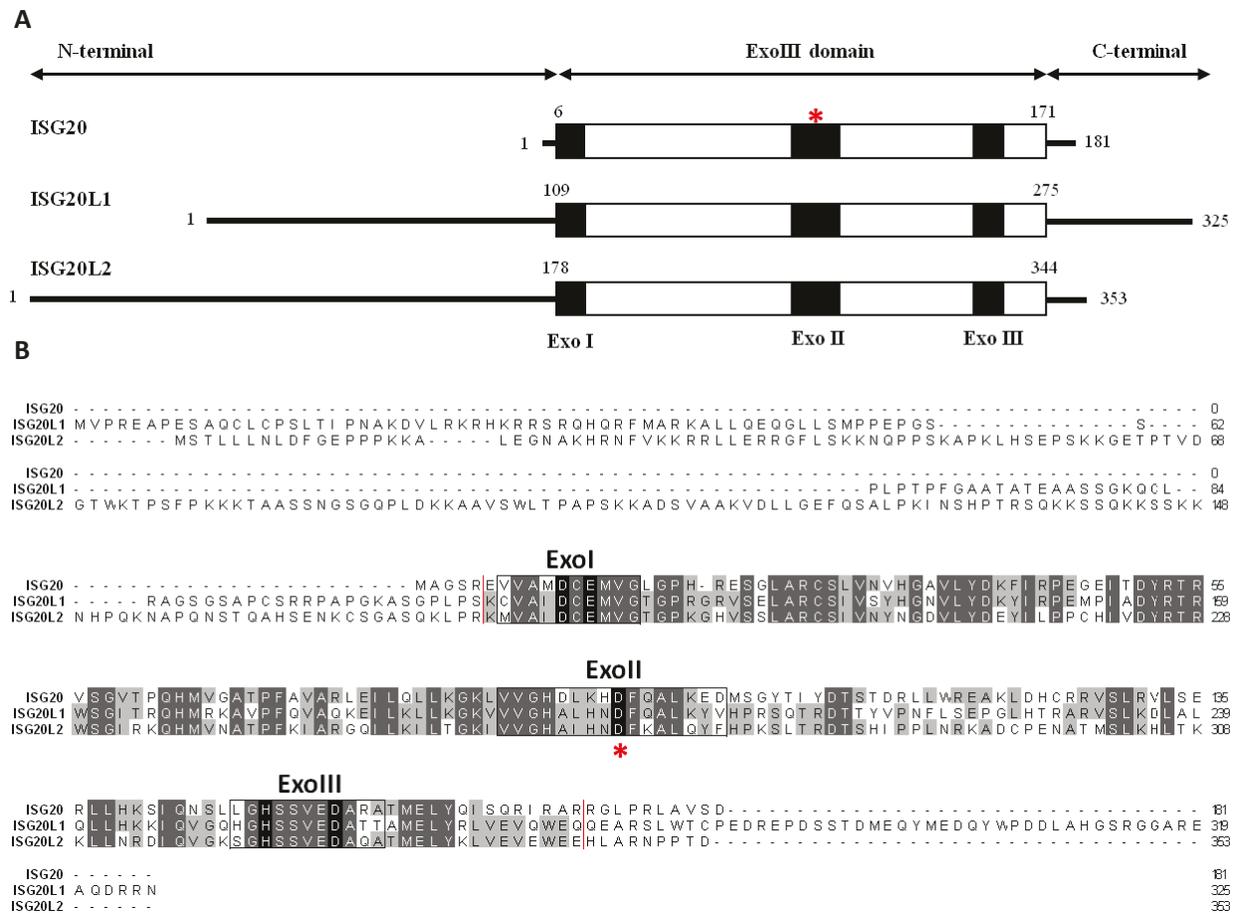


Figure IV-1, Alignment of ISG20, ISG20L1 and ISG20L2 (Adapt from Zhou et al, 2011).

A, Schematic alignment of ISG20, ISG20L1 and ISG20L2. The exonuclease domains are filled in black.

B, Amino acid sequence alignment of ISG20, ISG20L1 and ISG20L2. The exonuclease domains are bordered by red vertical line and the Exo motifs are boxed in black. The DEDDh conserved residues within the three motifs, Aspartate11, Glutamate13, Aspartate94, Aspartate154 and histidine149 are in black-shaded letters. The heavy or light grey-shaded letters present the identical residues in all three proteins or in two of them, respectively. Similar residues are presented in light grey-shaded. The red asterisk () indicates the position of the most commonly used single point mutation in the catalytic residue of ISG20 (D94).*

IV.B Structure and function(s) of ISG20

The crystal structure of ISG20 was determined by Horio et al in 2004. The structure revealed strong similarities of the exonuclease domain to the corresponding domains of the ϵ subunit of *Escherichia coli* DNA polymerase III and *E. coli* exonuclease I (Horio et al, 2004), that despite low sequence identity among their nuclease domains.

However, apart from a few differences of loop segments in size, twist and direction, the most interesting distinction between ISG20 and the two DNases is that ISG20 recognizes the sugar moiety of the nucleotide in a slightly different manner from the two DNases. Indeed, the carbonyl oxygen of Met14 and Arg53 in the active site of ISG20 accommodate hydrogen bonds of ribose 2'-OH, which is the character of the UMP ribose. UMP and dTMP (deoxythymidine monophosphate) are products of the exonuclease cleavage on RNA and DNA, respectively. The ribose 2'-OH binding preference of these two residues may explain the preference of ISG20 for RNA over DNA substrates (Nguyen et al, 2001).

It is therefore not surprising that ISG20 displays strong RNase activity *in vitro*. The exonuclease properties of ISG20 have been thoroughly examined *in vitro* using bacterially purified his-tag-ISG20. Under these conditions, ISG20 displays a 3'-5' exonuclease activity on RNA substrates without any apparent preference for their length in that both short and long RNAs are equally well digested. However, the presence of stem-loops in the RNA substrate caused a ~40-fold reduction in the degradative ability of ISG20 indicating that the structure of the RNA substrate may modulate its susceptibility to ISG20 in cells.

IV.C Subcellular localization and potential cellular functions of ISG20

Using confocal immunofluorescence microscopy as well as electronic microscopy, Espert et al showed that, despite a diffused cytoplasmic distribution (Espert et al, 2003&2006; Zhou et al, 2011), ISG20 could be found concentrated in the nucleus both in the nucleolus as well as in Cajal Bodies (CBs) (Espert et al, 2006).

Nucleoli and Cajal Bodies are the meeting places and factories for ribonucleoprotein (RNP), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and small cajal body-specific RNAs (ScaRNAs) formation (Raska et al, 2006; Cioce and Lamond, 2005). Such RNAs play capital and multiple features in the cell physiology and are involved in numerous functions such as splicing, ribosome biogenesis and telomere maintenance (Dundr and Misteli, 2010; Machyna et al, 2013). A number of CB-specific proteins have been found to be associated to these structures. Since these proteomic studies had not been conducted in IFN-stimulated conditions, the association of ISG20 with these structures could not be verified with this method. Among the proteins associated to CB, the survival motor neuron (SMN) protein is an essential component of the spliceosomal machinery and is involved in the assembly of small nuclear ribonucleoproteins (snRNPs) (Pellizzoni et al, 2002; Battle et al, 2006). In cells, along with processes of snRNPs assembly and maturation, SMN translocalizes from the cytoplasm to the nucleus, where it concentrates in CBs and gems (Gems stand for Gemini of CBs, where concentrated SMN is frequently found neighbouring or overlapping CBs) (Liu and Dreyfuss, 1996; Pellizzoni et al, 2002; Han et al, 2016).

An interaction between SMN and ISG20 was demonstrated by co-immunoprecipitation (coIP) analysis (Espert et al, 2006). Specifically, the interaction between ISG20 and SMN could be detected in nuclear extracts but not in cytoplasm extracts, supporting the possibility of a physical interaction between these two proteins in CB. To further strengthen the connection between ISG20, SMN, snRNPs and CBs, Espert et al also demonstrated that spliceosomal U1 and U2 snRNAs, and U3 snoRNA, which are essential part of ribonucleoproteic complexes, could be co-immunoprecipitated with ISG20. These results strongly suggesting that ISG20 might be associated with nuclear SMN complex in CBs and involved in later stages of snRNP maturation.

It must however be said, that the localization in CB requires extensive permeabilization and loss of ISG20 at other locations within the cell so that the specific localization of ISG20 to CB must be considered with precaution. Indeed, although ISG20 is distributed in nucleus, there's no doubt that both endogenous and ectopically expressed ISG20 displays a rather diffused localization within the cell (Espert et al, 2003&2006; Zhou et al, 2011).

In contrast to ISG20, both ISG20L1 and ISG20L2 present a nuclear localization promoted by their N-terminal domain (Lee et al, 2005; Couté et al, 2008; Zhou et al, 2011). However, an earlier study showed that both GFP fused ISGL1 and ISG20L2 were concentrated in nucleoli, but not in CBs (Couté et al, 2008). ISG20L2 interacts with more than 20 ribosome-associated proteins and is involved in ribosome biogenesis (Couté et al, 2008). Overexpressing or silencing ISG20L2 induces a specific decrease or increase of the 12 S precursor ribosomal RNA (rRNA), respectively. Since 12 S rRNA is the precursor of 5.8S rRNA and the latter is a component of the large subunit of eukaryotic ribosome, ISG20L2 may plays a role in translation through regulation of 5.8S rRNA maturation (Couté et al, 2008). ISG20L1 has instead been shown to possess a DNase activity rather than an RNase one. Such DNase activity might be required for efficient DNA fragmentation and enhancement of p53-dependent apoptosis which are at the moment the only activities attributed to this protein (Lee et al, 2005).

IV.D ISG20 displays broad antiviral activities

The literature on ISG20 is not very extensive at this moment and a large number of studies published on the subject originate from Dr. Nadir Mechti's laboratory (Gongora et al, 1997; Espert et al, 2003&2004; Imaizumi et al, 2008; Kato et al, 2003; Hashimoto et al, 2000). Considering that ISG20 is an exonuclease that prefers RNA substrates, most efforts have been directed towards RNA viruses. Various RNA viruses have been found to be inhibited by ISG20 in cell culture (Table IV-1) with a few exceptions such as the severe acute respiratory syndrome coronavirus (SARS-CoV).

The antiviral activity of ISG20 was correlated to a decrease in viral RNA and to corroborate the importance of the RNase activity of ISG20 in its antiviral effects, a catalytic-deficient mutant (D94A) lost its capacity to interfere with viral replication. However, a deeper analysis of the literature indicates that the antiviral effect of ISG20 is not always correlated with a decrease in the amount of viral RNA in the cell, indicating that mechanism alternative to RNA degradation may be at play. Below, I have tried to separately describe results present in the literature for specific viruses.

Viruses	Family	Genome	Replication place	Cells/Inhibition	Reference
Adenovirus	<i>Adenoviridae</i>	dsDNA	Nucleus	HeLa/-	Espert et al, 2003
Severe acute respiratory syndrome coronavirus (SARS-CoV)	<i>Coronaviridae</i>	+ssRNA	Cytoplasm	Huh7.5/-	Zhou et al, 2011
Vesicular stomatitis virus (VSV)	<i>Rhabdoviridae</i>	-ssRNA	Cytoplasm	HeLa/+; PKR/RNase L/Mx ^{-/-} MEF	Espert et al, 2003
Influenza A virus (IAV)	<i>Orthomyxoviridae</i>	-ssRNA	Nucleus	HeLa/+	Espert et al, 2003
Encephalomyocarditis virus (EMCV)	<i>Picornaviridae</i>	+ssRNA	Cytoplasm	HeLa/+	Espert et al, 2003
Hepatitis A virus (HAV)				Huh7.5/+; HEK293/+	Zhou et al, 2011
Hepatitis C virus (HCV)	<i>Flaviviridae</i>	+ssRNA	Cytoplasm	HEK293/+; Huh7/7.5/+	Jiang et al, 2008; Zhou et al, 2011
West Nile virus (WNV)				HEK293/+	Jiang et al, 2010
Bovine viral diarrhoea virus (BVDV)				MDBK/+	Zhou et al, 2011
Dengue virus (DENV)				HEK293/+	Jiang et al, 2010
Yellow fever virus (YFV)				Huh7.5/-; HEK293/+	Zhou et al, 2011
Sindbis Virus (SINV)	<i>Togaviridae</i>	+ssRNA	Cytoplasm	MEF/+; RNase L / PKR ^{-/-} mice/+	Zhang et al, 2007
Human immunodeficiency virus-1 (HIV-1)	<i>Retroviridae</i>	+ssRNA	Nucleus (integrated)	CEM/+; PBMC/+	Espert et al, 2005

Table IV-1, Viruses inhibited by ISG20.

Viral genome: dsDNA, double-strand DNA; -ssRNA/ +ssRNA, negative/ positive single-strand RNA; Inhibition, -/+ , No / Yes.

Vesicular stomatitis virus (VSV)

(Espert et al, 2003).

Espert et al demonstrated that ISG20 inhibits VSV replication by suppressing viral RNA accumulation and viral protein production. The authors showed that both transient and stable express of ISG20 restricted VSV replication in HeLa cells, while the catalytic mutated D94G (ISG20m) displayed no protective effect and actually led to a significant enhancement of VSV replication. The drop in viral RNA accumulation in ISG20 infected cells supported the hypothesis that ISG20 acts by degrading viral RNA.

In addition to these tests, the authors also tested the antiviral activity of ISG20 in MEFs triply absent of three IFN induced antiviral factors, PKR, RNase L and Mx. In these cells, the ectopic expression of ISG20

led to unchanged anti-VSV replication kinetics, suggesting that the anti-VSV effect of ISG20 doesn't require the functional PKR, RNase L, or Mx pathway.

Human immunodeficiency virus-1 (HIV-1)

(Espert et al, 2005).

The effect of ISG20 on HIV replication was examined using a particular experimental setting in which ISG20 itself was inserted into the *nef* gene of a modified HIV-1 proviral pNL4-3 DNA. Recombinant viruses were produced from transfected HEK293, normalized and then used to infect target cells for replication kinetics analysis. The delayed replication kinetics of the recombinant HIV-1 virus expressing ISG20 was observed in both T cells and PBMCs, suggesting that the anti-HIV-1 activity was mediated by ISG20. Again, the restriction was dependent on the exonuclease activity of ISG20, since the replication kinetics of ISG20m-recombinant viruses was similar to controls. In this case however, ISG20 does not seem to alter transcription from the HIV-1 long terminal repeat (LTR) promoter indicating that in this case viral RNA accumulation was unaffected by ISG20.

Sindbis Virus (SINV)

(Zhang et al, 2007).

Sindbis virus is a member of *Alphavirus* genus of the family *Togaviridae*. *Alphaviruses* are sensitive to activated type I IFN responses or prior treated IFN α/β (Ryman et al, 2000; Despres et al, 1995). Searching for specific ISG that could mediate this effect, brought Zhang et al to examine several candidates including ISG20. Ectopic expression of ISG20 in mice significantly prolonged the survival time, and overexpression of ISG20 in mouse embryonic fibroblasts (MEFs) restricted the virus replication, while knock-down ISG20 in MEFs increased their permissivity to SINV replication. Indicating the important role ISG20 played in prevent mouse from SINV infection. Moreover, although ISG20 is contributed to IFN-mediated resistance against *Alphaviruses*, expression of directly delivered viral mRNA into such IFN- α/β -treated cells were inhibited without observation of RNA degradation. Thus, the inhibition of *Alphaviruses* expression was apparently not related with ribonuclease activity of ISG20 (Ryman et al, 2005).

Hepatitis C virus (HCV)

(Jiang et al, 2008; Zhou et al, 2011).

Although HCV can avoid triggering host intracellular innate response in hepatocytes via proteolytic processing of RIG-I/MDA5 adaptor protein MAVS and the TLR3 adaptor protein TRIF (Gale et al, 2005; Meylan et al, 2005; Li et al, 2005b), stimulation of these cells by both IFN and HCV infection promotes the up-regulation of a range of interferon (IFN)-stimulated genes (ISGs) including ISG20. These ISGs might therefore influence the course and outcome of HCV infection (Su et al, 2002; Bigger et al, 2004; MacParland et al, 2015; Lanford et al, 2006; Marcello et al, 2006; Jiang et al, 2008).

Two groups have investigated the anti-HCV effect of ISG20 after overexpressing in hepatic cell lines. By using HCV subgenomic replicons (genotype 1b), Jiang et al demonstrated that ISG20 significantly reduces the intracellular amounts of HCV RNA in a time-dependent manner, in contrast to the ISG20m (Jiang et al, 2008). A second study also examined the role of ISG20 in HCV replication and identified a clear antiviral effect. Also in this case, the antiviral effect seemed also dependent on exonuclease activity of ISG20, since the ISG20m lost its antiviral ability. However, in contrast to Jiang et al, this study didn't observe the decrease of HCV RNA (both JFH1 of genotype 2a and HCV-N of genotype 1b) by ISG20 (Zhou et al, 2011).

West Nile virus (WNV) and Dengue virus (DENV)

(Jiang et al, 2010).

WNV, DENV, yellow fever virus (YFV) and Zika virus that cause a recently outbreak, are all mosquito-borne viruses (genus *Flaviviruses*, family of *Flaviviridae*). By employing the similar strategies of investigation on ISG20 and HCV (Jiang et al, 2008). Restriction of WNV and DENV by overexpressed ISG20 were also observed. To determine which viral replication steps disrupted by ISG20, colony formation assay and replicon transient transfection assay based on mRNA transfection were applied to bypass the early steps, since transfected RNA can be directly translated or replicated (Lo et al, 2003; Puig-Basagoiti et al, 2005). ISG20, but not the mutant, significantly inhibited the formation of WNV positive colony. ISG20 selectively inhibited the later-phase expression of transfected DENV-1 Rluc mRNA which stands for translation of replicated replicons, but not the early-phase that responds to translation of input replicon RNA (Jiang et al, 2010; Puig-Basagoiti et al, 2005). Taken together, these assays indicated that ISG20 doesn't affect the early steps of infection, but rather inhibit steps in viral RNA and/or protein amplification of WNV and DENV.

Bovine viral diarrhoea virus (BVDV), Yellow fever virus (YFV), Hepatitis A virus (HAV), Encephalomyocarditis virus (EMCV), Influenza A virus (IAV), Severe acute respiratory syndrome coronavirus (SARS-CoV) and Adenovirus

(Espert et al, 2003 ; Zhou et al, 2011).

These viruses are introduced together here because the inhibitory effects of ISG20 were provided with no further information on the antiviral mechanism underlying inhibition.

In the paper authored by Zhou et al, five positive ssRNA viruses were, include HCV, BVDV and YFV of *Flaviviridae* family, HAV of *Piconaviridae*, and SARS-CoV from *Coronaviridae*. BVDV shares many similarities with HCV. Madin-Darby bovine kidney (MDBK) cells stably expressing human ISG20 also presented antiviral activity against BVDV similar to HCV. However, antiviral activity of ISG20 against YFV was cell-type dependent, stable expression of ISG20 in HEK293 cells, but not Huh7.5, inhibits the replication of YFV. Replication of both RNA replicon and cell culture-derived HAV-18f was also inhibited by ISG20 in both hepatic (Huh7.5) and non-hepatic (HEK293) cells.

By using the same stable cell line, Espert et al also observed the antiviral activity of ISG20 against EMCV and IAV. However, the inhibition observed in the case of IAV was less efficient than the one observed for

VSV, and even weaker/borderline in the case of EMCV. This work mainly studied on antiviral mechanism on VSV, they didn't go further on EMCV and IAV.

SARS-CoV is the representative virus of the family *Coronaviridae*, it caused an outbreak from 2002 to 2003 in Asia. Another member of this family, Middle East respiratory syndrome coronavirus (MERS-CoV) also spreaded in more than 20 contries recently. Experimental infection of SARS-CoV also induces the expression of ISG20 in lungs of macaques (de Lang et al, 2007) likely due to the raise of interferon responses. However, no inhibition of this virus was observed with presence of ISG20 (Zhou et al, 2011).

At present, Adenovirus is the only DNA bearing virus tested for ISG20. However, comparing with many RNA viruses inhibited by ISG20, no inhibition was observed in HeLa cells for a derivative adenovirus strain bearing a β -galactosidase reporter gene (Espert et al, 2003). Given that ISG20 is an exonuclease with higher RNA specificity, absence of antiviral effects on a DNA virus may not be totally unexpected.

Overview of the antiviral effects of ISG20

Evidence for an antiviral role of ISG20 is rather strong at present in the literature, given that the ectopic expression of this protein in different cell lines inhibits a large spectrum of viruses as discussed above. In addition, the fact that this protein presents a readily detectable RNase activity and that the exonuclease inactive mutant D94A is devoid of antiviral activity, concur in the hypothesis that ISG20 blocks viral replication by degrading the viral RNA.

While strong evidence exist for this model, antiviral effects of ISG20 have been shown also in the absence of degradation of viral RNA. In addition, if critically examined, the fact that during viral replication, less RNA is observed is criticizable. Indeed, if fewer cells are infected by the virus, an overall population will display less viral RNA, but this may or may not be related to direct RNA degradation by ISG20.

Besides, while it is true that the D94A mutant loses its antiviral activity, this mutation may disrupt other functions of the protein or even change its intracellular localization and so forth.

The results we have obtained indicate that the antiviral activity of ISG20 does not strictly correlate with its capacity to degrade RNA, as several mutants were constructed that lost their antiviral properties despite a robust RNase capacity *in vitro*. Instead, during VSV infection we have found that the major inhibition exerted by ISG20 resulted only in a very minor decrease in viral RNA but in a strong decrease in their translation. In a separate but convergent line of experiments, we have generated *isg20* knock-out mice. These mice are perfectly viable, which is expected in case of an IFN-regulated gene. When challenged with VSV *in vivo*, this mice display higher replication therefore confirming *in vivo*, the antiviral role of ISG20 in limiting viral spread.

Methods

Plasmids and reagents

Wild-type ISG20 was cloned with a N-term Flag-tag into a pcDNA3 vector. Mutants were produced by a two step overlapping PCR method, then re-inserted into a pcDNA3 vector as BamHI / XbaI fragments. Flag-ISG20s were also subcloned to pRetroX-Tight-Pur vector (pRetroX-Tight-Pur and pRetroX-TetON-Neo) to produce stable-inducible cells. For a detailed list of primers see Supplementary-table 1 and 2.

Flag-TREX1-pcDNA3, HA-Ubiquitin, SAMHD1-vsVg expressing plasmids were constructed by standard molecular biology techniques for this study by our laboratory. Luciferase reporters are gifts from the laboratory of Théophile OHLMANN (CIRI, ENS-Lyon), are listed in the table below. The following plasmids have been described before: viral mini-genome coding GFP (Naldini GFP); HIV-1 Gag-Pol (8.2); VSVg; replication-competent HIV-1 proviral clone X4-tropic NL4-3; HIV-1 Gag; destabilized GFP.

<i>Mono-cistronics</i>	
Reporters	5'UTR (Cap- or IRES-dependent)
pGlobin-intron-RLuc	Globin (Cap-)/ Renilla Luciferase
pGlobin-RLuc	Globin (Cap-)/ Renilla Luciferase
pcMyc-RLuc	cMyc (Cap- or IRES-)/ Renilla Luciferase
pIGF1R-RLuc	IGF1R (IRES-)/ Renilla Luciferase
pVSV-Rluc	VSV (Cap-)/ Renilla Luciferase
pHCV-Rluc	HCV (IRES-)/ Renilla Luciferase
pCrPV-Rluc	CrPV (IRES-)/ Renilla Luciferase

<i>Bi-cistronics</i>		
Reporters	5'UTR (first cistron)	5'UTR (second cistron)
Bi-Glo-Fluc-cMyc-RLuc	Globin (Cap-)/ Firefly Luciferase	cMyc (IRES-)/ Renilla Luciferase
Bi-Glo-Fluc-EMCV-RLuc	Globin (Cap-)/ Firefly Luciferase	EMCV (IRES-)/ Renilla Luciferase
Bi-Glo-Fluc-PV-RLuc	Globin (Cap-)/ Firefly Luciferase	PV (IRES-)/ Renilla Luciferase

For Western blot and immunofluorescence analyses, the following antibodies were used: anti-Tubulin (Sigma), anti-Flag (F7425, Sigma), anti-M2-Flag (Sigma), anti-N-term GFP (Sigma), anti-HA (Sigma), anti-Vsvg (Sigma), anti-Gag/p24 (clone 183-H5C from the AIDS Reagents Program of the NIH), anti-CEBP (Abcam), anti-mSin3A (Abcam), anti-RPS6 (Abcam), anti-Env (for WB: #ab21179, Abcam); anti-ISG20 (Proteintech), anti-Akt (Cell Signaling Technology).

Transfections

For DNA transfection, cells were grown in complete DMEM medium until 70-80% sub-confluence was reached. The total amount of DNAs for transfection in each 100mm dish, or each well of 6-well, 12-well and 24-well plate were 20µg, 4µg, 2µg and 1µg, respectively. Cells were collected at 24h or 48h post transfection. Unless indicated, all transfections were carried out by calcium phosphate/HBS in HEK293T

cells. When specified lipofectamine 2000 reagent (Invitrogen) was also used for cotransfection assays according to the manufacturer's instructions.

For mRNA transfection, HEK293T cells were resuspended in Eppendorf tubes and 100 000 cells were electroporated by Neon electroporator (Invitrogen) with 200ng of *in vitro* transcribed mRNA in 10µl of total volume (settings of 1150V, 20ms, 2 pauses). Cells were then transferred into 500µl pure RPMI medium (Gibco) and cultured for 1h in the cell incubator.

Establishing stable cell lines inducible by Doxycycline

pRetroX Tight plasmid allows the production of MLV retroviral vectors that are DOX-inducible. To produce them, ISG20s-pRetroX and Tet-ON-pRetroX were packaged into MLV virions by cotransfection with MLV-Gag-pol and VSV-G into HEK293T cells (according to Protocol-1). The virions were normalized by exogenous-RT activity and used to challenge cells by co-infection. Positive cells were selected by puromycin (Sigma) and G418.

HEK293T and HeLaP4 were cultured in DMEM high glucose medium plus 10% Foetal Bovine Serum (FBS) and 1% Penicillin/ Streptomycin (p/s). Jurkat and SupT1 were cultured in RPMI medium plus 10% FBS, 1% P/S, 1×Glutamine, and 1x Hepers. THP-1 was grown in similar medium of Jurkat with additional 1× 2-Mercaptoethanol and 1× MEM NEAA (minimum essential medium, non-essential amino acids). Jurkat, SupT1 and THP-1 were cultured in bottle. All cells were obtained from the CelluloNet facility of the UMS3444 Biosciences Gerland.

Exonuclease activity assays

Flag-ISG20s were transfected by calcium phosphate into HEK293T cells. Forty eight hours post transfection cells were collected and subjected to immunoprecipitation (IP). Immunoprecipitates were then incubated with exogenously provided nucleic acid (Protocol-2), prior to analysis on agarose gel and quantify by Multi Gauge V3.0 software (Fujifilm).

Confocal microscopy analyses

Stably transduced ISG20-HeLa cells were grown on coverslips and induced by DOX. Twenty-four hours post DOX treatment, cells were transfected with destabilized GFP for an additional 24 hours, prior to fixation and analysis. Cells on the coverslips were fixed by Formalin, permeabilized with PBS-0.5% Triton X-100, incubated with anti-Flag (F3165, Sigma) and anti-GFP (G1544, Sigma), then with donkey anti-rabbit IgG, Alexa Fluor® 594 conjugate (A-21207, Life) and donkey anti-mouse IgG, Alexa Fluor® 488 conjugate (A-21202, Life). DAPI-containing mounting medium was finally used (DAPI Fluoromount G, Southern biotech). Images were acquired using a spectral Zeiss LSM710 confocal microscope and analyzed with the Fiji software.

Luciferase assays

Luciferase reporter DNAs were cotransfected with ISG20s in triplicate in HEK293T cells (placed in 12-well plate). Twenty-four hours afterwards, cells were washed once with PBS and collected into Eppendorf tubes. One fifth of the cells were lysed and subjected to *Firefly* or *Renilla* luciferase assays (Luciferase Assay System or *Renilla* Luciferase Assay System, Promega; Mithras LB 940 Multimode Microplate Reader, Berthold). The remaining fractions of the cells were used for RNA extraction and quantitative real-time PCR (qPCR) assay.

Luciferase mRNA constructs were *in vitro* transcribed from the reporter DNAs by T7 promoter, then used to transfect ISG20s expressed HEK293T cells by electroporation (Neon Transfection System Kit, Invitrogen). For each condition, the mRNA was electroporated in quadruplicate and analyzed for luciferase analyses as well as RNA extraction and quantification.

When available in both monocistronic and bicistronic vectors, the *Renilla* luciferase was used to normalize for Firefly activity.

DNA/ RNA extraction and quantitative reverse-transcription PCR assay

DNA was extracted according to Protocol-3.

Cellular total RNA was extracted by Trizol Reagent according to the manufacturer's instructions (Invitrogen). Cytoplasmic and nuclear fraction were separated and purified by Trizol Reagent, according to Protocol-4. The extracted RNA was treated with DNase to remove the contaminated DNA. For RNA extraction of luciferase assay, the NucleoSpin RNA kit (Macherey-Nagel) was used according to the manual. The RNAs were reverse-transcribed to cDNA by qScript cDNA SuperMix (Quanta BioSciences).

DNA and cDNA from RNA were quantified by qPCR performed on a StepOnePlus Real-time PCR system (Applied Biosystems) using the FastStart Universal SYBR Green Master mix (Roche Diagnostics). *Actin* and *Hprt1* was used for normalization, the primers used for qPCR are listed in Supplementary-table 3.

VSV and HIV-1 replication in ISG20 expressing cells

VSV-GFP (Indiana serotype) was kindly provided by Joanna Brunel and Denis Gerlier (CIRI, ENS-Lyon). This virus expresses the GFP reported in fusion with the M protein and is replication competent. ISG20 stable cells were induced by DOX at optimized concentrations for 48h, then challenged with VSV-GFP. The percentage of GFP positive was determined at different time post infection by fluorescence-activated cell sorting (FACS) assay (BD Biosciences).

HIV-1 (strain NL4-3, lab-adapted, X4-tropic virus) viruses were obtained by transfection of the HIV-1 proviral clone NL4-3 into HEK293T cells. ISG20 stable HeLaP4 (stably expressing the CD4/CXCR4 receptors), Jurkat and SupT1 cells (T cell that already present the appropriate HIV-1 receptor/co-receptor) induced by DOX, or HeLaP4 transiently transfected with ISG20 were challenged HIV-1 NL4-3. Viral spread was measured through the accumulation of viral particles in the supernatant of infected cells every 2-3 days by exo-RT activity.

Translation assay

Transfected cells were washed and starved in 1ml/well Met-/Cys-free DMEM medium (#21013024, Gibco) for 1h. Media was then changed to 500µl fresh medium containing 20 µCi [³⁵S]Met/Cys for a pulse of 1h. Labeled cells were then lysed with RIPA buffer in the presence of protease inhibitors (Roche), centrifuged at 13 000 rpm for 10min to remove insoluble material and then immunoprecipitated with anti-M2-Flag beads (Sigma). After extensive washing, material was analyzed by SDS-PAGE gel and densitometric analysis (Typhoon 8600 system, GE healthcare).

For ISG20-HeLa cells, expression of ISG20 (and M1) was induced by 48h DOX-treatment. The cells was then directly starved and labelled by [³⁵S]Met/Cys, or pre-infected with VSV for indicated hours followed by the starving, labeling and the following steps. For both, IP were needless.

Generation of ISG20-knockout mice and infection of VSV in mice

ISG20-knockout mice were generated by clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (CRISPR/Cas). Briefly, a single ISG20 sgRNA was designed to target the mouse *isg20* ORF (5'-GATCACTAATACGACTCACTATAGGTGGGCCTCAAAGGGTGAGGTTTTAGAGCTAGAAAT-3'). *In Vitro* Transcription T7 Kit (TaKaRa) was used for transcription of both sgRNA and Cas9, the RNA products were purified and injected into mice (C57BL/6) zygotes. Screening was carried out by PCR on genomic DNA from F0 and F1 mice and sequencing. PCR products were ligated into T vector (TA cloning kit, TaKaRa) and sequenced using the primers of 5'-TTTCTGAGGGTCGCCAA-3' (Forward) and 5'-TGTACTTGTCATACAGGACT-3' (Reverse). The Cas9 endonuclease-mediated genome editing led to a deletion of 17 bases (₅₉AAAGGGTGAGTGGCCTC₇₅) that disrupt the mouse ISG20. All animal experiments performed according to the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and the Animal Investigation Committee of East China Normal University.

For *in vivo* studies of VSV replication, age- and sex-matched groups of littermate mice were peritoneally infected with VSV (1×10⁸ pfu per mouse) and mice were sacrificed 12 h after infection. Spleen, liver and lung were obtained from the mice, the total RNA was extracted and quantified by qRT-PCR.

Results

I. Construction of a large panel of ISG20 mutants and examination of their expression

All studies on ISG20 made use so far of a single ISG20 mutant (D94G). This mutation lays in the enzymatic site of ISG20 and as a result the D94G-ISG20 mutant is catalytically-deficient. Given that the antiviral activity of ISG20 was not always linked to RNase activity, we wondered whether a more extensive mutagenesis could lead us to appreciate/investigate the functions of ISG20 in a deeper manner.

To this end, we first performed a sequence alignment of ISG20 orthologues retrieved from 12 species: human, apes (Western gorilla, Bonobo, Sumatran orangutan), old world monkeys (Rhesus macaque and Golden snub-nosed monkey), rodents (mouse and rat), bovids (cattle and goat), dog and cat (Figure 1).

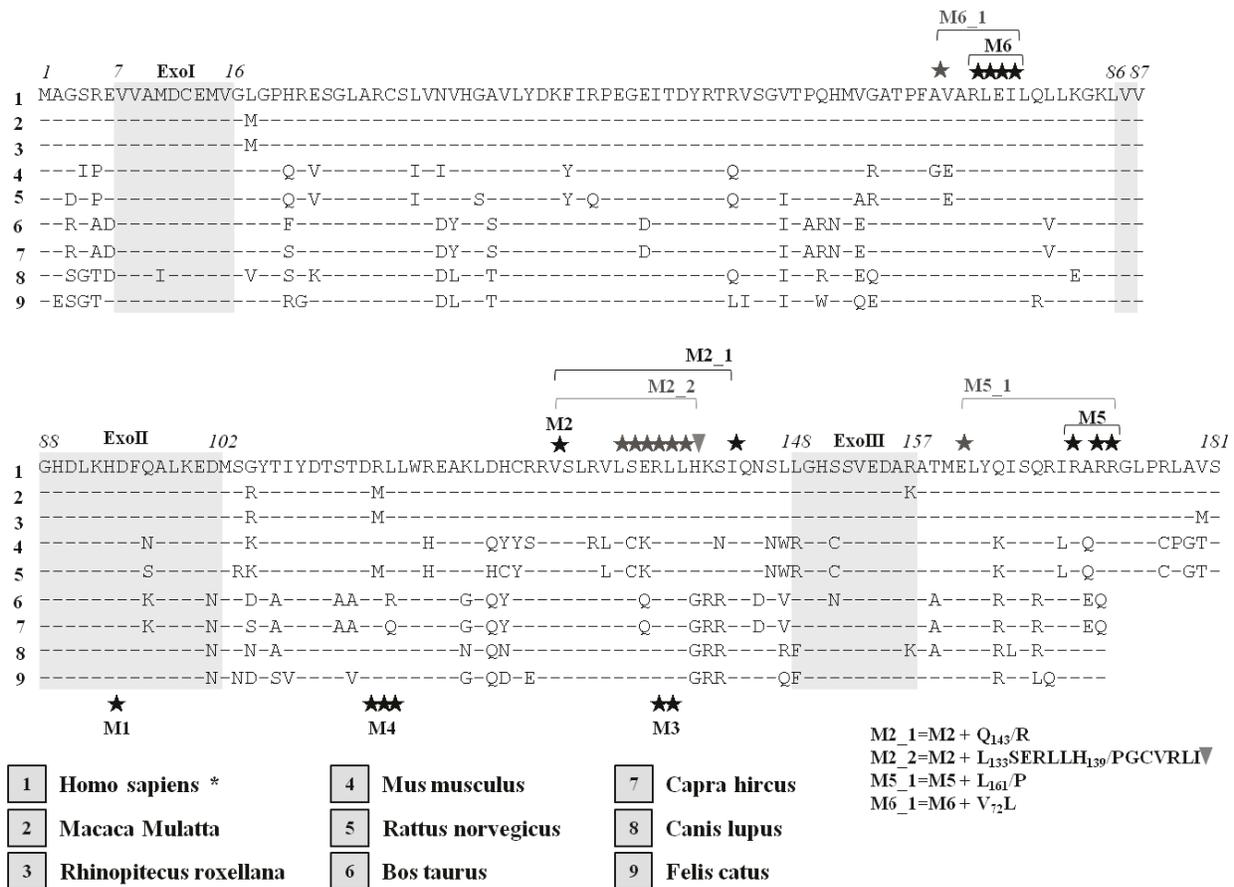


Figure 1. Alignment of ISG20 orthologues and mutants.

Amino acids sequence alignment of ISG20s orthologues of 12 species (shown 9 sequences of indicated species). *Gorilla gorilla, Pan paniscus and Pongo abelii orangu are 100 identical to homo sapiens. The hyphens represent identical residues, while the non-identical residues are specified. The three Exo motifs are highlighted in gray background.

The black stars (★) indicate sites we originally designed and all mutated to Alanine (A), we also obtained some by-product constructs that have additional mutated sites are indicated by grey stars (★) and detailed at the lower-right-corner. Inverted triangle (▼) of M2_2 indicates the site of termination.

The amino acids sequences of ISG20 are highly conserved especially in primates, in which they are close to 100% identical (75% when compared to mouse). Based on the alignment, we selected several mutants representing: fully conserved sites (M1, M2, M3 and M6), sites conserved in human and monkeys but not in mouse (M5), and conserved in human and mouse but not in monkeys (M4). They also differed from length of mutation, from single residue (M1 and M2) to four residues (M6) (Figure 1). Some of these mutations were chosen as they targeted potential trafficking sites (M5), or dileucines indicating trafficking from membranes (M6 and M4), or potential phosphorylation sites (M2). All targeted residues were mutated to Alanine (A) (Figure 2A). Several other mutations were then retrieved which however did not express correctly (see below).

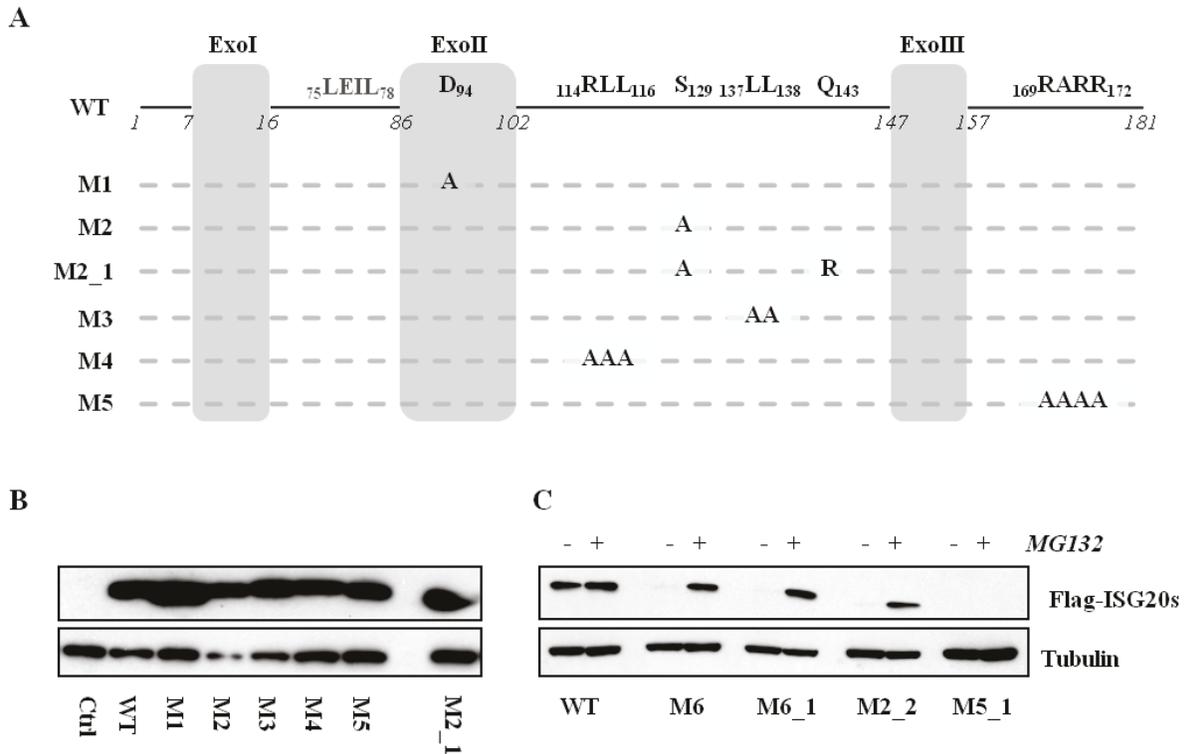


Figure 2. ISG20 mutants and their expression

(A) Graphical representation of ISG20 mutations we finally selected. Exo motifs are highlighted in gray. Indicated residues were all mutated to Alanine, except for mutant M2_1 which was obtained as a by-product of another mutant (M2) that presented an additional Q143R mutation. All ISG20s were fused with a Flag-tag at the N-terminus and cloned into a pcDNA3 backbone (Invitrogen).

(B) Expression of ISG20WT and mutants. HEK293T cells were transfected with ISG20s or pcDNA3 (control), the expressed proteins were analyzed by Western blot at 48h post transfection.

(C) The same procedure as in (C), but with a 14h treatment +/- MG132 prior to cells lysis.

Expression of these mutants was tested by transient transfection into HEK293T cells (Figure 2 A and 2B). Most of the ISG20 mutants exhibited good expression, although some were not expressed at detectable levels (M6, M6_1, M2_2, M5_1). To determine whether these mutants could be degraded in a proteasome dependent manner more rapidly or efficiently than the wild type, I performed the same transfections in the presence of MG132, which is a protease inhibitor (Figure 2C). Under these conditions, detectable expression could be observed for mutants M6, M6_1 and M2_3 indicating that these mutants were degraded via a proteasome dependent manner. However, the M5_1 mutant that contains an additional amino acid residue difference (L161P) with M5, was not detectable even in the presence of MG132 (Figure 2C). Although potentially interesting, these observations were not pursued further and my further studies focused on the mutants that could be detected.

II. ISG20 exhibits strong Ribonuclease activity *in vitro*

Like many members of DEDDh family, ISG20 was shown to possess both RNase and DNase activities *in vitro* (Nguyen et al, 2001). However, the antiviral specificity of ISG20 was confined to RNA viruses. We decided to characterize again the specificity of ISG20 for DNA, RNA, as well as DNA/RNA hybrid substrates. Instead of purifying ISG20 protein from transformed bacteria (Nguyen et al, 2001), our ISG20 with a N-terminal Flag-tag (Flag-ISG20) was immunoprecipitated (IP) from transfected cells with an anti-flag affinity gel. The protein, as well as its potential interactors, was then incubated with different nucleic acid substrates prior to analysis (Figure 3A and 3B). All double-strand poly I:C, ~100bp single-strand RNA and tRNA were significantly cleaved by ISG20, contrarily to single- or double-stranded, circular or linear DNA substrates (Figure 1B, S1B). Of note, no major RNA degradative activity could be noticed in cells expressing ISG20 at the total cellular RNA level including 28S, 18S and tRNA, indicating a form of regulation of the RNase activity of this protein in cells (Figure S1A).

Poly I:C is a well-known dsRNA that mimics viral dsRNA and is often used to induce antiviral responses and IFN signaling. Next, substrates were provided in the form of short oligos of different length in either RNA or DNA forms (20 or 15 nt) in single or double stranded form, blunted or protruding ends, as well as DNA/RNA hybrids (Figure 3C). Under our conditions, we found that RNA substrates were efficiently degraded irrespectively of their form, again contrarily to DNA. A decrease in RNA/DNA hybrids was also observed but more moderate than with complete RNA only molecules. This small decrease may be due to the removal of the RNA from the hybrid, although this possibility has not been verified further.

Next, we focused on the RNase activity of the ISG20 mutants that were engineered here on a ssRNA20 substrate (Figure 3D). Under these conditions, all mutants, with the exception of the ISG20-M1 presented strong RNase activity, although a slight decrease could be observed for mutant M4 (Figure 3D). Of note, mutant M2_1 possesses a mutation in residue Q143 that lines on the catalytic site of ISG20 (Figure S2C). However, this mutant is still capable of RNA degradation, at least *in vitro*. Taken together, these results suggesting that the RNase ability of ISG20 is powerful and highly conserved among most ISG20 mutants.

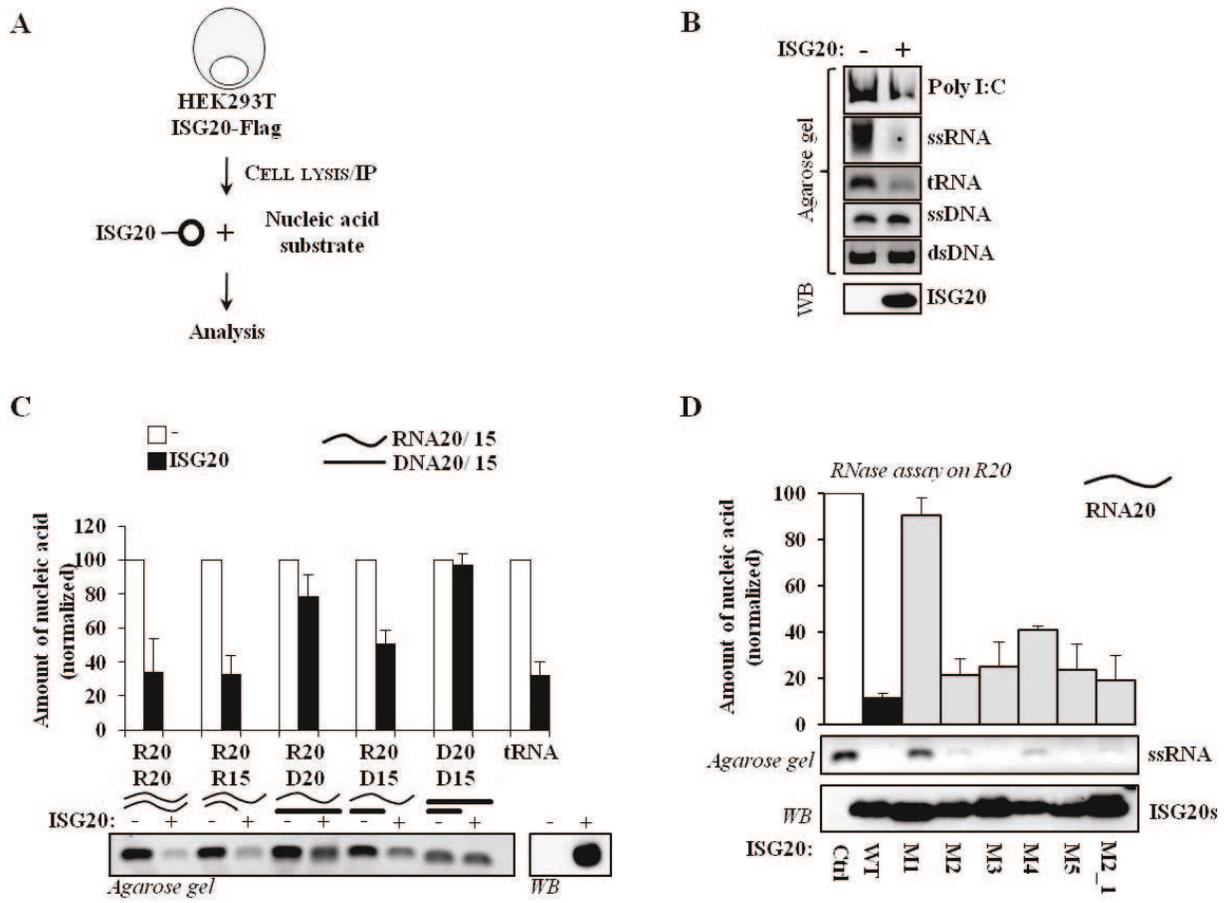


Figure 3. ISG20 is a Ribonuclease in vitro.

(A) Experimental procedure. HEK293T cells were transfected with Flag-ISG20 or pcDNA (control) for 48h. Flag-ISG20 protein was immunoprecipitated from cell lysis by the anti-flag affinity gel, then incubated with nucleic strands at 37°C for 30 minutes or 1 hour. The reaction was then run on agarose gel and analyzed by densitometry.

(B) Incubation of precipitated ISG20 with RNA or DNA substrates.

(C) 20 or 15nt DNA or RNA were designed as paired oligonucleotides. To obtain substrates presenting blunt or overhang ends, pairing was obtained after denaturation and annealing. tRNA served as a positive control.

(D) The same procedure as in B was applied to ISG20WT and mutants. A single strand RNA substrate was used (RNA20).

Pictures of representative agarose gels or WB are presented. -/+ represent control group transfected with pcDNA3 or treatment group transfected with ISG20 (Also applicable to the following figures unless otherwise indicated). Each panel represents one typical result from independent experiments, AVG and SEM.

III. The anti-viral properties of ISG20s do not strictly correlate with RNase activity

Previous studies on ISG20 already noticed that highly expressed ISG20 is toxic to cells and even bacteria bearing ISG20-expressing plasmids grew slower than controls. To bypass this problem, we established stable and doxycycline (DOX) inducible cells using retroviral-mediated transduction (MLV-pTightX-TetON) (Figure 3A). This procedure allowed us to obtain several cell lines with inducible and modulatable ISG20 expression. Several cell lines were therefore obtained (HeLaP4, Jurkat and THP-1 cells) and kinetics of induction and cell viability were established for each of them to determine the optimal conditions of expression of ISG20 (Figure 4B; Figure S2A for SupT1). At the concentrations of DOX chosen (from 0.1 to 10 µg/ml), the expression of ISG20 does not affect neither cell viability nor cell growth, allowing us to focus solely on its antiviral activities.

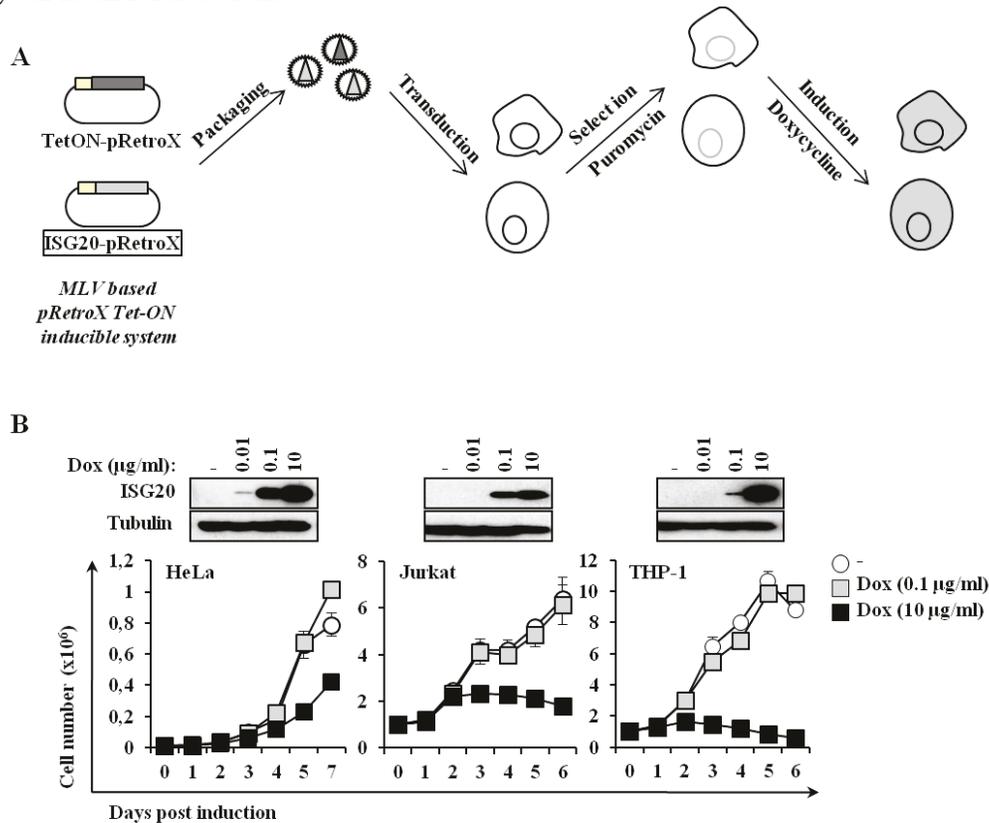


Figure 4. Establishment of ISG20 stable cell lines and optimization of expression conditions.

(A) Procedure used for the establishment of ISG20 stable and inducible cell lines. Flag-ISG20 (WT and mutants) was subcloned into MLV-based pRetroX plasmid. Both ISG20-pRetroX and Tet-ON-pRetroX were packaged into MLV retrovectors and then challenged cells by co-infection. Positive cells were selected with puromycin (or plus G418 in Jurkat, SupT1 and THP-1 cells). ISG20 was induced upon Doxycycline (DOX) treatment.

(B) Flag-ISG20 stable cell lines were induced by increased concentrations of DOX (0, 0.01, 0.1, 10 µg/ml), 48h later the expression of ISG20 were checked by WB. In concentrations of 0, 0.1 and 10 µg/ml, the growth of cells were analyzed by count cell numbers every day in one week. The curves were obtained from 2 independent experiments (n=2).

To this end, we challenged several cell lines in which the expression of ISG20 was induced with a replication competent VSV bearing a GFP reporter. As reported previously (Espert et al, 2003), the replication of VSV was inhibited upon expression of ISG20 in different cell lines (Figure 5).

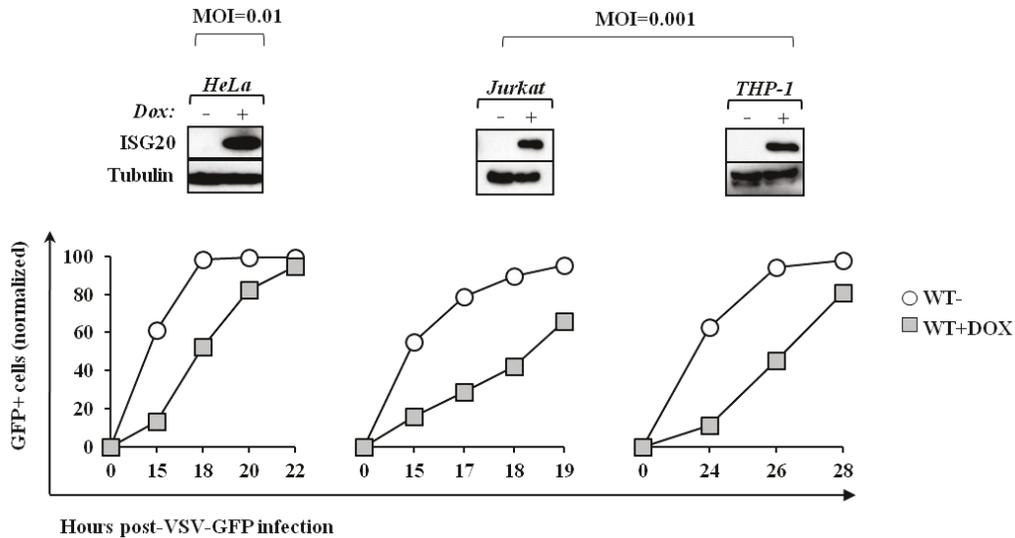


Figure 5. ISG20 restricts replication of VSV in several cell lines.

ISG20-stable cells were induced with DOX (1 μ g/ml) for 48h, then challenged by VSV-GFP viruses (with indicated MOI). Replication of VSV was quantified by measuring the GFP positive cells by flow cytometry at the indicated time points post-infection. Cells were collected at 48h after DOX treatment and subjected to WB to verify the expression of ISG20. The replication curves and WB represent one typical result of ≥ 2 independent experiments.

Next, ISG20 mutants were compared to WT in their ability to affect VSV replication (Figure 6A). Under these conditions, mutants M2 and M5 retained the similar anti-VSV activities than wild-type ISG20, while the remaining four mutants lost their restrictive properties. Of interest, in particular mutants M3, M4 and M2_1 display robust RNase capacities *in vitro*, despite loss of their antiviral phenotype. When analyzed for their location in the crystal structure of ISG20, two of these mutations impact a surface that is common and that may be of importance for a number of reasons such as protein localization, overall folding or even for the interaction with cellular partners (Figure 6C).

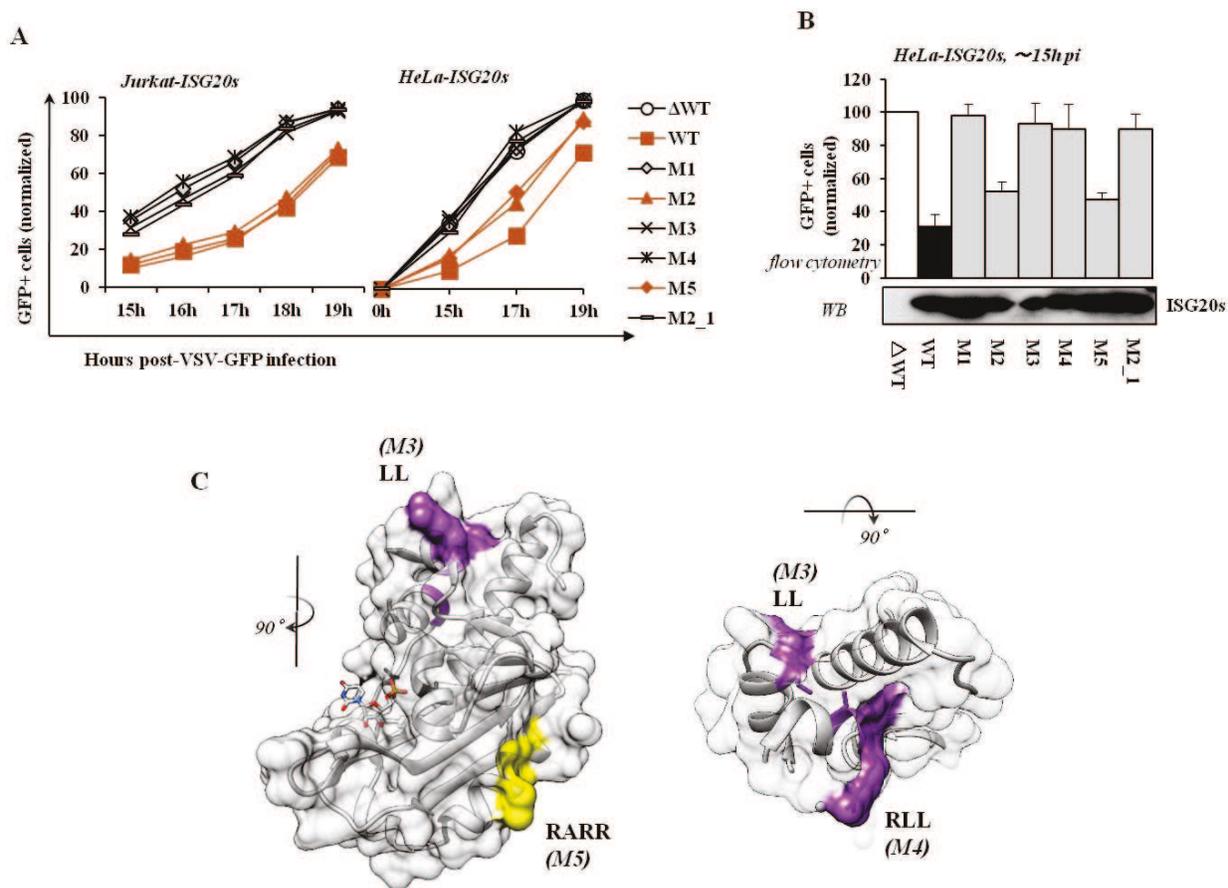


Figure 6. The anti-VSV abilities of ISG20 mutants.

(A) Stable Jurkat and HeLa cell lines inducible expressing ISG20 mutants were established as in Figure 3A. Cells were induced and infected in the same conditions as in Figure 3B. The replication curves of VSV were obtained from flow cytometry data ($n=2$).

(B) Data of one time point (Around 15h p.i.) from (A) was drew out and normalized by Δwt cells. Error bars represent the SEM ($n=3$);

(C) Structure of ISG20WT from two angles, the mutated sites of M3, M4 were depicted in purple, and the yellow one represent mutant M5. The model also depicts UMP at the active site of exonuclease domain.

IV. ISG20 Mutants display different intracellular distributions

Given that the replication of VSV takes place entirely in the cell cytoplasm, the different restriction abilities of the ISG20 mutants may depend on a different subcellular distribution. To investigate the potential correlation between VSV inhibition and subcellular localization, immunofluorescence assays were performed in HeLa stable cells upon DOX induction (Figure 7). WT ISG20 displayed a heterogeneous distribution that was already described in the literature. The ISG20 mutants analyzed here displayed changed intracellular distributions and in particular the mutant M2_1 became completely excluded from the cell nucleus. Despite these changes however, it was not possible to relate a particular distribution with the antiviral properties of ISG20, likely due to the fact that this analysis does not take into account the possible dynamics of ISG20. Therefore, this analysis underlines a broad intracellular presence of ISG20 within the cell, and indicates that multiple mutations in the protein can shift the distribution of ISG20 within the cell. At this stage, such changes could not be firmly associated to the protein antiviral properties.

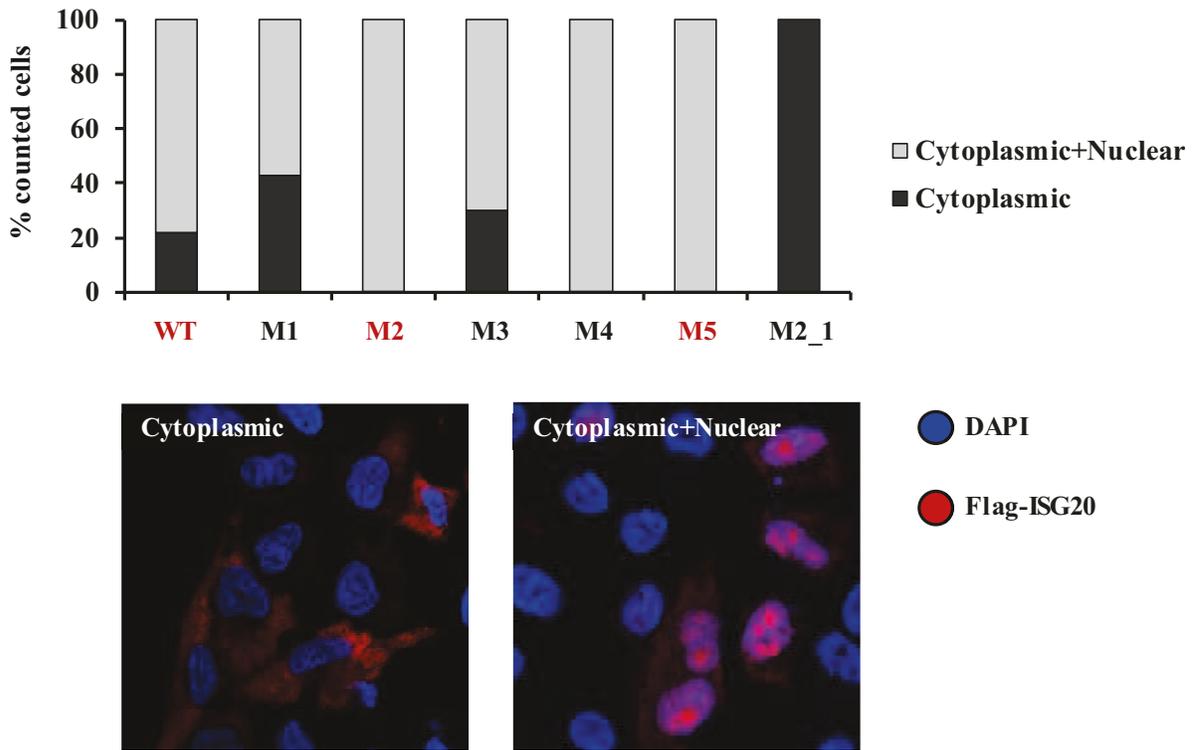


Figure 7. The subcellular localization of ISG20s.

Intracellular localization of inducible expressed Flag-ISG20s was examined by confocal microscopy in stable HeLa cells. The localizations were assigned into two types as indicated and represented as the merged photos in the lower panel. The percentages of 2 types in each ISG20 expressed cells were calculated from more than 100 counted cells per condition.

V. ISG20 blocks the expression of transfected DNAs through translational inhibition

During our experiments, we realized by immunofluorescence that there seemed to exist an exclusion between the presence of cells expressing WT ISG20 and GFP, upon co-transfection of DNAs coding the two proteins, or even in stable cells lines (Figure 8A). This was observed with WT IG20 and with another antiviral ISG20 mutant (M2), but not with an antiviral null mutant (M1), prompting us to investigate this further. When cells were co-transfected with WT ISG20 along with a reporter gene (GFP), we found a specific inhibition in the accumulation of GFP, but no difference in the accumulation of several endogenously expressed cellular proteins (Figure 8B). This observation was not dependent on the DNA transfection method, on the promoter used to express the protein or interest, on the presence or absence of introns, or on the kind of protein expressed (Figure S3A&B).

When cells co-transfected with DNAs coding GFP and ISG20 were analyzed, we did not observe notable changes in either DNA nor RNA levels, which could have been expected in light of the described RNase activity of ISG20 (Figure 8C). The finer separation of cell lysates in cytoplasmic and nuclear fractions also failed to reveal changes in the finer amounts of GFP-coding RNA as well as of small nuclear RNAs, that had been described to be associated to ISG20 (U1, U2, U3, Figure 8D).

Given these results, we turned naturally to translation. To determine whether ISG20 could act by affecting the translation of a reporter gene in the absence of changes in its mRNA, HEK293T cells were transfected with Flag-ISG20 along with a cellular protein of use in the laboratory (Flag-TREX1). After transfection, cells were metabolically labeled with [³⁵S]Met/Cys, then lysed and immunoprecipitated with anti-Flag beads, prior to SDS-PAGE analysis and phosphor-imager analysis (Figure 8E). Under these conditions, a strong decrease in the amount of reporter protein was observed in the presence of ISG20, indicative of a strong block in translation of the reporter in the presence of ISG20.

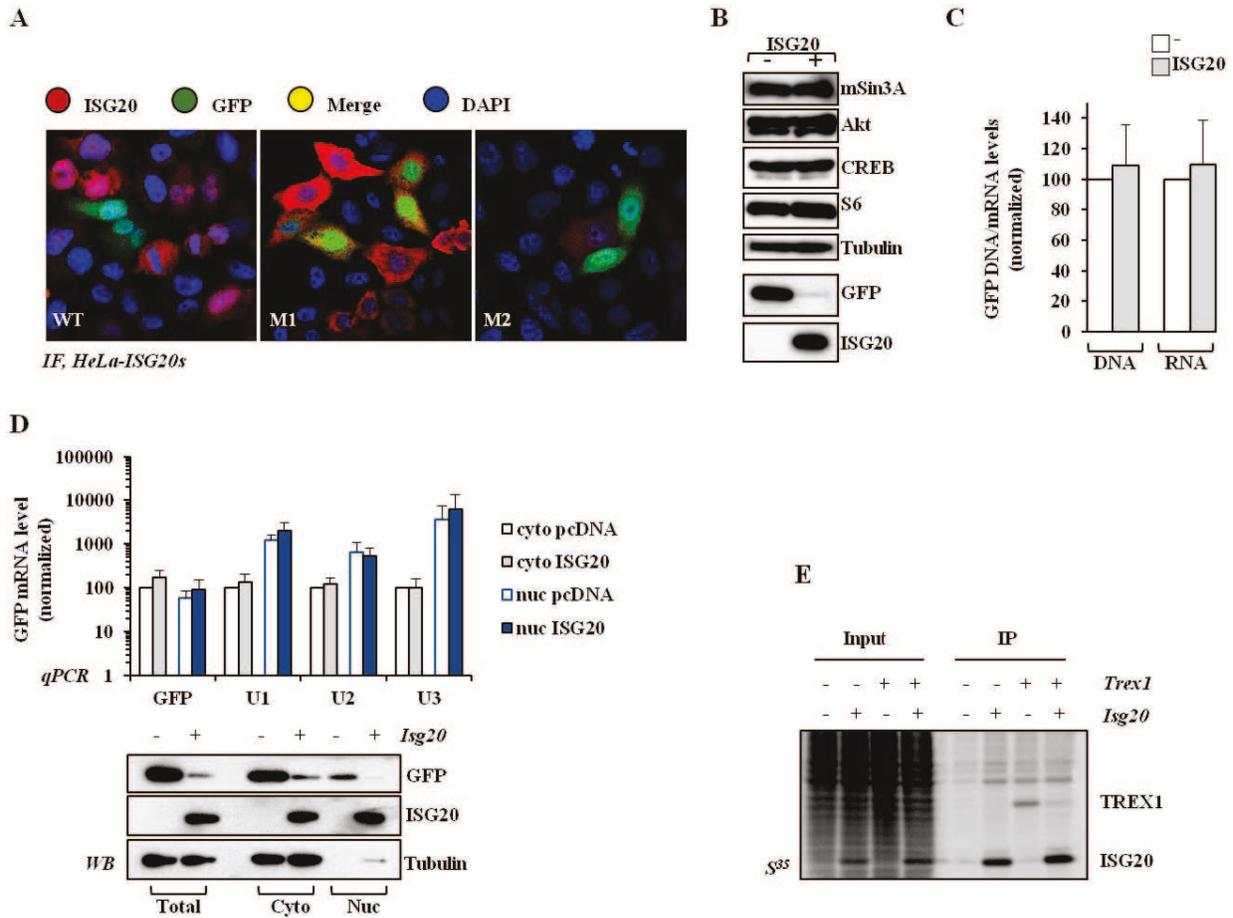


Figure 8. ISG20 inhibits the translation of transfected DNAs.

(A) HeLa stable cells bearing ISG20^{WT}, M1 or M2 were induced by DOX (1 μ g/ml), 24h later cells were transfected with GFP plasmid. Another 24h post transfection cells were fixed and subjected to immunofluorescence assay on ISG20, GFP and DAPI.

(B) HEK293T cells were cotransfected with Flag-ISG20 and destabilized GFP DNA, their expression as well as indicated cellular endogenous proteins were analyzed by WB ($n \geq 2$).

(C and D) HEK293T cells were cotransfected with ISG20 in addition of GFP for 48 hours. Cellular total DNA and RNA (C), or separated cytoplasmic and nuclear RNA (D) were extracted and analyzed by quantitative PCR (qPCR). Proteins of total lysate and separated fractions were analyzed by WB. U1, U2 and U3 are small nucleus or nucleolus RNAs which served as the indicators of nucleus-cytoplasm separation.

(E) HEK293T cells were cotransfected with Flag-ISG20 and Flag-TREX1, 48 hours p.t. cells were starved and labeled with [³⁵S]Met/Cys. The labeled cells were then lysed and immunoprecipitated by anti-Flag beads, proteins were separated in SDS-PAGE and visualized by a Typhoon 8600 system.

VI. The pattern of translational suppression by ISG20s completely overlaps the antiviral properties of the different ISG20 mutants

Since we demonstrated that ISG20 inhibits expression of cotransfected DNA at the translational level, we next determined the behavior of the different ISG20 mutants. Upon GFP cotransfection, mutants M2 and M5 retained a WT ability to inhibit the accumulation of the reporter protein, while the remaining mutants had lost it (Figure 9A). A similar effect could be observed using a different reporter protein (TREX1) and metabolic labeling (Figure 9B).

Taken together, these results indicate a perfect correlation between the antiviral properties of the different ISG20 mutants and the ability of these mutants to inhibit translation of reporter genes, strongly suggesting that the antiviral properties of ISG20 could relate to its ability to inhibit viral translation, rather than to degrade the viral RNA.

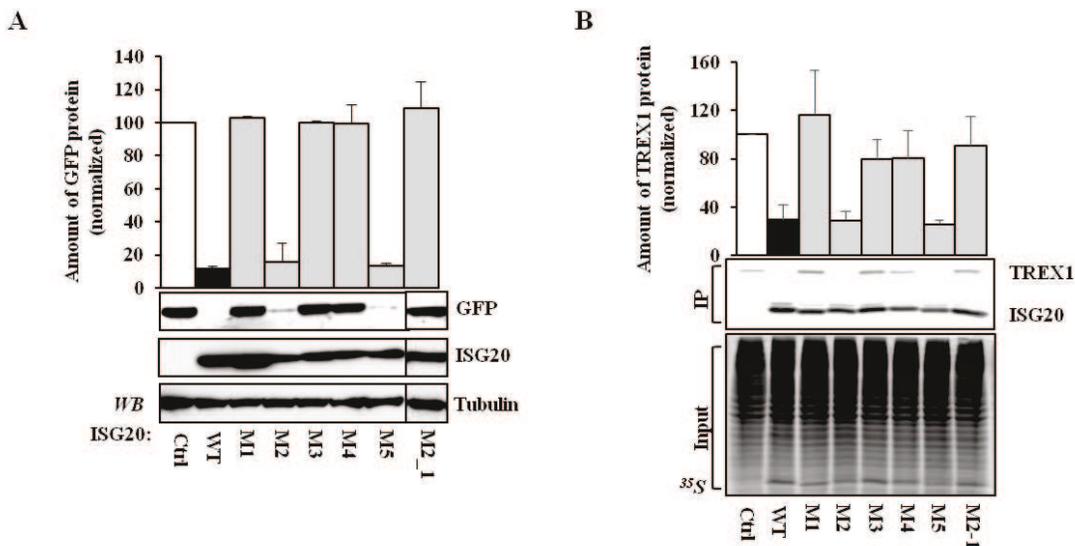


Figure 9. The translation suppression abilities of ISG20s are correlated with anti-VSV functions.

(A) HEK293T cells were cotransfected with GFP and ISG20 or mutants. 48h p.t. the expression of proteins was determined by WB, the amount of GFP protein was measured by density and showed in the column. The error bars indicate SEM of 3 independent repeats.

(B) The same procedure as in Figure 8E for all mutants, pcDNA3 and ISG20WT used as negative and positive control, respectively. The amount of TREX1 was measured by density and figured in the column ($n=3$).

VII. ISG20 results in a minor decrease of viral RNA but in a strong decrease of their translation

Next, we tried to determine whether ISG20 also affected the translation in the context of viral infection. To this end, cells stably expressing either WT or M1 ISG20 mutant were induced or not with DOX, then challenged with VSV-GFP virus for 16-18hrs prior to RNA extraction and quantification, WB analysis or [³⁵S]Met/Cys labeling according to the scheme provided in Figure 10A. In the presence of WT ISG20, a moderate reduction of the amount of gfp-coding mRNA was observed (Figure 10B). However, a more drastic effect was observed in the accumulation of either GFP or VSV-G proteins by WB. When cells were then metabolically labeled for 2 hours, a much stronger decrease in translation was observed (~10 fold, Figure 10C) in both GFP and VSV N protein confirming that translational inhibition by ISG20 occurred also in the context of viral replication. Of note, the moderate decrease in the amount of vRNA observed in ISG20-WT expressing cells could be due to the RNase activity of ISG20, or more simply from the fact that fewer cells are infected.

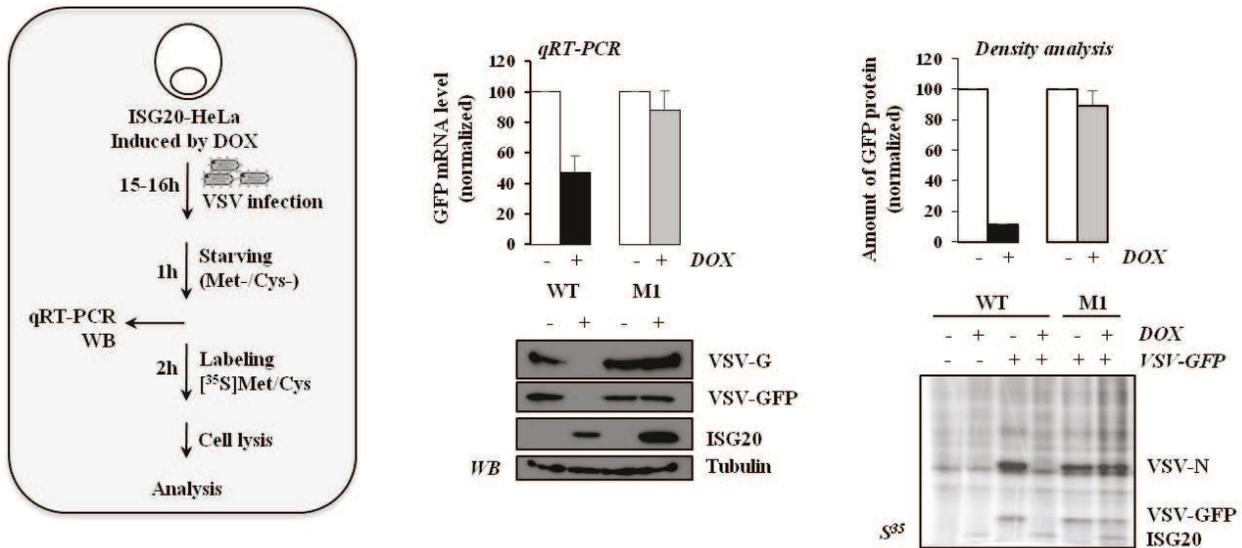


Figure 10: ISG20 results in a minor decrease of viral RNA but in a strong decrease of their translation.

(A) Experimental procedure. MycFlag-ISG20WT/M1 HeLa cells were treated with DOX (0.5 μ g/ml) for 48h to induce the expression of ISG20WT/M1 and then infected with VSV-GFP at MOI=0.1. At 16-18h p.i., cells were either harvested and analysed by qPCR and WB, or followed by starving and [³⁵S]Met/Cys labeling assay as described in Figure 8E, but without the step of IP.

(B) The mRNA level of virus carried Gfp and protein level of virus-encoded VSV-G and GFP were analyzed by qRT-PCR and WB, respectively. GFP mRNA level was normalized by cellular hprt1 mRNA (n=2).

(C) The translation of VSV-N, GFP and ISG20 were visualize through [³⁵S]Met/Cys labeling, and the amount of GFP was measured by density analysis, similar results have been obtained for amount of VSV-N (not shown, n=3).

VIII. ISG20 inhibits both cap- and IRESs-dependent translation

Translation can be schematically divided into initiation, elongation and termination. To determine whether ISG20 affected initiation, ISG20 was transfected with a series of reporter plasmids bearing either mono or bi-cistronic constructs and bearing different types of 5'UTRs. These 5'UTRs represent cellular/viral, cap-/IRES-mediated initiations, with different requirement of initiation factors (Figure 11A&B). In all the assays, luciferase activity was normalized by the amount of RNA. Under these conditions, ISG20 inhibited translation from both mono and bi-cistronic constructs (Figure 11C). The inhibition of IRES-driven reporters was less pronounced than in the case of the first cistron, however it was always observed. As expected the M1 mutant did not affect translation in all these cases.

The results obtained here indicate that translation initiation is likely not the major step affected by ISG20 as similar inhibitions are observed in the presence of 5' UTRs that rely on very different sets of initiation factors. To further support these results, ISG20 is also able to affect the translation from *in vitro* transcribed mRNA, directly transfected into ISG20-expressing cells (Figure 11D). These experiments are being repeated at the moment to lower the inevitable variability that we observe upon RNA transfection. However, the results displayed in the figure, clearly indicate that WT ISG20 also modulate translation of cytoplasmic RNA and this in the absence of notable variations in mRNA levels (Figure S4). Since no distinction of inhibition was observed among the panel of reporters displaying different translation initiation requirements, we tend to exclude a specific effect of ISG20 on translation initiation.

The future directions that may be taken to further pinpoint the exact molecular mechanism with which ISG20 interferes with translation will be presented in the Discussion section.

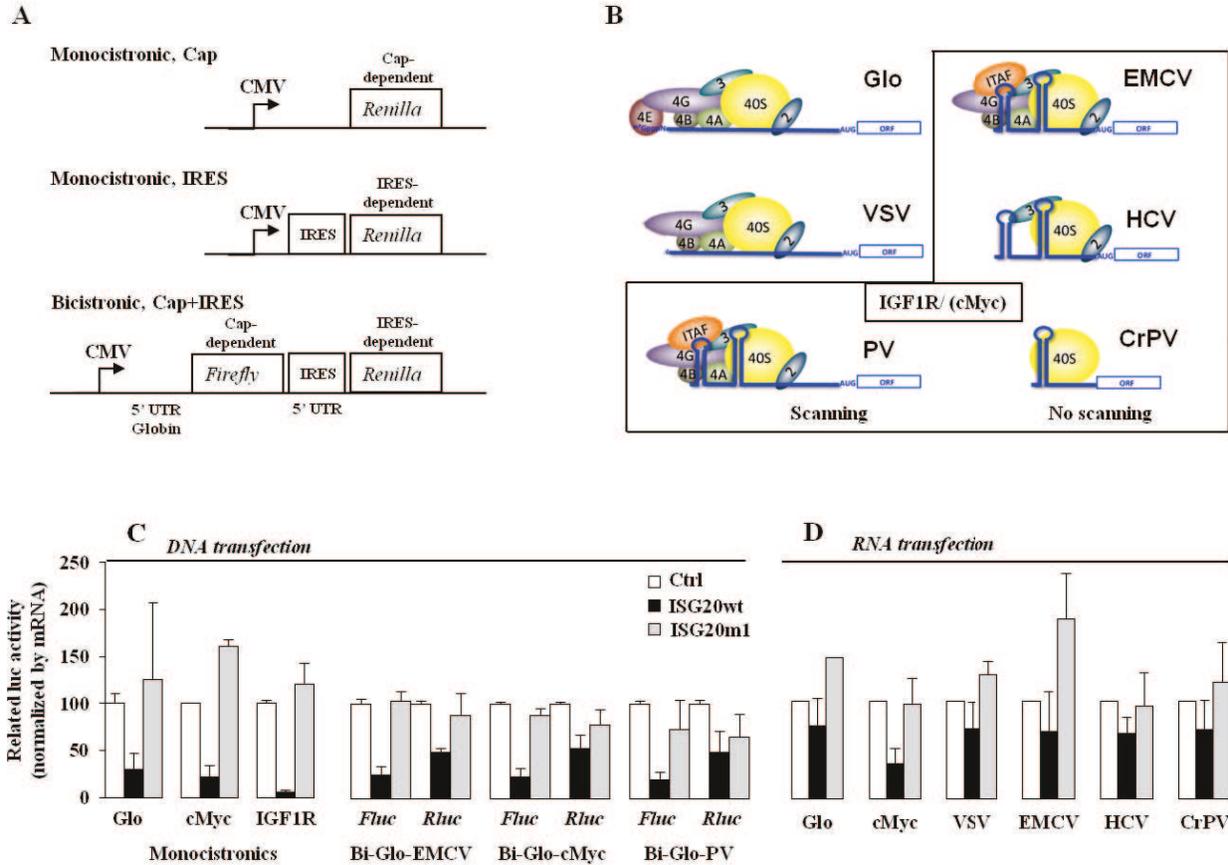


Figure 11. ISG20 inhibits both cap- and IRESs-dependent translation.

(A) Schematic representation of the mono- or bi-cistronic, cap- or IRES-dependent, Renilla (RLuc) or Firefly (Fluc) luciferase encoding reporters.

(B) Schematic representation of the initiation mechanisms of the 5'UTRs used in this assay (adapted from Picard-Jean et al, 2013). Inside the black frame are presented distinct IRES-dependent translation mechanisms.

(C) Indicated reporter DNAs was cotransfected with pcDNA (control), ISG20WT or M1 into HEK293T cells at a ratio of 1:7. Luciferase activities were measured 1 day p.t. and normalized for mRNA levels. Left panel, results of monocistronic reporters contain 5' UTR of Globin (cap), cMyc (cap/IRES), or IGF1R (IRES). Right panel, results of bicistronic reporters contain Fluc driven by 5' UTR of Globin (cap), and Rluc driven by IRES of EMCV, cMyc or PV.

(D) HEK293T cells were transfected with pcDNA (control), ISG20WT or M1. One day p.t., cells were resuspended in PBS 1× and transfected with mRNA of monocistronic cMyc, VSV, HCV, CrPV, or bi-Glo-EMCV, by electroporation. One hour p.t. cells were collected for luciferase assay or qRT-PCR assay. Luciferase activities were normalized by Rluc mRNA level.

For both DNA and RNA transfection, in each independent experiment, luciferase activity were averaged from triplicate repeats, while the Rluc mRNA levels were measured from duplicate (DNA transfection) and single sample (RNA transfection), respectively. The error bars represent more than 3 independent experiments.

IX. Generation of *isg20* knockout mice and susceptibility to VSV infection *in vivo*

To substantiate the antiviral role of ISG20 during viral infection, ISG20-knockout mice were generated by clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (CRISPR/Cas). Cas9 endonuclease-mediated genome editing led to a deletion of 17 bases (₅₉AAAGGGTGAGTGGCCTC₇₅) at the upstream of mouse ISG20 coding sequence (Figure 12A&B). Control and *Isg20*^{-/-} mice were challenged with VSV through peritoneal inoculation. The viral load in spleen, liver and lungs, as well as Hematoxylin and eosin staining were performed by our collaborators from my laboratory in China.

Isg20^{-/-} mice are fertile and viable as expected from the fact that ISG20 is an interferon regulated protein and therefore should not be expressed other than in the context of this specific anti-pathogen response. When challenged intraperitoneally with replicative VSV, knockout *isg20*^{-/-} mice displayed an accrued replication of VSV both in the spleen, liver and lungs (Figure 12D). VSV induced lung inflammation was also more severe in ISG20 knockout mice (Figure 12C). These results are in complete agreement with that from overexpression assays.

At present, we are characterizing more precisely the molecular mechanism underlying this inhibition by examining whether MEF of *isg20*^{-/-} mice display distinct translational abilities than control MEF.

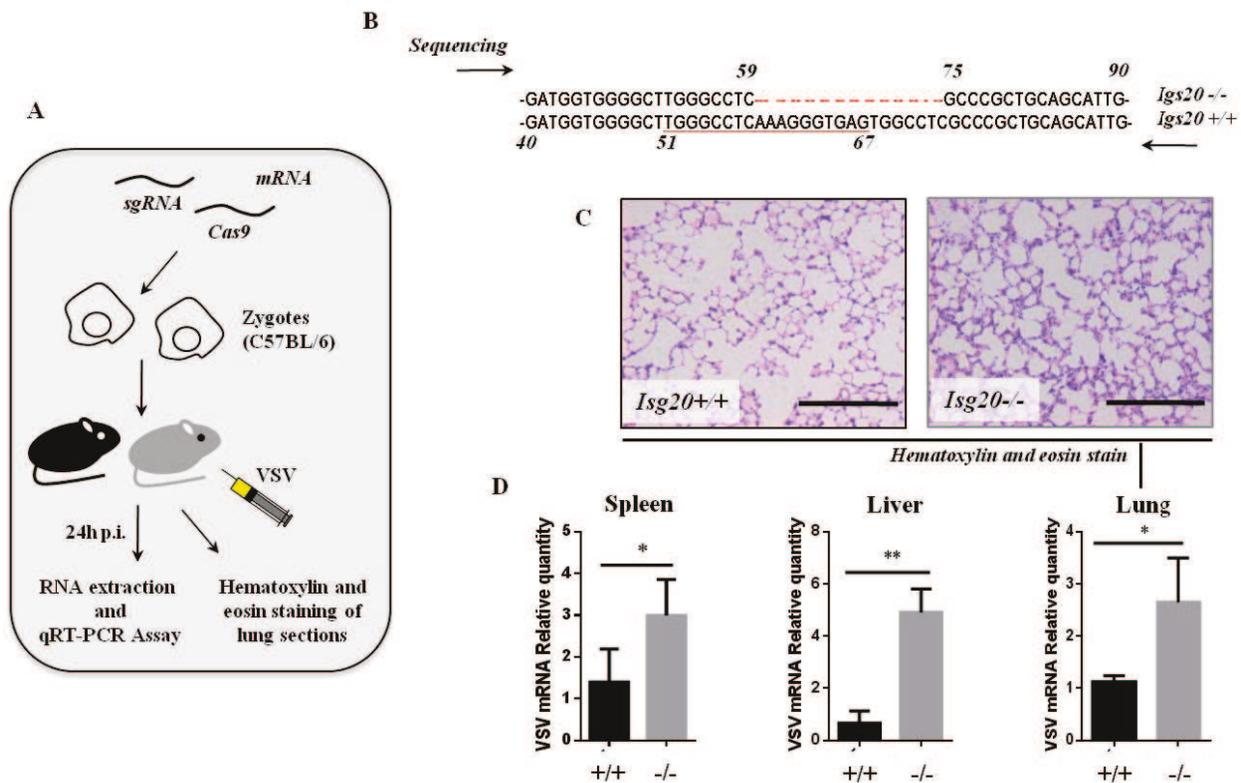


Figure 12. Enhanced replication of VSV in *ISG20*^{-/-} mice.

(A) Schematic representation of *ISG20*-knockout mice generation and VSV infection experiment. *In vitro* transcribed mRNA of *ISG20* target sgRNA and Cas9 were purified and injected to mouse zygotes, the KO mice were screened by DNA sequencing.

(B) Primers at the up- and down-stream of target sequence (underlined) were used for sequencing and the result revealed a deletion in KO mice genomic DNA are highlighted in red hyphens.

(C and D) Age- and sex-matched *Isg20*^{+/+} (n=3) and *Isg20*^{-/-} (n=3) mice were peritoneally injected with VSV (1×10^8 pfu/ mouse). Lung sections from infected mice were stained by hematoxylin and eosin. Viral load from spleen, liver and lung were quantitated by qRT-PCR analysis of VSV mRNA. Asterisks indicate statistical significance: ** $P < 0.01$, * $P < 0.05$.

X. ISG20 restricts HIV-1 replication in HeLa, SupT1 and Jurkat cells

To determine whether the antiviral properties of ISG20 could be observed also on other viruses, DOX-inducible cells were challenged with replication competent HIV-1. The extent of viral replication over days of infection was determined upon exogenous-reverse transcription activity. Under these conditions, HIV-1 replication was drastically and consistently reduced in the presence of ISG20 (Figure 13), indicating that the antiviral activity of ISG20 could broadly target different families of viruses.

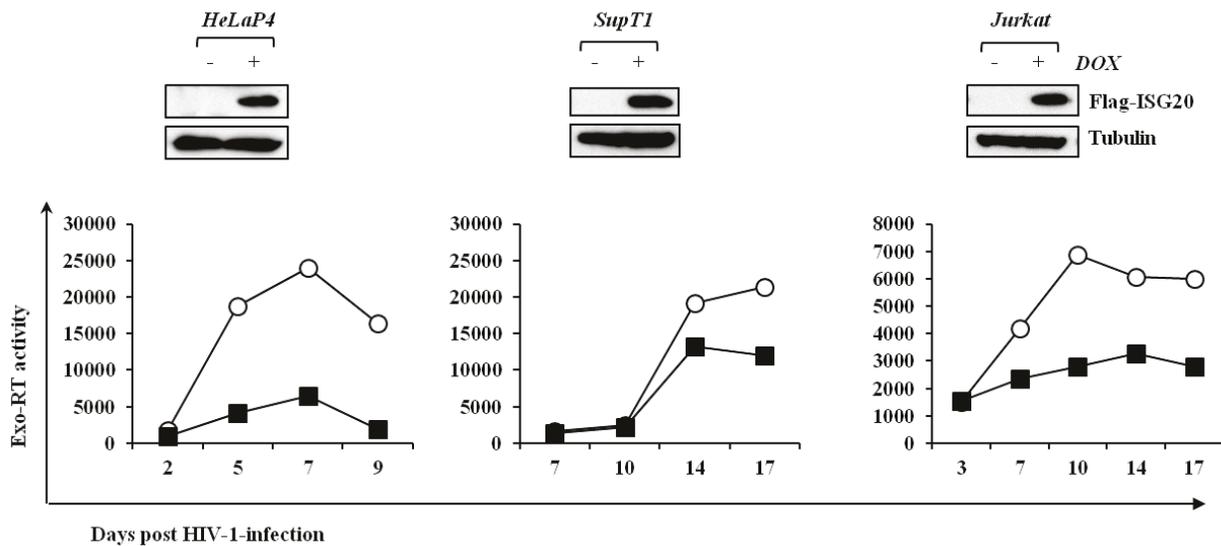


Figure 13. Overexpression of ISG20 restricts HIV-1 replication in HeLa, SupT1 and Jurkat cells.

HeLaP4, *SupT1* and *Jurkat* stable cells were induced by $1 \mu\text{g/ml}$ DOX for 48h, then challenged by HIV-1 replicative strain, NL4-3. The released virus from the supernatant of infected cells were monitored every 2-3 days, they were purified by ultracentrifugation through a 25% sucrose cushion, normalized by exogenous-RT (exo-RT). Induction of ISG20 by DOX was determined by Western blot. The replication curves show typical results from 3 repeats.

Discussion

In this study, we determine that ISG20 inhibits viral replication of VSV not by directly degrading the viral RNA but rather through a block in viral translation. The results we have obtained suggest that this mechanism of inhibition can potentially target diverse family of viruses as we have shown that ISG20 can also inhibit the replication of the HIV-1 retroviruses. The use of a large panel of ISG20 mutants allowed us to determine that the reported RNase activity of ISG20 is not the sole determinant of the antiviral activity of ISG20, indicating an antiviral mechanism likely more complex than previously expected. The fact that the antiviral properties of the ISG20 mutants tested here strictly correlated with the ability to inhibit translation reveals a novel mechanism that however remains to be further characterized. Finally, an *in vivo* experimental model (*isg20* $-/-$ mice) strongly corroborated the notion that ISG20 is an important player in the natural control of viral spread.

Up to ours, all the reported studies on ISG20 made use of a single point mutant, the catalytically inactive D94A (or G). This mutant is completely devoid of antiviral activity and such results were often taken as a proof that the exonuclease activity of ISG20 was the sole requisite for the protein's functions. The inclusion of a larger number of mutants, allowed us to determine that, although important, this activity is not the sole requisite necessary for the ability of ISG20 to block viral replication. Indeed, several mutants were identified that had lost their antiviral potential (M3, M4, M2_1) despite the presence of a strong RNase activity *ex vivo*. The fine analysis of the intracellular distribution of these mutants indicated changes among mutants although such changes could not be directly related to a phenotype. For instance, the M1 mutant loses its punctuated distribution in the nucleus (that is observed for about half of the cells expressing ISG20), the mutant M2_1 becomes overall excluded from the nucleus. However, mutants M4 and M5 display an identical distribution within the cell but are antiviral inactive and active, respectively. Therefore at this stage, the confocal microscopy analysis of ISG20 mutants that we have carried out must be regarded as revealing a complex and likely dynamic intracellular distribution of ISG20. Further studies will be necessary to formally demonstrate that the antiviral properties of ISG20 can be influenced by its specific intracellular distribution.

Our analysis however reveals that two mutants that have lost their antiviral properties map on a relatively narrow tridimensional surface, suggesting that this portion of the protein may be also important for the protein functions perhaps through the recruitment of additional cellular co-factors.

Overall, given that all our mutants display a robust RNase activity it is not possible to exclude the only enzymatic property of ISG20 as an important mechanism of antiviral inhibition. Our results however, exclude direct viral mRNA degradation as the major mechanism through which ISG20 functions.

In our study, we have not found evidence for a massive RNA degradation within the cell. This is intriguing because ISG20 presents a rather promiscuous distribution within the cell (i.e. it is present almost everywhere) and will have therefore large access (at least theoretically) to RNA in the nucleus or in the cytoplasm. The question of the specificity of the RNase activity of ISG20 has always been puzzling for studies that reported the direct degradation of viral RNA (why viral and not cellular RNA).

According to the data that we have gathered here, ISG20 does not degrade "*foreign*" mRNA in cells, but acts to diminish their translation. Several lines of evidence support this contention: no degradation is observed in mRNA of transfected plasmids or even transfected mRNAs, while a strong translational inhibition is present; only a moderate decrease in the amount of viral RNA is observed in the presence of

ISG20 during VSV infection. Of note, this small decrease can be caused by a lower number in infected cells (expected in this case) rather than by a true degradation of RNA.

The major inhibitory effect observed here occurs at translation. The use of several model mRNA displaying either cap-dependent or IRES-dependent translation seems to indicate that translation initiation is not affected by ISG20. At present, we ignore the exact step affected by ISG20, but experiments are ongoing to more precisely determine how ISG20 interferes with this process.

In particular, we are proceeding to ribosome profile experiments to further substantiate the translational inhibitory role of ISG20 by determining possible shifts in the distributions of reporter mRNAs in the presence/absence of this protein as well as determining whether ISG20 may be associated to a specific ribosomal fraction. The most interesting hypothesis will be that ISG20 blocks translation elongation. In the absence of further data it may be difficult to precisely pinpoint how precisely ISG20 works. However, one direction we will explore takes advantage of a previous study in the field of translation inhibition observed during VSV infection. VSV is a *Rhabdoviridae* whose cycle takes place entirely in the cell cytoplasm. The mRNAs of VSV appear to be translated according to a standard cap-dependent mechanism, which opens up to the question as to how cell host translational shut down can be observed at a certain point in virally-infected cells. It has been indeed shown that at late times post infection, most of the translational activities of the cell are diverted to the profit of the virus (i.e. on viral mRNAs). This mechanism of inhibition has not been the subject of intensive studies and is rather unexplained mechanistically.

A previous screen for ribosomal proteins that mediated the translation of VSV led to the identification of rpL40 as the sole required for the specific translation of this virus. This study also identified a certain number of cellular mRNAs whose translation was affected by the manipulation of the intracellular levels of rpL40 and suggested that under specific conditions, this protein may redirect or be used as fine translational regulator of specific classes of mRNAs and among them of viral RNA. One possible approach to explain the antiviral properties of ISG20 might be that ISG20 intersects rpL40 in a manner that impairs its recruitment in ribosomes that would be otherwise devoted to viral translation. At present we ignore whether this is the case. In the near future, we will clearly explore whether the antiviral effects of ISG20 can be reversed by the addition of increasing amounts of rpL40. In the likely possibility that this approach did not work, a future direction in the decortication of the antiviral mechanism of ISG20 will pass through the identification of the RNAs associated to this protein.

Which are the specificities of action of ISG20?

It is puzzling that we have been unable to identify possible specificities in the overall translation inhibition observed for ISG20 which poses the question as to how the cell distinguishes self from non self. At present, our working model is that this defense mechanism will firstly and more strongly interfere with those mRNAs that are the most abundant. During VSV infection, the extremely elevated levels of viral replication within the cell do cause the presence of a high amount of viral RNA that is translated. Our working hypothesis is therefore that ISG20 will block firstly whatever mRNAs are translating at higher levels (VSV ones as well as ectopically expressed genes). At a later time point translation inhibition may then hit also cellular genes and be likely responsible for the toxic effects observed in cells at high levels of ISG20. Under these

circumstances it will be of extreme importance to determine the fine regulation to which ISG20 is submitted during IFN responses to balance antiviral effects on one hand and cell survival on the other.

Finally, our study provided final evidence that ISG20 is important for viral modulation *in vivo* in a mouse KO experimental system. As expected from our experiments, the deletion of ISG20 increases viral replication and we are now using mouse embryonic fibroblasts (MEF) to determine whether, even in this case, inhibition of viral replication occurs at the translational level.

We believe this model will be of incredible use to further dissect the breadth and importance of ISG20 in antiviral responses directed against a large panel of pathogens.

Overall, the data presented here indicate that ISG20 is an important antiviral factor that inhibits translation and that can target at least two different families of viruses (*retroviruses* and *rhabdoviruses*). Given the apparent broadness of the mechanism through which ISG20 operates, it seems likely that ISG20 is also involved in the control of the replication of other viruses.

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Supplements

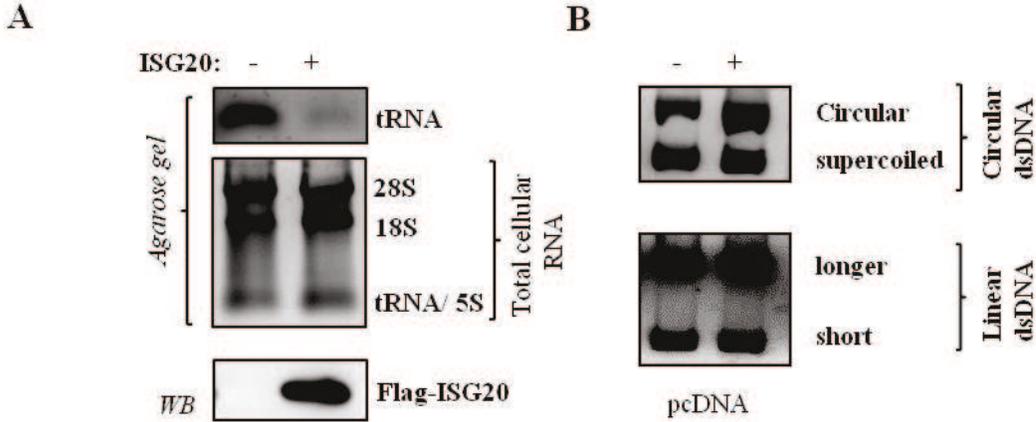


Figure S1. ISG20 doesn't digest DNAs or cellular total RNAs.

(A and B) Incubation of precipitated ISG20 with RNA or DNA substrates as described in Figure 3B. (A) Total cellular RNA was extracted from HeLa cells by Trizol reagent, and precipitated by isopropanol. The three bands from top to bottom represent 21S, 18S, tRNA/ 5S ribosomal RNA. (B) Circular and linear dsDNAs were circular pcDNA3 and pcDNA3 digested by PstI (two band, 4,8kb and 640bp).

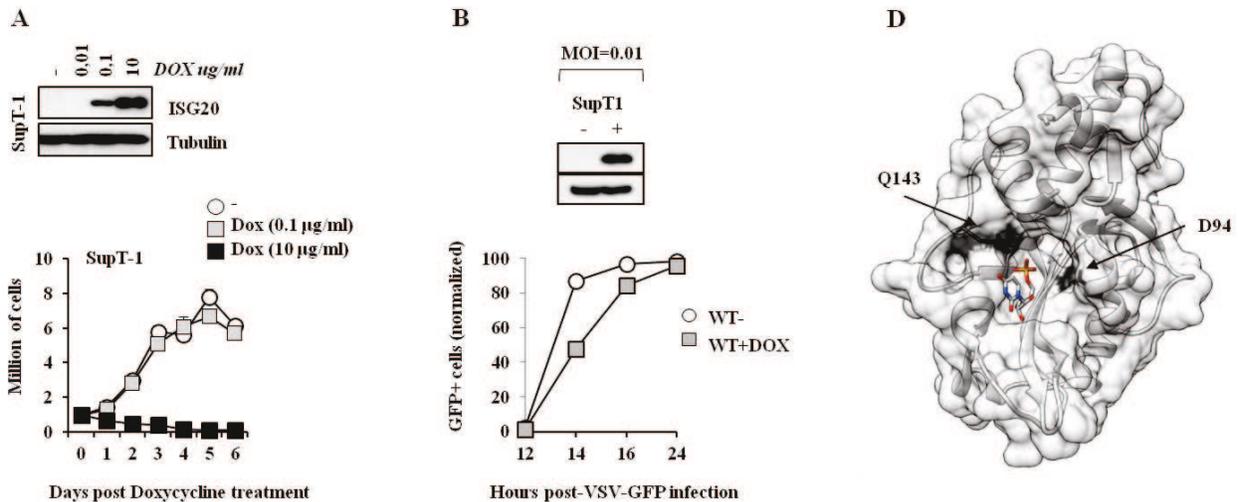
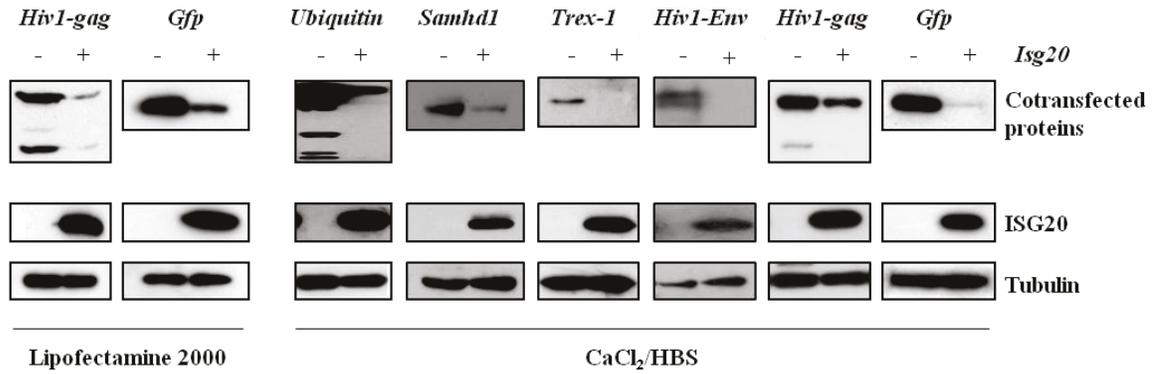


Figure S2. The anti-VSV abilities of ISG20s in SupT1 and Jurkat cells.

(A and B) Anti-VSV activity of ISG20 in SupT1 cells. (A) Optimized the DOX concentration for inducing ISG20 in SupT1 cells as described in Figure 4B. (B) ISG20-stable cells were induced with DOX (1 $\mu\text{g/ml}$) for 48h, then challenged by VSV-GFP viruses (MOI of 0.01) and followed the procedure as described in Figure 5 (n=2).

(C) Structure of ISG20WT from two angles, the arrows point at the residues of D94 and Q143, they locate at the opposite sites of exonuclease domain, the small molecular model was UMP.

A



B

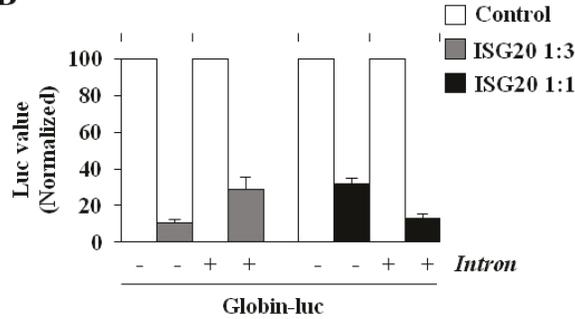


Figure S3. ISG20 inhibits expression of cotransfected DNAs.

(A) HEK293T cells were cotransfected with indicated plasmid and Flag-ISG20 (+) or pcDNA3 (-), 48h p.t. cells were collected for WB (n≥2). ISG20 plus destabilized GFP or HIV-1-Gag were also transfected by lipofectamine 2000 reagent in parallel, all rest were transfected by Calcium chloride/Hepes buffered saline method (n≥2).

(B) HEK293T cells were cotransfected with ISG20 and indicated reporters in ratio of 1:3 and 1:1, 24h later cells were lysed for luciferase assay. (B) Two reporters were used, both bearing luciferase gene under the cellular globin promoter, but one contains a cellular intron and another not (+/-). The data were analyzed from 2 independent experiments.

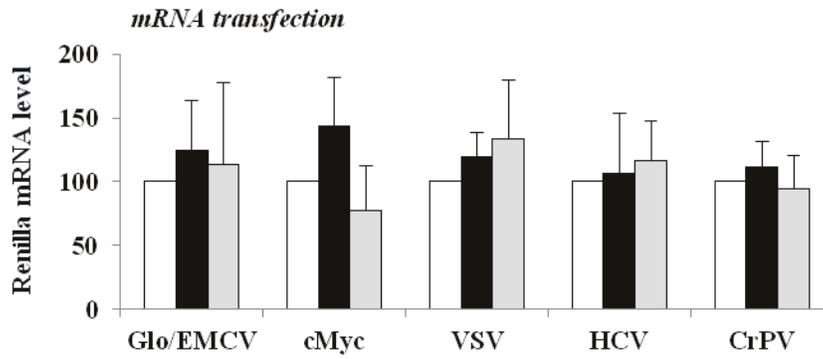


Figure S4. ISG20 leads to no significant change on RNA levels upon reporters mRNA transfection.

Corresponding to Figure 11D. For each independent experiment, each mRNA was electropotated in quadruplicate. The first one was kept for RNA extraction and quantification, while the rest three were went to luciferase analysis ($n \geq 3$).

Supplementary-table 1. Primers used for the cloning

Plasmids	Backbone	Digestion sites	Primes (5'-3')
Flag-ISG20s-pcDNA3	pcDNA3	BamHI XbaI	F: cgcgccggatccatggattacaaggatgacgacgataag R: cgcgctctagattaatctgacacagccaggc
Flag-ISG20s-pTightX	pTightX-pur	BamHI EcoRI	F: cgcgccggatccatggattacaaggatgacgacgataag R: cgcgcgaattcttaatctgacacagccaggc
Myc-Flag-ISG20s-pTightX	pTightX-pur	BamHI EcoRI	F: cgcgccggatccatggaacaaaaactcatctcagaagagg atctgggagattacaaggatgacg R: cgcgcgaattcttaatctgacacagccaggc

Supplementary-table 2. Primers used for introducing mutated sites into ISG20 cDNA

Primers of first step PCR:

D94AF: cctgaagcacg ctt ccaggcac	S129A F2: gcaggcgtg cc ctgcgg gtgc
D94AR: gtgctggaag gc gtgcttcagg	S129A R1: gcaccgcagg gc gacacgcctgc
LL138-9F: ggtgctgagtgagcgc gccgc gcacaaagc atccagaacagcc	
LL138-9R: ggctgttctggatgctcttg gcCggC gcgctcactcagcacc	
RLL114-6F: ctacgacagctcactgac ggcggc gtggcgtgagccaa gctgg	
RLL114-6R: ccagcttggcctcacgccac ggcggc gtcagtggacgtgctgtag	
RARR69-72F: ctatcaaatctccagagaatc gcagccgcgc agggctgccccgcctggc	
RARR69-72R: gccagggcgggcagccct ggcggc ggct gc gattctctgggagatttgatag	
LEIL76-9F: cacaccatttgcctggcca ggcagcggcgc gcagctctgaaagcaagc	
LEIL76-9R: gcttgccttcaggagetg ggcggc ctggccacggcaaatggtgtg	
The mutated nucleic acids are indicated in red bold letters.	

Primers of second step PCR:

F	BamHI Flag	CGCGCCGGATCCATGGATTACAAGGATGACGACGATAAG
R	XbaI	CGCGCTCTAGATTAACTCTGACACAGCCAGGC

Supplementary-table 3. Primers used for qPCR.

Primers	Sequences (5'-3')
GFP	F: gaacggcatcaaggtgaact; R: tgctcaggtagtggttgtcg
Renilla Luc	F: aggtgaagttcgtcgtccaacattatc; R: gaaacttcttggcaccttcaacaatagc
Hprt1	F: tgaccttgattttttgcatacc; R: cgagcaagacgttcagtct
Actin	F: tttcacggttggccttagg; R: aagatctggcaccacaccttct
U1	F: cttacctggcaggggagatacc; R: gcagtcgagttcccacatttgg
U2	F: gcttctcggccttttgctaag; R: caataccaggtc gatgcgtgg
U3	F: cgtgtagagcaccgaaaaccac; R: ctcccaatacggagagaagaacg

PROTOCOL-1. Virus production and purification

Adapted from Berger et al, 2011a

- 1) Cotransfection of MLV-Gag-Pol, ISG20s-pRetroX/ Tet-ON-pRetroX and VSV-G (8 μ g+8 μ g+4 μ g for each dish, 4 dishes of each) into HEK293T cells by calcium phosphate/HBS. Change medium 8-14h posttransfection.
- 2) Harvest the supernatants into 50ml falcon tubes, centrifuge at 2000 rpm for 10min, then filter (0,45 μ m) and transfer to a Becton ultracentrifuge tube.
- 3) Add 5 ml 25% TNE-sucrose into the bottom of the Becton tube with a Pipette Pasteur.
- 4) Equal the Becton-tubes by add PBS 1 \times , centrifuge at 28,000rpm, 4 $^{\circ}$ C for 1h30-2h00.
- 5) Remove the supernatant and resuspend the viral pellet with 200 μ l RPMI 1640 medium (add MgCl₂ 100mM/ml; dNTP 100mM/ml), scratch the bottom to detach the pellet and mix by pipetting. Shake slowly at 4 $^{\circ}$ C for 4h to overnight.
- 6) Collect the virions and stock in -80 $^{\circ}$ C freezer

Viral titers were normalized by Exo-RT activity before use.

PROTOCOL-2. *In vitro* Exonuclease activity assays

- 1) Transfection of pcDNA (control) / ISG20s (4µg in 6w plate) in HEK293T.
- 2) Two days posttransfection, wash cells twice by PBS, detach cells with Trypsin and collect them in separate eppendorf tubes.

ALL ON ICE FOR THE IP

- 3) Lyse cells in 600µl buffer SD-150mM-NaCl (with proteasome inhibitor). Sonicate, centrifuge 10 min to 12 000 rpm at 4°C.

In the meantime, prepare tubes contain 20µl M2-flag beads (Sigma) equilibrated with SD-150mM-NaCl buffer.

- 4) Collect the supernatant containing soluble proteins, transfer to tubes containing the equilibrated M2-flag beads. Incubate 2-3h at 4°C nutating.
- 5) Rinse 3 times with buffer SD, centrifuge at 2000rpm, 4°C, 5 min. Then resuspend in 25µl of buffer SD + 0.5mM MnCl₂ (add last to avoid precipitation)
- 6) Add the desired Nucleic Acid (1µg RNA/ 0,5µg DNA), incubate at 37°C for 30 or 60min.
- 7) Add 10µl RNA migration buffer or DNA migration buffer into each tube, mix and denature at 65°C for 5min and analyze by agarose gel migration, keep the beads for WB assay.
- 8) Quantify the nucleic acid bands by density (Multi Gauge V3.0 software, Fujifilm).

For conjugation of hybrid DNA/RNA

The oligos were ordered from Eurogentec (sequences see the table below), then diluted with water to 1µg/ul. Double helices were obtained by mixing the desired oligos in buffer SD-150mM-NaCl+0.5mM MnCl₂, prior to denaturation at 95°C for 5 min, and annealing at 50°C for 5 min.

RNA U 20nt	U	caaaugcauccuguacaugu			
RNA D 20nt	D	acauguacaggauugcauuug	DNA D 20nt	D	acaTgTcacaggaTgcaTTTg
RNA D 15nt	D	uacaggauugcauuug	DNA D 15nt	D	TacaggaTgcaTTTg

Buffer SD-150mM-NaCl:

50mM Tris pH7.4; 150mM NaCl; 0.5%Triton X100; Add proteasome inhibitor (Roche) one pill per 10ml

RNA migration buffer

50% Formamide (liquid); 25% Glycerol; 20mM Na₃PO₄ pH 7; 0.1% Bromophenol blue (powder)

PROTOCOL-3. Extract DNA from cultured cells

(Pre-treat cells with RQ1 DNase to remove plasmid DNA outside of cells)

For 100 000 cells

1) Resuspend cells with

100µl TpA (50mM KCl, 10mM TrisHCl pH8.3, 2.5mM MgCl₂)

+100µl TpB (0.5% NP-40, 0.5% Tween 20, 2.5mM MgCl₂)

+2.4µl protease K (PK) at 10mg/ml.

Incubate at 60°C for 1h to overnight.

2) Purify DNA by phenol/chloroform

- Add 200µl phenol/chloroform into each tube, vortex 20s
- Centrifuge 15min at max at room temperature (RT)
- Move the upper phase to the new tube (about 200ul), add 20ul NaAc 3M pH 5.3
- Add 220µl cold isopropanol, mix by invert several times
- Centrifuge 15min at max at 4°C
- Wash the pellet with 1ml cold 70% ethanol (EtOH)
- Centrifuge 10min at max at room temperature (RT)
- Dry the pellet, add 50µl H₂O to each tube
- Measure the concentration of DNA by Nanodrop 2000 (Thermo Scientific)

PROTOCOL-4. Cytoplasmic and nuclear RNA fractionation

- 1) Transfected cells are analyzed 48 hours after transfection.
- 2) Collect cell pellets and carefully resuspend in 175 μ l of cold RLNa lysis buffer, 5min on ice
- 3) Centrifuge lysate at 1200 g, 4°C for 5 min. Transfer supernatant (cytoplasm) to a new tube, add 1 ml of Trizol for RNA extraction, keep 10ul for WB assay.
- 4) Wash the pellet twice with 200 μ l of RLNa to eliminate residual cytoplasm.
- 5) Resuspend the pellet (nucleus) with 200 μ l RLNa, sonication on ice for 10s, then add 1ml of Trizol for RNA extraction, keep 10ul for WB assay.

RNA extraction and purification.

- Add 200 μ L of Chloroform
- Vortex (approximately 15 seconds)
- Spin at 4°C, 12 000G for 15 minutes
- Remove approx 500-600 μ L of clear aqueous phase and put into a clean eppendorf tube
- Add 500 μ L of Isopropanol, Vortex
- Leave on bench for 10 minutes
- Spin at 4°C, 12 000g for 10 minutes
- Remove supernate (approx 900 μ L)
- Add 900 μ L of 70% Ethanol
- Spin at 4°C, 12,000G for 5 minutes
- Remove Supernate carefully (approx 900 μ L)
- Leave on bench for 5-10 minutes to dry the Ethanol
- Add 20 μ L RNase free water
- Store at -80°C

RLNa (1 ml):

- 910 μ l Water
- 10 μ l Tris HCl (ph 8.1M); final: 10 mM
- 2 μ l NaCL (5M); final: 10 mM
- 60 μ l MgCl₂ (50 mM); final: 3 mM
- 5 μ l NP40; final: 0.5%
- 10 μ l DTT (100 mM); final: 1 mM (add at this moment)
- 2.5 μ l RNasin Plus (40 U/ul, Promega); final: 100U/ml (add at this moment)

Annexe

RESEARCH ARTICLE

The Frequency of Cytidine Editing of Viral DNA Is Differentially Influenced by Vpx and Nucleosides during HIV-1 or SIV_{MAC} Infection of Dendritic Cells

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Citation: Nguyen X-N, Barateau V, Wu N, Berger G, Cimarelli A (2015) The Frequency of Cytidine Editing of Viral DNA Is Differentially Influenced by Vpx and Nucleosides during HIV-1 or SIV_{MAC} Infection of Dendritic Cells. PLoS ONE 10(10): e0140561. doi:10.1371/journal.pone.0140561

Editor: Jean-Luc EPH Darlix, Institut National de la Santé et de la Recherche Médicale, FRANCE

Received: July 23, 2015

Accepted: September 28, 2015

Published: October 23, 2015

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Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by the Agence Nationale de la Recherche sur le Sida et les hépatites virales (ANRS), by the Fondation de la Recherche Médicale (FRM) and by Sidaction.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Two cellular factors are currently known to modulate lentiviral infection specifically in myeloid cells: SAMHD1 and APOBEC3A (A3A). SAMHD1 is a deoxynucleoside triphosphohydrolase that interferes with viral infection mostly by limiting the intracellular concentrations of dNTPs, while A3A is a cytidine deaminase that has been described to edit incoming vDNA. The restrictive phenotype of myeloid cells can be alleviated through the direct degradation of SAMHD1 by the HIV-2/SIV_{SM} Vpx protein or else, at least in the case of HIV-1, by the exogenous supplementation of nucleosides that artificially overcome the catabolic activity of SAMHD1 on dNTPs. Here, we have used Vpx and dNs to explore the relationship existing between vDNA cytidine deamination and SAMHD1 during HIV-1 or SIV_{MAC} infection of primary dendritic cells. Our results reveal an interesting inverse correlation between conditions that promote efficient infection of DCs and the extent of vDNA editing that may reflect the different susceptibility of vDNA to cytoplasmic effectors during the infection of myeloid cells.

Introduction

Circulating blood monocytes differentiate into macrophages and dendritic cells (DCs) in tissues, where they play instructive roles in adaptive immunity [1]. These properties make them appealing targets for pathogens such as primate lentiviruses that use them to spread to other cell types and to derail proper antiviral responses [2,3,4].

The infection of myeloid cells by primate lentiviruses is however hindered by a strong restriction that limits vDNA accumulation during the early phases of the viral life cycle [5,6,7,8,9,10,11,12,13,14,15]. A key player of this restriction is the sterile alpha motif-hydroxylase domain 1 protein (SAMHD1, [6,7,14]), a dGTP-dependent deoxynucleotide

triphosphohydrolase that maintains dNTPs at concentrations that are limiting for efficient reverse transcription [5,16,17,18,19,20]). Although data obtained from several laboratories indicates that SAMHD1 inhibits lentiviruses by modulating dNTPs levels, recent data suggests that this may not be the sole antiviral mechanism at play [21,22,23,24].

The lentiviral infection of myeloid cells is also affected by members of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 family (A3s) present in target cells and in particular by A3A, that leads to decreased accumulation of vDNA presenting accrued signatures of cytidine deamination [25,26,27].

A3s are single-stranded DNA-editing enzymes that can functionally inactivate retroviral genomes through extensive mutagenesis by promoting the deamination of cytidines to uracils (reviewed in [28,29]).

In the most commonly described and efficient mechanism of antiviral inhibition, A3s are packaged into viral particles in virus-producing cells to then deaminate vDNA as it forms during the following cycle of infection, when single-stranded DNA intermediates become transiently available. Under *wild type* conditions however, this mechanism of antiviral inhibition is counteracted by Vif, a non-structural viral protein that forces A3s degradation via the recruitment of an E3-ubiquitin ligase complex (reviewed in references [28,29]).

An additional and less studied mechanism of A3s-mediated viral inhibition seems to operate in myeloid cells. In these cells, data from our laboratory as well as others suggest that the pool of APOBEC3s present in target cells may directly influence vDNA accumulation and edit incoming vDNA [25,26,27,30]. At present, it remains unclear whether this low level of vDNA editing is directly antiviral, whether it correlates with the efficiency of infection and how it can be overall modulated.

In the study presented here, we have explored whether conditions that favor the efficient infection of primary human monocyte-derived dendritic cells (DCs) by either directly removing SAMHD1 (via SIV_{MAC} Vpx), or by counteracting its action on dNTPs (via dNs supplementation) could modulate the extent of vDNA cytidine deamination from the pool of A3 molecules present in target cells following HIV-1 or SIV_{MAC} infection.

Material and Methods

Cell culture and antibodies

HEK293T and HeLa cells were cultured in DMEM supplemented with 10% FCS. Human monocyte-derived immature dendritic cells (DCs) were obtained upon incubation of purified blood monocytes for 4 to 6 days in complete RPMI1640 plus GM-CSF and IL-4 at 100ng/ml (Eurobio). Briefly, human blood monocytes were first enriched from total white leukocytes by centrifugation through two consecutive gradients, the first on Ficoll and the second on Percoll. The monocyte-enriched fraction was then further purified following a negative selection procedure that removed contaminating T, B and NK cells (according the manufacturer's procedure, Miltenyi). This procedure routinely yields a monocyte cell population with purity around 92–95%, according to [31].

Primary human blood cells were obtained from discarded “leukopacks” (at the EFS of Lyon), the cells discarded from platelet donors. As leukopacks are obtained anonymously, gender, race, and age of donors are unknown to the investigator and inclusion of women, minorities or children cannot be determined. This research is exempt from approval. Written informed consent was obtained from blood donors so that their cells could be used for research purposes.

Anti-SAMHD1 (Ab67820) and anti-EF1 α (clone CBP-KK1) antibodies were respectively purchased from AbCam and Millipore. The anti-A3A antibody (clone ApoC17) was obtained through the AIDS Reagents and Reference Program of the NIH.

DNA constructs and viral production

HIV-1 and SIV_{MAC} derived retroviral vectors have been described before [8]. All vectors shared the same design and contained the same CMV-*gfp* sequence. For each virus, GFP-coding retroviral vectors were produced upon calcium phosphate transfection of HEK293T cells with DNA plasmids coding: the structural viral proteins Gag-Pro-Pol, the envelope derived from the Vesicular Stomatitis Virus protein G (VSVg) and a mini viral genome devoid of viral open reading frames, but bearing a CMV-*gfp* expression cassette (the respective ratio of the plasmids is 8:4:8, for a total of 20 µg per 10cm plate). Non-infectious SIV_{MAC} VLPs bearing Vpx (VLPs-Vpx) were similarly produced omitting the viral genome [31]. Virions were purified from the supernatant of transfected cells by ultracentrifugation at 28,000rpm for 2 hours through a 25% sucrose cushion and were then resuspended and normalized by infectious titers (on HeLa cells) or by protein content against standards of known infectivity (exo-RT activity). This assay measures the ability of virion-associated reverse transcriptase to incorporate ³²P-dTTP in an exogenous RNA:DNA substrate constituted by a poly-rA matrix and by an oligo dT primer [31].

Infections and flow cytometry analysis

Infections were carried out for 2 hours with multiplicities of infection (MOI) comprised between 0.5 and 1 on 1x10⁵ cells prior to flow cytometry analysis 3 days afterwards, or on 3x10⁵ cells for PCR. Nucleosides (dNs, SIGMA) were added at 500 µM during infection starting from 6 hours prior to infection for a total of 24 hours. This represents the optimal concentration to obtain a positive effect on lentiviral infectivity in DCs, as we previously published in [32]. To measure the kinetics of accumulation of functional viral genomes over time following infection, we employed an experimental setup that we have described in the past [33]. Briefly, reverse transcription is arrested through the addition of RT inhibitors at different times post infection prior to flow cytometry analysis 3 days after the initial infection. AZT/ddI were routinely used at 10 µg/ml each for both HIV and SIV. In some experiments, Nevirapine was used in the case of HIV-1 also at 10 µg/ml. No differences were observed when HIV-1 kinetics were performed in one or the other drug, so that the results were pooled together. All compounds were obtained from the AIDS Reagents Program of the NIH. For flow cytometry analysis, cells were analyzed three days after infection and the proportion of infected GFP-expressing cells was directly determined on a FACS Canto II (BD Biosciences). Dead cells were routinely excluded from the analysis by propidium iodide staining.

Quantitative PCRs and direct 3D-PCRs

Infections were carried out with viral preparations treated twice for 2 hours at 37°C with 20 U/ml RNase free DNase RQ1 (Promega). DNA extracted from infected cells was treated overnight with DpnI to remove plasmid DNA contaminants. Control infections were carried out in the presence of RT inhibitors. qPCRs were conducted twenty-four hours after infection using oligonucleotides specific for *gfp* carried by the viral genome (corresponding to full length viral DNA: GAACGGCATCAAGGTGAACT and TGCTCAGGTAGTGGTTGTCG). vDNA was then normalized for the amount of cellular DNA (actin: TTTTCACGGTTGGCCTTAGG and AAGATCTGGCACCACCTTCT). Quantitative PCRs were performed on a StepOnePlus Real-time PCR system (Applied Biosystems) using the FastStart Universal SYBR Green Master mix (Roche Diagnostics).

The protocol followed for *differential DNA denaturation PCR* (3D-PCRs) was modified with respect to the one previously described in [34], as follows. Infections of DCs were carried out with viral preparations treated as described above. Then, identical amounts of sample DNA were directly amplified using different denaturation DNA amplification temperatures

comprised between 94°C and 82°C using GoTaq (Promega) on a BioRad Thermal Cycler T100 for 35 cycles. Routinely, DNA obtained after amplification from fraction 4 representing the fraction amplified with the lowest denaturation temperature reproducibly detected (89°C) was cloned and individual colonies sequenced. In rare cases, sporadic amplification of vDNA from fraction 5 (denaturation temperatures of 87°C) was detected and in this case sequenced. For each donor, a minimum of 10 sequences x condition were routinely analyzed. For the PCR reaction, the following primers matching on *gfp* were used: forward, CAARCTRACCC-TRAAGTTCATC; reverse GTTGTGGYTGTTGTAGTTGTA where R = G/A and Y = C/T; yielding a 319 base pair fragment. Similar deamination frequencies were observed using distinct primers in GFP (data not shown).

Results

Effects of Vpx and dNs on the infection of DCs by HIV-1 and SIV_{MAC}ΔVpx viruses

To explore the potential relationship existing between the extent of vDNA deamination from cytoplasmic A3s and the efficacy of infection, DCs were challenged with a multiplicity of infection (MOI) of 0.5–1 of HIV-1 or SIV_{MAC} virions devoid of Vpx (SIV_{MAC}ΔVpx) in the presence or absence of nucleosides (dNs, added from 6 hours prior to infection to 18 hours afterwards, as in [16]), or virion-like particles bearing SIV_{MAC} Vpx (VLPs-Vpx, at an MOI-equivalent of 0.5, as determined by exogenous-RT assay against standards of known infectivity) and then either analyzed by flow cytometry or by 3D-PCR, as schematically presented in Fig 1A.

As well documented in the literature, VLPs-Vpx and dNs exert a positive effect during the infection of DCs: the former by directly forcing SAMHD1 degradation [6,7,14], while the latter by increasing the intracellular levels of dNTPs and therefore by overcoming the dNTPs triphosphohydrolysis antiviral activity of SAMHD1 [5,16,17,18]. However, while both Vpx and dNs have been shown to increase HIV-1 infectivity, dNs seem to fail to rescue the infectivity defect of a Vpx-deficient SIV_{MAC} virus [32,35], indicating possible mechanistic divergences between the action of Vpx and dNs during primate lentiviral infection.

Indeed as we and others have already described [32,35], while both Vpx and dNs exerted a positive effect on HIV-1 infectivity (from an average of 0.5–1% of GFP-positive cells for HIV-1 alone to an average of 30–40% and 15–25% in the presence of Vpx and dNs, respectively), only VLPs-Vpx but not dNs rescued the infectivity defect of SIV_{MAC}ΔVpx (Fig 1B, from an average of 0.01% to around 5–10%, in the presence of Vpx).

To determine the effects of infection on the intracellular levels of both SAMHD1 and A3A, cells were lysed twenty-four hours after viral challenge for WB analysis (Fig 1C), DCs undergoing infection displayed a drastic reduction in the amount of SAMHD1 upon co-treatment with VLPs-Vpx, but not dNs. In contrast, while infection with both HIV-1 and SIV_{MAC}ΔVpx (only HIV-1 is shown in the Fig) yielded to a small but reproducible increase of A3A in comparison to uninfected cells, co-treatment with VLPs-Vpx led to a dramatic increase in the intracellular levels of A3A. In light of the data presented later on in this study, we believe this is due to the detection by the cell of the dramatic accumulation of vDNA that occurs when infection is carried out in the presence of Vpx.

Next, standard PCR was used to examine the mutation rates present in vDNA synthesized during DCs infection under the different conditions. To this end, the classical differential DNA denaturation PCR (3D-PCR) protocol was modified, so that instead of two rounds of PCR [34] vDNA was directly amplified by 3D-PCR. vDNA corresponding to a denaturation temperature

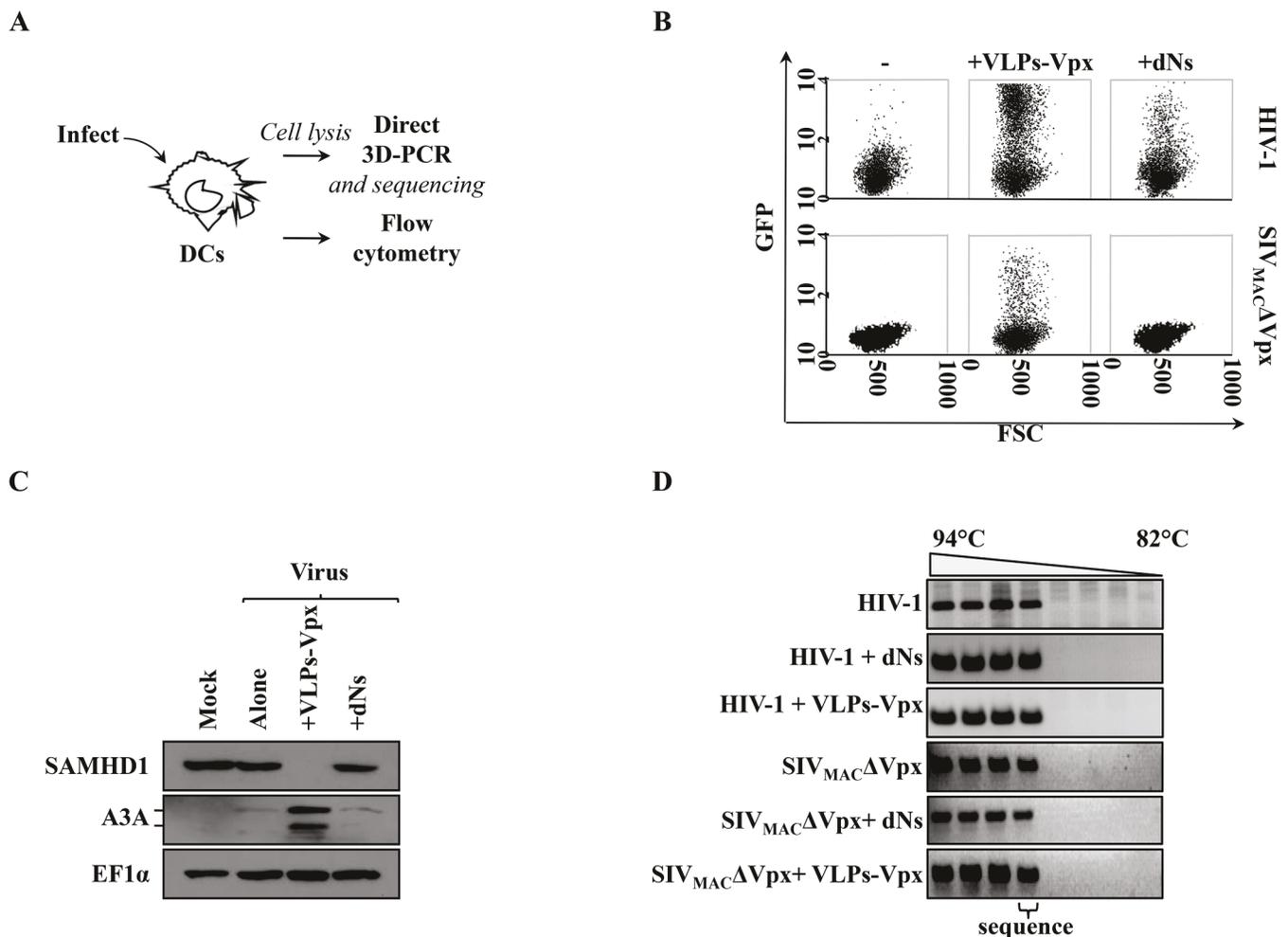


Fig 1. Experimental setup and susceptibility of DCs to HIV-1 and SIV_{MAC} infection in the presence of Vpx and dNs. A) DCs were differentiated by incubation of primary blood monocytes in GM-CSF/IL4 for 4 days prior to infection with VSVg-pseudotyped and exo-RT normalized HIV-1 and SIV_{MAC}ΔVpx viruses coding GFP at an MOI comprised between 0.5 and 1, according to the presented scheme. Cells were then either harvested 3 days later for flow cytometry analysis (B), or lysed twenty-four hours after infection for WB or DNA extraction and amplification (C and D, respectively). The results of the effects of Vpx and dNs on HIV-1 and SIV_{MAC} infection of DCs have been published in [32] and representative FACS panels are shown here only for clarity's sake from a total of more than 10 independent experiments conducted with cells of different donors. C) DCs were challenged with HIV-1 under the conditions specified in the figure prior to cell lysis twenty-four hours later and WB analysis. Note that the anti-A3A antibody used here recognizes two isoforms of the protein, the shorter one formed by translation at an internal ATG site. Similar results were obtained following infection with SIV_{MAC}ΔVpx (not shown). D) Identical amounts of cell lysates were amplified with primers specific for *gfp*, using denaturation temperatures ranging from 94°C to 82°C in a direct 3D-PCR. DNA amplified in the conditions of fraction 4 (denaturation temperature of 89°C) was cloned and individual clones sequenced. This fraction represents the lowest denaturation temperature at which DNA amplification is reproducibly observed. Representative agarose gel panels from 4 independent experiments are shown here.

doi:10.1371/journal.pone.0140561.g001

of 89°C (representing the border between reproducibly and sporadically detectable PCR product amplification, Fig 1D, fraction 4, as indicated) was then cloned and individual clones carrying the 319 nucleotide amplicon sequenced. The target sequence used was *gfp* that by its position on the viral genome corresponds to full length vDNA. This region is located downstream of the central polypurine tract-central termination sequence.

vDNA produced following infection of DCs displays clear signatures of cytidine specific editing

vDNA amplified in this manner following infection of DCs or HeLa cells as control was then sequenced and compared to the reference *gfp* with respect to the minus-strand (Fig 2A). vDNA synthesized after infection of HeLa cells with both HIV-1 and SIV_{MAC}ΔVpx displayed only a small number of mutations with respect to the reference sequence and more importantly, these mutations presented no discernable pattern. In contrast, 83% to 95% of mutations retrieved from vDNA amplified from infected DCs consisted of C>T transitions (in minus-strand DNA), typical fingerprints of A3s activity (Fig 2A). Although both Vpx and dNs are known to alter the intracellular concentration of dNTPs [5,16], it is highly unlikely that the particular skewing in C to T mutations observed here is due to alterations in RT fidelity that may follow changes in the intracellular concentration of dNTPs. Indeed, if this were the case, C to T mutations should have been equally distributed among minus- and plus-strand DNA. As a consequence, an equal proportion of C to T and G to A mutations (ie C to T mutations in the plus-strand) should have been present in minus-strand DNA, which is clearly not the case here. Given their exquisite concentration in the minus-strand DNA, these mutations are only compatible with the cytidine editing activity of members of the A3 family.

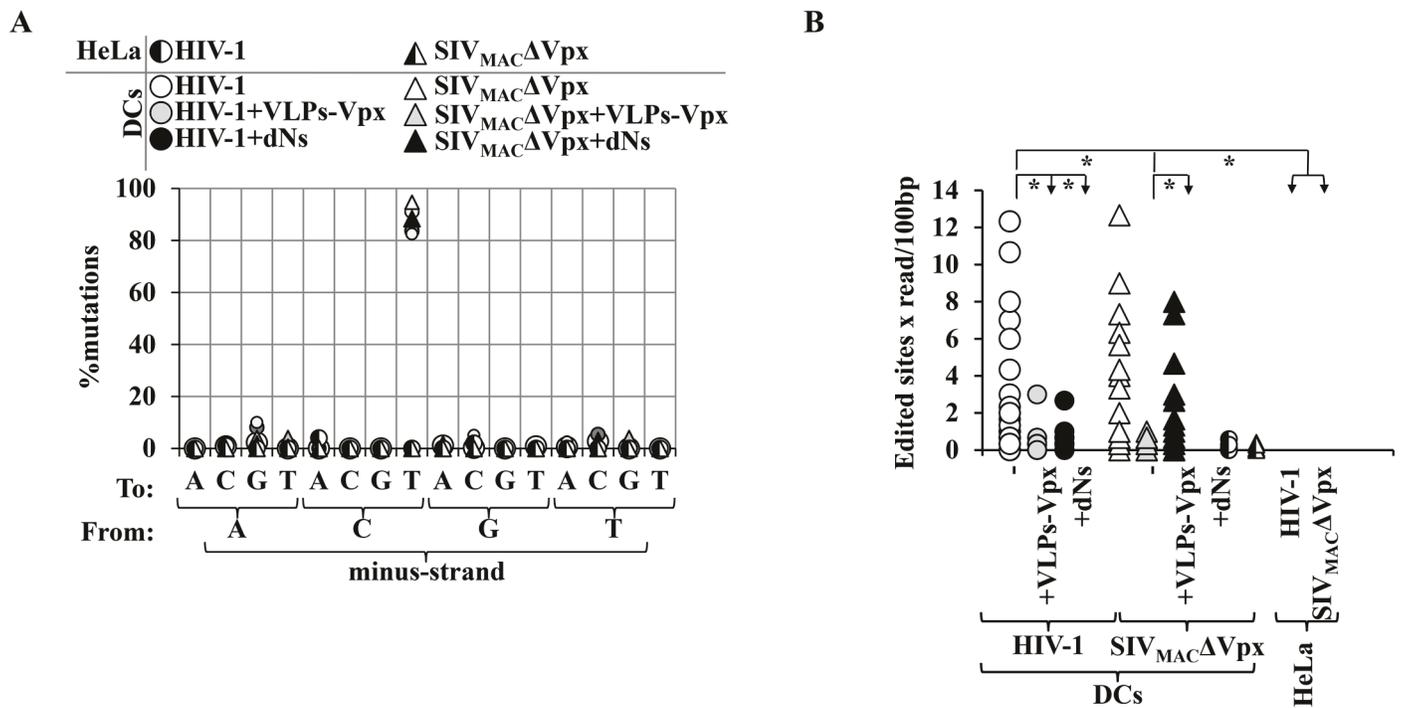


Fig 2. Extent of cytidine deamination following infection of DCs with HIV-1 and SIV_{MAC} in the presence of Vpx and dNs. A) For the analysis of mutations present in vDNA, vDNA amplified by direct 3D-PCR vDNA was cloned and individual colonies sequenced. A minimum of 10 individual colonies per condition and per independent experiment was analyzed (n = 4). The overall pattern of mutations retrieved with respect to the minus strand DNA reference sequence is shown here. As control, infections were carried out in HeLa cells, highly permissive to both HIV-1 and SIV_{MAC}. B) The graph presents the n° of edited cytidines/100bp in individual reads, in 4 independent donors/experiments. The asterisks indicate p ≤ 0.05 following a Student t test.

doi:10.1371/journal.pone.0140561.g002

The frequency of cytidine editing is differently modulated by Vpx and dNs during HIV-1 and SIV_{MAC}ΔVpx infection

The overall frequencies of edited cytidines ranged from 0.28 to 3.17/100 nucleotides across the different conditions and were therefore much lower than what can be observed upon direct incorporation of A3s into Vif-deficient virions [36]. However, interesting differences were noticed among conditions during both HIV-1 and SIV_{MAC} infection. HIV-1 and SIV_{MAC}ΔVpx vDNA displayed the highest average mutation rates (1.34 ± 0.5 and $3.17\pm 0.41/100$ nucleotides, respectively, Fig 2B) and addition of VLPs-Vpx consistently lowered these rates to 0.36 ± 0.03 and $0.37\pm 0.07/100$ nucleotides, respectively. In contrast, the frequency of cytidine deamination diminished significantly upon dNs supplementation only during HIV-1 infection ($0.28\pm 0.04/100$ nucleotides), while the decrease was more moderate and did not reach statistical significance in the case of SIV_{MAC}ΔVpx ($1.21\pm 0.57/100$ nucleotides).

Similar frequencies were observed using different primers in *gfp*, indicating the absence of skewing effects due to the choice of the primers (data not shown).

TC represents the major dinucleotide context of deaminated cytidines present on vDNA produced following infection of DCs

Upon close inspection of the dinucleotide context in which deaminated cytidines were present on vDNA, more than 60% of C>T transitions occurred in a TC dinucleotide context (Fig 3A), while the remaining were observed in a GC and CC (20% and 14%, respectively), the latter of which is evocative of A3G-based deamination [37,38,39,40,41]. This distribution remained relatively similar across conditions, although the absolute number of deaminated cytidines varied (as graphically presented through the size of the pies). All cytidines appeared susceptible to editing irrespectively of their physical location within the target sequence (Fig 3B).

Overall, the results presented above suggest that when infection occurs efficiently, vDNA is less susceptible to A3s. This is particularly striking for dNs that exert distinct effects on HIV-1 and SIV_{MAC}ΔVpx infection and rates of cytidine deamination.

Editing of vDNA by A3s may be influenced by the overall amount of vDNA produced during infection

As mentioned above, both VLPs-Vpx and dNs modulate vDNA accumulation [16,32,35] and can thus potentially influence the ratio between editing enzymes and their vDNA substrate. To determine whether this could be the case, the overall amount of vDNA synthesized during infection of DCs was determined by qPCR and used to calculate an expected average of editing. This value represents the editing frequency that could be expected, if this process were only influenced by vDNA levels. To this end, the editing frequency measured following HIV-1 infection was used as a reference and the remaining values were then calculated according to the following formula: expected frequency of deamination = average frequency/vDNA levels.

As already reported, VLPs-Vpx increased vDNA amounts following infection with both HIV-1 and SIV_{MAC}ΔVpx, while dNs promoted efficient vDNA accumulation mostly during HIV-1 infection (Fig 4A). When the average cytidine editing frequencies measured in this study for HIV-1 were corrected for the overall amount of vDNA present in the different conditions (Fig 4B), it appeared clear that vDNA produced in the presence of VLPs-Vpx and dNs (in this latter case only for HIV-1) ought to have accumulated 3 to 10 fold lower levels of editing than those that were instead measured experimentally. We believe that this discrepancy may be due to the fact that VLPs-Vpx (and dNs for HIV-1) lead not only to accrued vDNA accumulation, but also to an increase in A3A levels (see Fig 1C).

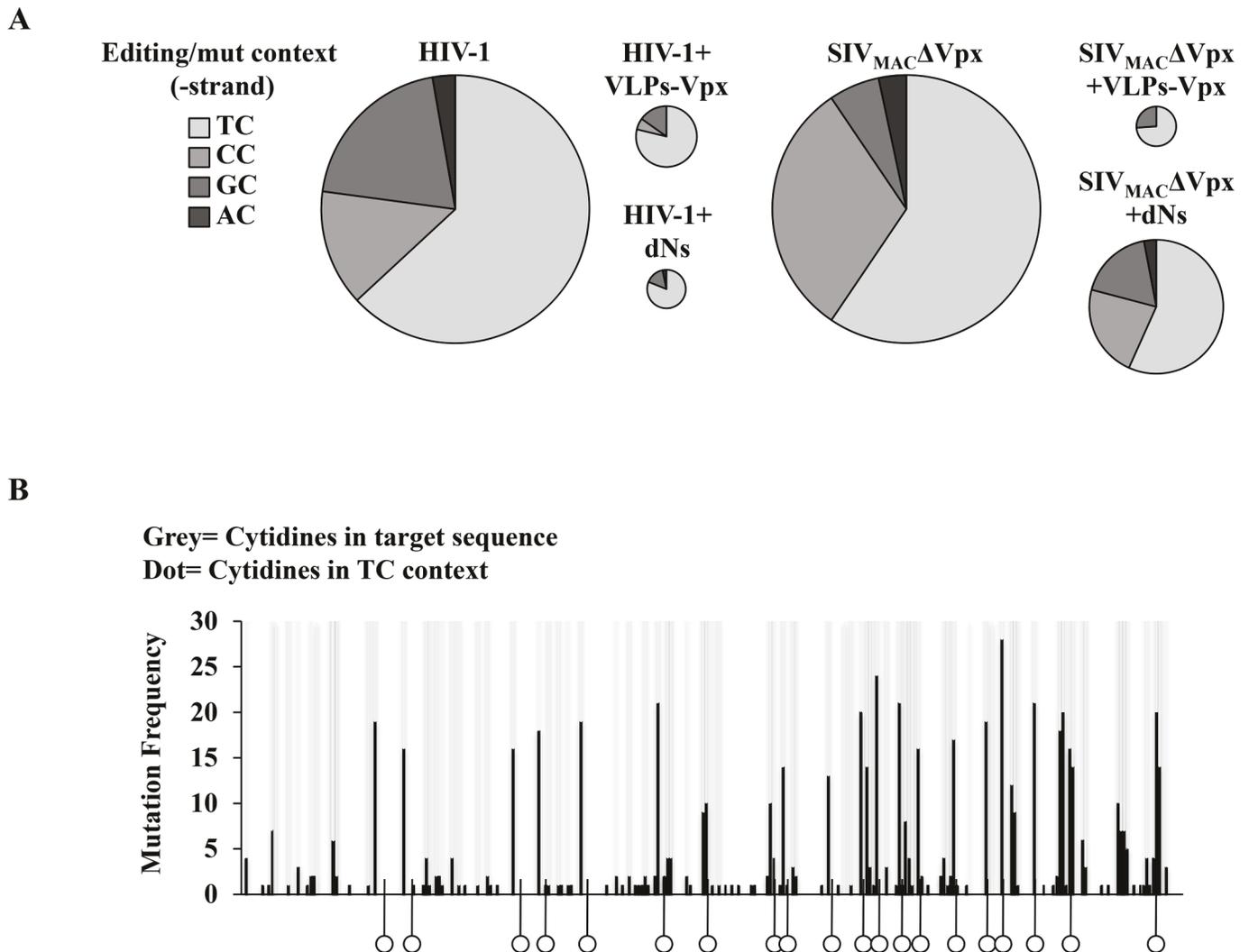


Fig 3. Cytidines present in a TC dinucleotide context account for the majority of editing signatures retrieved in vDNA produced following infection of DCs. A) The data presented in Figs 1 and 2 was analyzed to determine the dinucleotide context of mutated cytidines found in the different conditions. For each virus, the size of the pie is proportional to the absolute number of mutations. B) Spatial distribution of all the mutations obtained within the reference sequence. Cytidines present in a TC context are marked with a dot. The height of the black bars represents the frequency of mutations at a specific site.

doi:10.1371/journal.pone.0140561.g003

Conditions that promote efficient infection of DCs, promote the rapid completion of reverse transcription

A3s are single-stranded DNA editing enzymes. As such, it is conceivable that conditions that influence the speed of reverse transcription -and therefore the time required to pass from single- to double-stranded DNA may protect vDNA from the action of A3s. To determine whether this was the case, we measured the kinetics of reverse transcription of functional viral genomes through the addition of RT inhibitors at different times post infection, followed by flow cytometry 3 days later (functional genomes completed prior to the addition of the inhibitor will express the *gfp* reporter carried by the virus, as in [33], Fig 5A). Not all conditions

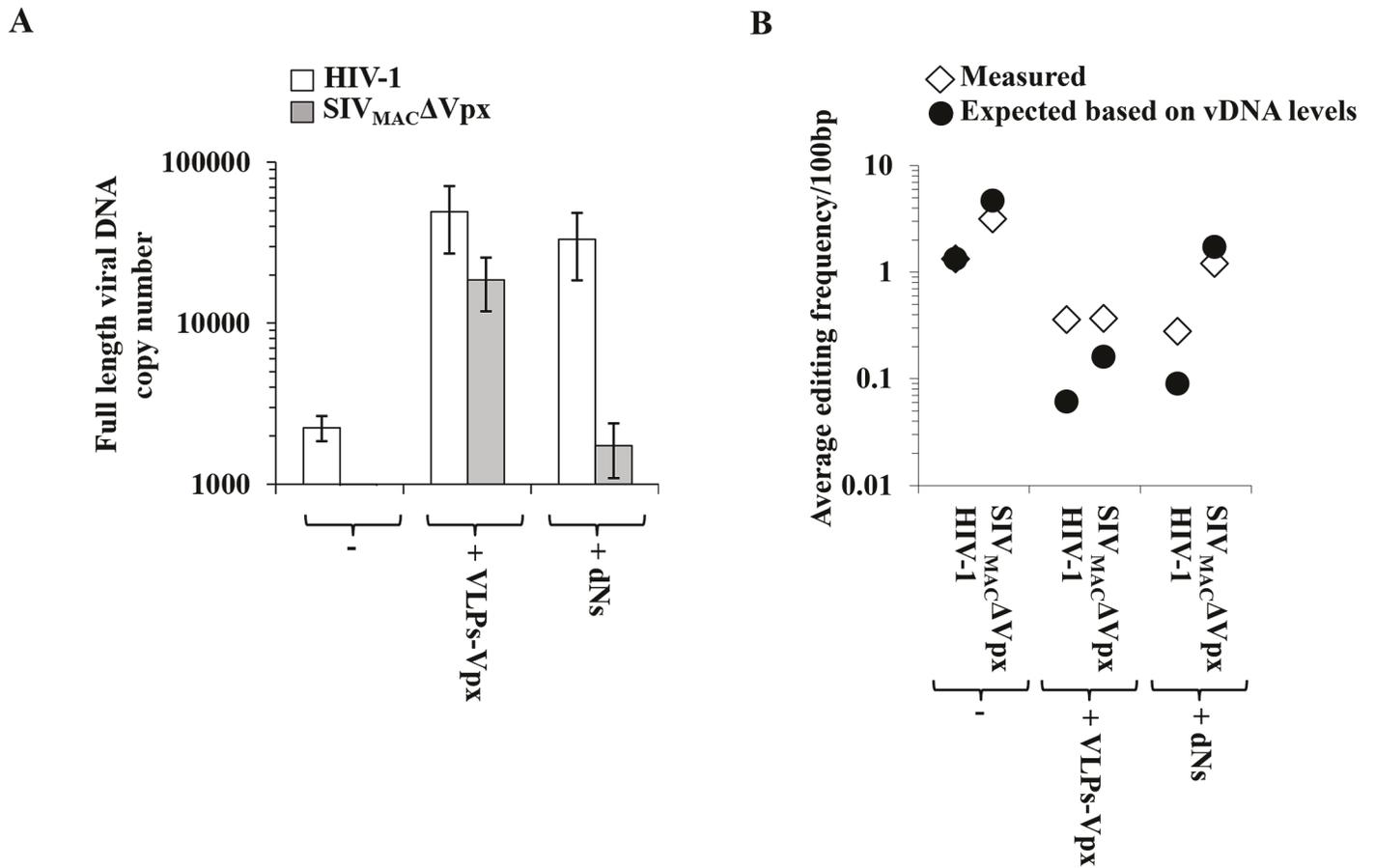


Fig 4. Analysis of the possible modulation between editing activity of A3s and overall vDNA levels. A) DCs were challenged with the indicated viruses and twenty-four hours post infection the amount of vDNA was quantified by qPCR using oligonucleotides specific for *gfp* carried on both HIV-1 and SIV_{MAC} genomes. B) The average frequencies of editing measured in the experiments of Fig 2 in the case of HIV-1 alone were then used to calculate an expected frequency of deamination following normalization for vDNA levels. This value represents the deamination that could be expected if this activity depended only on the amount of vDNA present during infection. Both expected and measured values are presented here.

doi:10.1371/journal.pone.0140561.g004

could be examined under this experimental setup, since this method relies on the measure of GFP-positive cells. However, both Vpx and dNs consistently accelerated the kinetics of HIV-1 reverse transcription and Vpx exerted similar effects in the case of SIV_{MAC} (Fig 5B), so that half of the infectious viral genomes were completed by 16 hours post infection, as opposed to longer time frame observed following WT HIV-1 infection.

Discussion

Previous studies have shown that A3A, a member of the A3 family that displays myeloid cell type specific expression, could directly target incoming vDNA, or even ectopically transfected DNA by editing cytidines present within a TC dinucleotide context [25,26,27]. In the present study, the implication of a particular A3 member was not investigated, given that we have been unable to obtain robust genetic silencing in DCs. However, we have examined the potential relationship existing between editing of vDNA, as it is exerted by A3s editing enzymes, and another cellular factor that strongly influences HIV infection of myeloid cells, SAMHD1.

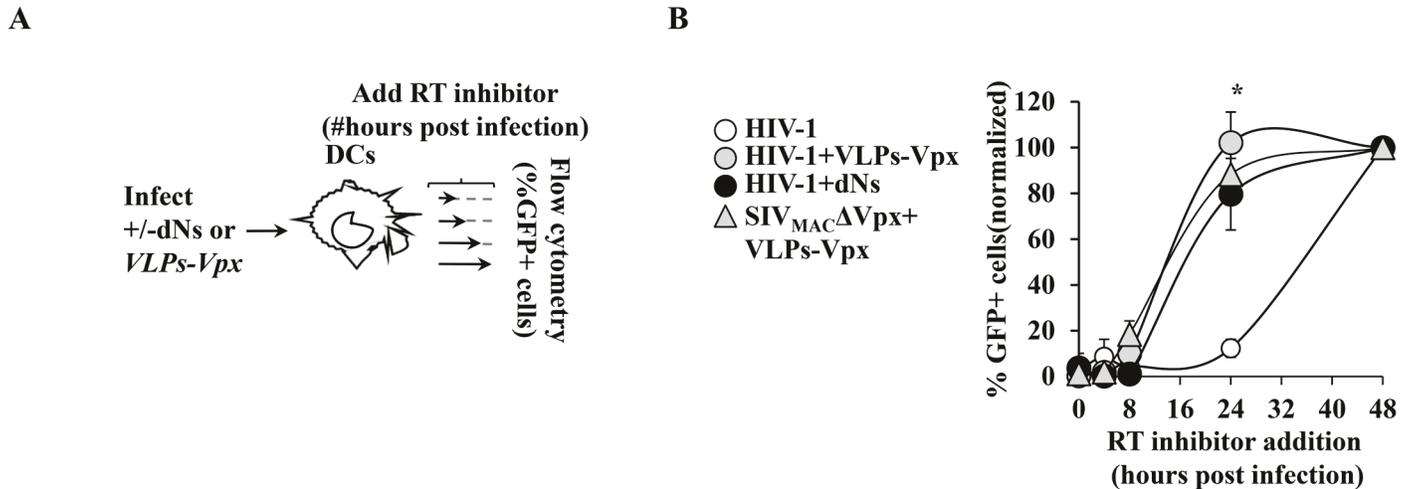


Fig 5. Analysis of the kinetics of reverse transcription in DCs. A) The kinetics of reverse transcription of functional viral genomes were measured according to the scheme presented in A in which RT inhibitors are added at different time post infection prior to flow cytometry analysis 3 days after the initial infection (routinely AZT/ddI at 10 μg/mL). B) Results are presented after normalization to samples in which the drugs had been omitted throughout the experiment (set to 100%). The graphs display averages and SEM obtained from 3 independent experiments and donors. Asterisks indicate $p \leq 0.05$ according to a Student t test.

doi:10.1371/journal.pone.0140561.g005

By examining vDNA accumulated in conditions that are well known to modulate the restriction specified by SAMHD1, we have been able to gather an interesting correlation between the efficiency of the infection process in DCs and the frequency of cytidine deamination of vDNA.

The mutations retrieved on vDNA produced during the infection of DCs consisted mostly of C to T mutations in the minus strand DNA, a pattern clearly identifiable as a signature of A3-mediated cytidine deamination. Neither an imbalance in dNTPs, nor differences in RT fidelity could otherwise explain this pattern and more importantly its exclusive concentration in only one of the two vDNA strands. Similarly, we believe it unlikely that SAMHD1 plays a more direct role in cytidine deamination, first because no such enzymatic activity has been described for this protein and second because cytidine editing lowers also upon dNs supplementation during HIV-1 infection, when SAMHD1 is present.

Cytidine editing occurs at higher frequencies in DCs than we had previously determined in macrophages (by a factor of 3 fold), in line with their lower susceptibility to viral infection. However, this frequency remains inarguably much lower than what can be observed upon the direct incorporation of A3s into Vif-deficient virion particles. If this suggests the absence of a direct antiviral effect due to mutagenesis, the presence of uracils on vDNA may be important to recruit other cellular factors onto vDNA with consequences that can range from its degradation to a more effective innate immune signaling. In support of the fact that deaminated vDNA can be at least in part degraded, we have reported that higher amounts of vDNA with lower deamination signatures accumulate in primary macrophages silenced for A3A [27].

Interestingly, the frequency of cytidine deamination of vDNA can be modulated by Vpx and dNs in what seems to be a virus-specific manner. In the case of HIV-1, the signatures of cytidine editing are significantly decreased in the presence of both Vpx and dNs, while in the case of SIV_{MAC}, this occurs only with Vpx. Apart from further stressing the existence of yet unappreciated mechanistic differences between Vpx and dNs during primate lentiviral

infection [32,35], these results strongly correlate (inversely) the success of infection to the extent of cytidine deamination of vDNA.

SAMHD1 is a major block to DCs infection and despite the fact that the mechanism/s of restriction remain debated [5,16,17,18,19,20] and [21,22,23,24], most studies agree on the fact that SAMHD1 is responsible for the low intracellular concentrations of dNTPs and that this situation can be reversed upon supplementation of Vpx or dNs [5,16,17,18,19,20]. In this respect, the fact that Vpx and dNs accelerate the kinetics of reverse transcription of HIV-1 vDNA is not unexpected and argue that -at least in the case of HIV-1- a major antiviral role of SAMHD1 is to slow down the kinetics of reverse transcription.

This property may already constitute a self-sufficient antiviral function *per se*. Furthermore, it may also be important to expose vDNA to a prolonged action of cytoplasmic effectors and particularly members of the A3 family that specifically require single-stranded reverse transcription products. Therefore, by slowing down the reverse transcription process, SAMHD1 may indirectly modulate the susceptibility of vDNA to A3-editing, thus explaining the inverse correlation observed here between editing on one hand and success of infection and speed of reverse transcription on the other.

Lastly, we believe it likely that the overall editing of vDNA is also influenced by the ratio between A3s editing enzymes and their substrates, i.e. vDNA. It is easy to envision that an excess of vDNA could attenuate the antiviral activity of A3s by dilution of vDNA in the cell. This hypothesis could explain the lower accumulation of editing signatures in vDNA produced in the presence of VLPs-Vpx. However, it is interesting to note that VLPs-Vpx also increase substantially the intracellular levels of A3A. We believe this is due to the fact that Vpx promotes high vDNA levels that are detected by the cell and lead to an increase in the expression of several antiviral genes, among which A3A. Therefore, if an increase in vDNA could in principle lead to the dilution of the antiviral effect of A3s, this is likely balanced by the concomitant increase in A3s.

Conclusions

Altogether, the results shown here suggest the existence of an inverse correlation between efficient infection and exposure of vDNA to A3 editing in DCs. By examining the effects that Vpx or dNs play during viral infection, the results presented here suggest a model in which efficient infection of DCs is promoted through a faster kinetics of reverse transcription that in turn may protect vDNA from cytoplasmic effectors like A3s by reducing the time of exposure of single-strand vDNA.

Acknowledgments

We thank Jeanine Bernaud and Dominique Rigal for help with blood sample collection. We are indebted to the NIH AIDS reagent program for the indicated material. AC is a CNRS researcher. XNN received the support of the FRM and of Sidaction. NW is a recipient of the China Scholarship Council PhD fellowship.

Author Contributions

Conceived and designed the experiments: XN GB AC. Performed the experiments: XN VB GB NW. Analyzed the data: GB AC. Contributed reagents/materials/analysis tools: XN VB. Wrote the paper: AC.

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